Université de Montréal

ÉTUDE DES GÈNES D'*ACTINOBACILLUS PLEUROPNEUMONIAE* EXPRIMÉS EN CONDITIONS D'INFECTION

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RÉSUMÉ

Actinobacillus pleuropneumoniae est l'agent étiologique de la pleuropneumonie porcine. La bactérie se transmet par voies aériennes et contacts directs. Plusieurs facteurs de virulence ont été identifiés, nommément les polysaccharides capsulaires, les lipopolysaccharide, les exotoxines ApxI à IV et de nombreux mécanismes d'acquisition du fer. Aucun vaccin efficace contre tous les sérotypes de la bactérie n'a encore été élaboré.

Afin de mieux comprendre de quelle façon *A. pleuropneumoniae* régule la transcription de ses nombreux facteurs de virulence et de découvrir de nouvelles cibles potentielles pour l'élaboration de vaccins efficaces, le profil transcriptomique de la bactérie a été étudié dans des conditions simulant l'infection ainsi qu'à la suite d'une infection naturelle aiguë chez l'animal. Des biopuces de première et de seconde génération (AppChip1 et AppChip2) comportant respectivement 2025 cadres de lecture ouverts (ORF) de la version préliminaire du génome *d'A. pleuropneumoniae* sérotype 5b souche L20 et 2033 ORF de la version finale annotée du même génome ont été utilisées. Dans un premier temps, des expériences réalisées dans des conditions de concentration restreinte en fer ont permis d'identifier 210 gènes différentiellement exprimés, dont 92 étaient surexprimés. Plusieurs nouveaux mécanismes d'acquisition du fer ont pu être identifiés, incluant un système homologue au système YfeABCD de *Yersinia pestis*, impliqué dans l'acquisition du fer chélaté, ainsi que des gènes homologues aux composantes du système HmbR de *Neisseria meningitidis* impliqué dans l'acquisition du fer à partir de l'hémoglobine.

Dans des conditions de culture permettant la formation de biofilms, les gènes *tadC* et *tadD* d'un opéron *tad* (« **t**ight **a**dherence **l**ocus ») putatif, les gènes *pgaBC* impliqués dans la synthèse d'un polysaccharide de la matrice du biofilm ainsi que deux gènes présentant de fortes homologies avec un gène codant pour l'adhésine auto-transporteur Hsf retrouvée chez *Haemophilus influenzae* ont montré une surexpression significative. Plusieurs de ces gènes ont également été retrouvés lors

d'expériences réalisées avec des cellules épithéliales d'origine pulmonaire en culture, qui ont permis d'identifier 170 gènes différentiellement exprimés après la croissance planctonique au-dessus des cellules, et 131 autres suite à l'adhésion à ces cellules. Parmis les gènes surexprimés, les gènes *tadB* et *rcpA* de l'opéron *tad* putatif, les gènes *pgaBC* ainsi que le gène codant pour l'homologue d'Hsf ont été retrouvés. En présence de liquide de lavage broncho-alvéolaire (BALF), 156 gènes ont montré un profil d'expression modifié, et le gène *apxIVA*, identifié comme étant surexprimé, a pu être détecté pour la première fois dans des conditions de croissance *in vitro*. Finalement, des expériences visant à déterminer les gènes utilisés directement chez l'animal en phase aiguë de la pleuropneumonie porcine ont permis d'identifier 150 gènes qui étaient différentiellement exprimés. En plus d'identifier des gènes d'un possible opéron codant pour un fimbriae de type IV, 3 des 72 gènes surexprimés sont conservés chez tous les sérotypes d'*A. pleuropneumoniae* et codent pour des protéines ou lipoprotéines de surface.

Nos expériences ont permis d'identifier plusieurs nouveaux facteurs de virulence potentiels chez *A. pleuropneumoniae* ainsi que plusieurs nouvelles cibles potentielles pour l'élaboration de vaccins efficaces contre tous les sérotypes.

Mots clés : *Actinobacillus pleuropneumoniae*, trancriptomique, fer, biofilm, culture cellulaire, liquide de lavage broncho-alvéolaire, *in vivo*, vaccin

ABSTRACT

Actinobacillus pleuropneumoniae is the etiological agent of porcine pleuropneumonia. Transmission of the disease occurs through direct contact or aerosols. The bacteria possess many virulence factors, namely capsular polysaccharides, lipopolysaccharides, four Apx toxins and iron acquisition mechanisms. To this day, an efficient cross-serotype vaccine has yet to be developed.

In order to investigate regulation mechanisms in *A. pleuropneumoniae* and to identify new potential targets for the synthesis of subunit vaccines, the transcriptomic profile of the bacteria under conditions that simulate the infection and following a natural acute infection *in vivo* were studied. The experiences relied on first and second generation microarrays (AppChip1 and AppChip2) designed using 2025 ORFs of the draft version of the *A. pleuropneumoniae* serotype 5b strain L20 genome and 2033 ORFs of the final and annotated version of the same genome respectively. First, experiments were conducted under iron-restricted conditions and 210 genes were deemed differentially expressed, 92 of which were up-regulated. Some new putative iron acquisition mechanisms were identified, including genes homologous to those of the *Yersinia pestis* YfeABCD chelated-iron acquisition system, as well as other genes homologous to components of the HmbR iron uptake from hemoglobin system of *Neisseria meningitidis*.

When cultured in conditions promoting biofilm production, genes *tadC* and *tadD* from a putative tad (« **t**ight **a**dherence **l**ocus ») operon, genes *pgaABC* involved in the biosynthesis of a polysaccharide of the biofilm matrix as well as two ORFs encoding a putative autotransporter adhesins similar to the *Haemophilus influenzae* Hsf adhesin were all significantly overexpressed. Many of these genes were also overexpressed when lung epithelial cells were infected with *A. pleuropneumoniae*. While 170 genes were differentially expressed after planktonic growth in the culture medium above the cells, another 131 were identified following direct adhesion to the

cells. Genes *tadB* and *rcpA* of the tad locus, as well as genes *pgaBC* and an ORF coding for the Hsf homolog where all found among overexpressed genes. When *A. pleuropneumoniae* was cultured in contact with broncho-alveolar lavage fluids (BALF), 156 genes were significantly differentially expressed and gene *apxIVA*, which was up-regulated, was detected for the first time during in *in vitro* growth conditions. Finally, experiments were conducted *in vivo* in animals naturally infected with *A. pleuropneumoniae* in the late stage of the acute phase in order to identify genes that are expressed during the infection of the natural host. While 150 genes were deemed differentially expressed, genes *apxIVA* as well as two genes from an operon coding for a putative type IV fimbriae were up-regulated. Out of those 72 genes that were overexpressed, 3 encode proteins or lipoproteins of the outer membrane which are conserved among all serotypes of the bacteria.

Overall, we were able to identify several new potential virulence factors for *A. pleuropneumoniae* in the course of our experiments, as well as several new potential targets for the elaboration of an efficient cross-serotype vaccine.

Key words: *Actinobacillus pleuropneumoniae*, trancriptomic, iron, biofilm, cell culture, BALF, *in vivo*, vaccine

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LISTE DES SIGLES ET ABBRÉVIATIONS

a.a. :	acides aminés
aa-dUTP :	amino-allyl déoxyuracyltriphosphate
ABC :	« ATP binding cassette », cassette liant l'ATP
ADN :	acide déoxyribonucléique
ADNc :	acide déoxyribonucléique complémentaire
ADNg :	acide déoxyribonucléique génomique
AFLP :	« Amplified fragment length polymorphism », polymorphisme de longueur de fragments amplifiés
AMPc :	adénine monophosphate cyclique
ANOVA :	« analysis of variation », analyse de variation
ARN :	acide ribonucléique
ARNa :	acide ribonucléique amplifié
ARNm :	acide ribonucléique messager
ARNs :	petits ARN régulateurs
ARNt :	ARN de transfert
ATP :	adénine triphosphate
ATPase :	adénine triphosphatase
BALF :	« Bronchoalveolar lavage fluids », fluides de lavages bronchoalvéolaires
BCAA :	« Branched chain amino acids », acides aminés à chaînes ramifiées
BHI :	« Brain-heart infusion »
CAST :	« Cluster affinity search technic »
CCAC :	« Canadian Council on Animal Care »
CDC :	« Center for Disease Control »
CFS :	« Cell-free supernatant », surnageant sans cellules
CFU :	« Colony forming unit », unités formatrices de colonies
CGH :	« Comparative genomic hybridizations », hybridations génomiques comparatives
ChIP :	« Chromatin Immunoprecipitation », immunoprécipitation de chromatine

CI :	« Competitive index », index compétitif
Cl :	chlore
cm :	centimètre
CM :	« Cytoplasmic membrane », membrane cytoplasmique
CO_2 :	dioxyde de carbone
CRM :	« Comprehensive Microbial Ressource »
CRP :	« Cyclic AMP receptor protein », protéine récepteur de l'AMPc
Cu :	cuivre
Cy :	cyanine fluorescente
DAP :	acide diaminopimélique
DIVA :	« Differenciating infected from vaccinated animals »
DMEM :	« Dubelcco's modified Eagle's medium »
DMSO :	diméthylsulfoxide
DNase :	déoxyribonucléase
EDDHA :	« ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid) »
EDTA :	« ethylenediamine dihydroxyphenyl acetic acid »
ELISA :	« Enzyme-linked immunosorbent assay »
FDR :	« False discovery rate »
Fe :	fer
FHA :	« Filamentous hemagglutinin/adhesin »
FQRNT :	Fond Québécois de Recherche pour la Nature et la Technologie
GTP :	guanine triphosphate
h :	heures
H :	hydrogène
HPS :	« hematoxylin phloxin saffron »
IgA, IgG :	immunoglobuline de type A ou de type G
IL :	interleukine
IM :	intra-musculaire
IN :	intra-nasal
IP :	intra-péritonéal
IPTG :	« Isopropyl β-D-1-thiogalactopyranoside »

IVET :	« *In vivo* expression technology », technologie d'expression *in vivo*
kDa :	kilodalton
KDO :	« 3-Deoxy-D-*manno*-oct-2-ulosonic acid »
kg :	kilogramme
K-MC :	« K-median clustering »
LB :	Luria-Bertani
LDH :	lactate déhydrogénase
LOWESS :	« Locally-weighted linear regression »
LPS :	lipopolysaccharide
M :	molaire
MAQC :	« Microarray quality control »
MEM :	« Minimum essential medium »
Mg :	magnésium
MH :	Muller-Hinton
min :	minutes
MLEE :	« Multilocus enzyme electrophoresis »
Mn :	manganèse
MOI :	« Multiplicity of infection », multiplicité d'infection
nm :	nanomètre
Na :	sodium
NAD :	nicotidamide dinucléotide
NCBI :	« National Center for Biotechnology Information »
NHS :	N-hydroxysuccinimidyl
NPTr :	« Newborn pig tracheal »
NRC :	« National Research Center »
O/N :	« overnight », pendant la nuit
OD :	« optical density », densité optique
OM :	« Outer membrane », membrane externe
OMP :	« Outer membrane protein », protéines de la membrane externe
pb :	paires de base
Pb :	plomb

PBS : « phosphate-buffered saline », saline tamponnée au phosphate
PDIM : phthiolocérol dimycocérosate
PGA : poly-N-acétylglucosamine
ppGpp : guanosine 3'-diphosphate,5'-bispyrophosphate
pppGpp : guanosine 3'-diphosphate,5'-triphosphate
PTS : « Phospho-transfer system »
ORF : « Open reading frame », cadre de lecture ouvert
PCR : « Polymerase chain reaction », réaction en chaîne de la polymérase
qRT-PCR « quantitative reverse transcription PCR », transcription inverse et PCR quantitatif
RBC : « Red blood cells », globules rouges
Rib : riboflavine
RNase : ribonucléase
RT-PCR DD : « Reverse transcription PCR differential display »
RTX : « Repeat in toxin », toxine avec séquence répétée
S : soufre
SAM : « Significance analysis of microarray »
SC : sous-cutanée
SCOTS : « Selective capture of transcribed sequences », capture sélective des séquences transcrites
SDS : sodium dodécyl sulfate
SELDI : « Surface Enhanced Laser Desorption Ionisation »
Sid : sidérophore
SJPL : « St.Jude porcine lung »
STM : « Signature-tagged mutagenesis », mutagénèse avec étiquette de signature
TAT : « Twin-arginin translocation »
TBS : « Tris-buffered saline », saline tamponnée au Tris
TCA : « Tricarboxylic acid », acide tricarboxylique
TCP : « Toxin corregulated pilus », pili corrégulé avec une toxine
TIGR : « The Institude for Genomic Research »

TMAO :	triméthylamine N-oxide
TMB :	3, 3`, 5, 5`-tétraméthylbenzidine
TNP :	transposon
TSB :	« Tryptic soy broth »
µg :	microgramme
µl :	microlitre
µm :	micromètre
µM :	micromolaire
UV :	ultraviolets
V :	volt
WGA :	« Wheat-germ agglutinin », agglutinine de germe de blé
Zn :	zinc

DÉDICACE

Cette thèse est dédiée à toutes les personnes qui m'ont supporté, d'une façon ou d'une autre, au cours de toutes ces années.

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INTRODUCTION

La recherche et l'identification des composantes bactériennes jouant un rôle dans les processus infectieux demeure la pierre angulaire de la guerre entre scientifiques et bactéries pathogènes. La compréhension des mécanismes responsables des effets pathologiques permet l'élaboration de contre-stratégies sous la forme d'antibiotiques ciblant spécifiquement les cellules bactériennes, ou encore sous la forme de vaccins stimulant le développement d'une réponse immunologique. Dans le domaine de l'agriculture et de la production animalière commerciale, des pressions existent afin que l'utilisation d'antibiotiques soit réduite. Le développement de vaccins permettant de prévenir les infections tout en minimisant les effets néfastes sur le développement des animaux est donc souhaitable.

Jusqu'au milieu des années 90, la recherche de facteurs de virulence importants pour des bactéries pathogènes nécessitait la caractérisation de souches. Le processus était plutôt aléatoire, et une banque de mutants était criblée en sélectionnant des clones ayant perdus certains phénotypes. Du phénotype observé, il fallait ensuite remonter à la source de la mutation dans le génome bactérien afin d'identifier le gène muté. Une fois le gène identifié, il pouvait être cloné et exprimé, permettant ainsi de produire la protéine recherchée en grandes quantités. De manière générale, chaque composante bactérienne devait être isolée et caractérisée de manière individuelle. Le processus, bien qu'efficace, est long et fastidieux.

Avec l'avènement des technologies permettant le séquençage rapide de génomes complets, il est maintenant possible, pour un grand nombre de micro-organismes (1016 génomes microbiens complètement séquencés, 2176 en cours de séquençage en date du 2 décembre 2009), de démarrer la recherche de facteurs de virulence potentiels directement à partir des séquences génétiques (NCBI 2009). La construction de biopuces à ADN, des supports solides où sont imprimés des génomes complets soit sous la forme d'oligonucléotides ou de produits PCR représentant des gènes complets, est intimement lié aux efforts de séquençages. L'utilisation de biopuces, qui peut permettre, entre autres, d'identifier tous les gènes qui sont exprimés par une bactérie dans une condition précise, a permis de modifier les

stratégies utilisées pour la recherche de gènes importants exprimés en conditions expérimentales simulant l'environnement rencontré chez l'hôte, ou encore directement chez l'individu infecté.

Actinobacillus pleuropneumoniae, bactérie causant la pleuropneumonie porcine, possède 15 sérotypes différents, sur la base de différences dans les polysaccharides de surface, répartis en 2 biotypes en fonction de leur dépendance au NAD (nicotidamide dinucléotide) (Bossé et al. 2002). L'existence de ces nombreux sérotypes complique les efforts en ce qui concerne l'élaboration de vaccins permettant de protéger l'hôte porcin contre l'infection par tous les sérotypes retrouvés dans l'environnement (Ramjeet et al. 2008b). De plus, les mécaniques nécessaires à l'installation et au développement de l'infection sont toujours obscures à certains égards. Alors que le rôle des LPS (lipopolysaccharides) dans l'adhésion est connu, aucune adhésine de nature protéique n'a encore été identifiée (Jacques 2004) et le comportement de la bactérie à l'intérieur de l'hôte n'a pas encore été investigué. En 2007, une première séquence génomique complète pour *A. pleuropneumoniae*, basée sur la souche de référence L20 du sérotype 5b, a été officiellement publiée (Foote et al. 2008), et ce projet fut complémenté par l'élaboration d'une biopuce comportant des amplicons spécifiques à tous les cadres de lecture ouverts identifiés au sein de la séquence génomique.

Revue de Littérature

1. *ACTINOBACILLUS PLEUROPNEUMONIAE*

1.1 Généralités et caractéristiques biochimiques

Actinobacillus pleuropneumoniae est une bactérie appartenant à la famille des *Pasteurellaceae* (Taylor 1999), famille qui comprend plusieurs bactéries capables de coloniser les muqueuses des voies respiratoires et génitales. Auparavant classée dans le genre *Haemophilus* sous la nomenclature *H. pleuropneumoniae* ou *H. parahaemolyticus*, des analyses plus poussées au niveau génétique ont démontré que *H. pleuropneumoniae* présentait plus d'homologie de séquences avec *Actinobacillus lignieresii* qu'avec les autres membres du genre *Haemophilus* (Pohl et al. 1983). La bactérie fut donc rebaptisée *Actinobacillus pleuropneumoniae*, et elle est reconnue pour son implication dans la pleuropneumonie porcine, maladie dont elle est l'agent étiologique (Taylor 1999). Elle est considérée comme un parasite obligatoire du tractus respiratoire porcin (Bossé et al. 2002).

Actinobacillus pleuropneumoniae est une bactérie à Gram négatif encapsulée et non-sporulée. Sa morphologie est typique des coccobacilles, et la bactérie peut se présenter de façon isolée, en paires ou en courtes chaînes, et possède un métabolisme de type fermentatif. La plupart des membres de cette espèce ne pousse pas sur des milieux de culture riches, à moins que de la nicotidamine-adénine-dinucléotide (NAD ou facteur V) soit ajoutée au milieu. Ce facteur peut aussi être apporté par des colonies de staphylocoques, raison pour laquelle un phénomène de satellitisme peut être observé lorsque ces deux micro-organismes sont cultivés sur un même milieu de culture (Taylor 1999). Lorsqu'*A. pleuropneumoniae* est cultivé sur gélose sang, toujours en présence de staphylocoques, une zone d'hémolyse β est observable autour des colonies, et elle produit une zone d'hémolyse accrue à l'intérieur d'une zone d'hémolyse partielle par une souche β-toxigénique de *Staphylococcus aureus* (Nicolet 1970), phénomène connu sous le nom de phénomène de CAMP. Cette caractéristique est attribuable aux toxines cytolytiques Apx sécrétées par la bactérie (Frey et al. 1994).

On reconnaît l'existence de deux biotypes chez *A. pleuropneumoniae* : le biotype 1, qui est dépendant du NAD pour sa croissance, et le biotype 2, qui ne nécessite pas la présence de ce composé puisqu'il possède les voies métaboliques requises pour le synthétiser à partir de certains précurseurs pyrimydiques (O'Reilly et al. 1986). En plus de ces deux biotypes, plusieurs sérotypes différents ont été identifiés sur la base de différences au niveau des polysaccharides capsulaires (Dubreuil et al. 2000). Les sérotypes 1 à 12 et 15, sérotype récemment proposé (Blackall et al. 2002), sont retrouvés au sein du biotype 1, les sérotypes 1 et 5 étant subdivisés en deux sous-sérotypes, soit 1a, 1b, 5a et 5b. Seulement deux sérotypes ont été décrits pour le biotype 2, soient les sérotypes 13 et 14, mais des souches du biotype 2 ont aussi été isolées des sérotypes 2, 4, 7 et 9 (Schaller et al. 2001).

1.2 La pleuropneumonie porcine

Actinobacillus pleuropneumoniae est l'agent étiologique de la pleuropneumonie porcine, maladie qui se propage principalement par voies aériennes, surtout lors de contacts directs entre les animaux (Taylor 1999). Les sérotypes les plus souvent impliqués dans l'infection varient en fonction des régions : les sérotypes 1, 5 et 7 sont plus souvent retrouvés en Amérique du Nord, alors que le sérotype 2 est prédominant dans plusieurs pays européens (Jacques 2004). Les observations sur le terrain tendent à démontrer que les sérotypes 1, 5, 9, 10 et 11 sont plus virulents que les autres sérotypes du biotype 1, alors que les souches de biotype 2 seraient moins virulentes (Jacobsen et al. 1996; Taylor 1999).

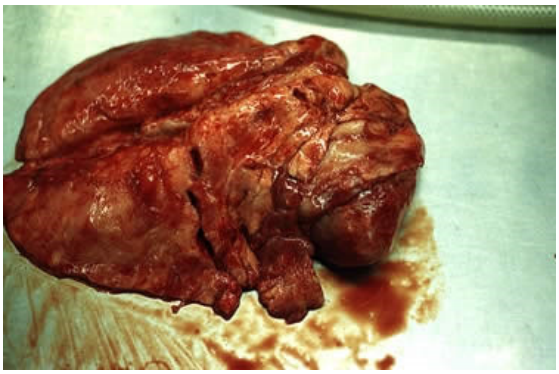

Fig. 1: Exemple de lésions pulmonaires typiquement causées par l'infection par *A. pleuropneumoniae*. (Dee 2008)

La pleuropneumonie porcine peut prendre plusieurs forme : la forme chronique, la forme aiguë et la forme suraiguë (Taylor 1999), tout dépendant du sérotype responsable de l'infection,

du statut immunitaire de l'hôte et du nombre de bactéries atteignant le site d'infection (Bossé et al. 2002). Les formes aiguë et suraiguë sont caractérisées par des taux de mortalité et de morbidité élevés (Taylor 1999), la première étant souvent fatale en moins de 48h (Dubreuil et al. 2000), et les bêtes présentent fréquemment des symptômes allant de la fièvre à l'augmentation du taux respiratoire, la toux et le reniflement, des difficultés respiratoires (dyspnée), l'anorexie, l'ataxie, des vomissements et la diarrhée (Bossé et al. 2002). Au niveau des poumons, l'infection est caractérisée par l'apparition de zones foncées de nécrose pulmonaire, parfois accompagnées de pleurésie fibrineuse (Taylor 1999) (Figure 1). Au niveau histopathologique, on observe l'apparition de zones d'hémorragie et d'infiltration de neutrophiles, de zones d'œdème ainsi que la présence d'exudats fibrineux. Les dommages au niveau des tissus sont attribuables autant aux toxines sécrétées par la bactérie qu'aux cytokines libérées par la réponse immunitaire de l'hôte (Bertram 1990).

Suite à l'infection aiguë, les animaux qui ont survécu peuvent devenir porteurs chroniques de la maladie (Bossé et al. 2002). La bactérie peut alors être retrouvée au niveau de certains focus nécrotiques ainsi que dans des abcès encapsulés, sous des zones de tissus connectifs fibrineux. Dans certains cas, la bactérie peut aussi être cultivée à partir des amygdales du porteur chronique. L'introduction de porteurs chroniques dans de nouvelles populations demeure une des principales voies de dissémination de la maladie entre différents cheptels (Taylor 1999).

Une expérience réalisée *in vivo* avec une souche d'*A. pleuropneumoniae* de sérotype 2 a permis de visualiser les effets de l'infection par *A. pleuropneumoniae* peu de temps après l'adhésion (Dom et al. 1994). Ainsi, seulement trente minutes après infection, des observations par microscopie électronique ont permis de montrer que 95% des bactéries présentes étaient retrouvées associées avec l'épithélium des cellules alvéolaires pulmonaires ou les cellules ciliées des bronchioles terminales (Figure 2). Plus tard, soit 90 à 180 minutes après l'infection, des focus d'infection de 1 à 5 mm sont observables et peuvent être séparés en trois zones disctinctes :

1. une zone centrale, où les structures primaires des poumons sont détruites. Les cellules bactériennes sont retrouvées en association avec des cellules infiltrées et libres dans l'exudat.
2. une zone intermédiaire, où les structures pulmonaires sont toujours visibles. La vaste majorité des bactéries présentes sont associées aux cellules pulmonaires.
3. une zone externe, où aucune lésion histologique n'est visible, mais où il est possible de détecter la présence de bactéries.

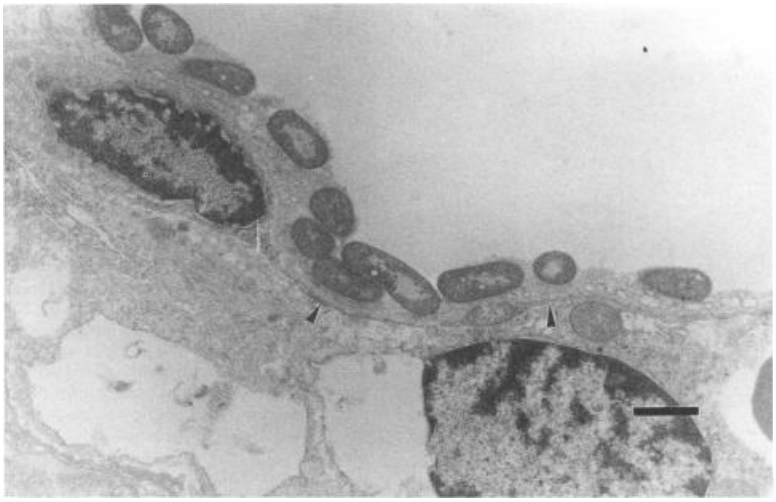

Fig. 2: Association d'*A. pleuropneumoniae* sérotype 2 avec les cellules épithéliales des alvéoles pulmonaires 30 minutes après l'infection visualisée par microscopie électronique. Les bactéries (batônnets) ont leur côté le plus long aligné de façon parallèle aux cellules épithéliales alvéolaires. Tiré de Dom & al., 1994.

1.3 Les facteurs de virulence d'*Actinobacillus pleuropneumoniae*

Bien que les dommages physiologiques ainsi que les symptômes de l'infection à *A. pleuropneumoniae* aient été étudiés et documentés extensivement, la pathogenèse de la pleuropneumonie porcine est complexe et les mécanismes l'entourant ne sont toujours pas totalement élucidés (Bossé et al. 2002). Chose certaine, beaucoup de composantes de la bactérie ont jusqu'à ce jour été mises en cause et sont considérées comme d'importants facteurs de virulence de la bactérie.

1.3.1 La capsule polysaccharidique

La synthèse de la capsule chez *A. pleuropneumoniae* dépend de la présence d'au moins deux opérons (Rioux et al. 2000) : l'opéron *cpxDCBA*, codant pour le système d'exportation des composantes capsulaires, et l'opéron *cps*, comprenant les gènes

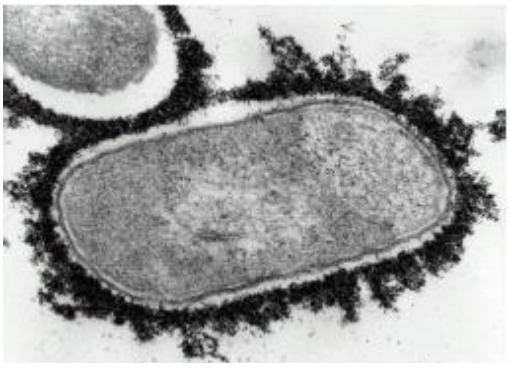

Fig. 3: Image par microscopie électronique de la capsule d'*A. pleuropneumoniae* sérotype 1 marquée à la ferritine polycationique. (Rioux & al. 2000)

cpsABCDE. L'opéron *cps* est situé directement en amont du gène *cpxD* et sur le brin opposé (Bandara et al. 2003), et code pour des protéines présentant de faibles similarités au niveau de leurs séquences en acides aminés avec des enzymes de type glycosyltransférases (Rioux et al. 2000). La composition des capsules des sérotypes 1 à 12 a été élucidée et varie considérablement entre ces sérotypes (Perry et al. 1990). Suite à la découverte de souches appartenant aux sérotypes 13, 14 et 15, d'autres études ont permis de déterminer à leur tour les structures capsulaires de ces sérotypes (MacLean et al. 2004; Perry et al. 2004; Perry et al. 2005). Les structures identifiées sont variables entre les différents sérotypes, malgré la présence de certaines similarités qui pourraient être responsables de certaines réactions sérologiques croisées entre certains sérotypes (Gutierrez et al. 1991), et ces divergences se réflètent aussi sur le plan génétique. Des études de génomiques comparatives effectuées par notre groupe avec des souches de référence représentant tous les sérotypes d'*A. pleuropneumoniae* ont mis en évidence que les locus impliqués dans la synthèse et la sécrétion des polysaccharides capsulaires divergent grandement au niveau de leurs séquences (Gouré et al. 2009).

Le rôle de la capsule *d'A. pleuropneumoniae* dans la virulence de la bactérie ne peut être remis en doute, plusieurs études ayant en effet démontré que des mutants acapsulaires sont atténués chez le porc (Rioux et al. 2000; Bandara et al. 2003). Les premières hypothèses formulées concernant la fonction *in vivo* de la capsule polysaccharidique reliaient cette dernière à l'adhésion aux tissus de l'hôte ainsi qu'à la résistance au sérum qui est observée chez les souches virulentes d'*A. pleuropneumoniae*, probablement via la formation d'une barrière physique en surface de la bactérie (Figure 3). Cependant, il a été démontré que des mutants de synthèse de capsule d'*A. pleuropneumoniae* montrent une adhérence accrue envers différents

types cellulaires (Jacques et al. 1991; Rioux et al. 2000), ce qui porte à croire que la capsule polysaccharidique d'*A. pleuropneumoniae* masque les adhésines de la bactérie. Alors que Ward et Inzana avaient démontré une certaine implication de la capsule dans la résistance au sérum (Ward et al. 1994), et ensuite qu'un mutant acapsulaire d'*A. pleuropneumoniae* sérotype 5a devenait sensible au sérum de porc (Ward et al. 1998), Rioux et al. ont démontré que tel n'était pas le cas pour le mutant acapsulaire du sérotype 1 (Rioux et al. 2000). Cette variation entre sérotypes serait possiblement due aux différences existant entre la composition des lipopolysaccharides (LPS) de ces deux sérotypes. Une étude a démontré qu'il y a une corrélation directe entre la quantité de polysaccharides capsulaires produits par des souches isogéniques d'*Actinobacillus pleuropneumoniae* et leur virulence chez l'animal (Bandara et al. 2003).

1.3.2 Les lipopolysaccharides (LPS) de la surface bactérienne

Les LPS forment de longues extensions extra-cellulaires polysaccharidiques ancrées à la membrane externe des bactéries à Gram négatif via leur région lipidique. Trois régions bien définies peuvent être identifiées (Miller et al. 2005)(Figure 4) :

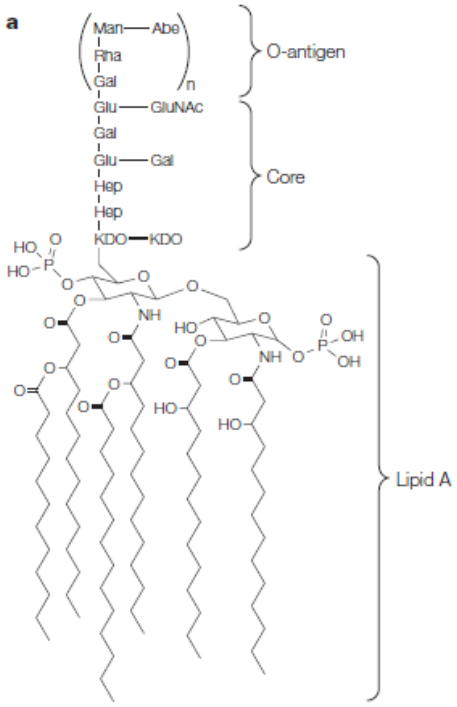

Fig. 4 : Structure des LPS des bactéries à Gram négatif.
Tiré de Miller et al. 2005.

1. Le lipide A : partie via laquelle le LPS est ancré à la surface de la cellule. Le lipide A est composé de deux dérivés de glucosamine avec des acides gras et des phosphates ou pyrophosphates. Il confère aux LPS sa propriété d'endotoxine.
2. Le noyau oligosaccharidique : partie centrale du LPS composée de sucres ayant souvent des structures inhabituelles ; on y retrouve entre autre du KDO (acide 2-céto-3-déoxyoctulosonique) ainsi que des heptoses. Le noyau oligosaccharidique comprend deux régions distinctes, soit le noyau interne, situé à proximité du lipide A, et le noyau externe, situé près de l'antigène-O.
3. L'antigène-O : partie polysaccharidique la plus externe du LPS, composée d'unités répétées. La structure et la composition de l'antigène-O ont été déterminées pour les sérotypes du biotype 1 d'*A. pleuropneumoniae* (Perry et al. 1990), et elles sont pratiquement toujours spécifiques au sérotype.

Les LPS des bactéries à Gram négatif sont surtout reconnus pour leur activité endotoxique. Cependant, chez *A. pleuropneumoniae*, c'est le rôle probable des LPS dans l'adhésion aux cellules de l'hôte, première étape du processus d'infection, qui retient surtout l'attention. Les LPS d'*A. pleuropneumoniae* seraient en effet impliqués dans l'adhérence aux cellules et au mucus du tractus respiratoire porcin ainsi qu'aux glycosphingolipides de l'hôte, présents sur les cellules épithéliales respiratoires (Bélanger et al. 1990; Bélanger et al. 1994a; Bélanger et al. 1994b; Paradis et al. 1994; Abul-Milh et al. 1999), en plus de pouvoir lier l'hémoglobine porcine (Bélanger et al. 1995; Archambault et al. 1999). Dans la même lignée, il a été observé que l'adhérence d'*A. pleuropneumoniae* à des sections congelées de trachée porcine est inhibée par la présence d'anticorps monoclonaux dirigés contre l'antigène-O des LPS (Paradis et al. 1999), et que la création de mutations dans les gènes impliqués dans la synthèse des LPS affecte l'adhérence *in vitro* d'*A. pleuropneumoniae* (Rioux et al. 1999; Galarneau et al. 2000). Alors que les mutations dans l'antigène-O du LPS ne semblent pas affecter l'adhérence aux cellules du tractus respiratoire porcin, les mutations au sein du noyau oligosaccharidique affectent grandement l'adhésion. De façon générale, une étude de

profil électrophorétique a permis de mettre en évidence deux types de structures pour la région comprenant le noyau oligosaccharidique et le lipide A : un groupe présentant une migration sur gel plus lente que le LPS de référence de *Salmonella* Typhimurium RA (sérotypes 1, 6, 9 et 11), et un autre groupe montrant une migration similaire au LPS de référence (sérotypes 2-5, 7, 8, 10 et 12) (Jacques et al. 1996).

Récemment, la structure du noyau oligosaccharidique des sérotypes 1, 2, 5a et 5b a été élucidée (Michael et al. 2004) (Figure 5). Le squelette de base semble bien conservé entre ces sérotypes et la structure ne semble diverger que par quelques substitutions. Cette ressemblance structurelle confirme des résultats qui avaient été obtenus auparavant par Southern blot, alors qu'il avait été possible de détecter, chez les 12 sérotypes du biotype 1 (Labrie et al. 2002), trois ORFs impliqués dans la synthèse du noyau oligosaccharidique avec des sondes spécifiques aux séquences de ces ORFs chez *A. pleuropneumoniae* sérotype 1 (Paradis et al. 1994; Galarneau et al. 2000).

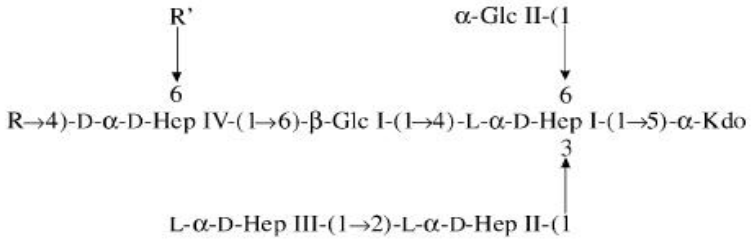

Fig. 5 : Structure du noyau oligosaccharidique d'*Actinobacillus pleuropneumoniae*.
- Sérotype 1 : R = (1*S*)-GalαNAc-(1→4,6)-α-Gal II-(1→3)-β-Gal I-(1→, R' = H
- Sérotype 2 : R = β-Glc III-(1→, R' = D-α-D-Hep V-(1→
- Sérotype 5a et 5b : R = H, R' = D-α-D-Hep V-(1→

Tiré de St.Michael & al. 2004.

Plus récemment, la mise en évidence de l'interaction de souches d'*A. pleuropneumoniae* de sérotype 1, 5b et 7 avec un phospholipide membranaire, le phosphatidyléthanolamine, a mené à l'élaboration d'un premier modèle hypothétique de l'adhésion d'*A. pleuropneumoniae* aux cellules de l'hôte (Jeannotte et al. 2003; Jacques 2004). L'interaction de faible affinité d'*A. pleuropneumoniae* avec le phosphatidyléthanolamine, qui, tel que démontré lors de la comparaison de

l'adhésion de divers mutants LPS envers ce phospholipide, s'effectue via l'antigène-O, serait en effet la première étape dans le processus d'adhésion de la bactérie. Suite à cette première adhésion, *A. pleuropneumoniae* aurait recours au noyau oligosaccharidique de son LPS et/ou à d'autres adhésines de surface pour interagir avec plus d'affinité avec des récepteurs lipidiques ou protéiques présents à la surface de la cellule cible (Jacques 2004).

1.3.3 Fimbriae

La présence de structures fimbriaires à la surface d'*A. pleuropneumoniae* a d'abord été observée par microscopie électronique lors de deux expériences indépendantes (Utrera et al. 1991; Dom et al. 1994) (Figure 6). Quelques années plus tard, une protéine de 17 kDa dont la séquence en acides aminés était identique aux séquences des protéines sous-unitaires des fimbriae de type 4 retrouvées entre autres chez *Haemophilus influenzae*, *Moraxella bovis*, *Neisseria gonorrhoeae*, *Neisseria meningitidis* et *Pseudomonas aeruginosa*, a été isolée à partir de cultures d'*A. pleuropneumoniae* en condition de microaérophilie (Zhang et al. 2000). La

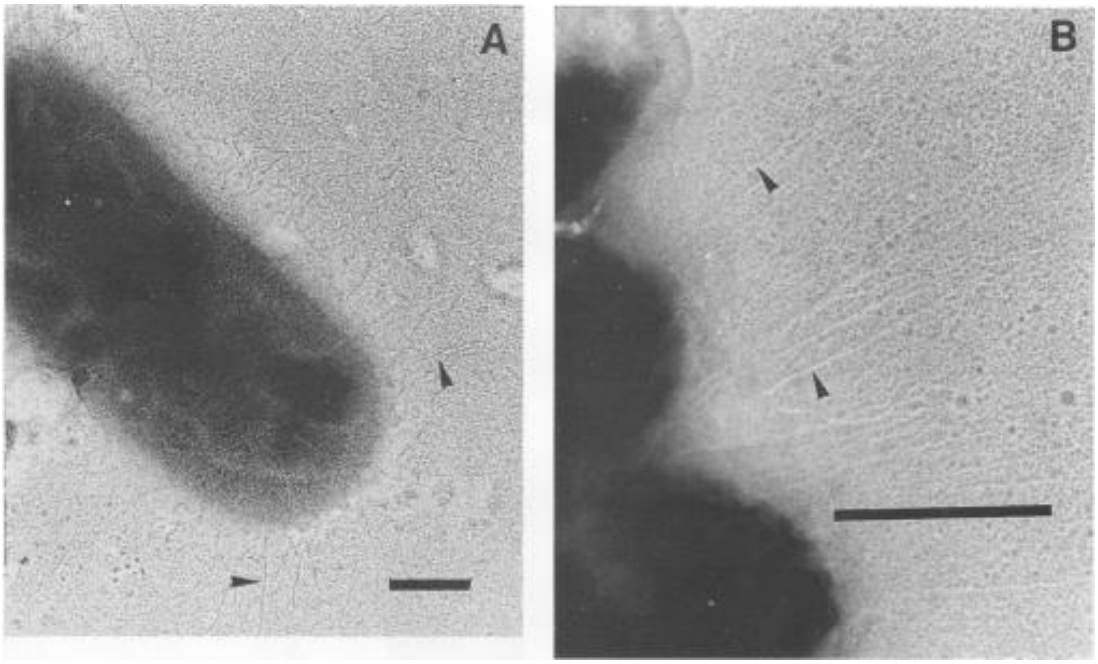

Fig. 6 : Appendices fimbriaires observables en surface d'*A. pleuropneumoniae* par microscopie électronique. Les fimbriae ont une longueur variant de 100 à 300 nm, sont larges d'approximativement 1nm et leur distribution est péritriche. (Tiré de Dom & al. 1994)

découverte de ce type de fimbriae chez *A. pleuropneumoniae* constitue un événement important étant donné la vaste étendue de fonctions qui leur sont attribuées. En effet, ces fimbriae, qui partagent de grandes ressemblances avec les systèmes de sécrétion de type II et de type III (Donnenberg 2000) (Figure 7), pourraient jouer un rôle dans l'acquisition d'ADN exogène, l'exportation de protéines, la motilité, l'infection par des phages et, ce qui pourrait présenter un intérêt plus particulier chez *A. pleuropneumoniae*, l'adhésion aux cellules cibles (Boekema et al. 2004b).

L'opéron *afpABCD*, qui coderait pour ce système chez *A. pleuropneumoniae,* a été cloné (Stevenson et al. 2003) et il a par la suite été démontré que ce système, bien qu'il ne soit pas induit *in vitro* dans une variété de conditions de culture, est effectivement transcrit lorsque la bactérie adhère à des cellules épithéliales pulmonaires en culture et lors de l'infection de l'animal (Boekema et al. 2004b). Le fait que la transcription de l'opéron *afp* nécessite des conditions de croissance bien précises et qu'il soit actif *in vivo* témoignent de son importance dans l'infection par *A. pleuropneumoniae*.

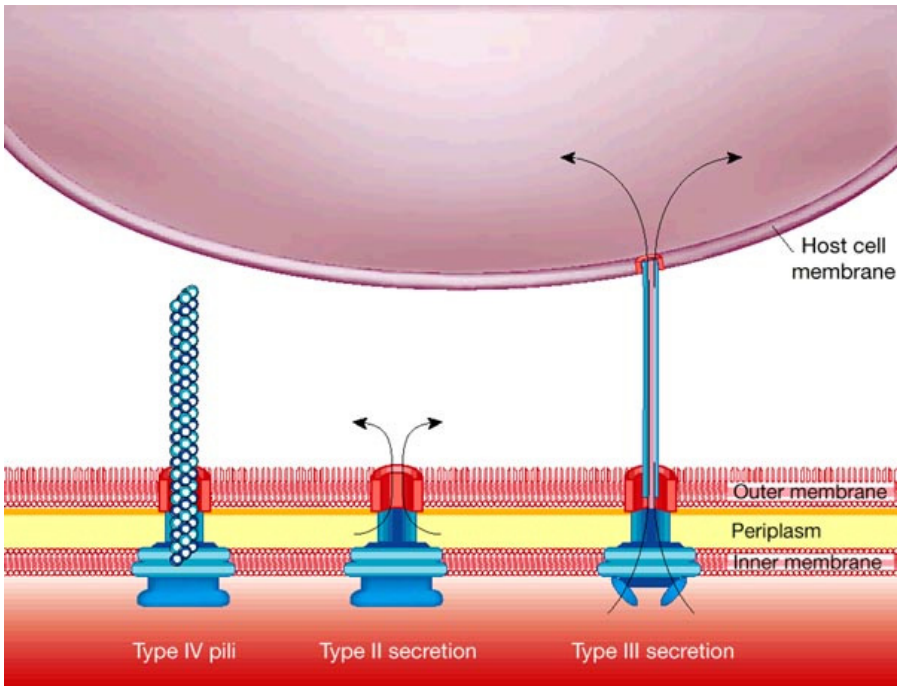

Fig. 7: Représentation schématique des structures associées aux fimbriae de type IV, ainsi qu'aux systèmes de sécrétion de type II et III. Les trois structures possèdent des porines centrales servant à la sécrétion de sous-unités dans l'espace extra-cellulaire. Tiré de Donnenberg & al. 2000.

1.3.4 Formation de biofilms

La formation de biofilms par *A. pleuropneumoniae* est étudiée depuis peu malgré que de plus en plus d'études montrent une association souvent claire entre la formation d'un biofilm et la virulence bactérienne, surtout dans les cas d'infections persistantes (Costerton et al. 1999; Ghigo 2003). Les biofilms sont des structures macroscopiques formées par l'aggrégation de bactéries en communautés parfois complexes (Figure 8). En plus de permettre l'attachement à de multiples surfaces, biotiques ou abiotiques, ce mode de croissance représente une forme de résistance accrue face aux conditions environnementales. Enrobées dans une matrice d'exopolysaccharides, les bactéries sont à l'abri, par exemple, des antibiotiques, des désinfectants ou encore des composantes du système immunitaire de l'hôte.

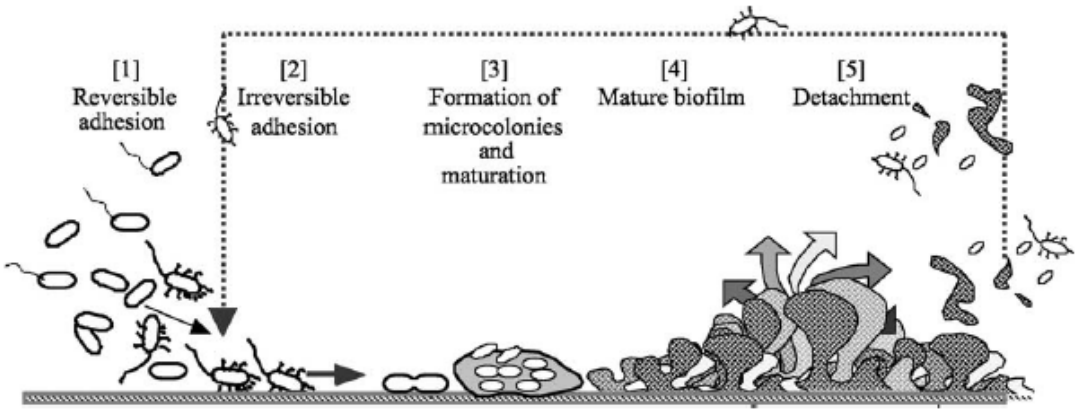

Fig.8: Étapes de la formation d'un biofilm sur une surface biotique ou abiotique. 1) Adhésion réversible des bactéries. 2) Adhésion irréversible. 3) Formation de micro-colonies et maturation du biofilm. 4) Biofilm mature. 5) Détachement et dissémination. Tiré de Ghigo & al. 2003.

Chez *A. pleuropneumoniae*, les premiers signes de production probable de biofilms sont venus avec la découverte des gènes *pgaABCD* au sein de son génome (Kaplan et al. 2004b). Des homologues de ces gènes sont aussi retrouvés chez *E. coli*, où ils codent pour l'exopolysaccharide PGA, un polymère de résidus de N-acétyl-D-glucosamine liés en β(1,6). La structure du PGA d'*A. pleuropneumoniae* est pratiquement identique à celle retrouvée chez *E. coli* (Izano et al. 2007). Contrairement aux souches de référence, qui ne forment souvent pas de biofilms (à

l'exception de la souche L20 de sérotype 5b et la souche 56513 de sérotype 11), la majorité des isolats frais d'*A. pleuropneumoniae* produisent des biofilms (Kaplan et al. 2005). Ce phénotype est ensuite perdu lorsque la bactérie est cultivée de façon répétée en bouillon nutritif. Comme c'est le cas pour *Staphylococcus epidermidis* (Kaplan et al. 2004a), le biofilm d'*A. pleuropneumoniae* peut être dissout suite à un traitement à la dispersine B (DspB), une enzyme produite par *Aggregatibacter actinomycetemcomitans* et *A. pleuropneumoniae* (Izano et al. 2007). La régulation de la formation de biofilm chez *A. pleuropneumoniae* est affectée par ArcA, un régulateur impliqué dans la réponse aux conditions anaérobies (Buettner et al. 2008b), LuxS, une protéine impliquée dans les phénomènes de « quorum-sensing » (Li et al. 2008), H-NS, une protéine de type histone (Li et al. 2008), ainsi que par AasP, une sérine protéase auto-transporteure (Tegetmeyer et al. 2009).

1.3.5 Mécanismes d'acquisition du fer

1.3.5.1 Généralités

Le fer est un composé essentiel à plusieurs voies métaboliques biologiques puisqu'il entre dans la composition de plusieurs molécules clés telles que les cytochromes, impliqués dans la génération d'ATP, la ribonucléotide nucléase, impliquée dans la synthèse de l'ADN, et plusieurs autres enzymes liées au métabolisme (Koster 2001). Sa présence au sein d'enzymes impliquées dans des réactions d'oxydoréduction est essentielle aux réactions entourant la respiration membranaire (Braun 2001). L'accomplissement de toutes ces fonctions nécessitent la présence d'environ 10^5 à 10^6 ions ferriques (Fe^{3+}) par cellule bactérienne (Wandersman et al. 2000; Braun 2001). Cependant, malgré que le fer soit le quatrième métal en importance sur Terre, il n'en demeure pas moins que, en conditions physiologiques, le fer est un élément limitant pour la croissance bactérienne. En effet, en condition aérobie, à un pH avoisinant 7, le fer est présent sous forme de Fe^{2+} et forme de gros complexes insolubles. La concentration en fer libre chute à environ 10^{-9} M (Ratledge et al. 2000), alors que les bactéries sont généralement plus confortables dans un

environnement présentant une concentration en fer libre d'environ 10^{-7} M (Braun 2001).

Dans les systèmes biologiques, la situation est encore plus dramatique : la très grande majorité du fer total est intracellulaire (Clarke et al. 2001) et la faible quantité de fer présente dans les liquides extra-cellulaires est complexée par des molécules spécialisées. Le fer intra-cellulaire est séquestré par des composés hémiques, les centres fer-soufre de plusieurs protéines ainsi que par la ferritine, une molécule d'entreposage (Clarke et al. 2001). Dans le milieu extra-cellulaire, le fer ferrique libre présent dans le sérum et les liquides sécrétoires est rapidement lié respectivement par la transferrine et la lactoferrine (Braun 2001). On le retrouve aussi parfois lié au citrate. Alors que 78% du fer total est contenu dans l'hémoglobine des érythrocytes et l'hème de la myoglobine (Braun 2001), les bactéries ne peuvent même pas compter sur la lyse accidentelle de ces molécules pour combler leurs besoins en fer : d'autres molécules spécialisées présentent dans le sang se chargent respectivement de transporter l'hémoglobine (haptoglobine) et l'hème (albumine et hémopexine) rapidement vers le foie (Wandersman et al. 2000) où ils seront éliminés. La combinaison de tout ces éléments a pour effet de faire chuter la concentration de fer libre dans les liquides extra-cellulaires à environ 10^{-24} M (Braun 2001).

Face à ces conditions, les bactéries ont dû développer divers procédés afin de combler leurs besoins en fer. La plupart des bactéries pathogènes possèdent la capacité d'exploiter le fer emprisonné par les différentes protéines de l'hôte chargées du transport du fer. Ainsi, certaines peuvent exploiter le fer lié à la transferrine et à la lactoferrine, ainsi que le fer provenant de l'hème de l'hémoglobine ou des complexes hémoglobine-haptoglobine, et plusieurs bactéries peuvent s'alimenter en fer via l'utilisation de composés chélateurs de fer appelés sidérophores (Andrews et al. 2003). Ces deux types de stratégies sont employés par *A. pleuropneumoniae* afin de combler ses besoins en fer (Bossé et al. 2002) (Figure 9), et seront expliqués plus en détail dans les sections qui viennent.

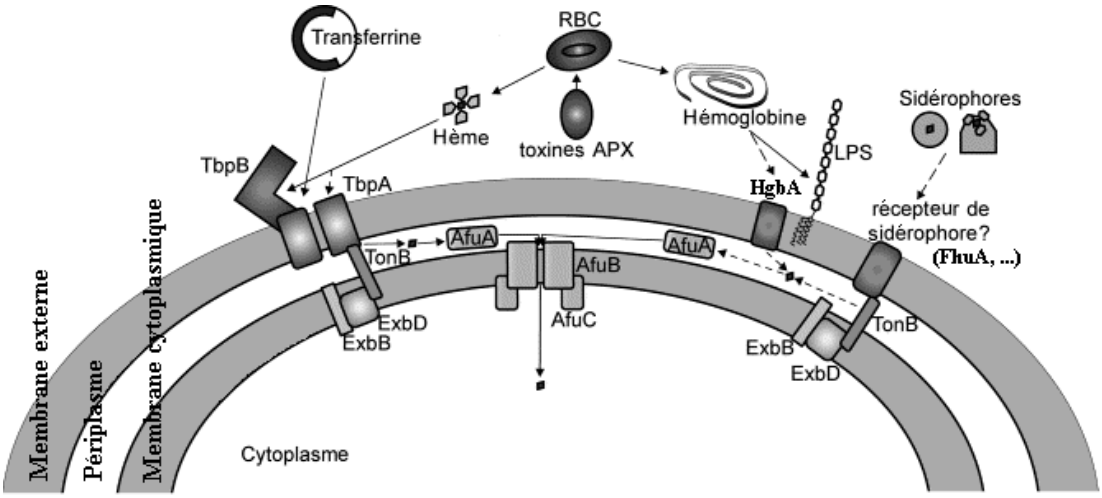

Fig. 9: Représentation schématique des différents mécanismes d'acquisition du fer retrouvés chez *Actinobacillus pleuropneumoniae*. RBC : « Red Blood Cell », globules rouges. Adapté de Bossé & al. 2002

1.3.5.2 Utilisation de la transferrine porcine

La transferrine est une molécule glycoprotéique monomérique de 80 kDa retrouvée dans le sérum (Cornelissen 2003). Sa fonction principale est de transporter le fer aux différentes cellules de l'hôte et, par le fait même, elle contribue à réduire la concentration en fer libre dans le sérum (Baker et al. 2002). Présente à une concentration d'environ 25 μM (Brock 1994), seulement 30% des molécules sont saturées en fer (Weinberg 1978), ce qui signifie qu'elle est majoritairement présente sous la forme d'apotransferrine. Sa structure bilobée lui permet de lier deux ions ferriques, soit un par lobe, qui sont stabilisés par la présence d'un anion carbonate. Chez les mammifères, l'acquisition de la transferrine par les cellules est effectuée grâce à un récepteur bipartite, composé entre autre d'une lipoprotéine transmembranaire (Aisen et al. 2001). Après liaison à la transferrine, cette lipoprotéine est internalisée et le fer est libéré dans les vésicules endocytiques, possiblement grâce à l'acidification du milieu. La transferrine, alors libérée de ses ions ferriques, est alors retournée vers la surface pour être sécrétée dans le milieu extra-cellulaire et accomplir de nouveaux cycles de capture d'ions ferriques.

Différentes bactéries pathogènes membres des familles *Neisseriaceae*, *Pasteurellaceae* (dont *A. pleuropneumoniae*) et *Moraxellaceae* sont capables d'utiliser la transferrine comme source de fer (Cornelissen 2003), et le système d'acquisition développé par ces bactéries partage plusieurs similarités avec le système utilisé par les mammifères, à commencer par la nature du récepteur impliqué. Le transport de la transferrine chez les bactéries implique deux protéines de surface : TbpA, une protéine transmembranaire, et TbpB, une lipoprotéine. Ce système est également présent chez *A. pleuropneumoniae* (Jacques 2004).

Des études menées avec *N. meningitidis* et *N. gonorrhoeae*, deux autres espèces bactériennes appartenant à la famille des Pasteureallaceae, ont permis d'obtenir plus d'information quant à la structure de TbpA et TbpB. TbpA est une protéine de 100 kDa qui partage beaucoup de similarités de séquence avec les protéines FepA et FhuA (Legrain et al. 1993). La structure proposée pour TbpA est celle d'un baril-β antiparallèle avec 22 domaines transmembranaires et dont le canal central serait bloqué par un domaine globulaire (Boulton et al. 2000), ce qui correspond bien au modèle établit jusqu'à présent pour les trois récepteurs de sidérophores (FepA, FecA et FhuA) dont la structure cristalline a été établie (Figure 12). Comme dans le cas de ces récepteurs, l'énergie nécessaire au transport catalysé par TpbA dépendrait du complexe TonB. TbpB, pour sa part, est une lipoprotéine retrouvée sous deux isotypes différents, un de 65 kDa (isotype I) et un de 90 kDa (isotype II), ancrée à la membrane externe via son extrémité N-terminale lipidique (Gray-Owen et al. 1996). Il a été démontré que TpbB interagit en surface avec TbpA (Boulton et al. 1998).

Chez la plupart des bactéries où les gènes codant pour ces protéines ont été retrouvés, ils sont génétiquement liés et le gène *tpbB* précède généralement le gène *tbpA* (Cornelissen 2003). Il y a évidemment quelques exceptions à ce principe, parmi lesquelles les bactéries *N. gonorrhoeae* et *N. meningitidis*, chez qui les deux gènes sont séparés par une région de 86 pb formant possiblement, après la transcription, une structure secondaire (Cornelissen et al. 1992). Chez *Moraxella catarrhalis*, c'est

un ORF de fonction inconnu qui sépare les deux gènes qui sont, par ailleurs, retrouvés dans un ordre inversé (Myers et al. 1998).

Bien que le mécanisme général d'acquisition du fer à partir de la transferrine soit connu, la suite précise des évènements se produisants au niveau de la surface de la bactérie est toujours inconnue. Et même si la structure du récepteur TbpA partage beaucoup de similarités avec celle des récepteurs de ferri-sidérophores, il existe une différence majeure en ce qui concerne l'utilisation du fer lié à la transferrine par rapport à l'utilisation du fer lié aux sidérophores : dans le cas de la transferrine, seulement l'ion ferrique traverse le récepteur membranaire, ce qui implique que le récepteur doit posséder un mécanisme pour extraire l'ion ferrique de la transferrine (Simonson et al. 1982). Étant donnée cette différence, il est donc possible que les interactions au niveau moléculaire donnant lieu à l'internalisation de l'ion ferrique soient différentes de celles menant à l'internalisation d'un complexe ferri-sidérophore. Le processus général par lequel les bactéries utilisent la transferrine peut donc être divisé en plusieurs étapes (Perkins-Balding et al. 2004) :

1. Liaison à haute affinité de la transferrine
2. Détachement de l'ion ferrique de la transferrine
3. Transport de l'ion ferrique à l'intérieur du cytoplasme

Dans un premier temps, la liaison à la transferrine serait effectuée par la protéine TbpA, qui lie la transferrine avec une affinité similaire à celle du récepteur de la transferrine présent chez les mammifères (Cornelissen et al. 1996). Fait surprenant, TbpA possède une plus forte affinité pour l'apotransferrine que pour l'holotransferrine (Krell et al. 2003). *In vivo*, ce phénomène n'a rien d'avantageux puisque seulement 30% de la transferrine en circulation est saturée en fer et donc la forme prédominante est l'apotransferrine. C'est à ce niveau que l'action de TbpB se fait sentir. En effet, bien que des mutants de *N. meningitidis* pour *tbpB* conservent leur capacité de croître avec la transferrine comme seule source de fer, leur croissance est plus lente que celle d'une souche sauvage possédant les deux gènes (Renauld-Mongenie et al. 2004). De plus, il a été démontré que, en présence de

TbpB, la spécificité de TbpA est altérée et la protéine lie alors préférentiellement l'holotransferrine (Krell et al. 2003). TbpB permettrait donc au récepteur TpbA de faire la distinction entre l'holotransferrine et l'apotransferrine, augmentant du même coup l'efficacité de l'acquisition du fer à partir de ce composé. En fait, TbpB possède elle-aussi la capacité de lier la transferrine, comme en font foi la présence de deux domaines de liaison à la transferrine dans sa séquence protéique (Renauld-Mongenie et al. 1997; Retzer et al. 1999) ainsi que la purification de ces deux protéines lors d'expériences d'isolation par affinité avec de la transferrine biotinylée (Schryvers et al. 1988). Il est à noter que, pour les souches de l'isotype I, la présence de TbpB est absolument nécessaire au transport de la transferrine. Cependant, la forte majorité (82%) des souches de *N. meningitidis* isolées appartiennent à l'isotype II (Rokbi et al. 2000).

Différents mécanismes ont été proposés pour l'extraction du fer de la transferrine. Schryvers et Stojiljkovic, en 1999 (Schryvers et al. 1999), ont suggéré que la liaison de la transferrine par le récepteur bactérien entraînerait la séparation des deux lobes de la transferrine, diminuant ainsi son affinité pour l'ion ferrique. Une étude par Gómez et al. en 1998 (Gomez et al. 1998) a aussi démontré que les protéines réceptrices purifiées TbpA et TbpB pouvaient, en solution, promouvoir l'échange d'ions ferriques entre la transferrine et la protéine périplasmique FbpA (voir système *afu* plus loin). Par contre, la nature réelle du mécanisme entourant la libération de l'ion ferrique n'a toujours pas été élucidée et il a été démontré que TbpB seule en solution ne pouvait promouvoir le transfert de l'ion ferrique vers FbpA (Nemish et al. 2003).

Ce système d'acquisition requiert, chez *A. pleuropneumoniae*, la présence de deux protéines : une lipoprotéine de 60 kDa nommée TbpB (ou TfbA), ainsi qu'une protéine de la membrane externe de 100 kDa nommée TbpA (ou TfbB). Il a été démontré que la lipoprotéine TbpB d'*A. pleuropneumoniae* peut aussi lier l'hémine (Gerlach et al. 1992). De plus, TbpB présente de grandes divergences entre les

différents sérotypes d'*A. pleuropneumoniae* et permet d'induire une réponse immunitaire protectrice spécifique aux différents sérotypes (Wilke et al. 1997).

1.3.5.3 Utilisation de l'hémoglobine porcine

L'hème, ou Fe-protoporphyrine IX, est un groupement prosthétique retrouvé au cœur de plusieurs enzymes (Wandersman et al. 2004), et son importance pour les bactéries est reflétée par la mobilisation de 10% des réserves de fer et d'importantes ressources énergétiques pour la synthèse de cet élément (Matzanke et al. 1991). L'hème est composé d'un noyau tétrapyrrole, ou porphyrine, entourant un atome de fer (Figure 10), et peut être utilisé en laboratoire comme source de fer ou de porphyrine par un grand nombre de bactéries (Wandersman et al. 2004). Cependant, chez l'hôte, l'hème est rarement retrouvé libre à cause de sa forte toxicité. Cette molécule hydrophobe a en effet tendance à déstabiliser les membranes et à promouvoir des réactions redox non-enzymatiques. Dans les liquides physiologiques, l'hème est donc lié par diverses protéines, protégeant ainsi l'organisme des effets toxiques de ce composé.

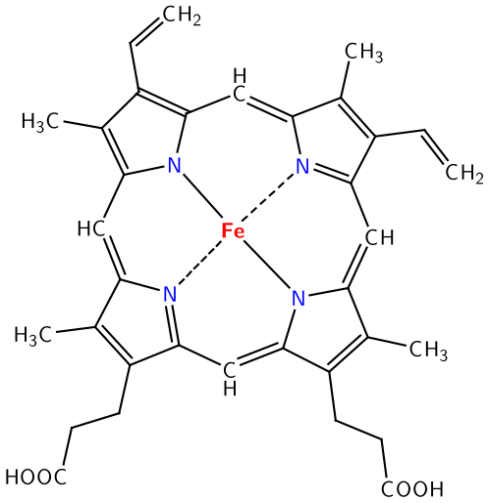

Fig. 10 : Structure de l'hème. Adapté de (Caughey et al. 1975)

Les récepteurs de surface chargés de la liaison aux composés hémiques peuvent être classés dans deux catégories : les récepteurs pouvant lier plusieurs composés hémiques et les récepteurs spécifiques pour certains composés (Wandersman et al. 2000). Ces derniers récepteurs sont surtout retrouvés chez les bactéries pathogènes qui ont évolué en association étroite avec leur hôte (Ratledge et al. 2000). De façon générale, il est fort probable que la structure de ces récepteurs soit fortement similaire à celle des récepteurs de sidérophore (Wandersman et al. 2000). Cependant, il existe au moins une exception à cette règle : le système Hpu de *N. meningitidis*, spécifique aux complexes haptoglobine-

hémoglobine, comprend un récepteur bipartite. HpuB est un récepteur TonB dépendant alors que HpuA est une lipoprotéine (Ratledge et al. 2000). La structure de ces récepteurs est donc plutôt semblable à celle des récepteurs pour la transferrine et la lactoferrine, mais l'interaction existant entre ces deux protéines n'a pas encore été caractérisée et n'est donc possiblement pas semblable à celle existant entre TbpA et TbpB.

Comme dans le cas de la transferrine et de la lactoferrine, les récepteurs de surface impliqués dans l'acquisition du fer à partir de composés hémiques doivent d'abord détacher en surface l'hème de la protéine complexe à laquelle il est attaché. La majorité de ces récepteurs sont aussi TonB dépendants, et le transport de l'hème au cytoplasme, où il peut être directement incorporé aux enzymes ou dégradé pour libérer son atome de fer, nécessite l'intervention d'une protéine de transport périplasmique à cause de l'insolubilité de l'hème et sa tendance à s'aggréger.

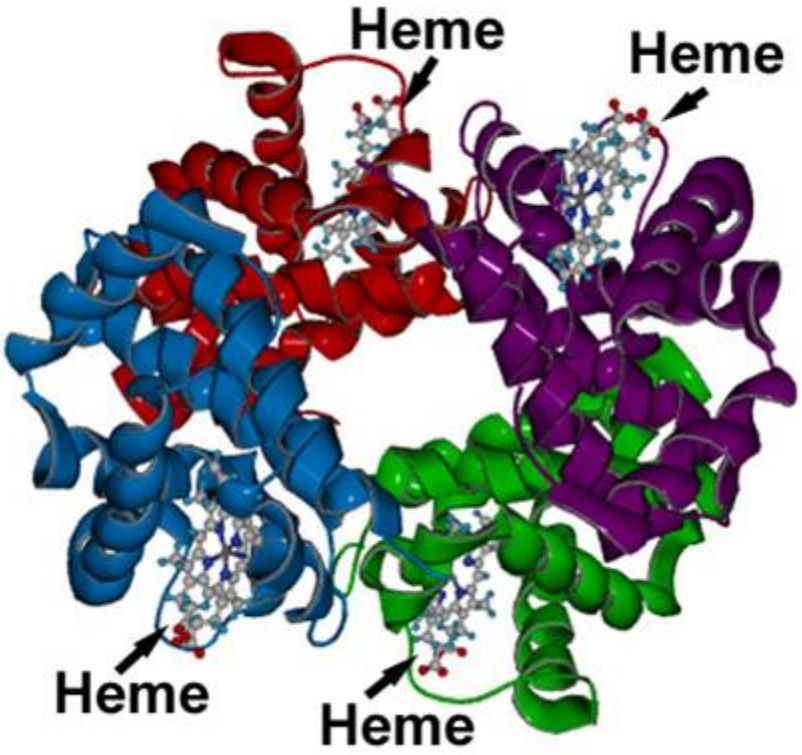

Fig. 11: Structure de l'hémoglobine. (Fermi et al. 1984)

Chez A. pleuropneumoniae, bien qu'il ait été démontré que les LPS d'isolats de tous les sérotypes peuvent lier l'hémoglobine porcine (Bélanger et al. 1995), l'acquisition du fer à partir de l'hémoglobine semble aussi être médiée par des récepteurs protéiques. Des expériences réalisées à l'aide de colonnes d'affinité avec billes d'agarose liées à de l'hémoglobine ont permis d'isoler, à partir de préparations de protéines de la membrane externe obtenues de cultures d'*A. pleuropneumoniae* en milieu déficient en fer, deux protéines possédant la capacité de lier l'hémoglobine porcine (Figure 11) de même que l'hémine (Archambault et al. 2003) : une protéine de 75 kDa possédant des homologies de séquences avec des protéines de la membrane externe TonB dépendantes conservées chez plusieurs bactéries Gram négatives, ainsi qu'une protéine de 105 kDa présentant une forte homologie avec la protéine liant l'hémoglobine HgbA, présente chez d'autres *Pasteurellaceae* (Jacques 2004). La présence de cette protéine est confirmée pour les sérotypes 1 à 12 d'*A. pleuropneumoniae*, et la génération d'un mutant ne possédant pas le gène *hgbA* a mené à l'abolition de l'acquisition du fer à partir de l'hémoglobine (Shakarji et al. 2006). La délétion de ce gène chez une souche de sérotype 1 a de plus entraîné une réduction dans la virulence par rapport à la souche-mère.

1.3.5.4 Les sidérophores et le système Fhu

Les sidérophores sont des agents chélateurs de faible poids moléculaires possédant une forte affinité pour le fer sous forme ferrique, et le système le mieux étudié est sans l'ombre d'un doute celui de l'acquisition du ferrichrome par les protéines Fhu d'*Escherichia coli* (Andrews et al. 2003). Ils sont sécrétés en grande quantité par plusieurs espèces bactériennes et fongiques ainsi que par certaines plantes, en périodes de carence en fer et sont considérés comme étant la principale voie de solubilisation et d'acquisition du fer par les micro-organismes (Drechsel et al. 1998). Ils sont généralement classifiés en trois grands groupes en fonction du groupement chimique effectuant la liaison avec l'atome ferrique (Andrews et al. 2003; Wandersman et al. 2004):

1. les catéchols (ex. : entérobactine)

2. les hydroxamates (ex. : aérobactine, ferrichrome)
3. les α-hydroxycarboxylates (ex. : pyocheline de *P. aeruginosa*)

Alors que les fungi synthétisent majoritairement des sidérophores de type hydroxamates, les trois grands groupes sont retrouvés au niveau des bactéries (Drechsel et al. 1998). Environ 500 sidérophores ont été caractérisés à ce jour avec des constantes de dissociation pour le fer variant de 10^{22} à 10^{50} (Ratledge et al. 2000), soit des valeurs similaires ou supérieures aux constantes de dissociation de la transferrine et de la lactoferrine (~ 10^{20}). Cette forte affinité des sidérophores pour le fer ferrique leur permettrait de soutirer le fer transporté par la transferrine et la lactoferrine, ainsi que de solubiliser les sels ferriques insolubles et les hydroxydes de fer ($Fe(OH)_3$), bien que ce soit insuffisant pour capturer le fer des centres hémiques (Ratledge et al. 2000). Une fois chargés en fer, les sidérophores sont capturés par les cellules bactériennes via des récepteurs membranaires à haute affinité et sont internalisés. Le fer est ensuite soutiré du sidérophore par des processus pouvant entraîner ou non la dégradation du sidérophore. Dans le cas où sa structure ne serait pas affectée, le sidérophore pourra être recyclé et effectuer d'autres cycles de capture d'ions ferriques. Il est à noter que, bien que les sidérophores soient considérés comme d'importants facteurs de virulence chez les bactéries pathogènes, peu d'expériences ont effectivement démontré que des mutants ne produisant pas de sidérophores étaient avirulents (Ratledge et al. 2000).

Les récepteurs membranaires responsables de la capture des sidérophores chargés possèdent une forte affinité pour leurs sidérophores respectifs et les trois récepteurs dont la structure crystalline a été établie jusqu'à présent, soit FepA, le récepteur de l'entérobactine, FecA, le récepteur du citrate ferrique, et FhuA, le récepteur du ferrichrome, possèdent des structures semblables (Andrews et al. 2003) (Figure 12). Dans les trois cas, il s'agit de protéines de type baril β de 22 feuillets formant un tube traversant la membrane externe. Le canal central des récepteurs est bloqué par un domaine globulaire de 60 acides aminés en N-terminal de la protéine. La liaison du sidérophore à son récepteur engendre un changement conformationnel dans la structure du récepteur menant, éventuellement, au déplacement de l'extension N-

terminale bloquant le canal, ce qui permet le passage du complexe ferri-sidérophore au périplasme.

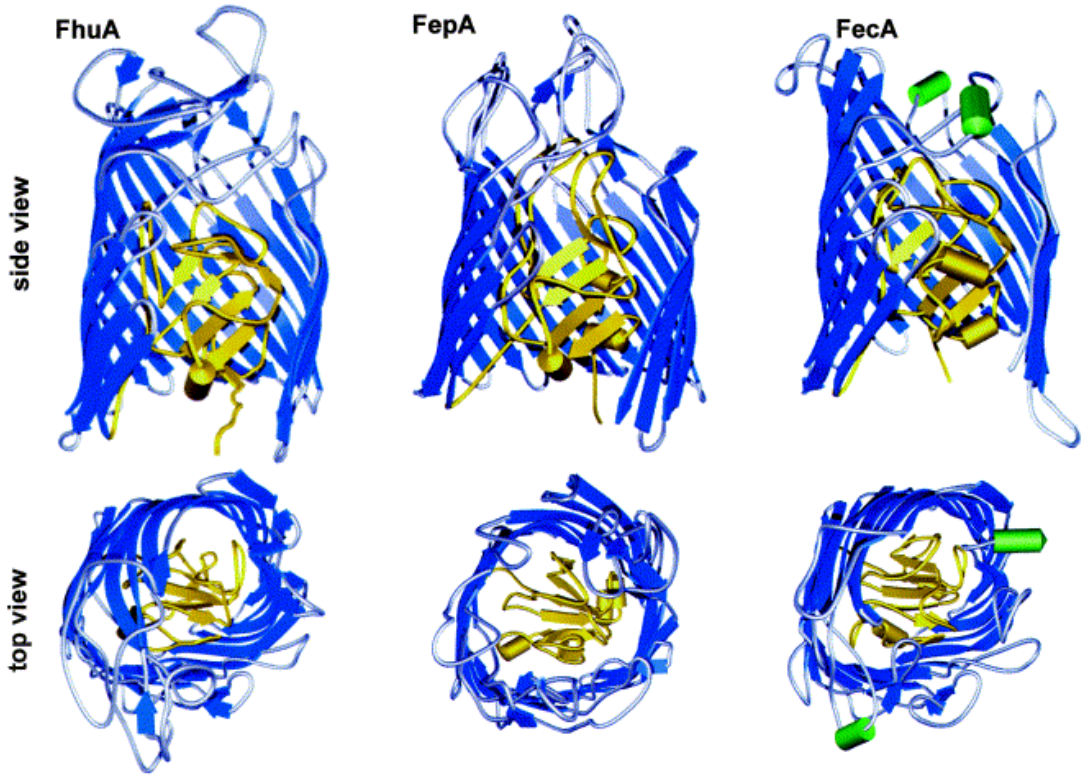

Fig.12 : Structure crystalline des récepteurs de sidérophores FhuA, FepA et FecA d'*Escherichia coli*. (Braun et al. 2002). En jaune, le domaine globulaire bloquant le canal, en bleu les 22 feuillets ß transmembranaires des récepteurs.

Fait intéressant, l'affinité de ces récepteurs pour les deux formes du sidérophore, c'est-à-dire la forme chargée et la forme non-chargée aussi appelée aposidérophore, est sensiblement la même, ce qui soulève des questions sur le mécanisme responsable de la discrimination entre ces deux formes pour l'internalisation (Wandersman et al. 2004). L'existence d'un tel mécanisme a été étudiée dans trois systèmes différents : la pyoverdine de *Pseudomonas aeruginosa*, le système Fec de *E. coli* et l'amonabactine de *Aeromonas hydrophila*.

I. *In vivo*, le récepteur FpvA de *P. aeruginosa*, spécifique pour la pyoverdine, est lié à l'apopyoverdine (Schalk et al. 2001). L'apopyoverdine serait déplacée lors de l'activation du récepteur par la protéine TonB1, et il y aurait alors compétition entre les deux formes du sidérophore pour la liaison au récepteur

(Clement et al. 2004). Dans cette compétition, le sidérophore chargé part avec une longueur d'avance puisqu'il lie FpvA beaucoup plus rapidement que l'aposidérophore. Les avantages procurés par un tel mécanisme sont toujours obscures, mais une étude récente (Yue et al. 2003) démontre que le récepteur du citrate ferrique (FecA) chez *E. coli* lie lui aussi son aposidérophore. Ces deux récepteurs partagent un autre point en commun : dans les deux cas, la liaison de l'aposidérophore sur son récepteur influence positivement l'expression des gènes responsables de l'acquisition des sidérophores (Gensberg et al. 1992; Braun 1997). Puisque chez *P. aeruginosa* la liaison de la pyoverdine ou de l'apopyoverdine à FpvA dépend de la concentration relative de chacune, la liaison prédominante de l'aposidérophore à FpvA pourrait être un moyen pour la bactérie de détecter la présence limitée de fer dans le milieu de croissance (Schalk et al. 2002).

II. *Aeromonas hydrophila* synthétise quatre sidérophores de type bis-catécholate qui sont regroupés sous l'appellation d'amonabactine (Telford et al. 1998). Fait surprenant, lorsque la bactérie est mise en présence de desferrioxamine et de TREN-1,2-HOPO, deux sidérophores, le transport de fer par les quatre amonabactines est totalement inhibé, ce qui implique que ces quatres sidérophores, en plus de plusieurs sidérophores exogènes tels que l'entérobactine et le ferrichrome, utilisent le même mécanisme de transport (Stintzi et al. 2000). Des études subséquentes suggèrent que *A. hydrophila* ne possède qu'un seul récepteur de surface, lié en permanence avec une forte affinité par un aposidérophore. Il se produirait, en surface de la cellule, un échange d'ions ferriques entre cet aposidérophore et différents types de sidérophores présents dans le milieu. Il est clair qu'un tel mécanisme, qui permet l'utilisation de plusieurs sources de fer à partir d'un seul et unique récepteur, représente un fort avantage puisque la bactérie peut alors acquérir du fer rapidement dès qu'elle le rencontre, sans avoir besoin d'induire au préalable la production d'un récepteur spécifique à la source de fer rencontrée.

De façon générale, trois mécanismes distincts ont été proposés pour la capture et l'internalisation des sidérophores (Figure 13) : la capture directe du sidérophore chargé par un récepteur spécifique pour cette forme du sidérophore, l'échange d'ions ferriques entre deux sidérophores (« siderophore shuttle ») et le remplacement de l'aposidérophore par la forme chargée (Schalk et al. 2002).

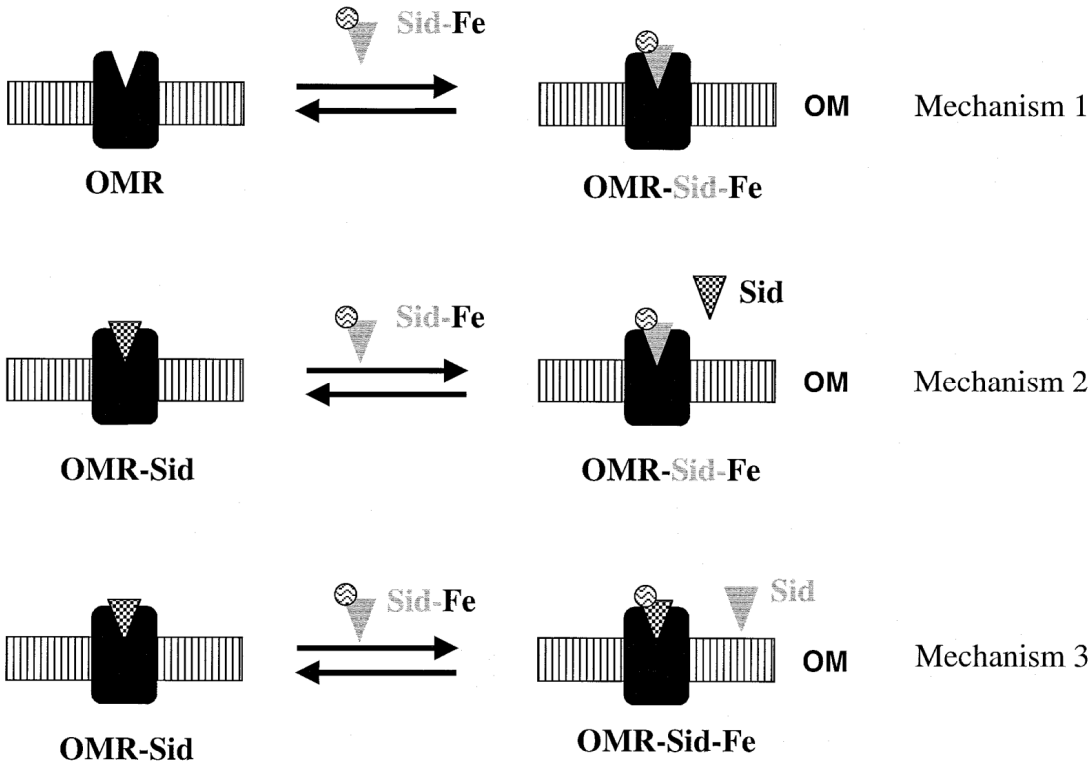

Fig. 13: Trois modèles proposés pour la capture et l'internalisation des sidérophores :
1. Capture du sidérophore chargé par un récepteur spécifique (ex. : système Fhu)
2. Remplacement de l'aposidéphore sur le récepteur par la forme chargée (ex. : PvdA)
3. Échange des ions ferriques entre deux sidérophores (siderophore shuttle) (*A. hydrophila*)

OMR : Outter Membrane Receptor, Sid : aposidérophore, Sid-Fe : sidérophore chargé
Adapté de (Schalk et al. 2002)

Le ferrichrome est un sidérophore de type hydroxamate qui est synthétisé et sécrété par plusieurs espèces fongiques. Bien que ne possédant pas les gènes requis pour la synthèse de cette molécule, plusieurs espèces bactériennes possèdent des récepteurs spécifiques au ferrichrome. En 1996, Diarra et al. ont démontré que la presque totalité des souches d'*A. pleuropneumoniae* possédait la capacité d'utiliser le fer lié au ferrichrome (Diarra et al. 1996). Par la suite, Mikael et al. ont réussi à cloner et

séquencer les gènes de l'opéron Fhu (Ferric Hydroxamate Uptake), impliqué dans l'acquisition du fer à partir du ferrichrome (Mikael et al. 2002).

L'opéron Fhu *d'A. pleuropneumoniae* est composé, dans l'ordre, des gènes *fhuC*, *fhuD*, *fhuB* et *fhuA*, encodant respectivement :

- une protéine de la membrane cytoplasmique de 28,5 kDa contenant un motif de liaison à l'ATP et au GTP appartenant à la famille des transporteurs ABC
- une protéine périplasmique de 35,6 kDa responsable du transport du ferrichrome ferrique de la membrane externe à la membrane cytoplasmique
- une autre protéine de la membrane cytoplasmique de 69,4 kDa, appartenant elle-aussi à la famille des transporteurs ABC
- un récepteur de la membrane externe de 77 kDa spécifique pour le ferrichrome.

La structure tri-dimensionnelle du récepteur FhuA, avec ses 22 brins β transmembranaires, ses 11 boucles extra-cellulaires et ses 10 boucles périplasmiques, est similaire à la structure du récepteur FhuA d'*E. coli*. Des expériences de fusion de FhuA avec une étiquette hexa-histidine (« His tag ») ont permis de démontrer que la protéine FhuA était reconnue par le sérum d'un porc infecté par une souche sauvage de sérotype 1 d'*A. pleuropneumoniae*, confirmant ainsi que la protéine FhuA est bel et bien exprimée *in vivo* lors de l'infection (Mikael et al. 2002). Cependant, d'autres expériences ont démontré par la suite que le niveau d'expression de FhuA n'était pas régulé par le niveau de fer présent dans le milieu de culture contrairement à ce qui est observé chez *E. coli* (Mikael et al. 2003), et que la délétion du gène *fhuA* n'a pas d'effet significatif sur la virulence d'*A. pleuropneumoniae*.

Malgré leur grande utilité en période de carence en fer, les récepteurs de sidérophore ne sont pas exprimés constitutivement par les bactéries qui les possèdent. Ces récepteurs, en plus de participer à la capture des sidérophores chargés, sont aussi la porte d'entrée pour de nombreux antibiotiques et phages (Wandersman et al. 2004).

Le récepteur FhuA, par exemple, en plus de servir de récepteur pour le ferrichrome et la ferricrocine, deux sidérophores, il permet l'internalisation de l'albomycine, un sidérophore conjugué à un antibiotique, ainsi que le transport de la rifamycine CGP4832, un antibiotique sans fer qui ne présente aucune ressemblance structurelle avec le ferrichrome (Wandersman et al. 2004), et de la colicine M (Endriss et al. 2004), une exotoxine produite par certaines souches d'*E. coli*. Il est aussi la cible des phages T5, T1, phi80 et UC-1 (Killmann et al. 2002).

D'autres expériences ont été réalisées afin de déterminer si *A. pleuropneumoniae* est capable de synthétiser ses propres sidérophores. Lorsque Diarra et al. ont testé l'utilisation de différentes sources de fer par *A. pleuropneumoniae*, ils ont constaté que deux des souches testées, soit des souches des sérotype 1 et 5, sécrétaient dans le milieu de culture un agent chélateur de fer (Diarra et al. 1996). Ce sidérophore endogène potentiel *d'A. pleuropneumoniae* ne posséde cependant pas une structure correspondant aux structures bien établies pour les trois grands groupes de sidérophores, empêchant ainsi sa détection par les tests d'Arnow et de Csaky, spécifiques respectivement pour les sidérophores de type catéchol et hydroxamate.

1.3.5.5 Les deux systèmes TonB d'A. pleuropneumoniae

Il a été établi que l'énergie nécessaire au transport des ions ferriques vers le périplasme à partir des différents récepteurs de surface est fournie par le gradient électrochimique de la membrane cytoplasmique, grâce au complexe transducteur d'énergie composé des protéines TonB, ExbB et ExbD (Larsen et al. 1994; Higgs et al. 1998), trois protéines de la membrane cytoplasmique. Alors que ExbB et ExbD sont deux protéines intégrale*s* de la membrane cytoplasmique, TonB est majoritairement périplasmique, n'étant liée à la membrane cytoplasmique que par son extrémité N-terminale hydrophobe. Ceci lui permet d'interagir avec les domaines périplasmiques des protéines de la membrane externe (Andrews et al. 2003). TonB, ExbB et ExbD interagissent ensemble avec une stochiométrie de 1/7/2 (Higgs et al. 2002). Des preuves récentes semblent démontrer que la partie C-terminale

périplasmique de TonB forme des dimères (Sauter et al. 2003). Le rôle de cette dimérisation dans le processus de transduction d'énergie est toujours indéterminé.

Lors de la liaison du substrat au récepteur de surface, il se produit des changements conformationnels, entre autre au niveau des boucles extracellulaires de la protéine, qui se referment autour du substrat, et des régions périplasmiques (Wandersman et al. 2004). En N-terminal des récepteurs de sidérophores, on retrouve une région de 5 acides aminés appelée « boîte TonB » qui, comme son nom l'indique, interagit avec l'extension périplasmique de TonB (Figure 14). Les mouvements périplasmiques induits par la liaison du ferri-sidérophore à son récepteur amèneraient le fonctionnement du complexe TonB (Wandersman et al. 2004) et la production, par ExbB et ExbD, d'une forme énergisée de TonB (Andrews et al. 2003). La transduction de cette énergie au complexe récepteur – ferri-sidérophore permettrait l'internalisation de ce dernier.

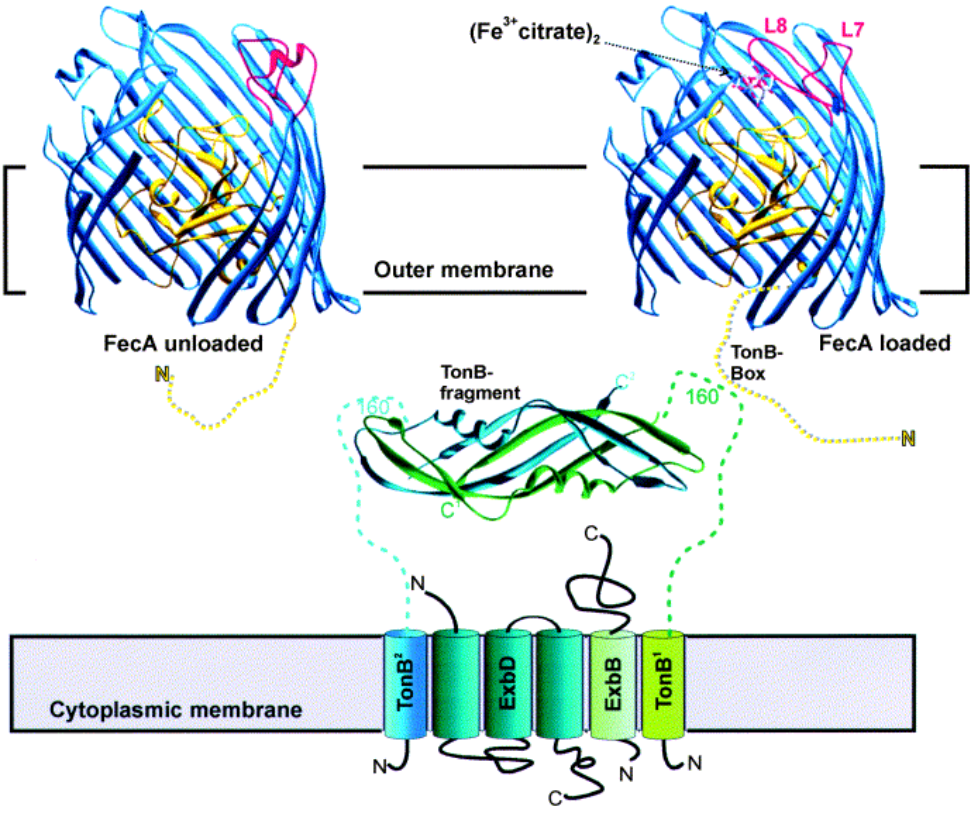

Fig. 14: Interaction de TonB avec les récepteurs de surface chargés (Braun et al. 2002).

La boîte TonB présente sur les récepteurs de sidérophores est essentielle à l'utilisation de ces substrats, des expériences de mutagénèse sur la protéine FhuA d' *E. coli* ayant démontré que la perte de cette boîte abolissait les fonctions des récepteurs de surface (Endriss et al. 2003). Par contre, encore chez *E. coli*, la délétion du domaine globulaire et de la boîte TonB par la même occasion transforme FhuA en porine pour les grosses molécules hydrophiles de moins de 1500 Da (Braun 2003). Plus surprenant, ce récepteur incomplet permet toujours le passage du ferrichrome et cette activité semble toujours dépendre d'interactions avec TonB, ce qui pourrait indiquer la présence de sites de liaison pour TonB sur le baril-β de FhuA.

Chez *A. pleuropneumoniae*, les gènes *tbpA* et *tbpB* sont liés et cotranscrits avec les gènes *exbB* et *exbD*, et on retrouve le gène *tonB* en amont des gènes *exbBD*. Ces gènes pourraient donc être retrouvés sur le même transcrit (Tonpitak et al. 2000), et les gènes *exbB* et *exbD* de ce système TonB-ExbB-ExbD semblent essentiels à l'utilisation du fer lié à la transferrine. Ces composantes ont été renommées TonB1-ExbB1-ExbD1 suite à la découverte lors d'une étude STM (« Signature Tagged Mutagenesis ») réalisée chez le porc d'un deuxième système TonB (Sheehan et al. 2003). L'analyse des séquences d'ADN a permis de découvrir une deuxième série de gènes *exbBD* (*exbB2*, *exbD2*) en amont de ce deuxième gène *tonB* (*tonB2*). L'ordre des gènes présents dans ce deuxième système TonB (*exbB2-exbD2-tonB2*) ressemble à celui retrouvé chez d'autres *Pasteurellaceae*, et présente de bonnes similarités de séquences avec les gènes des micro-organismes de cette famille. Le système TonB1 qui présente quant à lui une organisation génétique similaire à celle retrouvée chez *Pseudomonas* et les similarité de séquences avec les autres Pasteurellaceae sont moins importantes, ce qui porte à croire que TonB2 est le système endogène d'*A. pleuropneumoniae* (Beddek et al. 2004). Tout comme le système TonB1, TonB2 est retrouvé chez les sérotypes 1 à 15 et sa transcription est accrue en conditions limitantes en fer. Étrangement, le gène *tonB2* semble être requis pour l'utilisation du fer lié à la transferrine. Bien qu'un mutant *tonB1* perde aussi sa capacité d'utilisé la transferrine, il a été démontré que cette mutation a aussi un effet polaire sur la transcription de *exbB1* et *exbD1*, reconnus comme étant essentiels à l'utilisation du

fer lié à la transferrine. Finalement, alors qu'une mutation incapacitante dans le gène *tonB2* affecte grandement la virulence *in vivo* d'*A. pleuropneumoniae*, le même type de mutation dans le gène *tonB1* n'atténue en rien la virulence pour une même dose d'infection (Beddek et al. 2004).

1.3.5.6 Système Afu

Une fois dans le périplasme, les ions ferriques obtenus via l'utilisation de la transferrine ou de l'hémoglobine de l'hôte ou encore suite à la capture directe d'hémine, de noyaux hémiques ou encore d'ions ferriques libres, doivent traverser la membrane cytoplasmique. Chez *A. pleuropneumoniae*, le système Afu est dédié à cette tâche (Bossé et al. 2002). Cloné et séquencé par Chin et al. en 1996, l'opéron *afu* est situé directement en amont mais transcrit dans la direction opposé du locus *apxICABD* codant pour la toxine hémolytique ApxI (Chin et al. 1996). L'opéron *afu* est composé des gènes *afuA*, *afuB* et *afuC*, codant respectivement pour :

- une protéine périplasmique liant les ions ferriques
- une protéine intégrale de la membrane cytoplasmique avec deux motifs consensus des protéines de type perméases
- une protéine hydrophile retrouvée à la surface interne de la membrane cytoplasmique et possédant des motifs de liaison à l'ATP

Sans surprise, les gènes de l'opéron *afu* sont sous le contrôle du répresseur transcriptionnel Fur (Hsu et al. 2003). L'opéron *afu* d'*A. pleuropneumoniae* présente de forte similarités avec les opérons *sfuABC* de *Serratia marcescens*, *hitABC* d'*H. influenzae*, *fbpABC* de *N. gonorrhoeae* et *yfuABC* de *Yersinia enterocolitica*, tous impliqués dans le transport vers le cytoplasme des ions ferriques périplasmiques.

1.3.5.7 Régulation transcriptionnelle : Fur et ryhB

Bien que le fer soit un élément essentiel à la croissance bactérienne, il n'en demeure pas moins que c'est un élément extrêmement toxique en condition aérobie et sa

concentration à l'intérieur du cytoplasme doit donc être conservée à l'intérieur de certaines limites acceptables (Touati 2000). La toxicité du fer provient de la tendance qu'ont les espèces réactives de l'oxygène, des dérivatifs partiellement réduits de l'oxygène moléculaire produits naturellement par le métabolisme aérobie et parmi lesquelles on retrouve le peroxyde (H_2O_2) et l'ion superoxyde (O_2^-) (Fridovich 1995), à oxyder les ions ferriques (Andrews et al. 2003) (Figure 15). Les radicaux hydroxyles formés par cette réaction, appelée réaction de Fenton, sont extrêmement réactifs et toxiques et peuvent endommager pratiquement toutes les macromolécules biologiques (Touati 2000).

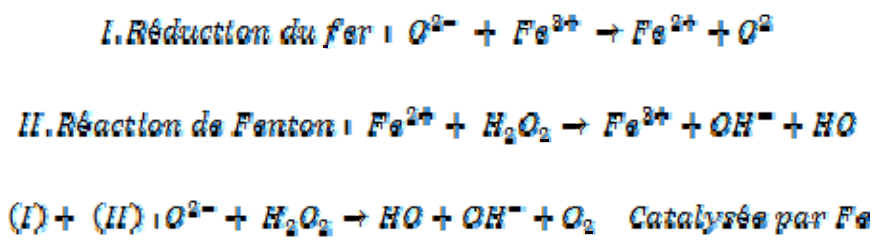

Fig. 15: Réactions intracellulaires en présence d'ions ferriques et de formes réactives de l'oxygène (Andrews et al. 2003). Dans la réaction de réduction du fer ainsi que dans la réaction de Fenton, les termes O_2^- et H_2O_2 présents dans la partie gauche de l'équation sont interchangeables.

Deux expériences de mutagénèse réalisées sur *E. coli* viennent appuyer l'existence de ces réactions. Dans un premier, une mutation inactivant le gène *fur* et dérégulant ainsi le métabolisme du fer augmente la sensibilité au stress oxydatif, possiblement à cause de l'augmentation de la concentration intra-cellulaire d'ions ferriques libres (Touati 2000). De plus, les effets de cette mutation peuvent être annulés par la création d'une mutation inactivante dans le gène *tonB* (empêchant ainsi l'acquisition de fer) ou par la surexpression de la ferritine, protéine responsable de l'entreposage intra-cellulaire du fer. Une deuxième expérience d'inactivation, cette fois-ci sur les gènes *sodA* et *sodB*, responsables de la synthèse de la superoxyde dismutase, enzyme responsable de l'élimination des ions O_2^-, cause l'accumulation de dommages au niveau de l'ADN (Imlay et al. 1991). Puisque l'ion superoxyde ne peut, par lui-même, affecter l'ADN, ceci pointe vers l'implication du fer via la réaction de Fenton.

Afin d'éviter de telles réactions, les bactéries possèdent certains mécanismes permettant d'entreposer les ions ferriques de façon sécuritaire. À l'intérieur d'une bactérie, il est possible de retrouver jusqu'à trois types de molécules d'entreposage différents : les ferritines, qui sont aussi retrouvées chez les eucaryotes, les bactérioferritines, qui contiennent des groupements hèmes et qui sont retrouvées uniquement chez les bactéries, ainsi que les protéines Dps, retrouvées chez les procaryotes (Andrews et al. 2003). Ces protéines sont composées de 24 (ferritines et bactérioferritines) ou 12 (Dps) sous-unités identiques formant une sphère au centre de laquelle peuvent être stockés jusqu'à 3000 ions ferriques dans le cas des ferritines et bactérioferritines. Néanmoins, la présence de mécanismes transcriptionnels efficaces permet de réagir plus rapidement aux variations du niveau intra-cellulaire d'ions ferriques.

La découverte par Hantke en 1981 d'un mutant d'*E. coli* exprimant constitutivement tous les gènes normalement inhibés par la présence de fer dans le milieu de culture a constitué une avancée majeure dans l'étude des mécanismes d'acquisition du fer par les bactéries (Hantke 1981; Escolar et al. 1999). L'existence de ce mutant, nommé *fur* pour « ferric uptake regulation », suggérait fortement qu'un seul élément était responsable de la régulation transcriptionnelle des gènes impliqués dans l'acquisition du fer. Chez ce mutant *fur*, la synthèse d'une protéine de 19 kDa était diminuée (Hantke 1981). Le gène responsable de la synthèse de cette protéine de 19 kDa, subséquement baptisée Fur, a depuis été cloné (Hantke 1984) et séquencé (Schaffer et al. 1985), et de nombreuses études ont permis d'élucider son mécanisme d'action. De plus, diverses expériences ont permis de mettre en évidence sa participation à d'autres fonctions non-reliées au métabolisme du fer, comme par exemple la réponse au choc acide (Hall et al. 1996), la défense contre les espèces réactives de l'oxygène (Niederhoffer et al. 1990), la chémotaxie (Karjalainen et al. 1991), les voies métaboliques (Hantke 1987), la bioluminescence (Makemson et al. 1992) et la production de toxines et autres facteurs de virulence (Litwin et al. 1993). Ce dernier point n'est pas nécessairement surprenant puisque le fer est généralement présent en

quantités limitantes dans les tissus et fluides de l'hôte. La faible concentration en fer agirait donc comme un signal indiquant à la bactérie son entrée chez l'hôte.

La protéine Fur agit comme un répresseur positif et exerce son action sur plus de 90 gènes (Andrews et al. 2003). En solution, elle est retrouvée sous forme d'homodimère composé de deux sous-unités de 17 kDa (Coy et al. 1991). Dans un milieu riche en fer, l'homodimère Fur lie un ion ferreux (Fe^{2+}) par sous-unité (Bagg et al. 1987), et la liaison de cette ion ferreux augmente l'affinité de la protéine pour son site de liaison sur l'ADN d'environ 1000 fois. Le site de liaison de Fur est situé au niveau du promoteur des gènes réprimés, entre les régions –35 et –10. La présence de Fur au niveau de ces régions bloque donc l'accès de l'ARN polymérase à ces sites, empêchant ainsi la synthèse d'ARNm. L'étude des séquences promotrices affectées par Fur a permis de déterminer une séquence palindromique consensus de 19 pb appelée boîte Fur.

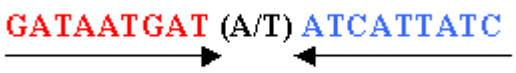

Fig. 16: Première interprétation de la boîte Fur. Un dimère Fur se lie sur chaque côté de la boîte. (Andrews et al. 2003)

Dans un premier temps, le modèle de liaison le plus accepté prônait la liaison d'un dimère Fur par séquence répétée au sein de la boîte Fur (Figure 16). Cependant, il a été démontré par des expériences d'empreinte à la DNase ainsi que par microscopie électronique, entre autres, que Fur a tendance a polymérisé le long du double-brin d'ADN à la manière d'un tire-bouchon, jusque dans des régions qui ne semblent pas présenter de similarités avec la boîte Fur (de Lorenzo et al. 1988a; Le Cam et al. 1994) (Figure 17). De telles observations ont donné lieu à des réinterprétations de la boîte Fur. Le modèle le plus accepté aujourd'hui est celui de Lavrrar et al., qui suggèrent un site de liaison Fur composé de deux motifs de 13 pb « 6-1-6 » superposés (Lavrrar et al. 2002). Ce modèle permet de bien expliquer la

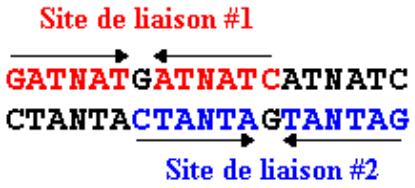

Fig. 17: Réinterprétation de la boîte Fur par Lavrrar et al. Deux dimères Fur se lient sur des faces opposées de la double-hélice à des sites déplacés d'environ un demi-tour d'hélice. (Lavrrar et al. 2002)

disposition en tire-bouchon des dimères Fur autour du double-brin d'ADN, et chaque addition d'un hexamère permet la liaison d'un dimère Fur supplémentaire.

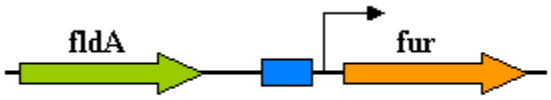

Fig. 18: Organisation génétique de l'opéron bicistronique *fldA/fur*. Le gène fur possède son propre promoteur au sein duquel on retrouve une boîte Fur.

Le gène *fur* est retrouvé au sein d'un opéron bicistronique directement en aval du gène *fldA* (Andrews et al. 2003) (Figure 18) codant pour la flavodoxine, une protéine qui pourrait possiblement être impliquée dans la réduction du fer cytosolique ($Fe^{3+} \rightarrow Fe^{2+}$) et qui fournirait ainsi son substrat à la protéine Fur. Bien que la transcription à partir du promoteur *fldA* permette aussi la transcription du gène *fur*, ce dernier possède néanmoins son propre promoteur, situé dans la région intergénique, et contenant une boîte Fur (Andrews et al. 2003). La protéine Fur régule donc son propre niveau d'expression.

Fait intéressant, il a aussi été mis en évidence que différents facteurs de transcription connus pour leur implication dans la réponse au stress oxydatif stimulent aussi l'expression de *fur*, suggérant ainsi un rôle quelconque de Fur dans la réponse au stress oxydatif. Une étude de Zheng et al. démontre que la transcription de *fldA* et *fur* est induite par le système SoxR-SoxS, système modulant la réponse aux composés générant des ions superoxydes, alors que OxyR, un facteur de transcription responsable de la réponse à des niveaux élevés de peroxyde d'hydrogène (H_2O_2), stimule la transcription de *fur* seulement (Zheng et al. 1999). L'augmentation de la synthèse de Fur en conditions de stress oxydatif permettrait d'augmenter la capacité de liaison des ions Fe^{2+} du cytoplasme, ce qui pourrait permettre de contrer les effets oxydatifs toxiques causés par le fer libre (Andrews et al. 2003). Il est aussi suggéré que Fur pourrait catalyser l'élimination du peroxyde d'hydrogène grâce à son ion Fe^{2+}, qui alternerait ainsi entre les formes Fe^{2+} et Fe^{3+} (Zheng et al. 1999). Il est donc possible que Fur possède plus qu'une fonction de répresseur transcriptionnel.

En plus de cette régulation par OxyR et SoxRS, une expérience par De Lorenzo et al. en 1988 semble démontrer que la synthèse de Fur est aussi régulée par la protéine réceptrice de l'AMPc (CRP) (De Lorenzo et al. 1988b). Bien que les méthodes expérimentales de cette étude aient depuis été remises en doute (Andrews et al. 2003), des études informatiques indiquent que CRP et MarA, une autre protéine régulatrice impliquée, entre autres, dans les phénomènes de résistances multiple à des composés antibiotiques, pourraient se lier à l'intérieur de la région de l'opéron *fldA/fur* (Zheng et al. 1999).

Jusqu'à ce jour, plusieurs gènes dont le niveau de production est induit par Fur ont été identifiés (Andrews et al. 2003). Par contre, contrairement à ce qui se produit dans le cas des gènes réprimés par Fur, aucune boîte Fur n'a pu être identifiée dans les régions promotrices de ces gènes et le mécanisme d'action de Fur sur la transcription de ces gènes est longtemps resté obscur. Récemment, un gène codant pour une petite molécule d'ARN non-codante (ARNs) et dont la synthèse est réprimée par Fur a été identifiée (Masse et al. 2002). Cette petite molécule d'ARN, nommée RyhB, permet lorsqu'elle est exprimée de réguler négativement la synthèse d'au moins six protéines qui avaient toutes été identifiées auparavant comme étant positivement régulées par Fur, même si le mécanisme de cette régulation était inconnu (Masse et al. 2002). Il est désormais clair que l'effet de Fur sur la transcription de ces gènes est indirect, via la répression de l'expression de RyhB. Les produits de ces six gènes codent pour des protéines pouvant lier le fer à l'intérieur de la cellule bactérienne :

- les gènes *bfr* et *ftn* codent respectivement pour la bactérioferritine et la ferritine
- le gène *sodB*, chez *E. coli*, code pour la seule des trois superoxyde dismutases de cet organisme utilisant un atome de fer dans son site actif
- les gènes *acnA*, codant pour l'aconitase, *fumA*, codant pour la fumarase, ainsi que l'opéron *sdhCDAB* codant pour la succinate déhydrogénase, codent pour trois enzymes impliquées dans le cycle des acides tricarboxyliques (TCA)

possédant aussi des groupements fer-soufre de type $[4Fe\text{-}4S]^{2+}$ au sein de leurs sites actifs

Grâce à RyhB, la cellule bactérienne se donne un moyen de contrôler la synthèse de composés non-essentiels utilisant des atomes de fer en période de carence. Le mode d'action de RyhB n'a pas encore été parfaitement élucidé, mais il a été démontré que RyhB n'interfère pas avec l'initiation de la transcription. La majorité des ARNs régulateurs interagissent avec l'ARNm des gènes dont ils modifient l'expression via des régions de complémentarité de séquence, empêchant ainsi l'élongation de la chaîne polypeptidique lors de la traduction. L'action de ces ARNs dépend aussi de la présence de la protéine Hfq, une protéine liant l'ARN et qui agirait comme chaperonne pour l'ARN (Moller et al. 2002; Zhang et al. 2002). Cependant, la possibilité d'une interaction directe avec l'ADN qui bloquerait l'élongation de l'ARNm n'est pas écartée dans le cas de RyhB (Masse et al. 2002).

1.3.6 Toxines RTX : ApxI à ApxIV

Les toxines RTX (Repeats in ToXin) sont sécrétées par diverses bactéries pathogènes Gram-négatives (Lally et al. 1999) et sont particulièrement répandues chez les *Pasteurellaceae* (Frey et al. 2002). Génétiquement parlant, la majorité des toxines de cette famille présente une organisation similaire classique de quatre gènes, *rtxCABD* (trancrits dans cette ordre) codant respectivement pour :

- *rtxC* : une protéine de transport de groupements acyl, responsable de l'acylation post-transcriptionnelle de la protéine native
- *rtxA* : la prétoxine
- *rtxB* et *rtxD* : la machinerie de sécrétion (Frey 1995). Ces deux protéines appartiennent respectivement à la famille des transporteurs ABC et à la famille des protéines de fusion membranaire (Lally et al. 1999). La sécrétion se fait grâce à la reconnaissance d'une séquence signal au niveau des 30 à 50 premiers acides aminés en C-terminal de la protéine (Hughes et al. 1992a; Hughes et al. 1992b).

Les toxines RTX ont typiquement une masse moléculaire allant de 100 à 200 kDa (Frey et al. 2002). La caractéristique principale des toxines de la famille des RTX est la présence d'un motif riche en glycine de 9 acides aminés répété de six à quarante fois (GGXGXDX[L/I/V/W/Y/F]X) dans la protéine de structure RtxA (Lally et al. 1999). Les RTX sont divisées en deux groupes :

1. les hémolysines (ex. : HlyA, *E. coli* et ApxI, *A. pleuropneumoniae*), qui sont toxiques pour un vaste éventail de cellules provenant de diverses origines
2. les toxines spécifiques (cytolysines), (ex. : LtxA, *Aggregatibacter actinomycetemcomitans*), qui attaquent certains types cellulaires d'espèces spécifiques

Les toxines de la famille RTX sont solubles et s'attaquent aux membranes de leurs cellules cibles en y formant des pores, par un mécanisme qui reste encore inconnu. L'insertion des toxines dans la membrane cible dépendrait d'un changement de conformation de la protéine (Lally et al. 1999)(Figure 19). Alors que dans la forme soluble de la toxine les résidus hydrophobes seraient séquestrés vers l'intérieur de la protéine, ils seraient au contraire exposés vers l'extérieur suite à l'insertion de la toxine dans la membrane biologique. Ce changement de conformation pourrait

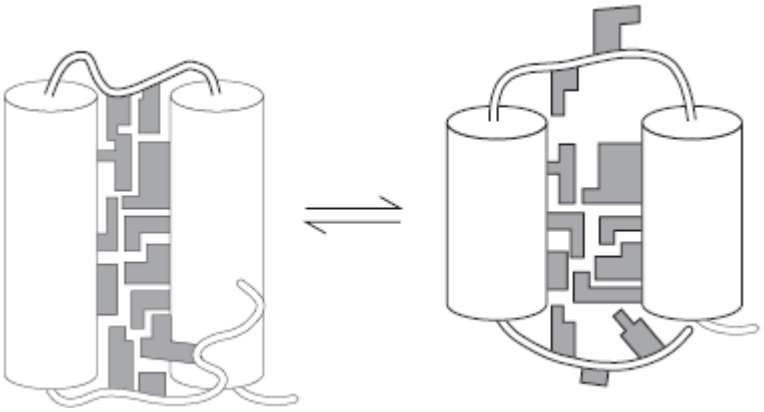

Fig. 19 : Changement de conformation pouvant se produire dans la structure des toxines RTX au niveau des motifs hydrophobes (gris) lors de leur insertion dans la membrane. (Tiré de Lally 1999)

hypothétiquement être dû à des forces électrostatiques ou encore à l'interaction de la toxine avec un récepteur de surface sur la membrane.

Dans le cas de LtxA, une toxine spécifique aux leucocytes, ce récepteur a été découvert grâce à l'utilisation d'anticorps monoclonaux (Lally et al. 1997). Il a ainsi été démontré que l'utilisation d'anticorps spécifiques aux molécules CD11a et CD18, deux sous-unités de la molécule LFA-1 associées à la plupart des leucocytes circulants, éliminait l'effet cytolytique. Ceci expliquerait, par le fait même, la spécificité de la toxine. Les toxines non-spécifiques, quant à elles, pourraient se lier à la surface des cellules-cibles grâce à des interactions électrostatiques. Le domaine riche en glycine, qui caractérise la famille des RTX, possède d'ailleurs une grande affinité pour les ions Ca^{2+}, et il a été démontré que ces ions sont impliqués dans l'adhésion aux érythrocytes, en collaboration avec des acides aminés portant des résidus lipidiques (Hughes et al. 1992b).

Une fois intégrée à la membrane, la toxine se polymérise afin de former des pores de 0,9 à 2 nm (Bhakdi et al. 1989; Clinkenbeard et al. 1989; Bhakdi et al. 1993). Tout dépendamment de la concentration de toxines présentes dans le milieu, la mort cellulaire se produira soit par nécrose, soit par apoptose (Lally et al. 1999). En présence de concentrations plus faibles de toxines RTX, les pores formés par les RTX entraînent un débalancement ionique qui mène, lorsque les fonctions mitochondriales sont suffisamment perturbées, à l'enclenchement des mécanismes de l'apoptose (Kroemer et al. 1998; Lally et al. 1999). Au contraire, lorsque les cellules-cibles sont incubées avec de fortes concentrations de toxines, la mort se produit par nécrose, le très grand nombre de pores formés entraînant le déversement des composantes cellulaires dans le milieu extra-cellulaire (Jonas et al. 1993).

Au cours des dernières années, plusieurs expériences ont été menées afin de déterminer le rôle potentiel des LPS dans l'action des toxines RTX (Ramjeet et al. 2008a). Les questions ont commencé à faire surface alors que les laboratoires tentant de purifier les toxines faisaient souvent face à des problèmes de contamination par

des LPS. Ainsi, certains effets biologiques attribués jusqu'à présent aux toxines RTX, comme par exemple l'induction *in vivo* de médiateurs de l'inflammation (Frey et al. 2002), pourraient en fait s'expliquer par un effet coopératif entre les RTX et les LPS (Bhakdi et al. 1990; Walmrath et al. 1994; Stevens et al. 1995). L'existence de mutants pour le noyau oligosaccharidique du LPS ayant une capacité réduite de production ou de sécrétion de toxines actives semblent accréditer cette thèse (Stanley et al. 1993; Bauer et al. 1997). Au cours d'une étude récente avec la leucotoxine de *Pasteurella multocida* (Li et al. 1999), les auteurs ont co-isolé LPS et leucotoxine par immunoprécipitation. Le rôle exact du LPS dans cette association n'a pas encore été éclairci, mais les auteurs estiment que le LPS pourrait contribuer à stabiliser la structure de la toxine.

Jusqu'à ce jour, quatre toxines de type Apx ont été identifiées chez les différentes souches de référence d'*A. pleuropneumoniae* (Figure 20):

- ApxI : Toxine de 105 kDa fortement hémolytique et cytolytique. Toutes les souches de référence sauf celle du sérotype 3 possèdent les gènes de sécrétion de la toxine (*apxIBD*), alors que l'opéron entier (*apxICABD*) n'est retrouvé que chez les sérotypes 1, 5 (a et b), 9, 10 et 11. La toxine lie le calcium avec une haute affinité et cet élément est requis pour l'activité hémolytique (Frey et al. 1991).

- ApxII : Toxine de 103 à 105 kDa, faiblement hémolytique et cytotoxique. Produite par toutes les souches de référence sauf celle du sérotype 10. L'opéron *apxII* comporte le gène activateur *apxIIC* ainsi que le gène de structure *apxIIA*, mais aucun appareil de sécrétion n'est présent. La sécrétion semble s'effectuer via l'appareil de sécrétion de la toxine ApxI (produit des gènes *apxIBD*) (Frey 1995).

- ApxIII : Toxine de 120 kDa ne possédant pas d'activité hémolytique mais qui est fortement cytotoxique envers les macrophages alvéolaires et les

neutrophiles (Kamp et al. 1991; Rycroft et al. 1991). Elle est produite et sécrétée par les sérotypes 2-3-4-6-7-8 du biotype 1. Les souches du biotype 2 ne possèdent pas la toxine (Beck et al. 1994).

Sérotype	ApxI		ApxII	ApxIII		ApxIV
	CA	BD	CA	CA	BD	A
1	■	■	■			■
2		■	■	■	■	■
3			■	■	■	■
4		■	■	■	■	■
5	■	■	■			■
6		■	■	■	■	■
7		■	■	■	■	■
8		■	■	■	■	■
9	■	■	■			■
10	■	■				■
11	■	■	■			■
12		■	■			■
13		■	■			■
14		■	■			■
15		■	■			■

Fig. 20: Distribution des différents gènes Apx parmi les différents sérotypes d'*A. pleuropneumoniae*.

- ApxIV : Découverte plus récemment, cette quatrième toxine a été retrouvée chez toutes les souches de référence du biotype 1 et semble exprimée uniquement *in vivo* (Schaller et al. 1999). Contrairement aux trois autres toxines Apx, les gènes codant pour la protéine activatrice (*apxIVC*) et pour le système de sécrétion (*apxIVBD*) n'ont pas été retrouvées. Cependant, un ORF est présent en amont du gène *apxIVA* qui semble essentiel pour la faible activité hémolytique de la toxine. La toxine ApxIV semble être spécifique à l'espèce *A. pleuropneumoniae* plutôt que spécifique de certains sérotypes (Schaller et al. 2001), faisant ainsi de cette protéine un outil potentiel pour le sérodiagnostique des infections à *A. pleuropneumoniae* (Dreyfus et al. 2004). Elle présente de grandes ressemblances avec la toxine RTX FrpC régulée par le fer de *N. meningitidis* (Schaller et al. 1999).

Les sérotypes 1, 5, 9 et 11 étant ceux qui sont le plus souvent associés à de fortes épidémies caractérisées par une forte mortalité et des lésions pulmonaires majeures, ils sont généralement considérés comme étant les plus virulents (Frey 1995). Tous ces sérotypes ont une chose en commun : ils produisent la toxine ApxI. Il est dès lors tentant d'associer la présence de la toxine ApxI à un phénotype de virulence accrue. Cependant, il faut aussi garder à l'esprit que le sérotype le plus souvent retrouvé dans les cas de pleuropneumonie porcine dans la plupart des pays européens est le sérotype 2, qui produit et sécrète les toxines ApxII, III et IV. En Chine, le sérotype prédominant est le 7, qui ne produit que les toxines ApxII et IV (Bei et al. 2005). Bien qu'il soit difficile d'évaluer la contribution relative de chacune des toxines à l'infection, une chose reste certaine : la présence des toxines Apx est indispensable à l'infection. Il a de plus été démontré que l'inoculation endobronchiale de toxines recombinantes pouvait, à elle seule, entraîner l'apparition de lésions typiques de cas de pleuropneumonie porcine (Kamp et al. 1997). Une étude récente démontre que, pour le sérotype 1 d'*A. pleuropneumoniae*, l'induction de lésions pathologiques avec une certaine constance requière la présence des toxines ApxI et ApxII, des mutants ne produisant que l'une ou l'autre des deux toxines causant l'apparition de symptômes cliniques plus modérés (Boekema et al. 2004a). Les mutants ne possédant aucune des deux toxines ne causaient aucune lésion visible. De plus, la contribution de la toxine ApxII au développement de lésions semblait légèrement plus important que celle de la toxine ApxI, contrairement à ce que les résultats d'études différentes pouvait laisser croire (Kamp et al. 1991; Kamp et al. 1997). De tels types d'études n'ont pas été menés avec ApxIII et ApxIV, de sorte qu'il est impossible de spéculer sur le rôle précis de ces toxines.

En plus d'être requis pour l'activité des toxines, les ions Ca^{2+} libres seraient aussi impliqués dans la régulation de la production de la toxine ApxI (Frey et al. 1991; Gygi et al. 1992; Hsu et al. 2003). Il a aussi été montré que la phase de croissance influence la production de toxines, celle-ci étant maximale lors de la phase stationnaire, alors que la densité bactérienne est élevée (Jarma et al. 2004b), et que la présence ou l'absence d'oxygène ne semble pas influencer la production de la toxine

(Jarma et al. 2004a). De plus, Hsu et al. ont établi un lien possible entre l'activité hémolytique et Fur, le répresseur transcriptionnel sensible aux concentration d'ions ferriques (Hsu et al. 2003). Combinées ensemble, ces données ont plusieurs implications :

- Les toxines semblent être exprimées surtout *in vivo* : ceci a été démontré par plusieurs études (Cho et al. 2001), et cette présomption semble renforcée par l'expression accrue en phase stationnaire, qui peut, d'une certaine façon, être associée à un stress. De plus, l'expression tant en milieu aérobie qu'anaérobie signifie que la production des toxines n'est pas affectée dans les poumons alors qu'il peut y avoir formation de zones anoxiques localisées suite à la formation des lésions.
- L'utilité des toxines pour la bactérie pourrait être liée à l'acquisition du fer : le fer est un élément limitant *in vivo* pour la croissance bactérienne. L'hémolyse par les toxines Apx pourrait mener à la libération de composés hémiques dans le milieu, permettant ainsi à la bactérie de combler ses besoins en ions ferriques (Hsu et al. 2003)(Figure 9).

1.3.7 Protéases

Bien qu'il soit généralement accepté que les toxines Apx sont largement responsables de la formation de lésions lors d'infections à *A. pleuropneumoniae*, il est probable que d'autres composantes aient aussi leur rôle à jouer. La sécrétion de protéases est souvent observée chez les bactéries pathogènes (Miyoshi et al. 2000), et *A. pleuropneumoniae* ne fait pas exception. Ainsi, au moins 6 métallo-protéases différentes ont pu être détectées dans des surnageants de culture ainsi que dans des extraits cellulaires de la bactérie (Negrete-Abascal et al. 1994). Ces protéases ont pu mener à la dégradation efficace de la gélatine porcine, des IgA porcins et, à un moindre niveau, de l'hémoglobine.

Des expériences ultérieures ont aussi démontré que les protéases de haut poids moléculaire, une fois purifiées, sont en fait des multimères d'une sous-unités de 47

kDa, qu'elles sont présentent dans tous les sérotypes, qu'elles sont actives contre les IgG porcins, et qu'elles peuvent être reconnues par du sérum porcin immun (Negrete-Abascal et al. 1998). Une métallo-protéase au zinc de plus petit poids moléculaire (24kDa), aussi retrouvée chez tous les sérotypes de biotype 1, a aussi été purifiée et semble active contre l'actine *in vitro* (Garcia-Cuellar et al. 2000). Il a de plus été démontré que des protéases étaient relarguées dans le milieu de culture via le relarguage de vésicules membranaires, vésicules comprenant aussi des toxines Apx (Negrete-Abascal et al. 2000) (Figure 21). Finalement, une métallo-protéase de 101 kDa a pu être isolée, et sa présence *in vivo* a aussi été démontrée (Garcia Gonzalez et al. 2004).

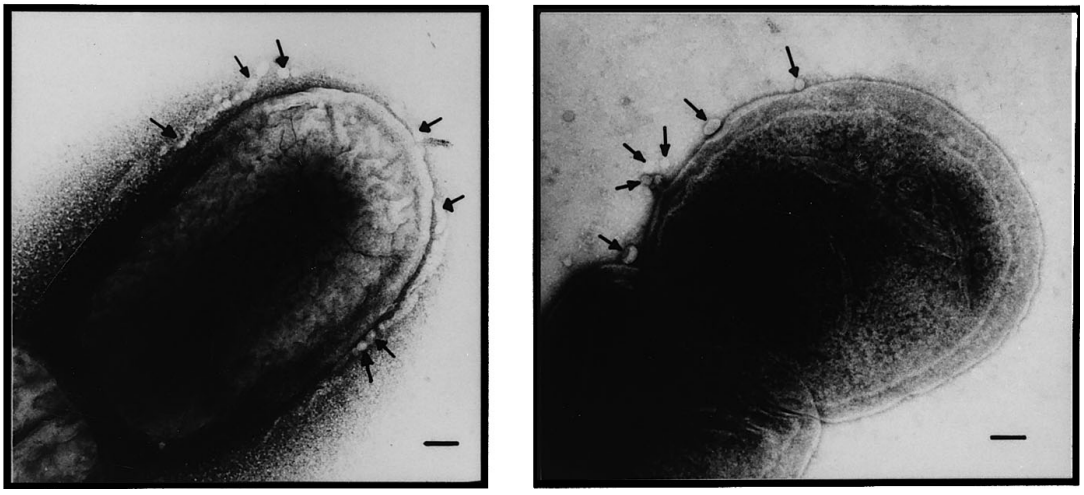

Fig. 21: Présence de vésicules contenant des protéases et des toxines Apx à la surface d'*A. pleuropneumoniae*. Tiré de Negrete-Abascal & al. 2000.

Plus récemment, une sérine protéase autotransporteur sécrétée nommée AasP a été identifiée (Baltes et al. 2007), et cette enzyme serait impliquée dans le clivage de la lipoprotéine OmlA, dont la fonction est inconnue (Ali et al. 2008). Au final, toutes ces études suggèrent que, malgré le peu d'informations dont nous disposons, des protéases sont bel et bien sécrétées par *A. pleuropneumoniae* et leur activité contre les IgA et les IgG suggèrent un rôle important dans le processus d'infection.

1.3.8 Uréase

Biochimiquement, *A. pleuropneumoniae* peut être distingué des autres *Pasteurellaceae* dépendants du NAD (facteur V) par son hémolyse, sa réaction positive au test de CAMP ainsi que par sa forte production d'uréase (Blanchard et al. 1993). Cette enzyme, qui catalyse la conversion de l'urée en ammoniaque et dioxyde de carbone, est considérée comme un facteur de virulence important chez plusieurs bactéries gastrointestinales ou urinaires (Collins et al. 1993), et est aussi présente chez plusieurs bactéries des voies respiratoires (Monack et al. 1993; Reyrat et al. 1996). L'ammoniaque créée par la réaction que catalyse l'uréase est directement cytotoxique en plus de promouvoir différents processus inflammatoires. L'uréase d'*A. pleuropneumoniae*, codée par les opérons *ureABC* (protéines de structure) et *ureEFGD* (protéines accessoires) (Bossé et al. 1997), ne semblait d'abord pas essentielle au processus d'infection puisque des souches où l'enzyme était naturellement absente ou encore inactivée par la présence d'une mutation ne se sont pas montrées moins virulentes que leurs souches parentales (Blanchard et al. 1993; Tascon Cabrero et al. 1997). Néanmoins, dans un modèle expérimental d'infection initiale avec une faible dose de bactéries (10^3cfu/ml), un mutant au niveau du gène *ureG* n'a pu coloniser efficacement les poumons de son hôte (Bossé et al. 2000). De plus, il fut par la suite montré qu'un mutant *ureC*, comparativement à sa souche parentale, ne pouvait être récupéré des poumons d'un animal trois semaines après une infection expérimentale, et que les animaux infectés par ce mutant présentaient une réponse immunitaire spécifique à *A. pleuropneumoniae* accrue (Baltes et al. 2001). Les auteurs de cette étude ont donc posé l'hypothèse que l'uréase produite par *A. pleuropneumoniae* pourrait nuire suffisamment à l'établissement d'une réponse immunitaire adéquate afin de permettre l'établissement d'une infection persistante.

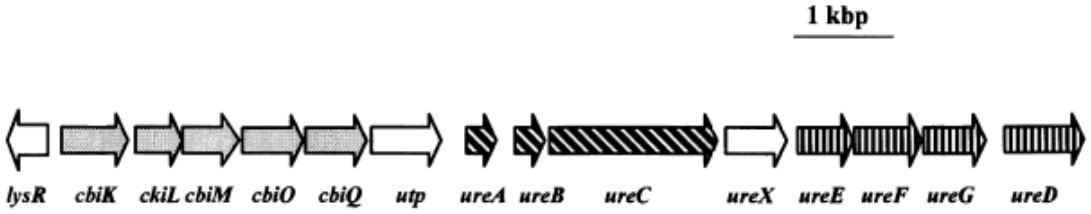

Fig. 22: Structure génétique des opérons *ure* et *cbi*. Tiré de Bosse & al. 2000

Comme beaucoup d'autres uréases, l'uréase d'*A. pleuropneumoniae* est articulée au tour d'un centre actif portant des ions de nickel (Bossé et al. 1997). Il n'est donc pas surprenant de constater la présence des gènes *cbiKLMQO*, codant pour un système de perméase pour le nickel, en amont de l'opéron *ureABC* (Bossé et al. 2000; Bossé et al. 2001) (Figure 22).

1.3.9 Superoxyde dismutase (SOD)

Les métallo-enzymes superoxide dismutases sont présentes autant chez les eucaryotes que chez les procaryotes, et servent à catalyser la conversion des radicaux anioniques superoxide générés lors du métabolisme aérobie en oxygène et peroxide d'hydrogène afin d'éviter l'accumulation de radicaux libres cytotoxiques (Kroll et al. 1995). Chez *A. pleuropneumoniae*, une séquence codant pour une SOD contenant du cuivre et du zinc ([Cu,Zn]-SOD) a d'abord été détectée par PCR (Kroll et al. 1995) avant que le gène *sodC* ne soit cloné et caractérisé (Langford et al. 1996). La [Cu,Zn]-SOD encodée par *sodC* est sécrétée dans le périplasme, comme en fait foi la présence d'un peptide leader en N-terminal de la protéine, où elle pourrait potentiellement jouer un rôle de bouclier contre la poussée oxidative des macrophages de l'hôte (Forest et al. 2000). Ceci contraste avec l'activité des SOD bactériennes au manganèse ou au fer, encodées par les gènes *sodA* et *sodB*, qui, de par leur localisation dans le cytoplasme, exercent vraiment un rôle dans la protection face au métabolisme aérobie. Néanmoins, il a été démontré expérimentalement qu'un mutant *sodC* d'*A. pleuropneumoniae*, bien qu'il soit plus sensible au superoxide *in vitro*, n'est pas atténué dans le cadre d'une infection expérimentale (Sheehan et al. 2000).

1.3.10 Enzymes du métabolisme anaérobie

Au cours de la dernière décennie, plusieurs publications ont démontré l'importance de certaines enzymes impliquées dans le métabolisme anaérobie pour la virulence d'*A. pleuropneumoniae*. Considérant qu'au cours de l'infection, surtout dans le cas

où la maladie évolue vers la forme chronique, les lésions nécrotiques au sein des tissus pulmonaires porcins forment un environnement sans oxygène, l'importance possible de ce type de métabolisme n'est pas surprenante. Dans un premier temps, l'enzyme dimethyl sulfoxide (DMSO) reductase, DmsA, a été identifiée suite à une expérience au cours de laquelle des ADNc d'*A. pleuropneumoniae* obtenus suite à la culture dans un milieu de croissance standard ont été soustraits d'une banque d'ADNc obtenue suite à la mise en contact de cultures bactériennes avec du liquide de lavages bronco-alvéolaires (BALF) (Hennig et al. 1999; Baltes et al. 2003). Un mutant *ΔdmsA* fut construit, et des infections expérimentales ont démontré son atténuation *in vivo*. Chez *E. coli*, les protéines DmsABC du complexe DMSO réductases permettent l'utilisation du DMSO comme accepteur final d'électrons au sein de la chaîne respiratoire lors de la respiration anaérobie (Weiner et al. 1992).

Par la suite, après qu'il fut démontré que deux autres enzymes du métabolisme anaérobie n'avaient aucune influence sur le développement de la pleuropneumonie porcine (Baltes et al. 2004b), une autre enzyme potentiellement importante pour la survie en anaérobiose et dont l'expression était stimulée par le BALF a été identifiée (Jacobsen et al. 2005). L'enzyme AspA (aspartate-ammoniaque lyase) permet la synthèse du fumarate, un composé qui peut servir d'accepteur final d'électron lors de la respiration anaérobie. Présent chez tous les sérotypes d'*A. pleuropneumoniae*, le gène *aspA*, lorsque muté, mène à l'atténuation *in vivo* de la souche mutante. Le fait qu'un mutant *ΔdmsAΔaspA* ne puisse être ré-isolé des cavités pulmonaires suite à l'infection a mené les auteurs de l'étude à conclure que les enzymes du métabolisme anaérobie sont importantes pour la persistence *in vivo*. Cette hypothèse fut renforcée lorsqu'il fut démontré qu'un mutant *ΔhlyX*, gène codant pour le régulateur global anaérobie HlyX, colonisait les poumons porcins en moins grand nombre, et que cette souche ne pouvait, elle non plus, persister *in vivo* (Baltes et al. 2005).

Outre HlyX, l'adaptation aux conditions anaérobies requière la présence du système à deux composantes ArcA-ArcB. Ce système existe également chez *A. pleuropneumoniae*. À l'instar de ce qui se produit avec un mutant *ΔhlyX*, un mutant

ΔarcA est atténué *in vivo* (Buettner et al. 2008b). Il fut aussi démontré que ce mutant présentait une capacité de production de biofilms réduite. Depuis, les régulons ArcA et HlyX ont tous deux été déterminés, et deux autres facteurs de virulence ont été suggérés (Buettner et al. 2008a; Buettner et al. 2009). Tout d'abord, l'analyse du régulon ArcA a mené à l'identification de l'enzyme fumarate réductase, encodée par les gènes *frdABCD*, qui permet l'utilisation du fumarate comme accepteur final d'électron. Un mutant *Δfrd* a été construit, et est lui aussi atténué *in vivo*. Finalement, le gène codant pour une protéine régulée par le fer (*frpB*) fut identifié au sein du régulon HlyX, et l'atténuation d'un mutant *ΔfrpB* fut aussi démontrée.

1.4 Traitement et prévention de la pleuropneumonie porcine

Bien qu'il soit possible de traiter les animaux atteints de pleuropneumonie porcine, il est vital, vu l'évolution rapide de la maladie, que les traitements commencent suffisament rapidement suite à l'infection. De façon générale, des résistances aux tétracyclines, aux sulfonamides, à la gentamicine, la streptomycine, la tiamuline, la kanamycine, la lyncomycine, la tyolosine, l'ampicilline, l'amoxicilline, l'acide nalixidique, au thiamphénicole et au chloramphénicole ont été détectées chez *A. pleuropneumoniae*, mais les isolats sont généralement sensible au ceftiofur (Kim et al. 2001; Chang et al. 2002; Gutierrez-Martin et al. 2006; Morioka et al. 2008).

Devant l'apparition de plus en plus fréquente de gènes de résistance chez plusieurs bactéries pathogènes, et devant le risque que représente les pathogènes animaux dans le transfert de ces gènes à des pathogènes humains (*A. pleuropneumoniae* serait d'ailleurs responsable du transfert du gène de résistance pour la bêta-lactamase ROB-1 à une souche humaine d'*H. influenzae* (Livrelli et al. 1993)), beaucoup d'efforts ont été investis dans l'élaboration d'un vaccin pouvant prévenir l'infection par *A. pleuropneumoniae* (pour une revue détaillée, voir Ramjeet et al. 2008b, Annexe 1). Cependant, la présence de nombreux sérotypes pour cette bactérie nuit grandement au développement d'un tel vaccin. Ainsi, alors qu'un animal survivant à une infection naturelle devient résistant à une ré-infection autant par une souche de même

sérotype ou que par une souche de sérotype différent, aucun vaccin permettant de protéger efficacement les animaux contre tous les sérotypes existants d'*A. pleuropneumoniae* n'a encore été développé (Ramjeet et al. 2008b).

La première génération de vaccins contre *A. pleuropneumoniae* était composée de bactéries complètes inactivées (bactérines), souvent cultivées dans des conditions de culture simulant, jusqu'à un certain point, l'environnement *in vivo* (Ramjeet et al. 2008b). Par contre, ce type de vaccin ne conférait qu'une protection limitée, avec seulement une faible réduction de mortalité (Jolie et al. 1995; Furesz et al. 1997). Plusieurs facteurs peuvent expliquer l'inefficacité de ces vaccins :

- l'altération possible d'antigènes de surface par les techniques chimiques ou physiques utilisées pour inactiver les bactéries
- l'absence de sécrétion, par les bactéries inactivées, de composantes parfois fortement immunogéniques

Ce dernier point est particulièrement important dans le cas *d'A. pleuropneumoniae* puisque les toxines Apx sont très immunogènes et essentielles pour le développement d'une réponse immunitaire efficace (Seah et al. 2002).

Suite aux différents échecs rencontrés avec les bactérines, les recherches se sont concentrées sur l'élaboration de vaccins sous-unitaires. À ce chapitre, plusieurs facteurs de virulence ont été testés pour ce qui est de leur potentiel immunoprotecteur (voir Tableau I, Annexe I). La présence de multiples sérotypes ayant chacun leur propre version des diverses protéines et lipoprotéines de surface retrouvées chez *A. pleuropneumoniae* représente cependant un obstacle majeur à l'élaboration d'un vaccin sous-unitaire efficace. Ainsi, alors que les vaccins sous-unitaires permettent souvent de bien protéger les animaux infectés par la souche ayant servi à l'élaboration du vaccin ou encore par une souche du même sérotype, ces animaux demeurent néanmoins sensibles à l'infection par une souche de sérotype différent. Aussi, certains facteurs de virulence qu'il pourrait être intéressant d'inclure dans un

vaccin sous-unitaire ne sont exprimés que dans l'environnement *in vivo* (Ramjeet et al. 2008b). De plus, le fait qu'aucune adhésine protéique n'ait encore été formellement identifiée chez *A. pleuropneumoniae* empêche les chercheurs de cibler spécifiquement cette étape cruciale de l'infection (Haesebrouck et al. 2004).

Malgré les nombreux désavantages qui sont associés aux vaccins vivants atténués, dont les risques de réversion à l'état pathogène ainsi que les retards de croissance occasionnés pas l'atténuation inadéquate des bactéries, les insuccès rencontrés dans la recherche de vaccins sous-unitaires ont poussé certains chercheurs à explorer cette avenue (Ramjeet et al. 2008b) (Tableau II, Annexe I). Contrairement aux vaccins sous-unitaires, qui doivent cibler une protéine accessible au système immunitaire, l'utilisation de souches atténuées permet de cibler, dans le processus de mutagénèse, des gènes liés au métabolisme avec pour but de rendre la souche mutante non-virulente mais néanmoins viable et fortement immunogénique. Il est en effet souhaitable que la bactérie puisse persister un certain temps à l'intérieur de son hôte, tout en minimisant les effets sur son développement, afin qu'une réponse immunitaire forte puisse être stimulée. Il est aussi souhaitable que les souches vaccinales utilisées puissent permettre de différencier, sérologiquement, les animaux qui ont été vaccinés de ceux qui sont infectés par une souche virulente de la bactérie. En effet, l'utilisation d'une souche vivante atténuée fait en sorte que la réponse immunitaire de l'hôte génère des anticorps contre la majorité des antigènes acccessibles au système immunitaire, comme dans le cas d'une infection réelle.

L'introduction de marqueurs négatifs par élimination, chez une souche vaccinale, de protéines fortement immunogéniques, exprimées chez toutes les souches sans pour autant être essentielles dans l'élaboration d'une réponse immunitaire efficace, est à la base du concept DIVA (« **D**ifferentiating **I**nfected from **V**accinated **A**nimals ») (Maas et al. 2006b; Ramjeet et al. 2008b). Quelques vaccins de ce genre ont été générés chez *A. pleuropneumoniae*. Dans un premier temps, des mutants acapsulaires obtenus par mutations chimiques chez les sérotypes 1 et 5 ont montré un bon potentiel protecteur autant contre des infections avec des souches de même sérotypes

qu'avec des souches de sérotypes hétérologues (Inzana et al. 1993). L'absence de production d'anticorps dirigés contre la capsule polysaccharidique permet de différencier, par réaction ELISA, les animaux vaccinés des animaux infectés. Par la suite, une souche *ΔureC* et *ΔapxIIA* a été générée (Tonpitak et al. 2002), et quatre autres mutations furent ensuite ajoutées (*ΔdmsA*, *ΔhybB*, *ΔaspA*, *Δfur*) afin de diminuer l'étendue des lésions occasionées par le mutant double original (Maas et al. 2006a). Le mutant sextuple résultant est hautement atténué, persiste en petites quantités chez l'animal jusqu'à six semaines après l'immunisation, confère une bonne protection contre une ré-infection par un sérotype homologue (sérotype 2), mais a aussi montré une bonne protection lors d'infections avec une souche de sérotype hétérologue (sérotype 9).

1.5 Études de régulation génique et identification de gènes exprimés *in vivo* par *A. pleuropneumoniae*

Devant l'absence d'un vaccin vraiment efficace contre tous les sérotypes d'*A. pleuropneumoniae*, les chercheurs se sont tournés vers diverses méthodes visant à identifier les gènes qui sont préférentiellement transcrits au cours du processus infectieux. Ceci permet d'identifier des gènes dont les produits seront effectivement présents *in vivo*, et qui pourraient donc être de bonnes cibles pour l'élaboration de vaccins sous-unitaires. Différentes techniques ont été utilisées avec *A. pleuropneumoniae* : les techniques IVET, STM et SCOTS. Un article passant en revue l'utilisation de ces techniques chez *A. pleuropneumoniae* et le développement de vaccins a d'ailleurs été publié (Ramjeet et al. 2008b) (voir Annexe 1).

1.5.1 « *In Vivo* Expression Technology » (IVET)

La technique IVET est basée sur la recherche de promoteurs qui sont activés dans des circonstances précises, plus particulièrement dans le cadre d'expériences effectuées *in vivo* (Slauch et al. 1994). Le protocole original, d'abord appliqué chez *Salmonella* Typhimurium lors d'infections expérimentales de souris, nécessite

l'obtention d'une banque de promoteurs, sous la forme de fragments génomiques, ainsi qu'un mutant auxotrophe pour un gène essentiel à la survie *in vivo* de la bactérie. La banque de promoteurs est clonée en amont d'une copie intacte du gène muté sur un plasmide, avant que la construction ne soit ensuite introduite dans le génome bactérien via recombinaison homologue simple. Depuis l'utilisation d'un mutant *purA* chez *S.* Typhimurium, trois autres variations dans la technique IVET ont été développées (Angelichio et al. 2002)(Figure 23). La dernière de ces variations, soit l'utilisation du gène *tnpR* encodant une résolvase qui, une fois produite, mène à l'excision d'un gène de sélection, permet de contourner le principal défaut de la technique. En effet, dans sa forme initiale, la technique IVET ne permet d'identifier que les gènes qui sont transcrits tout au long du processus d'infection,

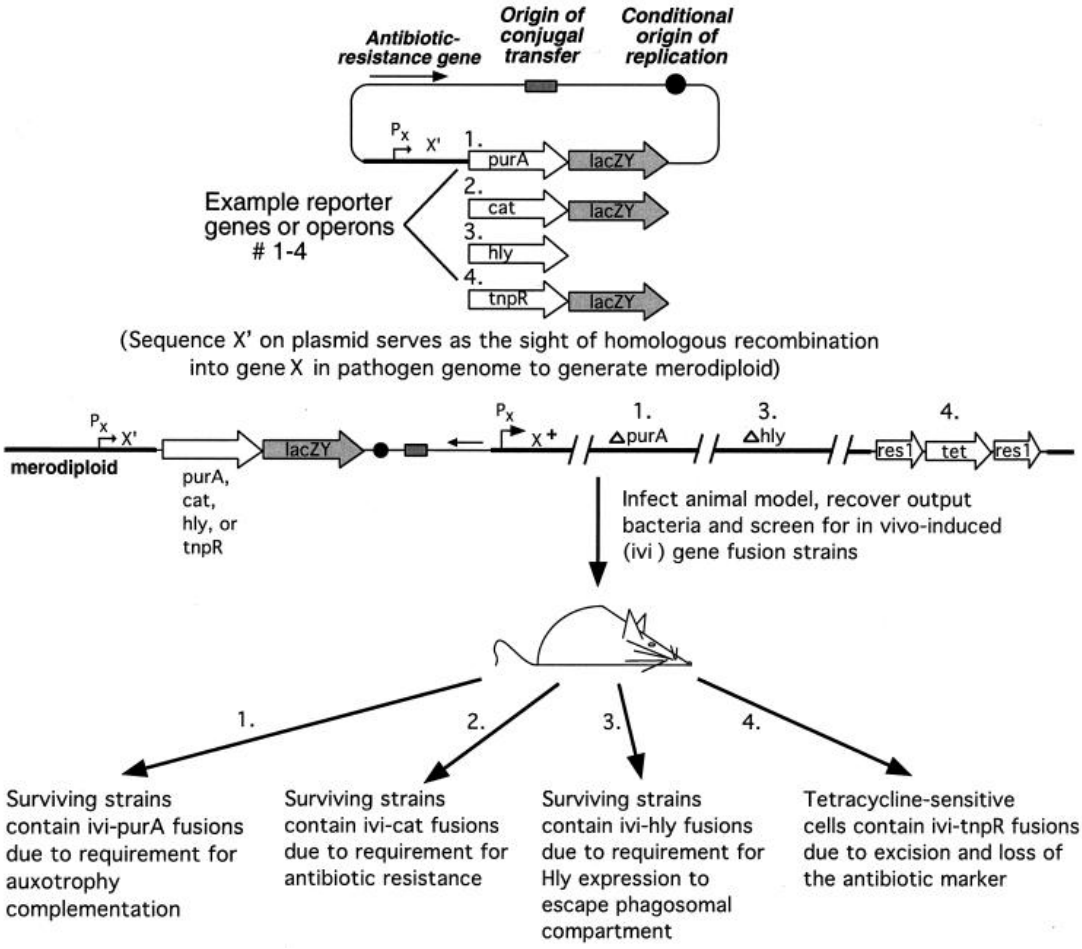

Fig.23: Représentation schématique des différentes variations existant pour la technique IVET. Tiré de Angelichio & al. 2002.

ignorant ainsi les gènes dont l'expression ne pourrait être que transitoire. Une autre

limitation d'IVET vient du fait que ce ne sont pas tous les gènes identifiés qui sont réellement utiles au cours du processus infectieux. Les gènes identifiés sont majoritairement impliqués dans des voies métaboliques.

Fuller et al. ont utilisé une banque de promoteurs composée de fragments de 0.4 à 1.0 kb en amont des gènes *ribBAH* de *Bacillus subtilis* avant d'introduire cette construction dans une souche Rib- d'*A. pleuropneumoniae*, déficiente pour la biosynthèse de la riboflavine (Fuller et al. 1999). Cette étude a permis de cribler plus de 2400 mutants Rib- lors d'infections expérimentales de 12 à 16h, mais a mené à l'identification de seulement 10 loci génétiques différents qui sont actifs *in vivo*. De ces 10 loci, baptisés *iviA-iviJ* (pour « *in vivo* induced »), seulement 6 ont montré des analogies de séquences avec des gènes connus et qui sont, pour la plupart, impliqués dans différentes voies métaboliques. Malgré tout, cette étude a mené à une découverte importante. Dans une étude subséquente, il a en effet été démontré que 8 des 10 loci *ivi* montraient une augmentation d'activité transcriptionnelle dans un milieu dépourvu d'acides aminés ramifiés (« Branched-Chained Amino-Acids », BCAA, comprenant la leucine, l'isoleucine et la valine). Leur concentration dans l'environnement pourrait potentiellement servir de signal pour l'induction de la synthèse d'ARNm codant pour des protéines impliquées dans la biosynthèse de ces acides aminés, et peut-être aussi pour des gènes potentiellement impliqués dans la virulence. Le gène codant pour la protéine Lrp (« Leucine-Responsive Protein ») chez *A. pleuropneumoniae* a par la suite été identifié et cloné (Wagner et al. 2007).

1.5.2 « Signature-Tagged Mutagenesis » (STM)

La technique STM a été mise au point par Hensel et al. en 1995, encore une fois dans le but d'identifier des gènes impliqués dans la survie *in vivo* de *S.* Typhimurium (Hensel et al. 1995). Afin de mettre au point le système STM, une banque de séquences variables de 40 pb, entourées par des bras invariables de 20 pb, a été insérée dans un transposon qui fut subséquemment utilisé afin de générer une banque

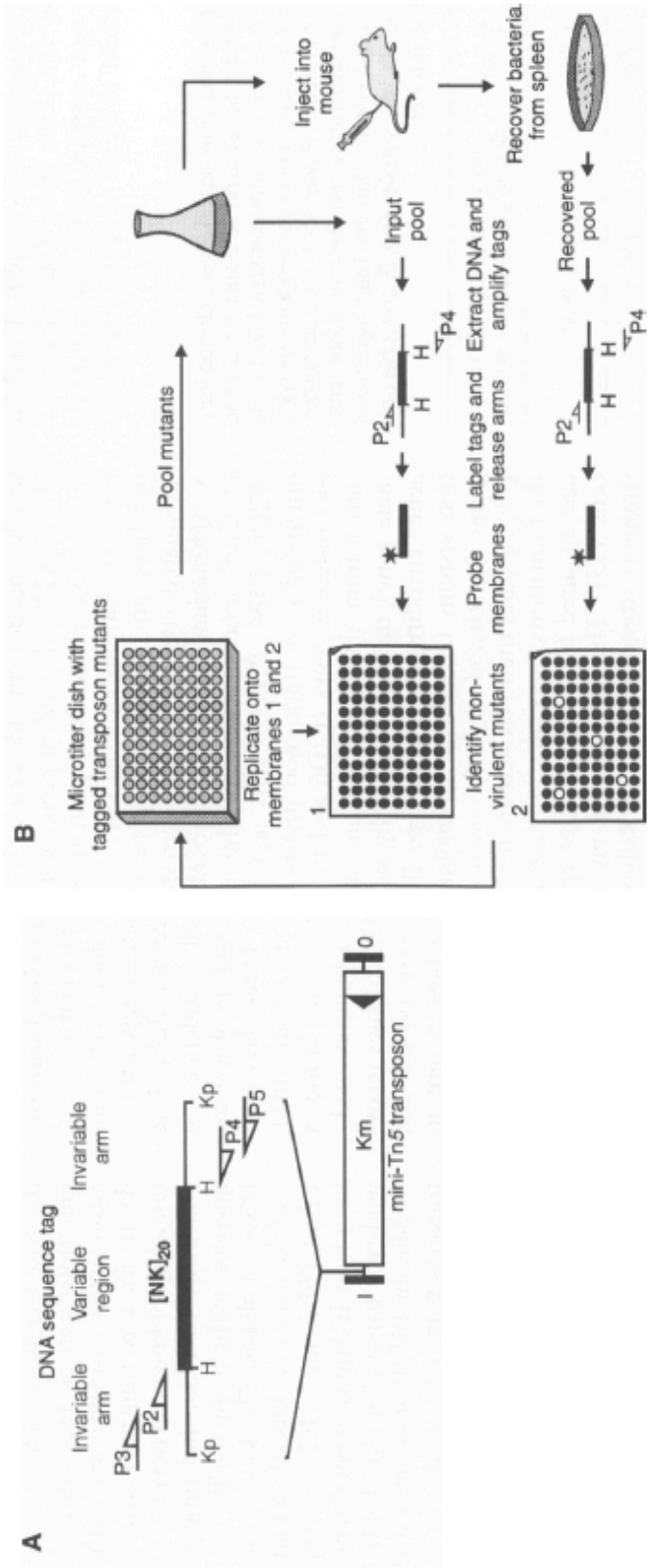

Fig.24: Représentation schématique du protocole original pour la technique STM. Tiré de Hensel & al. 1995.

de mutants de *S.* Typhimurium (Figure 24). Les mutants individuels ont ensuite été stockés dans des plaques 96 puits. Lors des expériences *in vivo*, les mutants d'une de ces plaques sont regroupés dans un même milieu afin d'être inoculés chez l'animal. En comparant les mutants qui sont présents dans le groupe de départ, et ceux qui sont récupérés suite à l'infection, il est possible de détecter ceux qui sont atténués, et donc porteurs de mutations dans des gènes essentiels pour la survie *in vivo*. Dans l'étude originale de Hensel et al., 40 mutants de *S.* Typhimurium ont été identifiés comme étants atténués *in vivo*. La technologie STM présente sensiblement les mêmes défauts que la technique IVET. La technique STM a été adaptée à plusieurs autres bactéries pathogènes. Chez *A. pleuropneumoniae*, deux groupes différents ont utilisé cette technique afin de découvrir des gènes potentiellement importants *in vivo*.

Dans une première expérience de criblage par STM, Fuller et al. ont identifié 110 mutants potentiellement atténués, portant des mutations dans 35 loci génétiques différents (Fuller et al. 2000b). De ce groupe de 35 mutants uniques, 20 ont présenté, lors d'infections expérimentales, un index compétitif (CI; [mutant cfu/wildtype cfu]$_{input}$/ mutant cfu/wildtype cfu]$_{output}$) permettant de croire qu'ils étaient significativement atténués *in vivo*. Sept de ces 20 mutants, incluant 4 mutants présentant des CI très bas (gènes *yaeE*, *fkpA*, *tig* et *HI0379*) et 3 mutants pour des gènes précédemment identifiés lors d'une étude avec *P. multocida* (*exbB*, *atpG* et *pnp*) (Fuller et al. 2000a), ont été testés lors de protocoles d'immunisation, avec des résultats mitigés : 3 des 7 mutants ont causé des mortalités lorsqu'administrés à fortes doses.

Contrairement à l'étude de Fuller (Fuller et al. 2000b), où le nombre de mutants était nettement insuffisant pour cribler la totalité des gènes (environ 2000) présents dans le génome d'*A. pleuropneumoniae*, Sheehan et al. ont criblé plus de 2064 mutants mini-*Tn*10 lors de leur étude STM en plus de maximiser le recouvrement des bactéries dans les tissus pulmonaires (Sheehan et al. 2003). Au total, 105 mutants ont été identifiés comme étant atténués lors d'au moins deux expériences STM, et les mutations ont été localisées dans 55 gènes uniques. Plusieurs des gènes porteurs de

mutations, comme par exemple ceux impliqués dans l'export de polysaccharides capsulaires, la biosynthèse de LPS et le transport du fer, étaient déjà connus pour leur rôle dans la virulence d'*A. pleuropneumoniae*. Seulement 3 des gènes identifiés dans cet étude, soit les gènes *tig*, *pnp* et *apvD/macA*, étaient communs avec l'étude STM de Fuller et al 2000b. Onze des 55 mutants isolés présentaient des problèmes de croissance *in vitro*, indiquant qu'ils étaient vraisemblablement porteurs de mutations dans des voies métaboliques importantes. Sur les 14 mutants pour lesquels l'index de compétitivité a été mesuré, 6 ne semblaient pas atténués lorsqu'inoculés à fortes doses en cultures pures. Plusieurs études STM ont obtenu des résultats similaires (Autret et al. 2001; Maroncle et al. 2002), et les auteurs posent l'hypothèse que les gènes mutés ont probablement un effet subtil sur la virulence qui peut ne pas être perçu lors d'inoculations à fortes doses ou dans une population moins diversifiée. Fait notable, l'expérience de Sheehan et al. a mené à l'identification du deuxième système TonB chez *A. pleuropneumoniae* mentionné précédemment (page 32) (Beddek et al. 2004).

1.5.3 « Selective Capture Of Transcribed Sequences » (SCOTS)

Un des problèmes majeurs retrouvés dans la plupart des études de virulence bactérienne conduites *in vivo* est l'isolement du matériel bactérien à partir d'échantillons mixtes d'éléments procaryotes et eucaryotes. Le protocole SCOTS, qui est en fait un protocole de sélection d'ADNc, permet de contourner ce problème. D'abord suggérée par Graham et al. en 1999 lors d'études sur *Mycobacterium tuberculosis*, la technique SCOTS permet d'isoler spécifiquement l'ADNc d'origine bactérienne dans un mélange complexe obtenu après la conversion en masse d'ARN de sources procaryotes et eucaryotes en ADNc, grâce à une hybridation avec de l'ADNg bactérien biotynilé préalablement bloqué avec de l'ADN ribosomal (Graham et al. 1999) (Figure 25). Le protocole offre de plus une certaine flexibilité : si des amorces avec des séquences conservées sont utilisées lors de la synthèse de l'ADNc, il devient possible d'amplifier par PCR l'ADNc obtenus avant le cycle de capture. Il est aussi possible de conduire plusieurs cycles de captures d'ADN et d'amplification.

Une fois l'ADNc bactérien obtenu à partir des tissus de l'hôte, un cycle de capture avec de l'ADNc bactérien provenant d'une condition contrôle est effectué. Ceci permet d'éliminer l'ADNc qui est commun aux deux conditions de croissance, et ainsi d'isoler spécifiquement l'ADNc provenant d'ARNm synthétisé préférentiellement dans la condition d'infection *in vivo*.

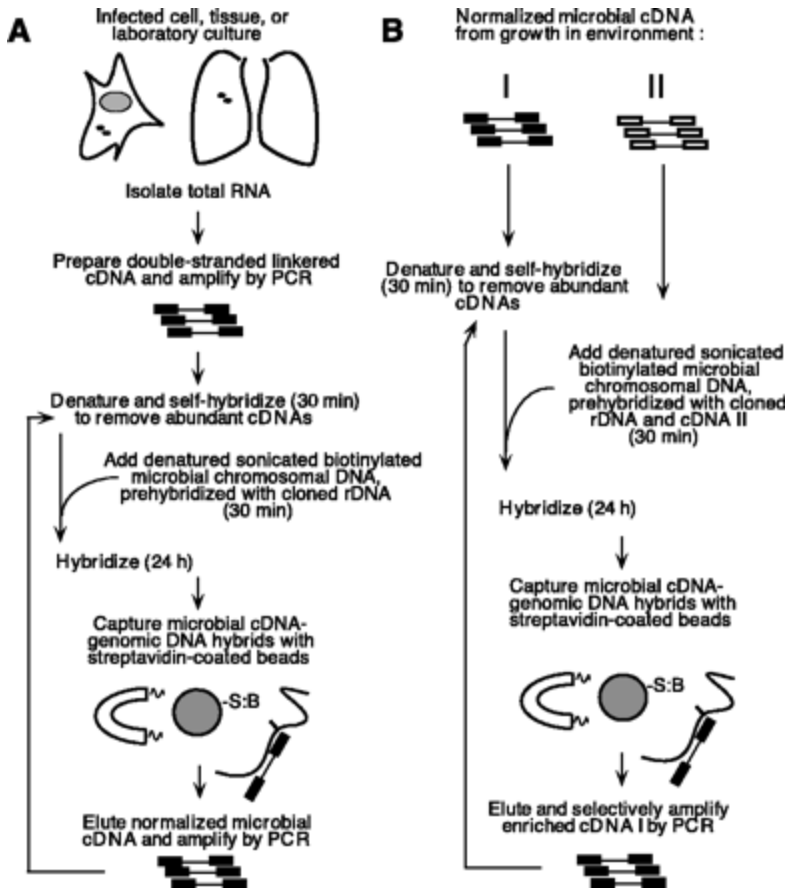

Fig. 25: Schéma original du protocole SCOTS. Tiré de Graham & al. 1999.

Bien que légèrement plus complexe que les méthodes IVET et STM, la méthode SCOTS présente néanmoins plusieurs avantages : en plus d'être applicable à toutes les espèces bactériennes dont l'ARN peut être isolé, la méthode ne dépend pas de l'existence de méthodes moléculaires pour ces espèces. Il n'est donc pas surprenant que ce protocole ait aussi été appliqué, à deux reprises, chez *A. pleuropneumoniae*. Partant de tissus nécrotiques de poumons porcins 7 jours après une infection expérimentale par *A. pleuropneumoniae*, Baltes et al. ont réusssi à identifier 46 gènes

spécifiquement exprimés *in vivo* (Baltes et al. 2004a). De ces 46 gènes, 20 avaient déjà été identifiés comme étant importants *in vivo* chez *A. pleuropneumoniae* ainsi que chez d'autres pathogènes. Parmis ces gènes, on retrouve : *apxIVA*, codant pour l'unité structurale de la quatrième toxine RTX, *apaA*, codant pour un transporteur ABC, *tbpB*, codant pour la sous-unité lipoprotéique du récepteur pour la transferrine porcine, et le gène *dmsA*, codant pour la diméthyl sulfoxide reductase. Parmi les autres facteurs de virulence identifiés, les gènes *hgbA*, codant pour le récepteur pour l'hémoglobine porcine, et le gène *hsf*, codant pour un auto-transporteur important chez *H. influenzae*, ont aussi été retrouvés.

Le même groupe a répété l'expérience trois ans plus tard, cette fois en utilisant des échantillons de tissus nécrotiques de poumons porcins récupérés 21 jours après une infection expérimentale par *A. pleuropneumoniae* (Baltes et al. 2007). À ce stade, l'infection peut être considérée comme chronique. Au total, 36 gènes ont été identifiés comme étant préférentiellement transcrits *in vivo*. De ces 36 gènes, 21 encodaient des protéines impliquées dans des voies métaboliques, et seulement 3 avaient été précédemment identifiés lors de l'étude SCOTS originale. Au niveau de la virulence, deux gènes ont surtout retenu l'attention. Le gène *hlyX* code pour une protéine homologue au régulateur anaérobie global Fnr d'*E. coli*. Puisque l'environnement est fort probablement dépourvu d'oxygène au sein des lésions nécrotiques à l'intérieur des tissus pulmonaires porcins, la présence de ce régulateur n'est pas surprenante. Tel que mentionné précédemment (page 49), un mutant Δ*hlyX* colonise d'ailleurs les poumons porcins en moins grand nombre(Baltes et al. 2005). De plus, plusieurs articles ont démontré, au cours de la dernière décennie, l'importance du métabolisme anaérobie pour la virulence d'*A. pleuropneumoniae* (Baltes et al. 2003; Baltes et al. 2004a; Baltes et al. 2005; Jacobsen et al. 2005). Finalement, cette expérience a aussi permis l'identification du gène *aasP*, codant pour une sérine protéase autotransporteur.

1.6 Projets de séquençage : les génomes complets

Les premiers efforts de séquençage génomique pour *A. pleuropneumoniae* ont été entamés en 2001, au sein de l'Université d'Oklahoma (USDA 2001). Ces efforts, bien qu'ils n'aient jamais été complétés et n'aient pas mené à des publications officielles, ont permis de voir apparaître les premières séquences génomiques d'*A. pleuropneumoniae* dans les bases de données du National Center for Biotechnology Information (NCBI). Depuis, trois séquences génomiques complètes ont été déterminées pour les souches de référence L20 (sérotype 5b), JL03 (sérotype 3) et AP76 (sérotype 7) (Foote et al. 2008; Xu et al. 2008; NCBI 2009), comportant respectivement 2017, 2097 et 2036 cadres de lecture ouverts codant potentiellement pour des protéines. Une comparaison entre les génomes des sérotypes 3 et 5b a permis de mettre en évidence une très forte similarité entre les séquences, avec quelques zones de disparités (Figure 26) (Xu et al. 2008) :

- un ilôt de séquences phagiques présent chez la souche L20
- une délétion des gènes *cysDHG* de l'opéron cys toujours chez la souche L20
- l'absence de 5 gènes (*rcpC*, *tadV*, *flp2*, *flp1* et *rhlB*) au sein de l'opéron *tad* de la souche JL03
- des différences marquées au sein des opérons codant pour les gènes de synthèse de la capsule (opéron *cps*) et les gènes de synthèse des LPS

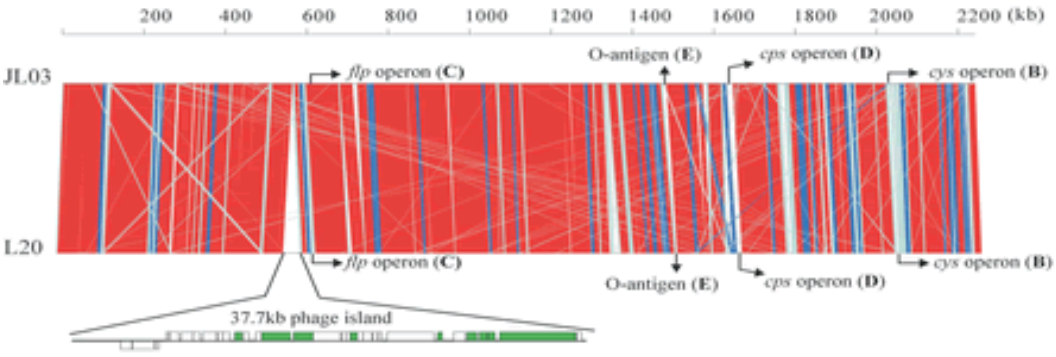

Fig. 26 : Zones de disparités entre les génomes des souches L20 et JL03. En rouge, les séquences hautement similaires de plus de 10 kb, en bleue celles de 5 à 10 kb, et en cyan celles de 1 à 5 kb. Tiré de Xu & al. 2008.

Ce dernier point n'est pas surprenant puisque le regroupement des différents isolats d'*A. pleuropneumoniae* en 15 sérotypes est effectué sur la base de différences existant au sein des polysaccharides capsulaires. De plus, des expériences effectuées avec une biopuce à ADN spécifique à la souche L20 du sérotype 5b a permis d'établir que les différentes souches d'*A. pleuropneumoniae* sont d'origine clonale

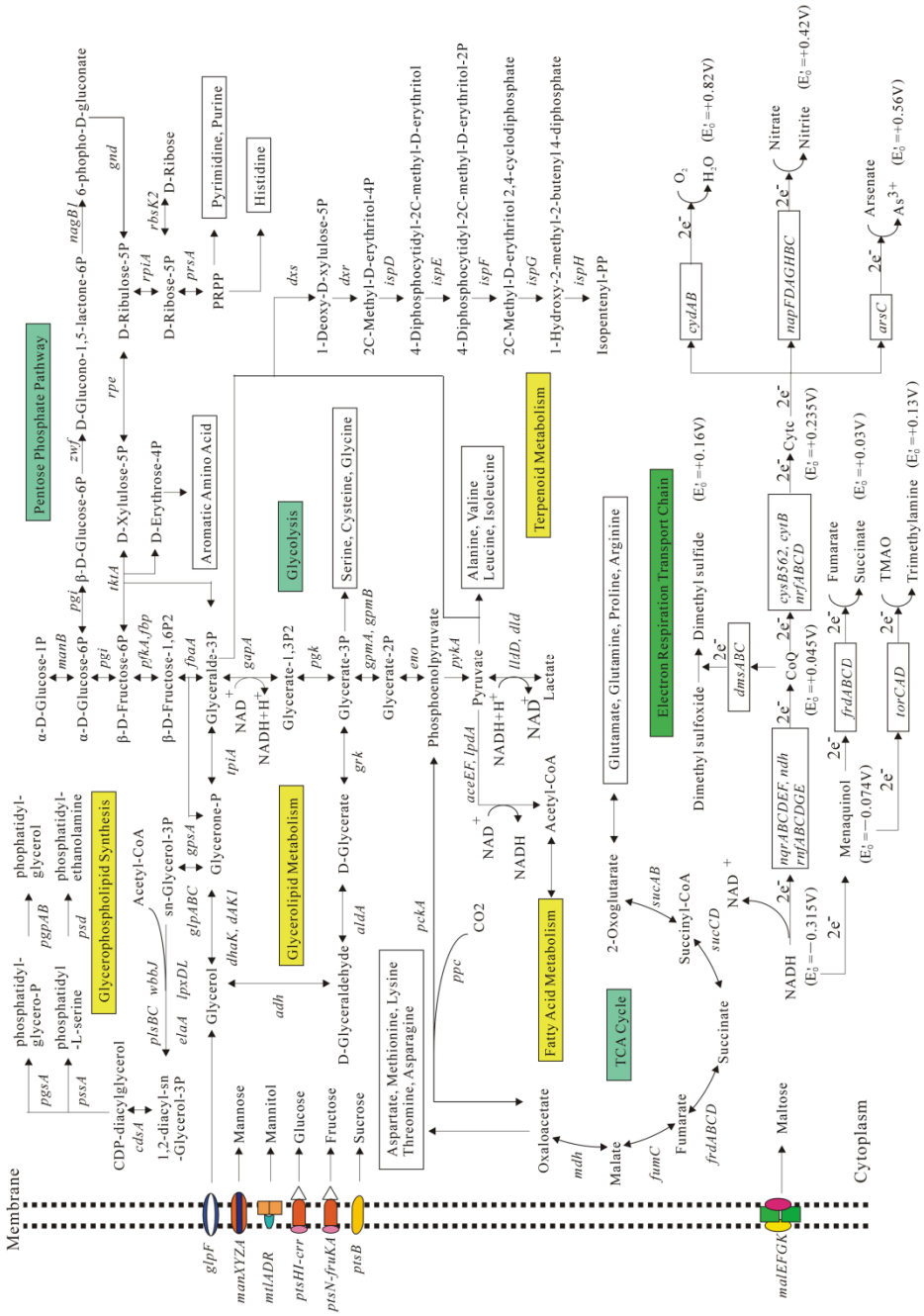

Fig. 27 : Représentation schématique des diverses voies métaboliques présentes chez *A. pleuropneumoniae* sérotype 3. Tiré de Xu & al. 2008.

(Gouré et al. 2009). L'analyse du génome d'*A. pleuropneumoniae* sérotype 3 a permis de confirmer sur le plan génétique plusieurs observations phénotypiques sur le plan métabolique, à savoir qu'*A. pleuropneumoniae* possède les enzymes nécessaires à l'utilisation de plusieurs sucres (mannose, mannitol, maltose, glucose, fructose et sucrose), peut générer de l'énergie autant par des mécanismes fermentatif que respiratoire, et que la respiration peut se faire autant en aérobie qu'en anaérobie (Figure 27) (Xu et al. 2008).

2. BIOPUCE A ADN

2.1 Historique et principe de base

Les biopuces d'ADN sont composées d'acides nucléiques cibles immobilisés sur un substrat solide servant à capturer des séquences homologues présentes dans des sondes fluorescentes (Bryant et al. 2004). La première utilisation de ce qu'on pourrait qualifier de biopuce d'ADN remonte à plus de vingt ans et à l'élaboration d'une expérience visant à identifier les gènes responsables de l'action antiprolifération de l'interféron (Kulesh et al. 1987). Le support utilisé était un papier filtre sur lequel était déposé une banque génomique des cellules HT1080, une lignée cellulaire de fibrosarcome humain, présente dans une souche d'*E. coli*. À l'époque, le recoupement des résultats obtenus, dans un premier temps, lors de la détermination des gènes affectés par l'interféron et, dans un second temps, ceux obtenus lors de la détermination des gènes actifs dans des cellules réplicatives ou quiescentes, avait permis d'identifier quatre ADNc, 1 qui était induit et 3 qui étaient réprimés, codants pour des gènes impliqués dans l'action antiproliférative de l'interféron.

Une expérience similaire fut aussi réalisée avec *E. coli* en 1993 à l'aide de membranes de nylon de 19 cm de côté comportant 441 zones d'hybridation correspondant à différentes sections du génome d'*E. coli* (Chuang et al. 1993). Les auteurs testèrent alors un grand nombre de conditions de croissance (induction à l'IPTG, choc thermique, choc osmotique, quantités restreintes de divers nutriments,

entrée dans la phase stationnaire de croissance, croissance anaérobie, croissance dans les tissus digestifs de souris gnobiotiques, ainsi que les effets de mutations pléïotropiques dans les gènes *rpoH*, *himA*, *topA* et *crp*) afin de déterminer la quantité d'évènements transcriptionnels liés à ces stimuli dans les diverses sections du génome d'*E. coli*.

À l'époque, deux facteurs limitaient énormément le développement de la technologie dont allaient naître les biopuces : l'absence de méthodes de séquençage à haut-débit et l'absence de techniques permettant de créer des puces à haute densité. Les données ont changé depuis. Depuis le séquençage du premier génome bactérien en 1995 (Fleischmann et al. 1995), les bases de données du « National Center for Biotechnology Information » ont accumulé des séquences d'ADN liées à plus de 5932 espèces vivantes, dont 1338 bactéries (NCBI) (Figure 28). En date du 2 décembre 2009, les bases de données du NCBI recensaient plus de 1016 génomes microbiens complètement séquencés, alors que 2176 autres génomes sont en cours de séquençage (NCBI 2009). La disponibilité de ces séquences génétiques permet maintenant le développement de biopuces beaucoup plus élaborées. De plus, le développement de machines capables soit d'appliquer des échantillons d'ADN sur des supports solides, soit d'effectuer la synthèse *in situ* d'oligonucléotides sur un support solide, a permis d'élaborer des biopuces d'ADN à très haute densité. Ainsi, alors que les précurseurs des biopuces actuelles pouvaient permettre d'aligner 421 zones d'hybridations sur une surface de 361 cm^2 (19 cm X 19 cm), les biopuces actuelles permettent d'aligner un génome entier,

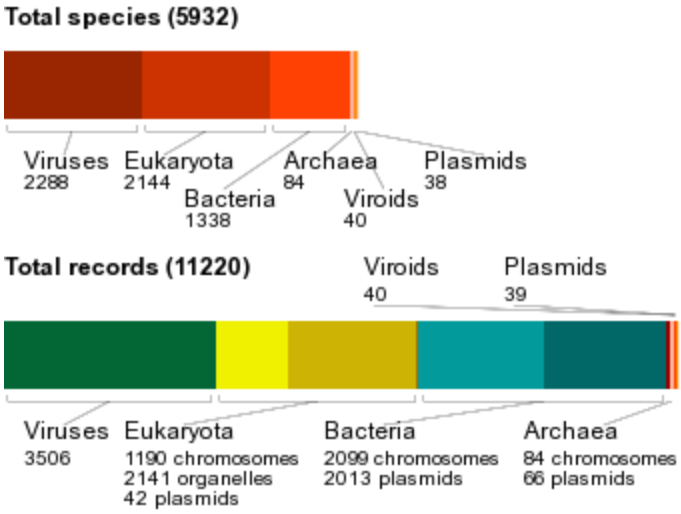

Fig. 28: Nombre d'espèces représentées par des séquences génomiques sur le site du « National Center for Biotechnology Informations » (NCBI). Tiré du site internet du NCBI, 2009.

soit sous formes de produits PCR, sois sous formes d'oligonucléotides, sur une surface de la grandeur d'une lame de microscope.

La visualisation des résultats obtenus suite à l'hybridation sur biopuces repose sur des techniques de marquages d'ADN ou d'ARN. Les marqueurs les plus fréquemment utilisés sont ceux de la famille des cyanines, plus particulièrement les cyanines-3 (Cy3) et les cyanines-5 (Cy5), qui ont la capacité d'émettre de la fluorescence suite à l'excitation par un rayon laser d'une longueur d'onde précise. Comme les deux marqueurs émettent aussi une lumière à une longueur d'onde

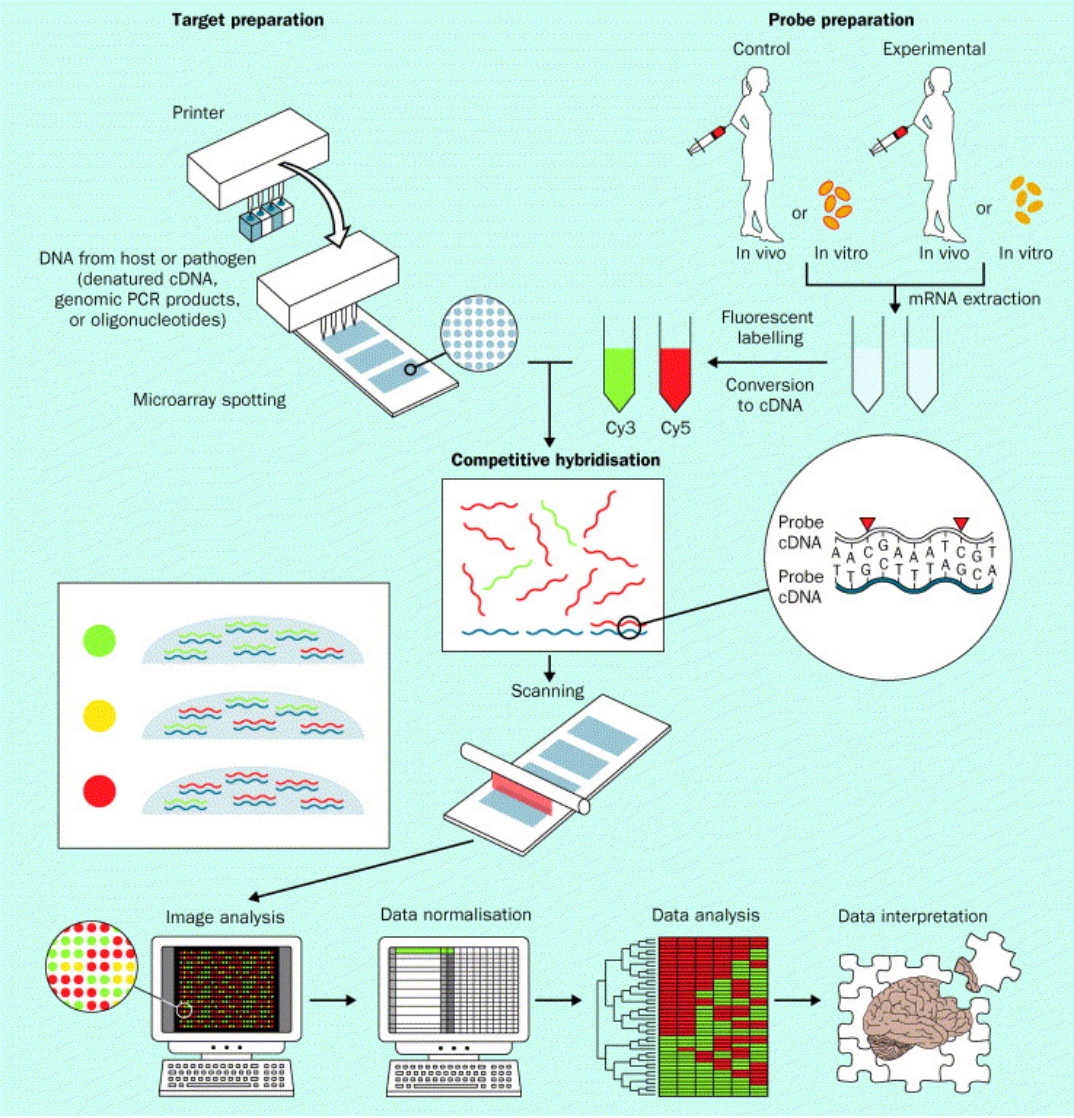

Fig. 29: Aperçu général des étapes expérimentales requises lors de l'utilisation de biopuces d'ADN dans le cadre d'expériences de profil transcriptomique. Tiré de Bryant & al,. 2004.

précise, il devient possible de quantifier de manière précise le signal associé à chaque marqueur (Figure 29). C'est aussi la raison pour laquelle plusieurs échantillons, s'ils sont marqués avec des marqueurs ayant des longueurs d'ondes d'émission différentes, peuvent être hybridés sur la même biopuce.

Les marqueurs Cy peuvent être fixés à l'échantillon génétique de manière chimique. La façon la plus simple est l'utilisation de nucléotides modifiés. Dans le cas d'expériences où l'on veut mesurer le niveau d'ARNm présent pour chaque gène dans une bactérie dans une condition précise (profil transcriptionnel), l'ARMm extrait est généralement transformé en ADNc par transcription inverse, et c'est au cours de cette étape que les nucléotides modifiés marqués sont incorporés à l'échantillon génétique.

2.2 Types de puces

De façon générale, on retrouve deux types différents de biopuces à ADN : des puces où l'information génétique, sous forme de produits PCR ou d'oligonucléotides, est « imprimée » sur le support solide, et des puces où l'information génétique est synthétisée directement sur le support sous forme d'oligonucléotides (Bryant et al. 2004). Plus souvent qu'autrement, et ce pour les deux types de biopuce, le support solide est une lame de microscope ou une plaquette de silicone.

2.2.1 Technique par impression : la biopuce classique

La première biopuce à être synthétisée via l'impression de fragments génétiques sur support solide était basée sur le génome d'*Arabidopsis thaliana*, petite plante à fleur possédant le plus petit génome eucaryote recensé jusqu'à présent (Schena et al. 1995). Dans une plaque de 96 puits, les auteurs ont regroupé 48 ADNc d'*A. thaliana* obtenus par PCR, en duplicata, et le contenu de ces 96 puits a été imprimé sur une lame de microscope recouverte de poly-L-lysine. Sur la lame, chaque point correspondant à un puit de la plaque a été imprimé à une distance de 500 μm du

suivant, et seulement 5 nl du produit PCR, à une concentration de 0.5 mg/ml était nécessaire par point et par lame. Cette biopuce a permis aux auteurs de détecter l'expression différentielle de 45 gènes chez différentes lignées cellulaires dérivés d'*A. thaliana*, mais aussi dans différentes conditions et dans différents tissus.

Cette technologie est particulièrement avantageuse pour les laboratoires qui veulent créer leurs propres biopuces à moindres coûts. Actuellement, il est possible d'incorporer jusqu'à 20000 zones d'hybridation sur les biopuces de ce type (Bryant et al. 2004).

2.2.2 Synthèse In Situ : les oligopuces

Le deuxième type de biopuces est issu d'une technologie commercialisée par la compagnie Affymetrix. Souvent appelées oligopuces, puisqu'elles sont en fait composées d'oligonucléotides plus ou moins longs représentants des sections de gènes ou de génomes, ces puces font appel à une technologie beaucoup plus complexe. La synthèse *in situ* d'oligonucléotides sur une surface solide allie la photolitographie et la chimie de la synthèse nucléotidique (Lockhart et al. 1996; Barone et al. 2001) (Figure 30). Le processus repose sur la présence, à la surface du support solide, de molécules permettant de faire le lien entre la surface et le premier oligonucléotide qui doit être ajouté, mais aussi sur l'ajout, entre chaque étape d'élongation de la chaîne d'oligonucléotides, d'un agent photo-sensible empêchant l'ajout supplémentaire de nucléotides. La photolitographie permet de dégrader cette substance photo-sensible entre chaque étape d'ajout de nucléotides seulement aux endroits précis où un nucléotide particulier doit être ajouté.

Cette technologie présente quelques avantages sur la méthode par impression : un plus grand nombre de points peut-être entassé sur une seule puce, soit jusqu'à 600 000, et plusieurs éléments différents peuvent être synthétisés pour un seul et unique gène. Cependant, la technologie impliquée est beaucoup plus dispendieuse.

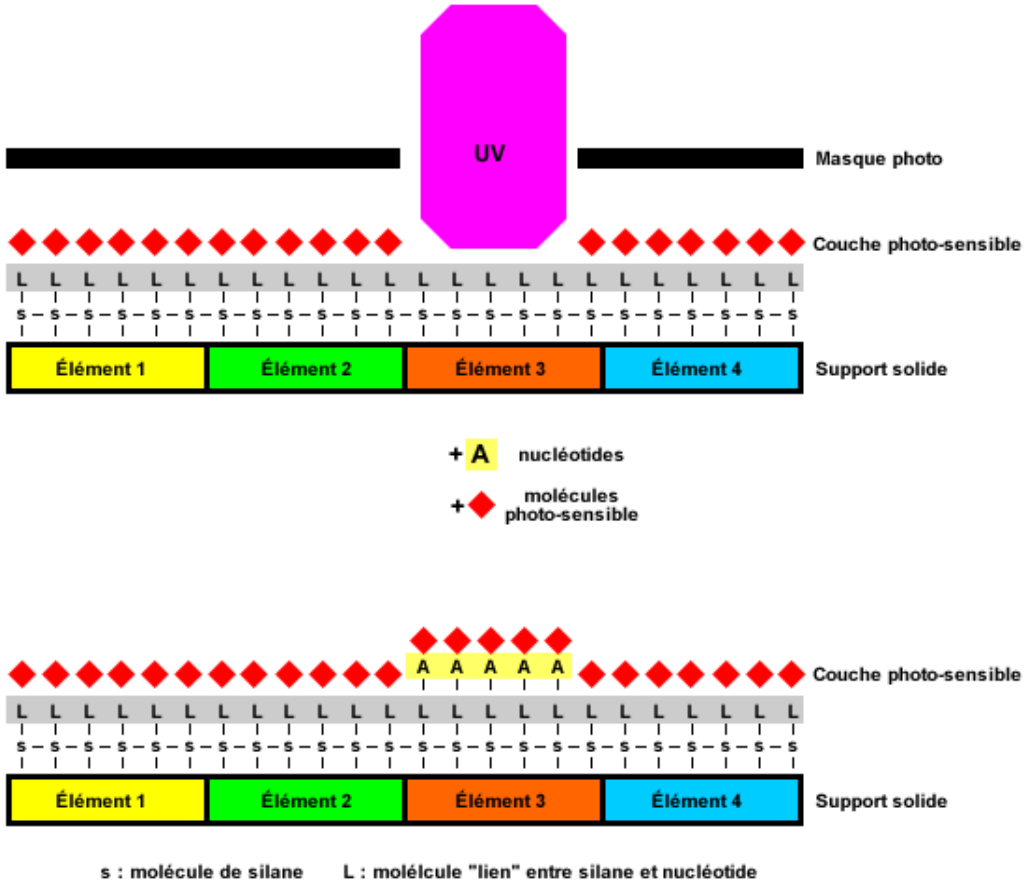

Fig. 30: Principe de synthèse d'oligonucléotides *in situ* utilisant la photolitographie. Inspiré et adapté de (Barone et al. 2001).

2.3 Types d'expériences et applications

Classiquement, il existe deux types majeurs d'expériences qui sont réalisées avec des biopuces : les expériences de génomique comparative, et les expériences d'expression génique ou de profil transcriptomique. La différence majeure entre ces expériences est le type de matériel qui est extrait de la bactérie pour procéder aux expériences : l'ADNg pour les expériences de génomique comparative, et l'ARNm pour les expériences d'expression génique. Lors d'expériences de génomique comparative, ou encore de profil génétique, l'objectif est de déterminer le contenu génétique d'une souche test par rapport à celui d'une souche de référence. Cette souche de référence est généralement celle dont le génome est imprimé sur la biopuce. Ce genre d'études est particulièrement utile avec les espèces pour lesquelles un très grand nombre de sérotypes ou de souches sont recensées. Les puces utilisées

pour ces expériences peuvent soit contenir une version complète du génome bactérien, comme celle utilisée par Taboada et al. afin de comparer différentes souches de *Campylobacter jejuni* (Taboada et al. 2004), ou encore simplement quelques gènes spécifiques à certaines souches. Par exemple, chez *E. coli*, on retrouve une grande quantité de souches non-pathogènes résidentes de la flore intestinale, mais aussi de nombreuses souches pathogènes appartenant à différents pathotypes et causant différentes pathologies. L'utilisation de biopuces d'ADN peut permettre de rapidement faire la distinction entre ces différentes souches, mais peut aussi permettre de détecter la présence de souches potentiellement virulentes dans l'environnement ainsi qu'au niveau de troupeaux d'animaux destinés à la consommation (Bekal et al. 2003).

Dans le cas d'études portant sur le profil transcriptomique, le but principal est l'observation des modifications qui se produisent au niveau de la synthèse d'ARNm suite à un changement environnemental, ou encore suite à l'apparition de mutations dans certains gènes. Alors qu'on récolte l'ADNg dans le cadre d'expériences de génomique comparative, l'ARNm est récolté pour les études transcriptomiques et les molécules récupérées dans une condition « contrôle » servent de référence lors de l'hybridation sur puce.

Les études transcriptomiques peuvent entre autres servir à étudier de manière approfondie les interactions hôte-pathogène dans le contexte de maladies infectieuses, et ce autant du point de vue de l'hôte que du point de vue de l'agent infectieux (Bryant et al. 2004). Il est donc possible de voir, à certains moments précis de l'infection, les diverses stratégies déployées par l'agent pathogène afin de circonvenir les défenses de l'hôte, ou encore les tactiques employées par l'hôte pour contenir l'infection.

2.4 Vaccinologie inverse (« Reverse Vaccinology »)

Le développement de la technologie des biopuces a permis de complémenter certaines nouvelles stratégies nées avec l'ère de la génomique, stratégies utiles, entre autre, pour la découverte de vaccins sous-unitaires. Classiquement, la recherche de cibles vaccinales impliquait qu'il fallait, suite à la croissance de la bactérie en laboratoire, séparer les composantes une à une, les identifier biochimiquement, sérologiquement ou génétiquement, avant de finalement tester leur potentiel immuno-protecteur (Rappuoli 2000). Le processus peut être très long, ne permet d'identifier que les antigènes qui peuvent être isolés et s'avère littéralement impossible si la bactérie étudiée ne peut être cultivée en laboratoire avec des méthodes conventionnelles. Le séquençage d'un nombre considérable de micro-organismes a permis d'inverser le processus de recherche d'antigènes : en partant directement des séquences nucléotidiques, il est possible d'utiliser des outils informatiques afin de générer une liste de candidats potentiellement intéressants (Figure 31). Par exemple, plusieurs algorithmes permettent de prédire avec une bonne efficacité, en se basant sur la présence de motifs conservés, quelles protéines seront exprimées à la surface de la bactérie. D'abord utilisée avec *N. meningitidis* (Pizza et al. 2000), cette stratégie a ensuite été reprise avec d'autres bactéries, dont *A. pleuropneumoniae* (Chung et al. 2007).

Dans cette optique, l'utilisation de biopuces à ADN peut permettre de cibler les candidats les plus intéressants de deux façons :

1- Dans le cas d'espèces bactériennes où des nombreuses souches et sérotypes existent, les expériences de génomique comparative peuvent permettre de déterminer quels gènes sont suffisament bien conservés à travers l'espèce entière, et donc de déterminer quelles protéines sont suffisament bien conservées pour servir de cibles vaccinales permettant de prévenir l'infection par toutes les souches.

2- Des expériences de profils transcriptionnels conduites dans des conditions simulant l'environnement rencontré par la bactérie chez l'hôte peuvent aider à déterminer quelles composantes sont potentiellement synthétisées *in vivo*.

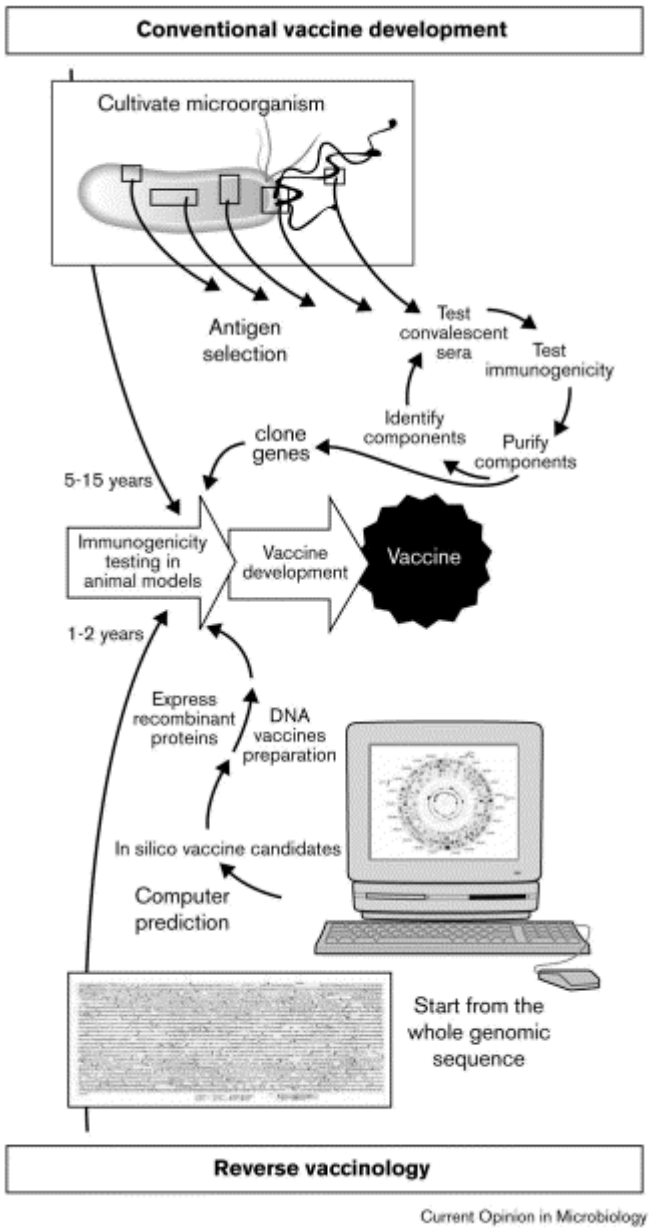

Fig. 31: Schéma comparatif entre les stratégies conventielles d'identification de cibles vaccinales et la vaccinologie inverse. Tiré de Rappuoli 2000.

2.5 Biopuces et expériences de profil transcriptionnel *in vivo*

2.5.1 Problématique

Malgré le potentiel énorme lié à l'utilisation de biopuces afin de mesurer l'expression des gènes d'agents pathogènes directement chez leur hôte, très peu d'expériences ont été conduites directement *in vivo* à ce jour. Ceci peut s'expliquer par la présence de plusieurs obstacles techniques qui doivent être surmontés afin de pouvoir travailler avec des échantillons recueillis *in vivo* (Hinton et al. 2004):

- les conditions expérimentales doivent permettre de recueillir un nombre suffisant de bactéries pour pouvoir obtenir des quantités d'ARN adéquates
- le matériel eucaryote doit être séparé du matériel procaryote, soit avant ou après l'extraction d'ARN
- l'extraction d'ARN doit se faire rapidement après l'échantillonnage, ou l'ARN doit être stabilisé rapidement en arrêtant la transcription ou la dégradation intra-cellulaire

Dans bien des cas, il est donc beaucoup plus facile de travailler avec des protocoles expérimentaux permettant de mimer, jusqu'à un certain point, l'environnement *in vivo*. Ainsi, dans plusieurs cas, des expériences sont conduites avec des lignées cellulaires immortalisées (Faucher et al. 2006; Waddell et al. 2007; Corcionivoschi et al. 2009; Jandu et al. 2009), ou encore avec des technologies *ex vivo* (Batisson et al. 2003; Silva et al. 2008). Bien que ce type d'expériences puisse permettre de maximiser le nombre de bactéries recueillies en augmentant la dose expérimentale en plus de permettre l'isolation rapide de l'ARN, la séparation du matériel eucaryote du matériel procaryote demeure problématique. Dans le cas des études conduites par Faucher et al. (Faucher et al. 2006), les expériences de biopuces ont été combinées avec le protocole SCOTS afin d'éliminer les transcrits eucaryotes, mais des rondes d'amplification par PCR ont aussi été ajoutées par la suite afin d'augmenter la quantité d'ARN bactérien obtenu. Plusieurs chercheurs ont soulevé des doutes sur

l'utilisation du PCR pour l'amplification d'échantillons destinés à être hybridés sur biopuce : l'amplification exponentielle par PCR peut en effet modifier la relation initiale existant entre deux échantillons (Kacharmina et al. 1999).

2.5.2 Amplification d'ARN : T7 polymérase et PCR balancé

Différentes méthodes ont été développées, au cours des dernières années afin de permettre l'amplification linéaire d'ARNm dans le but de générer des résultats de profil transcriptomique à partir de cellules uniques. La méthode la plus populaire est celle qui consiste à utiliser l'enzyme ARN polymérase T7 dans une réaction de transcription *in vitro* (Phillips et al. 1996; Kacharmina et al. 1999; Eberwine et al. 2001; Ginsberg et al. 2006) (Figure 32).

Classiquement, l'ARNm est d'abord transformé en ADN double-brin en tirant avantage de la présence de la queue poly-A existant à la partie 3' terminale de l'ARN messager eucaryote. L'utilisation d'une amorce poly-T, portant aussi un promoteur spécifique pour la T7 polymérase, permet donc la synthèse du premier brin d'ADN avec une transcriptase inverse, ce qui génère une molécule hybride ARN –ADN. La synthèse subséquente du deuxième brin s'effectue à l'aide d'une ADN polymérase, et l'ARN est digéré simultanément par une RNAse. Finalement, la réaction de transcription *in vitro*, menée par la T7 polymérase, permet de synthétiser de nouveaux fragments d'ARN, appelé ARNa, de façon linéaire. Il est à noter que pour amplifier de l'ARN d'origine bactérienne, il faut d'abord ajouter une queue poly-A aux ARNm à l'aide d'une poly(A) polymérase d'*E. coli* (Botero et al. 2005). Des variations de cette technique existent (Ginsberg 2005).

Une autre technique, baptisée « PCR balancé », permet de passer outre les limitations initiales reliées à l'utilisation du PCR (Makrigiorgos et al. 2002). Dans cette technique, les deux populations d'ADN (ou ADNc) à comparer sont digérer par la même enzyme avant d'être lié à deux séquences marqueurs distinctes. Ces deux séquences comportent une section commune (nommée P1) et des sections distinctes

(P2a et P2b). Les deux populations sont ensuites mélangées et amplifiées dans la même réaction grâce à une amorce spécifique à P1. Tout biais possiblement incorporé dans la réaction PCR est donc incorporé aux deux populations. Par la suite, il est possible de marquer différement, par incorporation de nucléotides fluorescents, les deux populations par élongation d'amorces à partir des séquences P2a et P2b respectivement.

Une comparaison récente de ces deux méthodes a permis de déterminer que les résultats générés suite à l'hybridation sur biopuce sont relativement fiables et reproductibles, mais seulement avec les séquences ayant un fort signal d'hybridation (Lang et al. 2009). Néanmoins, ces techniques demeurent relativement peu utilisées jusqu'à maintenant.

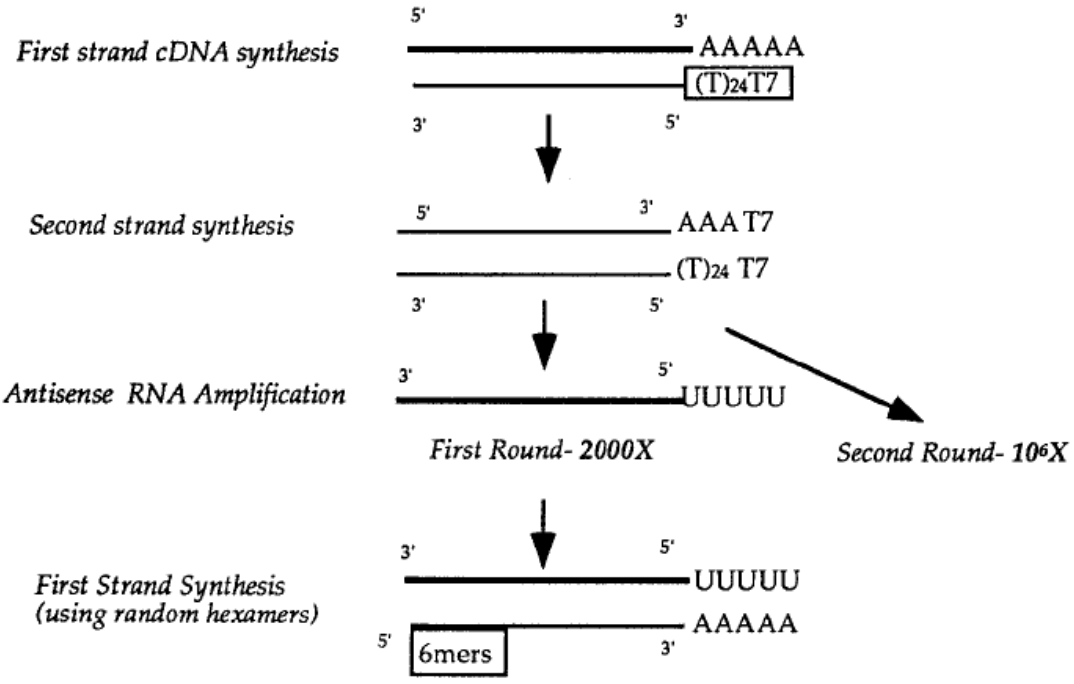

Fig. 32: Protocole d'amplification d'ARN bactérien. L'ARN bactérien doit d'abord être modifié avec une poly-A polymérase (étape non-illustrée) afin de lui ajouter une queue poly-adénylée. L'utilisation d'amorces poly-T comportant un promoteur pour l'enzyme T7 polymérase permet l'amplification subséquente linéaire de l'ARN ainsi modifiées. Tiré de Philips et al. 1996.

2.5.3 Expériences in vivo : infections expérimentales et naturelles

Alors que plusieurs laboratoires ont tenté d'élucider les mécanismes de réponse de l'hôte face à des agents infectieux (Li et al. 2009; Niewold et al. 2009; Sarson et al. 2009; Stockhammer et al. 2009), expériences qui ont d'ailleurs été réalisées chez le porc lors d'infections à *A. pleuropneumoniae* (Moser et al. 2004; Hedegaard et al. 2007), l'inverse n'a été tenté qu'à peu de reprises possiblement à cause des limitations techniques mentionnées précédemment. Aussi, dans les rares cas où des expériences ont été conduites, des modèles animaux sont utilisés et les protocoles d'infection sont élaborés minutieusement afin de contourner certains obstacles, plus particulièrement en ce qui concerne la quantité de matériel pouvant être recueilli suite à l'infection. Ainsi, afin de maximiser le nombre de bactéries pouvant être récupérées suite à l'infection, les chercheurs utilisent couramment des doses infectieuses supérieures à celles naturellement rencontrées par l'hôte, ou encore des voies d'inoculation différentes de celles normalement rencontrées lors d'infections naturelles. Quelques exemples tirés de la littérature :

- Talaat et al. 2004 : Afin d'étudier l'infection causée par *Mycobacterium tuberculosis*, les souris sont infectées avec 10^3 ou 10^5 cfu/ml en fonction du temps d'incubation (dose plus forte pour les temps plus courts) (Talaat et al. 2004). Selon les données de l'Agence de Santé Publique du Canada, la dose infectieuse pour *M. tuberculosis* est de 10 bacilles (Agence de la Santé Publique du Canada 2001).

- Snyder et al. 2004 : Lors de l'étude du transcriptome d'*E. coli* uropathogénique, le souris sont initiallement infectées avec 5×10^9 cfu par la voie transuréthrale, et les animaux sont ré-infectés après six jours pour garder la concentration de bactéries entre 2×10^6 et 3×10^7 par ml d'urine afin de maximiser les rendements en ARN (Snyder et al. 2004).

- Stintzi et al. 2004 : Pour étudier les infections causées par *Campylobacter jejuni* chez le lapin, des sections d'iléon de 20 cm sont ligaturées aux extrémités directement chez l'animal anesthésié et sont infectées avec 10^{11} cfu pendant 24 à 48 heures (Stintzi et al. 2005). Le milieu intestinal, qui est normalement dynamique, est donc complètement statique pour la durée de l'expérience.

On peut donc affirmer que, même dans le cas de protocoles expérimentaux utilisant l'hôte naturel ou encore un modèle animal, et malgré le fait que les données générées apportent beaucoup de réponses quant aux mécanismes impliqués dans la pathogénèse, souvent les conditions utilisées ne représentent que partiellement les conditions réelles d'infection. À ce chapitre, seulement trois études de profil transcriptomique d'agents bactériens ont été conduites, à ce jour, directement chez l'hôte lors d'une infection naturelle, dont deux ont porté sur le transcriptome complet des agents infectieux étudiés. Dans un premier temps, LaRocque et al. ont déterminé le trancriptome de *Vibrio cholerae*, agent responsable du choléra humain, après avoir récupéré les bactéries directement d'excrétions de patients malades dans les stades précoces et tardifs de l'infection (Larocque et al. 2005). Puisque l'infection causée par *V. cholerae* entraîne des vomissements, dans le stade précoce, et des diarrhées dans le stade tardif, et puisque la bactérie est présente en très grande quantité dans les deux types d'échantillons, la récolte d'une quantité de matériel acceptable n'est pas problématique. La comparaison des transcriptomes des phases précoce et tardive a permis d'identifier 42 gènes différentiellement exprimés entre les deux conditions de croissance, et de déterminer que le pili de type IV TCP (« **T**oxin **C**orregulated **P**ilus ») est plus fortement exprimé dans la phase précoce de l'infection.

Dans un deuxième temps, Son et al. ont comparé le transcriptome d'isolats de *Pseudomonas aeruginosa* recueillis directement d'excrétions pulmonaires d'un patient souffrant de fibrose kystique au transcriptome des mêmes isolats suite à la croissance sur un milieu de croissance défini (Son et al. 2007). Chez ce patient, l'infection à *P. aeruginosa* était chronique et durait depuis plusieurs années. Cette

période d'évolution à l'intérieur de l'hôte explique probablement pourquoi Son et al. ont identifié plus d'une centaine de gènes, dont plusieurs classiquement impliqués dans la pathogénèse de *P. aeruginosa* (dont les gènes *alg*, impliqués dans la biosynthèse de l'alginate nécessaire à la formation de biofilms), pour lesquels les mécanismes de régulation connus semblaient inopérants. Ainsi, ces gènes semblaient être dérégulés chez la souche isolée *in vivo* comparativement à ce qui pouvait être observé chez une souche de référence. Finalement, au cours d'une étude avec une biopuce ne comprenant que les gènes du régulon Fur de *N. gonorrhoeae*, Agarwal et al. ont pu démontrer que plusieurs gènes de ce régulon sont exprimés chez des patientes atteintes de gonorrhée (Agarwal et al. 2008).

2.6 Critiques sur l'utilisation des biopuces : « The MicroArray Quality Control (MAQC) Project »

Depuis 1995, la quantité de publications ayant un lien avec les biopuces à ADN a augmenté de façon exponentielle pour atteindre un grand total d'environ 25 000 (Coppee 2008). Au cours de cette période, l'enthousiasme initial soulevé par la technologie des biopuces a regressé quelque peu alors que plusieurs scientifiques eurent questionné la reproductibilité des résultats obtenus entre diverses plate-formes et différents laboratoires pour une même expérience. Par exemple, une expérience identique fut menée par trois groupes différents afin de découvrir des gènes importants pour le développement de trois lignées de cellules souches, et les résultats de ces études furent publiés pratiquement au même moment (Ivanova et al. 2002; Ramalho-Santos et al. 2002; Fortunel et al. 2003). Étrangement, malgré le fait que les mêmes conditions aient été utilisées pour les trois expériences, les listes de gènes obtenues étaient largement différentes.

Étant donné l'énorme potentiel de la technologie biopuce, un projet regroupant 137 chercheurs de 51 organisations différentes fut mis sur pied afin de mesurer la reproductibilité d'expériences de biopuce entre différentes plate-formes, et différents sites expérimentaux (Shi et al. 2006). Apr ès 1300 hybridations, il a été établi que les

éléments responsables de disparités entre différentes expériences sont la qualité de l'échantillon biologique ainsi que le facteur humain. Il fut démontré que différentes techniques et différentes plate-formes utilisées dans différents lieux peuvent aisément générer des résultats concordants en autant que les procédures soient bien standardisées et que le matériel utilisé soit de bonne qualité.

3. APPROCHE ET DEMARCHES EXPERIMENTALES

3.1. Étude de l'expression des gènes d'*Actinobacillus pleuropneumoniae* dans des conditions *in vitro* contrôlées simulant l'environnement *in vivo*

Bien que plusieurs facteurs de virulence importants aient été identifiés chez *A. pleuropneumoniae*, l'implication réelle de ces composantes dans le processus infectieux ainsi que les mécanismes régulatoires régissant leur expression demeurent nébuleux. Par exemple, malgré que les différentes toxines Apx soient connues et étudiées depuis plusieurs années, les mécanismes entourant l'expression des gènes nécessaires à la synthèse de ces protéines sont peu connus (voir section 1.3). D'autre part, il est connu que certains facteurs de virulence d'*A. pleuropneumoniae* sont plus fortement exprimés *in vivo* qu'*in vitro*, comme par exemple les toxines et les fimbriaes (voir section 1.3.6). De plus, aucune adhésine de nature protéique n'a été formellement identifiée jusqu'à présent.

Dans le but d'identifier les signaux pouvant intervenir dans la régulation des nombreux facteurs de virulences d'*A. pleuropneumoniae*, mais aussi afin d'identifier de nouveaux gènes importants pour la pathogénèse d'*A. pleuropneumoniae*, nous allons :

- déterminer le profil transcriptomique d'*A. pleuropneumoniae* dans des conditions de croissance simulant l'environnement rencontré chez l'hôte. Ces conditions incluent la croissance en conditions de restriction en fer en présence d'EDDHA, un agent chélateur spécifique aux ions ferriques, la croissance dans des conditions favorisant la formation d'un biofilm, la mise en contact avec du liquide de lavages broncho-alvéolaire et la croissance, lors d'expériences de culture cellulaire, dans le milieu de culture présent directement au-dessus de cellules pulmonaires épithéliales (cellules SJPL), ou directement suite à l'adhésion à ces cellules

- comparer les différentes listes de gènes générées afin d'identifier les gènes qui sont exprimés dans plusieurs ou toutes les conditions expérimentales

3.2. Étude de l'expression des gènes d'*Actinobacillus pleuropneumoniae* lors d'une infection naturelle chez l'hôte naturel

Plusieurs études ont été menées afin de découvrir des gènes importants pour la croissance et la survie d'*A. pleuropneumoniae* chez son hôte naturel, le porc (voir section 1.5). Via l'utilisation des protocoles IVET, STM et SCOTS chez l'hôte, il a été possible de découvrir l'existence d'un deuxième système TonB chez *A. pleuropneumoniae* (Beddek et al. 2004), ainsi que de mettre en évidence l'importance des enzymes du métabolisme anaérobique à différents stades de l'infection (Baltes et al. 2004a; Baltes et al. 2007). Cependant, toutes ces expériences ont été conduites dans le cadre d'infections expérimentales bien contrôlées, et à l'aide de moyens techniques ne permettant pas d'obtenir une vue globale des changements transcriptomiques se produisant chez la bactérie au cours de l'infection. Parallèlement à nos expériences, des résultats obtenus dans notre laboratoire ont permis de produire une liste de gènes conservés chez tous les sérotypes d'*A. pleuropneumoniae* (Gouré et al. 2009), alors que des expériences menées par des collaborateurs ont parmi d'identifier les protéines d'*A. pleuropneumoniae* qui sont présentes à la surface de la bactérie (Chung et al. 2007).

Afin d'obtenir un portrait plus complet des différents changements transcriptionnels induits par la croissance chez l'hôte, et aussi dans le but d'identifier les gènes exprimés au cours du processus d'infection afin de générer une liste de cibles potentielles pour l'élaboration de vaccins sous-unitaires, nous allons :

- isoler, à partir de lésions pulmonaires produites dans le cadre d'une infection naturelle à *A. pleupneumoniae*, la souche virulente afin de caractériser son contenu génétique par hybridations génomiques comparatives

- élaborer un protocole expérimental afin de pouvoir recueillir en quantités suffisantes de l'ARNm bactérien de bonne qualité directement des tissus porcins
- conduire des expériences de profil transcriptionnel afin de comparer les gènes qui sont transcrits *in vivo* à ceux qui sont transcrits dans un milieu riche *in vitro*
- comparer la liste des gènes découverts avec ceux identifiés lors d'expériences précédentes afin d'obtenir une liste de gènes conservés chez tous les sérotypes d'*A. pleuropneumoniae*, exprimés *in vivo* et qui codent pour des protéines ou lipoprotéines de surface

MÉTHODOLOGIE ET RÉSULTATS

Article 1

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Transcriptional profiling of *Actinobacillus pleuropneumoniae* under iron-restricted conditions

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§ Élaboration et réalisation des expériences de profil transcriptionnel, analyse des résultats et rédaction du manuscrit.

Abstract

Background

To better understand effects of iron restriction on *Actinobacillus pleuropneumoniae* and to identify new potential vaccine targets, we conducted transcript profiling studies using a DNA microarray containing all 2025 ORFs of the genome of *A. pleuropneumoniae* serotype 5b strain L20. This is the first study involving the use of microarray technology to monitor the transcriptome of *A. pleuropneumoniae* grown under iron restriction.

Results

Upon comparing growth of this pathogen in iron-sufficient *versus* iron-depleted medium, 210 genes were identified as being differentially expressed. Some genes (92) were identified as being up-regulated; many have confirmed or putative roles in iron acquisition, such as the genes coding for two TonB energy-transducing proteins and the hemoglobin receptor HgbA. Transcript profiling also led to identification of some new iron acquisition systems of *A. pleuropneumoniae*. Genes coding for a possible Yfe system (*yfeABCD*), implicated in the acquisition of chelated iron, were detected, as well as genes coding for a putative enterobactin-type siderophore receptor system. ORFs for homologs of the HmbR system of *Neisseria meningitidis* involved in iron acquisition from hemoglobin were significantly up-regulated. Down-regulated genes included many that encode proteins containing Fe-S clusters or that use heme as a cofactor. Supplementation of the culture medium with exogenous iron re-established the expression level of these genes.

Conclusions

We have used transcriptional profiling to generate a list of genes showing differential expression during iron restriction. This strategy enabled us to gain a better understanding of the metabolic changes occurring in response to this stress. Many new potential iron acquisition systems were identified, and further studies will have to be conducted to establish their role during iron restriction.

Background

Actinobacillus pleuropneumoniae, etiological agent of porcine pleuropneumonia, causes great commercial losses to the swine industry worldwide [1]. Transmission of this highly contagious disease that affects pigs of all ages occurs mostly by aerosol and close contact with infected animals [2]. During 24 to 48 hours of the acute phase of the disease, formation of extensive and fibrinohemorrhagic lung lesions is often fatal. Animals that survive the disease may become asymptomatic carriers of the bacteria, developing localized and necrotizing lesions associated with pleuritis [3]. Based on differences of capsular polysaccharides, fifteen serotypes have been identified: serotypes 1 to 12 and 15 belong to biotype 1, which is NAD-dependent; serotypes 13 and 14 are classified in biotype 2, which is NAD-independent [4]. In North America, serotypes 1, 5 and 7 are prevalent, while serotypes 2 and 9 are most commonly found in Europe.

Despite many years of research, the total complement of bacterial components that are involved in infection by *A. pleuropneumoniae* has yet to be identified. Several virulence factors have been proposed: capsular polysaccharides, lipopolysaccharides (LPS), Apx toxins and various iron acquisitions systems [2]. However, the overall contribution of each component to the infection process remains unclear. Although less virulent, an acapsular mutant was still serum-resistant, showed higher adhesion to piglet tracheal frozen sections and could still be re-isolated from lungs of infected animals [5]. LPS apparently plays a role in adhesion *in vivo,* as these molecules show *in vitro* adhesion to many biological components [4]. The Apx toxins contribute to development of lesions typically associated with the disease [6] and mutants missing Apx toxins are avirulent in pigs and mice [7]. However, different *A. pleuropneumoniae* serotypes secrete different sets of Apx toxins, and the relative contribution of the four different Apx toxins (ApxI to IV) is still not clear.

Low availability of iron in the host represents a major stress for bacterial pathogens and is considered a signal that leads to significant changes in cell processes. Iron

atoms are often linked with sulphur in Fe-S clusters in the catalytic core of enzymes involved in diverse functions such as respiration, ATP generation, and DNA replication and repair, which might account for this phenomenon. Iron is an essential element for almost every living organism. However in the host, molecules such as transferrin, lactoferrin, haptoglobin and hemoglobin in extra-cellular fluids bind free iron and iron-containing molecules very tightly [8]. While bacteria generally need free iron concentrations of about 10^{-7} M, its concentration may be 10^{-24} M in the mammalian host [9]. To counteract the effect of these iron-withholding mechanisms of the host, bacteria have evolved different iron acquisition systems, often relying on the secretion of siderophores, small (<1000 Da) molecules with high affinity for iron, or on surface receptors specific for iron-containing host proteins [10]. Studies in our laboratory have led to the identification, expression and characterization of the *A. pleuropneumoniae* hydroxamate siderophore receptor FhuA [11, 12] and a hemoglobin binding receptor HgbA [13]. *A. pleuropneumoniae* also possesses a transferrin receptor complex composed of two outer membrane (OM) proteins: a 100 kDa TbpA may form a transmembrane channel enabling transport of iron across the OM; a 60 kDa lipoprotein TbpB acts as an auxillary molecule [2, 14, 15]. Energization of these OM transporters relies on the transduction of the proton motive force from the cytoplasmic membrane (CM) by the TonB-ExbB-ExbD complex [16] that is anchored in the CM and spans the periplasm. In *A. pleuropneumoniae*, two different TonB systems have been identified: genes *tonB1-exbB1-exbD1* are transcriptionally linked to the *tbpA-tbpB* genes [17]; and a second system with genes *tonB-exbB2-exbD2* was also identified [18]. Transport of iron across the CM is apparently accomplished by the AfuABC ABC transporter [19]. It has also been shown that *A. pleuropneumoniae* can use exogenous siderophores and may be able to secrete an iron chelator in response to iron stress [20].

The ferric uptake regulator Fur protein has been identified in many pathogenic bacteria, including *A. pleuropneumoniae* [21]. Using Fe^{2+} as a cofactor, the Fur protein can interact with a specific sequence termed the Fur box in the promoter region of genes implicated in iron acquisition processes, thereby repressing

transcription. When iron becomes scarce, the Fur protein loses its cofactor and becomes inactive. The fact that transcription of some genes seems to be under positive control of active Fur protein [22, 23] was recently explained by the discovery of RyhB, a small non-coding RNA which belongs to the Fur regulon [24]. When transcribed, the RyhB RNA down-regulates the mRNA level of those genes that seemed to be positively regulated by Fur.

To better understand the mechanisms used by *A. pleuropneumoniae* that address iron restriction and to gain insights into strategies used by this pathogen under conditions mimicking the *in vivo* environment, we evaluated gene expression profiles of *A. pleuropneumoniae* grown under iron restriction. Our study identified 210 differentially expressed genes, of which 92 are up-regulated. Within the latter set, components of previously unrecognized iron acquisition systems were identified: a putative enterochelin-like siderophore receptor, a potential Yfe system for the acquisition of chelated iron, a putative hemoglobin acquisition system homologous to the *N. meningitidis* HmbR system, and a putative Fe^{2+}-specific porin system.

Results and Discussion

Microarray analysis of mRNA levels during growth of *A. pleuropneumoniae* under iron-restricted conditions

To assess the response of *A. pleuropneumoniae* to iron restriction, the reference strain S4074 was grown in BHI broth containing 50 μg/ml EDDHA, a concentration sufficient to cause iron restriction [11]. This strain was chosen because it is the strain that has been the most studied over time, but also because major problems were encountered with RNA extraction from strain L20. Preliminary CGH studies conducted in our lab showed that 95% of the genes of the *A. pleuropneumoniae* 5b L20 genome are conserved between both strains. Growth curves established the optimum growth phase for RNA extraction (data not shown). At 50 μg/ml of EDDHA, bacterial growth is almost completely inhibited within an hour of addition. By adding the iron chelator at an optical density of 0.1, iron-restricted cultures and

iron-rich cultures were harvested concurrently at an optical density of 0.3. Under these growth conditions, we identified 210 differentially expressed genes, with an estimated false discovery rate (FDR) of 3.22%: 118 were down-regulated (Table 1) and 92 were up-regulated (Table 2). In order to confirm that these variations were not caused by the chelator, control experiments where iron was supplemented to the restricted medium were conducted. Exogenous iron, in the form of $FeCl_3$, was added to a final concentration of 50 μg/ml to the iron-depleted medium. Growth curves indicated that this concentration of $FeCl_3$ was sufficient to promote growth at a similar level as in the BHI broth. Under these conditions, the expression pattern was highly similar to that seen in BHI broth: we identified only 30 differentially expressed genes, out of 2025, with an estimated FDR of 2.5%, 26 of which were up-regulated, while only 4 were down-regulated (data not-shown). Only 12 genes significantly differentially expressed in the iron-supplemented medium were identified as such in the iron-depleted *versus* BHI broth experiment, but with reversed levels of variation. Gene *lldD* (ap2032), which was up-regulated in the iron-depleted medium, was down-regulated in the iron-supplemented medium. Conversely, 11 genes that were down-regulated in the iron-depleted medium were up-regulated in the iron-supplemented medium. This indicates that the results obtained in the iron-depleted versus BHI broth experiment can be attributed to iron restriction, and not to another effect of the chelator.

Validation of microarray results by qRT-PCR

Seventeen genes, representing a wide range of $\log_2$ ratio values, were selected for transcript level analysis using qRT-PCR. Seven genes were overexpressed during iron restriction (*tonB1*, *hgbA*, *omp64*, *fetB2*, *apxIC*, PM0741, NMB1668); eight genes were repressed (*nrfA*, *nrfC*, *nfrE*, *ompW*, *dcuB2*, *dmsA*, *torA*, *ccmC*); two genes were not affected (*pedD*, *ap1465*). We also investigated the transcript level of the *exbB1*, *exbD1* and *tbpA* genes, all known to be transcriptionally linked to *tonB1* [17] and previously used as positive controls to assess iron restriction [12]. However, they were not present on the AppChip1 as this region of the genome was in one of the few unsequenced areas when the microarrrays were designed. In all cases, genes

that had been identified as up- or down-regulated with the microarrays were confirmed by the qRT-PCR experiments. The *exbB1*, *exbD1* and *tbpA* genes were also up-regulated. Genes not affected showed low level of variation during qRT-PCR analysis, and show good correlation with other results (Fig.1). Overall, there was good correlation between the $\log_2$ ratios measured by microarray and $\log_2$ ratios from qRT-PCR data (R^2=0.87). The $\log_2$ ratios observed with qRT-PCR were usually superior to those observed with the microarray. This outcome has been observed before [25, 26] and probably reflects the detection limit of microarrays as well as the complex normalization methods that are used prior to the analysis.

Genes expressed differentially under iron restriction

To evaluate the effect of iron restriction on the porcine pathogen *A. pleuropneumoniae*, we performed microarray hybridization experiments. Given that iron plays a vital role in metabolic pathways through its presence in the structure of numerous enzymes [27] and its implication in the regulation of genes associated with virulence [28], we recorded important changes in the transcriptome of the bacteria under iron-restricted conditions. A total of 210 genes showed differential expression and the functional classification of these genes provides a significant overview of changes occurring in the bacteria. Numerous microarray studies have investigated effects of iron restriction in many different pathogens, including *E. coli* [29], *H. pylori* [30], *H. parasuis* [31], *N. gonorrhoeae* [25], *N. meningitidis* [32], as well as *Pasteurella multocida* [33], a well known animal pathogen closely related to *A. pleuropneumoniae*. Many genes that were identified as being iron-regulated in the *P. multocida* study were homologs of some genes that were also identified in our study (Table 3), thus emphasizing the importance of their regulation during iron restriction. A common feature in all these studies is the high induction of genes related to iron acquisition as the products of these genes are essential for survival of the bacteria.

(i) Down-regulated genes. Down-regulated genes (Fig. 2) mostly belong to the functional class termed “Energy Metabolism”; 42 of the 118 repressed genes (35%) belong to this group, and they are amongst the most highly repressed. Almost all

these genes encode proteins with Fe-S clusters, that use heme molecules as cofactors, or that are activated by Fe^{2+} or other divalent cations. These include genes coding for the different subunits of formate dehydrogenase (*bisC, hybA, fdhE*), fumarate reductase (*frdABC*), nitrate reductase (*nfrABC*), nitrate/trimethylamine oxidoreductase (TMAO) I and III (*torAC* and *torYZ*), dimethyl sulfoxide reductase (*dmsA*) and glycerol-3-phosphate dehydrogenase (*glpAC*). These enzymes as well as numerous others that encode either cytochrome components or functional partners (*cydAB, ccp*), cytochrome maturation proteins (*ccmCF, nfrE*) or iron-sulfur electron transport proteins (*napF, hyaA, ykgF*), are all implicated in the electron transport respiratory chain, either as electron donor or acceptor during aerobic and anaerobic respiration. Other genes in this category are involved in pathways of sugar metabolism such as fermentation (*pflB, adh2*), glycolysis or gluconeogenesis (*pgk, pfkA, gapA*) and the TCA cycle (*fumC, maeA*).

Many genes that are assigned to this category have been demonstrated or proposed to be members of the FNR regulon. The *E. coli* FNR transcriptional regulator is an oxygen-responsive activator implicated in the switch from aerobic to anaerobic metabolism in facultative anaerobes [34]. The oxygen-sensing domain of the FNR protein contains a Fe-S cluster, which is likely oxidized under aerobic conditions, thereby inactivating the FNR protein. Genes coding for fumarate and nitrate reductase are known to be influenced by FNR [35], as well as genes coding for anaerobic enzymes involved in the utilization of alternative terminal electron acceptors such as TMAO [36]. Sequence analysis in *H. influenzae* has identified conserved FNR binding motifs upstream of the *cydAB* genes [37]. These genes are usually considered to be up-regulated by the presence of the FNR protein, but FNR has also been implicated in the down-regulation of genes involved in aerobic respiration, such as genes coding for aerobic enzymes like NADH dehydrogenase and cytochrome oxidase [36]. In our study, although the *A. pleuropneumoniae* FNR homolog HlyX was observed to be up-regulated, all other putative members of the FNR regulon were shown to be down-regulated. In recent studies, genes *aspA*, coding for aspartate ammonia lyase, and *dmsA*, encoding a dimethyl sulfoxide

reductase, were shown to be important for the virulence of *A. pleuropneumoniae* [38, 39]. Both these genes, which are apparently under HlyX regulation [40], also showed down-regulation in our experiments. These results might indicate that another factor could interfere with HlyX regulation, or counter-balance the HlyX-inducing effect during iron restriction. The fact that most of the affected genes code for enzymes containing an Fe-S cluster in their structures or use iron as an activator [41] could explain this effect. Since studies have shown that the *A. pleuropneumoniae* FNR homolog may be involved in the activation of genes coding for virulence factors [42] and is essential for full virulence [40], the observed up-regulation of HlyX is not unexpected. Precise characterisation of the HlyX regulon in *A. pleuropneumoniae* will provide a better view of its role in pleuropneumonia. In *E. coli*, a second regulatory system, the ArcA/ArcB two component system, has also been shown to sense oxygen levels [43]. In our system, the *baeS* gene product, which has 51% identity with the *P. multocida Pm70* ArcB protein, was also down-regulated, indicating that this system might be affected during iron restriction.

The overall picture of down-regulated genes shows that *A. pleuropneumoniae* has adopted strategies of economy for iron and energy. The principal components of the aerobic respiratory chain were all repressed, as well as key alternative final electron acceptors, probably since these processes implied extensive use of iron-containing enzymes. Genes involved in the synthesis of heme cofactors (*bioD1, hemA, hemC and hemN*) or quinones and menaquinones (*menA, menB* and *ispH*), which are important elements of the respiratory chain, showed down-regulation because lack of iron compromises these processes. Many components of the sugar phosphotransferase systems (PTSs) (*manX, hisS, ptsBHI*), which enable simultaneous transport and phosphorylation of sugars from phosphoenolpyruvate, as well as other genes involved in sugar transport (*dcuB1*, *dcuB2*, *rbsB*, *glpF*, *glpT*) were down-regulated under our experimental conditions. This outcome could hamper sugar uptake by the bacteria. Repression of the various PTSs might be caused by the repression of the *pfkA* gene, which codes for phosphofructokinase, a key enzyme in the pathway responsible for the conversion of glucose to phosphoenolpyruvate, and

which serves as the primary source of phosphate for activation of PTSs [44]. The product of the *mlc* gene, which shows 70% homology with a probable *Haemophilus ducreyi* sugar metabolism repressor and which was also down-regulated, might be implicated in this down-regulation of PTSs. This repressor has been shown to repress the transcription of many PTSs, and is subject to a negative auto-regulation [44].

Considering these metabolic deficiencies, it is significant that some enzymes with ATPase activity as well as others involved in processes that are not of primary importance in adapting to iron restriction were down-regulated. As an example, four genes *purA, purT, pyrD, pyrG* for enzymes with ATPase activity that belong to the "Purine, Pyrimidines, Nucleosides and Nucleotides" functional class were down-regulated. Since bacteria are growing in a stressful environment and their metabolism seems highly compromised, expression of genes involved in the biosynthesis of molecules useful for replication is not essential.

***(ii) Up-Regulated Genes*.** Many genes involved in cell metabolism were observed to be down-regulated by iron restriction, but cell metabolism was not highly represented in our set of up-regulated genes. Two genes showing high up-regulation during iron restriction were assigned to this category. The *lldD* gene showed a five-fold induction, and codes for L-lactate dehydrogenase, an enzyme required for conversion of lactic acid produced by fermentation to pyruvate. To compensate for defects of the respiratory chain, *A. pleuropneumoniae* might have started to rely on fermentation during iron restriction. The gene encoding the XylB xylose kinase involved in the degradation of xylose was also up-regulated. Considering that many PTSs were down-regulated, the use of this alternative sugar, for which PTS systems have seldom been implicated [45] may be reconciled. Several genes of the "Protein Fate" functional class also showed up-regulation. The two subunits of the Clp protease showed higher expression during iron restriction; this cytoplasmic protease is often involved in stress responses and protein quality control [46]. The genes *prlC* and *def*, encoding respectively an oligopeptidase and a peptide deformylase

responsible for the hydrolysis of the N-formyl group of nascent polypeptide chains [47], were also up-regulated. This might indicate a higher turnover rate for native proteins requiring iron molecules in their structure, which might be unable to fold correctly in the absence of iron. The last gene of the "Protein Fate" functional class to be up-regulated was *mopB*, which codes for co-chaperonin GroES. This co-chaperonin, essential for full function of GroEL, facilitates non-native protein folding [48]. Again, the absence of iron might cause the accumulation of incorrectly folded native oligopeptide chains, thereby leading to higher expression of the GroES co-chaperonin.

The major response of *A. pleuropneumoniae* to iron restriction was the induction of genes involved in iron transport, probably to counter-balance effects of EDDHA. Most genes with known functions, identified as up-regulated during iron restriction, were shown to be involved in iron acquisition and transport. The *tonB1* gene showed the highest level of up-regulation, and genes *exbB1*, *exbD1* and *tbpA* which are transcriptionally linked to *tonB1* were shown by qRT-PCR analysis to be also up-regulated. The *hgbA* gene was over-expressed, as well as the *hugZ* heme utilization protein which is located immediately upstream of *hgbA* [13]. Among other known *A. pleuropneumoniae* iron acquisition-related genes, *tonB2* also showed up-regulation, while genes of the *fhu* operon did not show any significant change in expression, in agreement with previous work done in our laboratory; expression of *fhuA* is not regulated by iron [12].

Previously unreported iron acquisition systems were also revealed by our experiments. We identified a gene cluster, composed of ORFs PM0741 and NMB1668, showing 43% identity with the HmbR hemoglobin receptor from *N. meningitidis*. The HmbR receptor was shown in *N. meningitidis* to be important for survival in an infant rat infection model [49]. HmbR binds hemoglobin with high affinity, is able to strip heme from hemoglobin and then transport it to the periplasm. In *N. meningitidis*, HmbR is subject to phase variation via frameshift mutations [50], and about half of all clinical isolates express HmbR [51]. In *A. pleuropneumoniae*,

the HgbA receptor has been shown to be responsible for iron acquisition from hemoglobin, and a mutant strain with an internal *hgbA* deletion could not grow in an iron-restricted medium supplemented with hemoglobin, albeit from different species [13]. Apparently HgbA is the sole hemoglobin receptor in *A. pleuropneumoniae* serotype 1, but it is not the sole hemoglobin binding protein that was identified in *A. pleuropneumoniae*. In the same study that lead to the identification of HgbA, a 75 kDa protein that could bind hemoglobin and hemin was also isolated [52]. The putative *A. pleuropneumoniae* HmbR has an estimated molecular weight of 76.7 kDa, and it is therefore tempting to speculate that those two proteins might share identity. In *N. meningitidis*, the *hmbR* gene is located downstream of *hemO* gene that codes for a heme oxygenase and that is considered essential for heme utilization by pathogenic *Neisseriae* [53]. No HemO homolog was found in the *A. pleuropneumonia*e genome, which might explain the apparent lack of iron acquisition from hemoglobin from other putative OM receptors than HgbA in *A. pleuropneumoniae*. Two other genes, located immediately downstream of the last NMB1668 ORF, and transcribed in the opposite direction, also showed up-regulation: ap2146 and ap2147; see Fig. 3. ORF ap2146 is predicted to code for the α subunit of a N-methylhydantoinase B/acetone carboxylase, while ORF ap2147 shares some region of homology with the periplasmic energy transducing protein TonB. Implication of the products of these ORFs in a potential iron-acquisition process involving the HmbR homolog remains to be assessed.

Our identification of a putative Yfe system was also of seminal interest. The Yfe system was first identified in *Y. pestis* and shown to allow chelated iron utilization in an *E. coli* mutant lacking enterobactin [54]. Two different operons encode the Yfe system, carrying genes *yfeABCD* and *yfeE* respectively; both operons were Fur-responsive. Later studies showed that the *yfeABCD* genes code for a periplasmic binding protein-dependent transport system belonging to the superfamily of ABC transporters [55], implicated in iron and manganese acquisition, and independent of TonB [56]. In *A. pleuropneumoniae*, homologs of components YfeABCD, showing respectively 63%, 76%, 75% and 66% homology with their counterparts in *P.*

multocida, showed up-regulation during iron restriction, but were not present on the same operon. Gene *yfeB* can be found immediately downstream of the *yfeA* gene, in the same area as two other ORFs that were up-regulated during iron restriction and that could be implicated in iron acquisition. These ORFs, which are annotated as *omp64*, show good homology (32%) to the *Moraxella catarrhalis* CopB OM protein. Meanwhile, the *yfeCD* genes are located 160 kb downstream of the last *omp64* ORF, and also show high homology with the corresponding *Y. pestis* Yfe proteins. The CopB protein has been implicated in iron acquisition from lactoferrin and transferrin; a mutant strain showed reduced ability to uptake iron from these proteins, with the more marked effect on transferrin-bound iron acquisition [57]. In *A. pleuropneumoniae*, proteins responsible for the utilization of transferrin-bound iron were first identified by affinity methods [58]. Later studies showed that the *tbpAB* genes from *A. pleuropneumoniae* are transcriptionally linked to genes *tonB1*, *exbB1* and *exbD1*, and these *exb* genes are essential for iron acquisition from transferrin [17]. It was also shown that both *tbp* genes are essential for iron acquisition from transferrin [59], and another recent study showed that a *tonB1* mutant cannot use porcine transferrin, but is not attenuated *in vivo* [18]. As with the *A. pleuropneumoniae* HmbR homolog, it would be interesting to examine the presence of the CopB homolog *in vitro* and *in vivo*, and possible effects of mutations in this gene. Although the best amino acid homology was with the CopB protein, the overall alignment of the *A. pleuropneumoniae omp64* gene product with the *M. catharralis* CopB protein is not strong, implying that Omp64 might have a role to play in iron acquisition, but its target might not be transferrin or lactoferrin. Another ORF (ap1453) also annotated *omp64* showed up-regulation in our experiments. This second Omp64 also shows homology with the CopB OM protein (57%), but the overall alignment with CopB seems superior to that of the other Omp64 (ap0300 – ap0301). The ap1453 Omp64 also shows homology with the *H. influenzae* heme-hemopexin utilization protein C (41%). We hypothesize that the YfeABCD-Omp64 (ap0300-ap0301) proteins are components of a new iron acquisition system in *A. pleuropneumoniae* that are located in the OM (Omp64) and in the CM (YfeBCD), as

determined by the PSORT algorithm [60]. The exact location of YfeA could not be determined precisely, but it is predicted not to be cytoplasmic.

Another cluster of genes was particularly interesting with regards to iron acquisition. Two ORFs, annotated *fetB2*, seem to encode a unique protein presenting similarities with members of the TroA superfamily of periplasmic metal binding proteins. Sequence analysis reveals homologies with other known or putative periplasmic binding proteins, some of which are involved in iron transport. Downstream, three putative genes appear to code for the components of an ABC transport system. One gene of this putative ABC transport system was also up-regulated: the *NMB1993* gene coding for a putative ATPase component. These components show homology with the Ceu system (*Campylobacter* Enterochelin Utilization), and prediction of localization with PSORT indicates that *fetB2* localizes to the periplasm, *NMB1993* to the CM and/or cytoplasm; the two other components, which are not over-expressed in our system, were predicted to be in the CM. We demonstrated [20, 61] that *A. pleuropneumoniae* uses different exogenous siderophores, including a catechol-type siderophore like enterochelin. Up to now, in *A. pleuropneumoniae*, the only identified siderophore OM receptor is FhuA, specific for ferric hydroxamates [11]. It is premature to conclude that the *fetB2* and *NMB1993* genes are part of this unidentified catechol-type siderophore acquisition system.

Three other up-regulated ORFs were identified as having a putative role in iron acquisition. ORFs *ccrA1*, ap0740 and ap0741 were classified as proteins of unknown function, but share homologies respectively with a family of periplasmic lipoproteins involved in iron transport, a family of iron-dependent peroxidase and a family of high affinity Fe^{2+}/Pb^{2+} permease. Since no clear homology with any known or characterized protein was established, hypotheses concerning their function and roles in iron acquisition have to be formulated with great care. Recently, Cowart showed [62] that bacterial reductases, by changing the state of free iron from Fe^{3+} to Fe^{2+}, could play a major role in iron acquisition. The presence of a

possible Fe^{2+} permease could indicate the existence of such a mechanism in *A. pleuropneumoniae*.

Considering that iron restriction conditions are encountered *in vivo*, we further examined the expression of known or putative virulence factors of *A. pleuropneumoniae* under such conditions. Aside from different iron acquisition systems, the Apx toxins are often regarded as essential for virulence of *A. pleuropneumoniae*. The Apx toxins are members of the RTX (Repeat in Toxin) family, and the genetic organization of the genes that are essential for the synthesis and secretion of the toxin generally follows the same order: *apxC*, *apxA*, *apxB* and *apxD*, which code respectively for the pretoxin activator, the pretoxin structure and the secretion apparatus [63]. These genes can be transcribed from two different transcripts: a major 3.5 kb transcript containing genes *apxICA*, and a minor 7.5 kb transcript with genes *apxICABD* [64]. During iron restriction, the first gene of the *apxI* operon, *apxIC*, showed slight up-regulation, but the three other genes were not over-expressed. The *A. pleuropneumoniae* strain used in this study possesses genes coding for the ApxI, II and IV toxins. Very little is known about the transcriptional regulation of the *apxI* operon, but it has been shown to be at least regulated by the combined activity of the Fur protein and calcium [21]. Under high calcium concentration, Fur seemed to act as an activator of the *apxI* operon, while it seemed to act as a repressor under low calcium concentration. Under our experimental conditions, it seems that Fur acts as a repressor since the *apxIC* gene was identified as being slightly up-regulated during iron-restriction, i.e. in the absence of Fur. The fact that it was the only gene of the *apxI* operon to show significant up-regulation might reflect the stringency of our analysis, but might also point towards the existence of fine post-transcriptional tuning of the *apxI* operon. The existence of such mechanisms of regulation could also explain why *apxIA* does not seem to be up-regulated, even though it is located on the same transcript as *apxIC*.

The *A. pleuropneumoniae ureC* has been implicated as a possible virulence factor, with a putative role in persistence of bacteria *in vivo* [65]. In our study, the *ureC*

gene was identified as being down-regulated during iron restriction. Since it was shown that this gene might have more effect in the late stage of the disease, it seems clear that other *in vivo* factors, as with *hlyX*, may influence the regulation of the *ureC* gene.

Three ORFs which had approximately two-fold induction during iron deficiency also warrant attention. The Ssa1 protein (Serotype 1-Specific Antigen) was first identified in *Mannheimia haemolytica* and was associated with the serotype switch occurring in the upper respiratory tract of bovines following stressful events, potentially leading to development of disease [66]. The protein was shown to localize to the OM [67]. Sequence homology research on the *A. pleuropneumoniae* Ssa1 protein led to identification of an autotransporter domain at the C-terminal extremity of the protein; the *A. pleuropneumoniae* Ssa1 was also classified in the family of subtilisin-like serine proteases, although no experimental evidence of this activity could be found. Recently, autotransporter proteins such as Ig proteases have been implicated in virulence [68]. Autotransporter proteins belonging to the serine protease family have been identified in various Gram-negative bacteria. *H. influenzae*, a close relative of *A. pleuropneumoniae*, possesses at least two: an IgA1 protease and the Hap protein which has been shown to be involved in adhesion. Little is known about the Ssa1 protease but its implication in virulence in *M. haemolytica* suggests that it could play a similar role in *A. pleuropneumoniae*.

Genes *ftsK* and *ftsA*, essential in the first steps of cell division, also showed higher expression during iron restriction. Considering that some genes involved in the "Purine, Pyrimidines, Nucleosides and Nucleotides" were down-regulated, this result was unexpected. However, it has been shown that the transcription of the *ftsZ* gene is subjected to regulation by antisense transcription of a 490-bp segment spanning the junction between the *ftsA* and *ftsZ* genes [69], which could probably explain the apparent overproduction of the *ftsA* mRNA. As for *ftsK*, although the protein is implicated in cell division, other functions have been suggested for this protein [70], and the observed up-regulation might not be linked with cell division.

Conclusions

In summary, the evaluation of differential gene expression in *A. pleuropneumoniae* during growth in an iron-restricted medium enabled us to gain a better understanding of the metabolic changes occurring in response to this stress. Transcript profiling using DNA microarrays is a powerful tool to determine the exact composition of the bacterial transcriptome in defined conditions, therefore leading to the putative identification of components that are essential during these conditions. It can also help identify components which are likely to be expressed during the infection process in the host, and that might be interesting targets for vaccines.

In the course of our study, many new potential iron acquisition systems were highlighted. Clearly, iron acquisition in *A. pleuropneumoniae* might rely on more systems that what was previously thought, and further studies will be necessary to evaluate the impact of these systems during the course of infection by *A. pleuropneumonia.*

Methods

Bacterial strains and growth conditions

Actinobacillus pleuropneumoniae serotype 1 strain S4074 was routinely grown on BHI medium supplemented with either 15 μg/ml (agar) or 5 μg/ml (broth) of NAD. For the microarray experiments, two flasks of BHI broth were inoculated with 500 μl of an overnight culture of *A. pleuropneumoniae* serotype 1 strain S4074 and grown at 37°C in an orbital shaker until an optical density of 0.1 was reached. To initiate iron restriction in one of the two cultures, EDDHA was added to a final concentration of 50 μg/ml. In the iron supplementation experiments, $FeCl_3$ was added to the iron depleted culture 5 min. after the addition of EDDHA to a final concentration of 50 μg/ml. The cultures were then re-incubated until they reached a final optical density of 0.3.

RNA extraction

RNA was harvested from cells at an optical density of 0.3. Ice-cold RNA degradation stop solution (95% ethanol, 5% buffer-saturated phenol), shown to effectively prevent RNA degradation and therefore preserve the integrity of the transcriptome [71], was added to the bacterial culture at a ratio of 1:10 (vol/vol). The sample was mixed by inversion, incubated on ice for 5 min, and then spun at 5000 g for 10 min to pellet the cells. Bacterial RNA isolation was then carried out using the QIAGEN RNeasy MiniKit. During the extraction, samples were subjected to an on-column DNase treatment, as suggested by the manufacturer. The RNA concentration, quality and integrity were assessed spectrophotometrically and by gel analyses.

Construction of the *A. pleuropneumoniae* 5b strain L20 microarray (AppChip1)

The draft genome sequence of *A. pleuropneumoniae* serotype 5b strain L20 [GenBank:CP000569] was used as a source of the genes used in this study. ORFs were identified using the Glimmer software package [72], and used to search for homologs among the bacterial gene subset of Genbank [73] using the BLASTP program [74]. PCR primers were designed for each of the 2025 ORFs of the genome of *A. pleuropneumoniae* using the Primer3 program [75] controlled by an automated script as described previously [76]. Primer-selection parameters were standardized and included a similar predicted melting temperature (60 ± 3°C), uniform length (25 nt), and a minimum amplicon size of 160 bp. Generation of PCR amplicons and fabrication of DNA microarrays were as described [76]. Details on the construction of this microarray (AppChip1) are available on the Institute for Biological Sciences website [77].

Microarray hybridizations

cDNA synthesis and microarray hybridizations were performed as described [78]. Briefly, equal amounts (15 μg) of test RNA and control RNA were used to set up a standard reverse transcription reaction using random octamers (BioCorp), SuperScript II (Invitrogen) and aminoallyl-dUTP (Sigma), and the resulting cDNA

was indirectly labelled using a monofunctional NHS-ester Cy3 or Cy5 dye (Amersham). The labelling efficiency was assessed spectrophotometrically. Labelled samples were then combined and added to the *A. pleuropneumoniae* 5b strain L20 microarray. Nine hybridizations were performed for the iron-restriction experiments, including three pairs of microarrays for which Cy3 and Cy5 dyes were swapped, while 4 hybridizations were conducted for the iron supplementation experiments. Data were submitted to the Gene Expression Omnibus [79] [GEO:GSE4674 and GSE6366]. All slides were scanned using a Perkin-Elmer ScanArray Express scanner.

Microarray analysis and bioinformatics

The TM4 suite of software from The Institute of Genomic Research was used for the whole microarray analysis [80]. First, raw data were generated using SpotFinder v.3.0.0 beta. The integrated intensities of each spot, equivalent to the sum of intensities of all unsaturated pixels in a spot, were quantified and the integrated intensity of the local background was subtracted for each spot. The same operation was performed with the median spot intensities. The spot detection threshold was set so that spots for which the integrated intensity was less than one standard deviation above the background median intensity were set to zero. Raw spot data were converted from integrated intensities to median spot intensities using TIGR's Express Converter software, the latter being less influenced by outlier values than integrated intensities.

Data were normalized with TIGR's MIDAS software tool using locally weighted linear regression (lowess) [81-83]. Spots with median intensities lower than 1000 were removed from the normalized data set. Intensities for duplicate spots were merged to generate the final normalized data set, subsequently analyzed using TIGR's TMEV microarray analysis tool. The Significance Analysis of Microarray (SAM) algorithm [84], which is implemented in TMEV, was used to generate a list of differentially expressed genes. During SAM analysis, a false discovery rate of 3.22% was estimated for the iron-depleted *versus* BHI broth experiment, while a

FDR of 2.51% was estimated for the iron-supplemented *versus* BHI broth experiment; this value estimates the proportion of genes likely to have been identified by chance. Functional classification of these genes was conducted using TIGR's Comprehensive Microbial Resource (CMR) [85]. Proteins were assigned to their corresponding pathways using the MetaCyc Metabolic Pathway Database [41]. Homologies were assessed using Blast tools [86] hosted on the NCBI and TIGR servers. Additional subcellular localization was determined with PSORTb [60]. Protein sequence alignments were performed using the ClustalW multiple sequence alignment algorithm [87].

Real-Time quantitative RT-PCR

Microarray results were verified by real-time quantitative RT-PCR (qRT-PCR), using the QuantiTect® SYBR® Green RT-PCR Kit (Qiagen). Reactions were performed with a 16-place Cepheid Smart Cycler® System in a total volume of 25 µl. Oligonucleotide primers (Table 4) were designed using Primer3 software [75]. To ensure that amplification with these primers resulted in single amplicon of the anticipated size, they were PCR tested before proceeding to qRT-PCR analysis. Primer pairs which amplified fragments of 195 to 205 bp with a melting temperature of 60°C were selected. Seventeen genes (7 up-regulated, 8 down-regulated, 2 non-significant) were selected for analysis. Relative expression of each gene as determined by qRT-PCR was normalized to that of the *ackA* gene which showed a stable level of expression throughout the different microarray experiments (data not shown). Prior to the qRT-PCR, the RNA samples were subjected to a DNase treatment with TURBO DNase (Ambion, Austin, TX) to avoid DNA contamination in the samples. Quantitative measures were obtained using the $2^{-\Delta\Delta C_T}$ method [88].

List of abbreviations

BHI : Brain Heart Infusion, CGH : Comparative Genomic Hybridization, EDDHA : ethylenediamine dihydroxyphenyl acetic acid, Fe-S : iron-sulfur, NAD :

Nicotinamide Adenosine Dinucleotide, Ig : Immunoglobulin, ORF : Open Reading Frame, TCA : Tricarboxylic Acid.

Authors' contributions

VD designed the transcript profiling experiments, carried out downstream data analysis, and drafted the manuscript. JHEN designed the AppChip1 and helped with the downstream data analysis. JWC and JH participated in the study design and revised the manuscript. MJ participated in the conception and supervised the design of the study and revised the manuscript. All authors read and approved the final manuscript.

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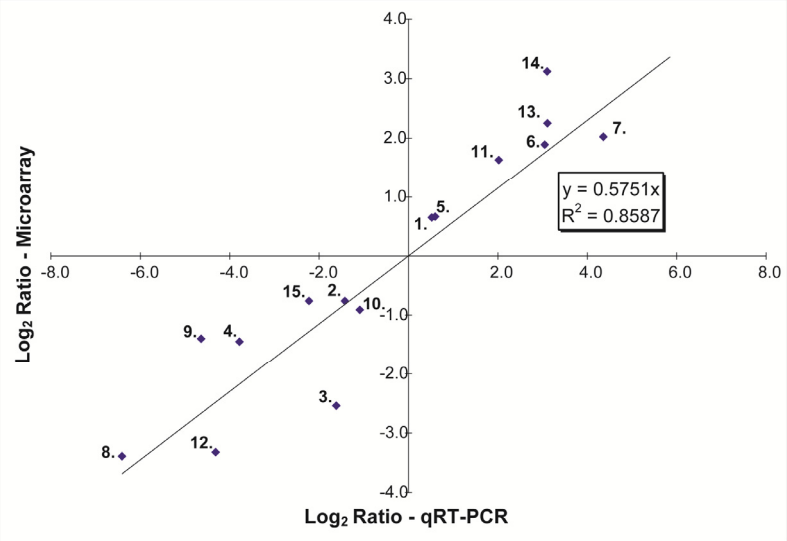

Figure 1 - Validation of microarray results by qRT-PCR

Seven up-regulated genes, eight down-regulated genes and two genes that did not show significant variation in the microarray experiments are presented. Mean $\log_2$ ratios obtained during qRT-PCR experiments are plotted against the mean $\log_2$ ratios obtained with the microarrays. Numbers on the graph refer to the gene numbers in Table 4.

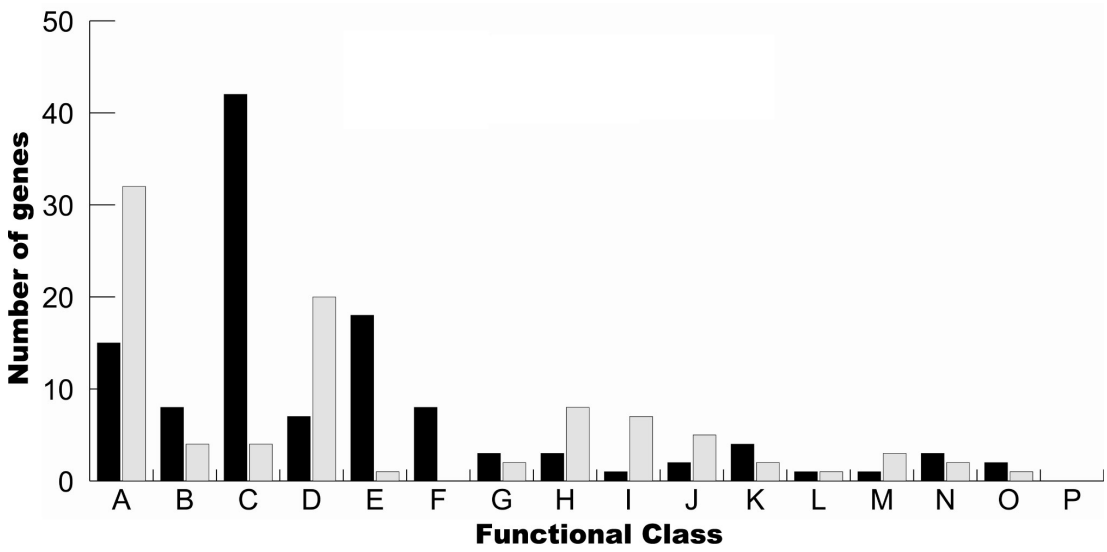

Figure 2 - Functional classification of the differentially expressed genes according to TIGRFAMs

Black and grey bars respectively represent down-regulated and up-regulated genes. A: Hypothetical proteins/Unclassified/Unkown; B: Biosynthesis of cofactors, prosthetic groups and carriers; C: Energy Metabolism; D: Transport and binding proteins: cations and iron; E: Transport and binding proteins: others; F: Purines, pyrimidines, nucleosides and nucleotides; G: Regulatory functions; H: Protein fate; I: Protein synthesis; J: Cellular processes; K: Cell envelope; L: Fatty acids and phospholipids metabolism; M: Amino acids biosynthesis; N: DNA metabolism; O: Central intermediary metabolism; P: Mobile and extrachromosomal element functions.

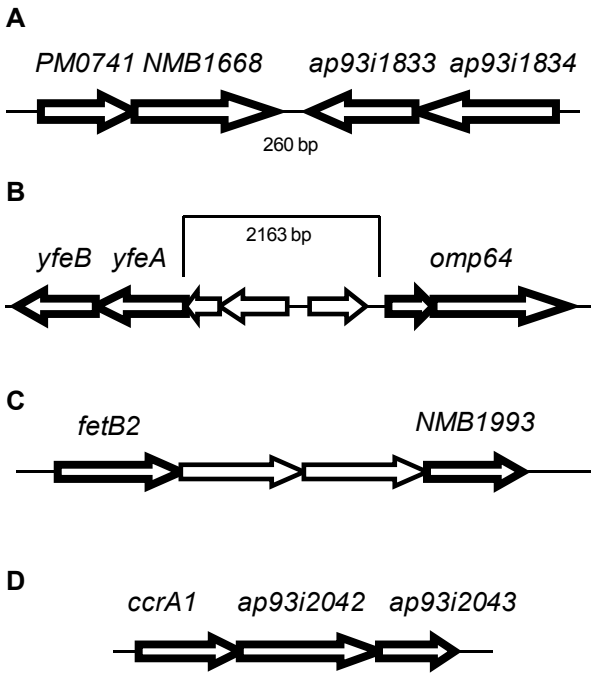

Figure 3 - Genetic organization of some gene clusters identified during this study

a) Genomic region of the *A. pleuropneumoniae* 5b strain L20 genome surrounding ORFs encoding for genes PM0741 and NMB1668. ORFs ap2146 and ap2147 are located 260 pb downstream of the NMB1668 ORF, and are transcribed in the opposite direction. b) *yfeAB* and *omp64* genes are separated by three ORFs that did not show differential expression. c) Genetic organization of a possible operon coding for a putative enterobactin-type ABC transporter system. The two ORFs separating *fetB2* and *NMB1993* are the putative cytoplasmic components of this hypothetical system. d) The Fe^{2+}/Pb^{2+} high affinity permease locus.

Tables

Table 1 - *A. pleuropneumoniae* genes which are down-regulated during iron restriction

Gene ID	Gene	Description	Fold Change
Hypothetical/Unclassified/Unknown			
ap0497	*engA*	putative GTP binding protein	-2.27
ap0491	*glnE*	unknown	-1.98
ap1365	*srmB*	uncharacterized conserved protein	-1.85
ap1538	*traC*	conserved hypothetical protein	-1.72
ap0677	*nfnB*	putative nitroreductase, FMN-dependent	-1.70
ap1779	*mscL*	conserved hypothetical protein	-1.69
ap0802	*dxr*	conserved hypothetical protein, distant homolog of PhoU	-1.58
ap0787	*cdsA*	putative transcriptional regulator	-1.54
ap0685	*mlc*	protein of unknown function	-1.53
ap1297^{+}	*sspA*	predicted iron-dependent peroxidase	-1.53
ap0973	*abgB*	possible metal dependent peptidase, unclassified	-1.48
ap1405	*nth*	possible sodium/sulphate transporter	-1.41
ap1725	*mviN*	uncharacterized membrane protein, putative virulence factor	-1.38
ap0622	*aroC*	*flp* operon protein C	-1.28
ap0989	*fstX*	conserved hypothetical protein	-1.27
Biosynthesis of cofactors			
ap0684	*bioD1*	probable dethiobiotin synthetase	-3.49
ap1624	*menA*	1,4-dihydroxy-2-naphthoateoctaphenyltransferase	-1.57
ap1131	*hemC*	porphobilinogen deaminase	-1.47
ap0447	*hemA*	glutamyl-tRNA reductase	-1.40
ap1080	*hemN*	oxygen-independent corproporphyrinogen III oxydase	-1.40
ap2005	*menB*	naphthoate synthase	-1.39
ap1684	*ispH*	hydroxymethylbutenyl pyrophosphate reductase	-1.37
ap2023	-	4-hydroxybenzoate synthetase	-1.31
Energy Metabolism			
ap0108^{+}	*nrfA*	nitrate reductase cytochrome c552	-10.48
ap1694^{+}	*frdA*	fumarate reductase flavoprotein subunit	-9.20
ap1693^{+}	*frdB*	fumarate reductase iron-sulfur protein	-7.86
ap1536	*ccp*	cytochrome C peroxidase	-6.61
ap0764^{+}	*torY*	nitrate/TMAO reductase, tetraheme cytochrome C subunit	-6.27
ap0996^{+}	*bisC*	nitrate-inducible formate dehydrogenase-N α subunit	-5.68
ap0997^{+}	*bisC*	nitrate-inducible formate dehydrogenase-N α subunit	-5.40
ap0762^{+}	*torZ*	trimethylamine-N-oxide reductase 2	-5.23
ap0998^{+}	*hybA*	formate dehydrogenase β subunit	-5.23
ap0498^{+}	*ykgF*	putative Fe-S electron transport protein	-4.78
ap1692^{+}	*frdC*	fumarate reductase 15kD hydrophobic protein	-4.67
ap1937	*fumC*	fumarate hydratase class II	-4.45
ap0499^{+}	*ykgE*	conserved putative dehydrogenase, Fe-S oxidoreductase	-4.38

ap1132^{+}	*adh2*	alcool dehydrogenase 2 dehydrogenase	-3.36
ap1163^{+}	*pflB*	formate acetyltransferase	-3.01
ap1221	*aspA*	aspartate ammonia-lyase	-2.78
ap1848^{+}	*dmsA*	dimethyl sulfoxyde reductase	-2.73
ap1222	*aspA*	aspartate ammonia-lyase	-2.69
ap0110^{+}	*nrfC*	nitrate reductase, Fe-S protein	-2.63
ap0380	*glgB*	1,4-α-glucan branching enzyme	-2.55
ap0414	*glpK*	putative glycerol kinase	-2.29
ap0109^{+}	*nrfB*	nitrate reductase, cytochrome C-type protein	-2.25
ap1255	*pfkA*	phosphofructokinase	-2.20
ap1486^{+}	*hyaA*	Ni-Fe hydrogenase I small subunit	-2.09
ap1525^{+}	*ccmF*	cytochrome C-type biogenesis protein	-1.98
ap1181^{+}	*nfrE*	cytochrome C-type biogenesis protein	-1.88
ap0418^{+}	*glpA*	anaerobic glycerol-3-phosphate dehydrogenase, subunit A	-1.76
ap0958^{+}	*sdaA*	L-serine dehydratase	-1.75
ap0420^{+}	*glpC*	anaerobic glycerol-3-phosphate dehydrogenase, subunit C	-1.72
ap1979	*torA*	trimethylamine oxydoreductase precursor	-1.70
ap1528^{+}	*ccmC*	cytochrome C-type biogenesis protein	-1.69
ap1000^{+}	*fdhE*	formate dehydrogenase formation protein	-1.62
ap0328^{+}	*cydB*	cytochrome D ubiquinol oxidase subunit II	-1.61
ap1588^{+}	*napF*	ferredoxin-type protein	-1.55
ap1402	*pgk*	phosphoglycerate kinase	-1.55
ap1585^{+}	*torC*	nitrate/TMAO reductase, tetraheme cytochrome C subunit	-1.53
ap0089	*dAK1*	dihydroxyacetone kinase	-1.51
ap0541	*maeA*	malate oxydoreductase	-1.46
ap0326^{+}	*cydA*	cytochrome D ubiquinol oxidase subunit I	-1.45
ap0484	*gapA*	glyceraldehyde-3-phosphate dehydrogenase	-1.35
ap1822	*atpH*	ATP synthase δ chain	-1.28
ap1116	*galK*	galactokinase	-1.26
Transport and binding proteins: cations and iron			
ap0169^{+}	*aopA*	NADH-ubiquinone oxidoreductase, Na^{+}-translocating A subunit (nqrA)	-2.38
ap0354	*nhaB*	Na^{+}/H^{+} antiporter protein	-2.14
ap0170^{+}	*nqrB*	NADH degydrogenase, Na^{+}-translocating B subunit	-2.09
ap0172^{+}	*nqrD*	NADH-ubiquinone oxidoreductase, Na^{+}-translocating D subunit	-2.05
ap0171^{+}	*nqrC*	NADH-ubiquinone oxidoreductase, Na^{+}-translocating C subunit	-2.02
ap1972	*nadR*	putative periplasmic binding protein, ABC metal ion uptake	-1.61
ap0173^{+}	*nqrE*	NADH-ubiquinone oxidoreductase, Na^{+}-translocating E subunit	-1.52
Transport and binding proteins: others			
ap1470	*dcuB2*	anaerobic C4-dicarboxylate membrane transporter	-5.81
ap0416	*glpT*	glycerol-3-phosphate transporter	-3.71
ap1835	*manX*	PTS system enzyme IIAB, mannose specific	-2.28
ap1548	*mMT1*	PTS system mannose-specific EII AB component	-1.83
ap1473	*ptsB*	PTS system, sucrose-specific IIBC component,	-1.67
ap1477	*ptsH*	PTS system phosphocarrier protein HPr	-1.65
ap1620	*glpF*	glycerol uptake facilitator	-1.56

ap1164	*focA*	probable formate transporter	-1.54
ap0924	*cydC*	ABC transporter involved in cytochrome bd biosynthesis	-1.51
ap1833	*hisS*	PTS system component IID, mannose specific	-1.48
ap1580	*rbsB*	galactose ABC transporter, periplasmic binding protein	-1.48
ap0886	*sapC*	peptide transport system permease protein	-1.39
ap1698	*dcuB1*	anaerobic C4-dicarboxylate transporter	-1.38
ap2065	*mscS*	small-conductance mechanosensitive channel	-1.37
ap1367	*PM0514*	permease of unknown function	-1.34
ap1478	*ptsI*	phosphoenolpuruvate PTS system enzyme I	-1.32
ap1463	*proP*	permease of the major facilitator superfamily	-1.32
ap1507	*artQ*	arginine transport system permease protein	-1.22
Purines, pyrimidines, nucleosides and nucleotides			
ap2022	*udp*	uridine phosphorylase	-2.21
ap1237	*purT*	phorphoribosyglycinamide formyltransferase 2	-1.67
ap0154	*pyrG*	CTP synthase	-1.54
ap1922	*cdpC*	2',3'-cyclic-nucleotide 2'-phosphodiesterase	-1.46
ap0862	*pyrD*	dihydroorotate dehydrogenase	-1.42
ap1204	*purA*	adenylosuccinate synthetase	-1.37
ap0863	*prsA*	ribose-phosphate pyrophosphokinase	-1.34
ap0729	*purE*	phosphoribosylaminoimidazole carboxylase catalytic subunit	-1.33
Regulatory functions			
ap1392	*ansB*	probable carbon starvation protein A, membrane bound	-2.59
ap1803	*glpR*	transcriptional regulator of sugars metabolism	-1.51
ap1048	*baeS*	sensory transduction histidine kinase	-1.36
Protein fate			
ap1485^{+}	*hypF*	Ni-Fe hydrogenase maturation protein	-2.39
ap2081	*lgt*	prolipoprotein diacylglyceryl transferase	-1.58
ap0428	*pepB*	peptidase B	-1.38
Protein synthesis			
ap0241	*thrS*	threonyl-tRNA synthetase	-1.40
Cellular processes			
ap0725	*uspA*	universal stress protein A	-1.59
ap0333	*tolB*	colicin tolerance protein	-1.29
Cell envelope			
ap1215	*ompW*	outer membrane protein W	-10.00
ap1156	*rplK*	COG5039: exopolysaccharide biosynthesis protein	-1.32
ap0021	*HI1139*	UDP-N-acetylmuramate-alanine ligase (murC)	-1.23
ap1154	*ushA*	glycosyltransferase involved in LPS biosynthesis	-1.19
Fatty acids and phospholipids metabolism			
ap2049	*accC*	biotin carboxylase	-1.24
Amino acids biosynthesis			
ap0351	*OB1054*	putative methionine synthase	-1.55

DNA metabolism			
ap1336	-	putative *hsdR*, type 1 site-specific restriction-modification system, R subunit	-1.54
ap0703	*alxA-hsdM*	type I restriction-modification system methylation subunit	-1.41
ap1247	*recQ*	ATP-dependent DNA helicase	-1.21
Central intermediary metabolism			
ap1787	*ureC*	urease α subunit	-1.45
ap1785	*ureE*	metallochaperone for urease	-1.22

[+] Genes coding for iron-containing proteins or proteins using Fe^{2+} as a cofactor.

Table 2 - *A. pleuropneumoniae* genes which are up-regulated during iron restriction

Gene ID	Gene	Description	Fold Change
Hypothetical/Unclassified/Unknown			
ap2147^{+}	-	possible N-methylhydantoinase B/acetone carboxylase, α subunit	6.22
ap0740	-	predicted iron-dependent peroxidase	3.56
ap2196	*PM1515*	protein of unknown function	3.40
ap0741^{+}	-	predicted high-affinity Fe^{2+}/Pb^{2+} permease	3.06
ap2146	-	possible N-methylhydantoinase B/acetone carboxylase, α subunit	2.88
ap0739^{+}	*ccrA1*	predicted periplasmic protein involved in iron transport	2.69
ap2014	*rpmJ1*	conserved hypothetical protein	2.01
ap0286	*nagB*	conserved hypothetical protein	1.85
ap1686	*araJ*	conserved hypothetical protein	1.84
ap2182	*rpsU*	conserved hypothetical protein	1.83
ap2207	*PM1452*	protein of unknown function	1.63
ap0035	-	hypothetical protein	1.62
ap1927	-	outer membrane lipoprotein A	1.59
ap1436	*NMA1782*	conserved hypothetical protein	1.57
ap0755	*aroA*	conserved hypothetical protein	1.55
ap0874	-	hypothetical protein	1.54
ap0143	*rplI*	HIT-like protein	1.51
ap0056	*typA*	predicted membrane GTPase involved in stress response	1.51
ap1364	*add*	conserved hypothetical protein	1.49
ap1252	*icc*	conserved putative lipoprotein	1.45
ap0371	*yrbK*	conserved hypothetical protein	1.43
ap0478	*HI0719*	conserved hypothetical protein	1.43
ap1444	*fimD*	conserved hypothetical protein	1.43
ap1598	*slyD*	hypothetical protein	1.41
ap0907	*HI1265*	conserved hypothetical protein	1.41
ap0375	*firA*	conserved hypothetical protein	1.39
ap0079	*comF*	conserved glutaredoxin-like protein	1.37
ap0329	*mlc*	conserved hypothetical protein	1.36
ap1664	*HI1720*	conserved hypothetical protein	1.34
ap0059	*dnaQ*	uncharacterized stress-induced protein	1.30
ap1172	*PM1281*	predicted permease	1.30
ap0324	*ureF*	conserved hypothetical protein	1.16
Biosynthesis of cofactors			
ap0423	*ribB*	riboflavin synthase α subunit	1.59
ap0422	*ribG*	riboflavin-specific deaminase	1.43
ap0947	*licA*	putative oxygen-independent coproporphyrinogen III oxidase (HemN)	1.37
ap1036	*fdx2*	ferredoxin	1.35
Energy metabolism			
ap2032	*lldD*	l-lactate dehydrogenase	4.98
ap1733	*xylB*	probable L-xylulose kinase (L-xylulokinase)	3.04

ap1424	*ndh*	NADH dehydrogenase	1.86
ap1363	*fldA*	flavodoxin	1.61
Transport and binding proteins: cations and iron			
ap1740^{+}	*tonB1*	energy transducing protein	8.71
ap2142^{+}	*PM0741*	outer membrane protein, Fe transport, hemoglobin	6.15
ap1175^{+}	*hgbA*	hemoglobin-binding protein precursor	5.89
ap1176^{+}	*hgbA*	hemoglobin-binding protein precursor	5.45
ap2144^{+}	*NMB1668*	hemoglobin receptor	4.78
ap1177^{+}	*hugZ*	heme utilization protein	4.14
ap0295^{+}	*yfeA*	iron (chelated) ABC transporter, periplasmic-binding protein	3.88
ap2143^{+}	*PM0741*	outer membrane protein, Fe transport, haemoglobin	3.66
ap0296^{+}	*yfeA*	iron (chelated) ABC transporter, periplasmic-binding protein	3.55
ap1453^{+}	*omp64*	outer membrane protein, TonB dependent receptor	3.09
ap0294^{+}	*yfeB*	iron (chelated) transporter, ATP-binding protein	2.98
ap2145^{+}	*NMB1668*	hemoglobin receptor	2.85
ap0300^{+}	*omp64*	outer membrane protein, TonB dependent receptor	2.07
ap0301^{+}	*omp64*	outer membrane protein, TonB dependent receptor	1.93
ap0797^{+}	*fetB2*	putative ferric enterobactin transporter binding protein	1.76
ap0796^{+}	*fetB2*	putative ferric enterobactin transporter binding protein	1.60
ap0082^{+}	*tonB2*	energy transducing protein	1.57
ap0801^{+}	*NMB1993*	iron(III) ABC transporter, ATP-binding protein	1.49
ap0144^{+}	*yfeD*	iron (chelated) transport system, membrane protein	1.47
ap0145^{+}	*yfeC*	iron (chelated) transport system, membrane protein	1.38
Transport and binding proteins : others			
ap1437	*NMA0994*	putative periplasmic protein	1.58
Regulatory functions			
ap0726	*hlyX*	FNR-like transcriptional regulator	2.63
ap0652	*HI0893*	transcriptionnal repressor Bm3R1	1.24
Protein fate			
ap0399	*ssa1*	subtilisin-like serine protease	2.36
ap0400	*ssa1*	subtilisin-like serine protease	2.22
ap0401	*ssa1*	subtilisin-like serine protease	2.01
ap1887	*def*	peptide deformylase	1.64
ap1432	*clpP*	ATP-dependent Clp protease	1.54
ap1160	*prlC*	oligopeptidase A	1.39
ap1134	*mopB*	heat-shock 10 protein GroES	1.36
ap1431	*clpX*	ATP-dependent Clp protease, ATP-binding ClpX subunit	1.20
Protein synthesis			
ap0337	*tdk*	probable tRNA-dihydrouridine synthase C	1.95
ap1295	*potD1*	probable pseudo-uridine synthase	1.35
ap1895	*rplK*	50S ribosomal protein L11	1.32
ap1305	*rplI*	50S ribosomal protein L9	1.31
ap1253	*rluD*	pseudo-uridine synthase	1.24
ap0245	*infC*	translation initiation factor IF-3	1.23
ap1666	*valS*	valyl-tRNA synthetase	1.22

Cellular processes			
ap0168	*napC*	transformation locus protein OrfG	1.62
ap1505	*HI1275*	tellurite resistance protein TehB	1.61
ap1606	*apxIC*	RTX-1 toxin determinant	1.57
ap0688	*ftsK*	cell division protein FtsK	1.27
ap0025	*ftsA*	cell division protein FtsA	1.22
Cell envelope			
ap0486	*mreB*	similar to rod shape-determining protein MreB	1.33
ap0507	*lapB*	putative membrane protein, virK family member	1.30
Fatty acids and phospholipids metabolism			
ap1649	*accA*	acetyl-CoA carboxylase carboxyl transferase α subunit	1.38
Amino acids biosynthesis			
ap2037	*ilvC*	ketol-acid reductoisomerase	2.38
ap1566	*gshA*	putative gluthatione biosynthesis bifunctionnal protein	1.51
ap0466	*argG*	argininosuccinate synthetase	1.24
DNA metabolism			
ap2148	*mutL*	DNA mismatch repair protein MutL	1.34
ap2202	*srmB*	ATP-dependent RNA helicase	1.19
Central intermediary metabolism			
ap1688	*HI0111*	gluthatione transferase	1.24

[+] Genes coding for proteins involved in iron transport

Table 3 - Iron regulated genes that are common between *A. pleuropneumoniae (App)* and *P. multocida (Pm)*

App Gene ID	Gene	*Pm* ORF	Description
Up-Regulated genes			
ap1453	*omp64*	576	CopB homolog, heme-hemopexin utilization protein C
ap2032	*lldD*	288	l-lactate dehydrogenase
ap0294	*yfeB*	399	chelated iron transport, ATP binding protein
ap0295-ap0296	*yfeA*	400	chelated iron transport, periplasmic binding protein
ap1739	*exbB*	1186	energy transducing protein
ap0145	*yfeC*	398	chelated iron transport, membrane protein
ap0726	*hlyX*	668	fnr-like transcriptional regulator
ap0144	*yfeD*	129	chelated iron transport, membrane protein
ap1175-ap1176	*hgbA*	741	hemoglobin-bindin protein precursor
ap1740 ap0082	*tonB1* *tonB2*	1188	energy transducing protein
ap0755	*aroA*	839	conserved hypothetical protein
ap1738	*exbD*	1187	biopolymer transport protein
ap0286	*nagB*	875	conserved hypothetical protein
ap1505	*HI1275*	656	tellurite resistance protein TehB
ap1363	*fldA*	353	flavodoxin
Down-Regulated genes			
ap0108	*nrfA*	1792	nitrate reductase cytochrome c552
ap1470 ap1698	*dcuB1* *dcuB2*	1434	anaerobic C4-dicarboxylate membrane transporter
ap0169-ap0173	*aopA, nqrBCDE*	1331	NADH : ubiquinone oxydoreductase
ap1937	*fumC*	823	fumarate hydratase class II
ap1588	*napF*	1592	ferredoxin-type protein
ap1822	*atpH*	1491	ATP synthase delta subunit
ap0996-ap0997	*bisC*	408-409	nitrate-inducible formate dehydrogenase-N α subunit
ap0725	*uspA*	1286	universal stress protein A
ap1478	*ptsI*	897	phosphoenolpyruvate PTS system enzyme I
ap1477	*ptsH*	898	phosphocarrier protein Hpr
ap1163	*pflB*	75	formate acetyltransferase
ap0684	*bioD1*	641	probable dethiobiotin synthetase
ap1402	*pgk*	1860	phosphoglycerate kinase
ap1848	*dmsA*	1754	dimethyl sulfoxide reductase
ap0998	*hybA*	407	formate dehydrogenase β subunit
ap0484	*gapA*	924	glyceraldehyde 3-phosphate dehydrogenase
ap1694-ap1692	*frdABC*	201-199	fumarate reductase
ap1132	*adh2*	1453	alcohol dehydrogenase 2
ap1215	*ompW*	331	outer membrane protein W

Table 4 - Oligonucleotide primers used for microarray results validation with qRT-PCR

#	Gene	Forward Primer	Reverse Primer
	ackA	CCTAAAACGGGTGACGAGAA	ACCGATAGCACCGATACTGG
17	*ap1465*	CGTAGCGCGTTCCGAATTAA	AACTGCCGTATTTGTCGTGC
2	*apxIC*	TGGTTATGGGCAAGTTCTCC	CAACTAGCGAGGCAACATCA
3	*ccmC*	ATACGGTTCTATGGCGGTTG	AAACAACACCAAAGCCGAAG
4	*dcuB2*	GGCTTTGAAGGCGTTACACT	GCCGGTAATTGCTCGTCTAA
5	*dmsA*	AACTGTGGTAGCCGTTGTCC	AATGCGGCAAACTGATAACG
	exbB1	CCGTTCATTGGGTTATTTGG	ACGGTTAAGGCGAGCAATTA
	exbD1	GGGCATTTATTTAGGCGAGA	TGAGTCACAAAGCCTATTTTCG
5	*fetB2*	CCGCTCTTGATATTCCGATG	TTCCAAGCGTTTGTTTGATG
6	*hgbA*	TGAATTTCGGGCAATTATGG	TCCGCTTTCTTCGCACTTAC
7	*NMB1668*	AAACGGATTTCGGCATACAC	CGTACCGGAGAACATTTCGT
8	*nrfA*	AAGAAAAACCGGCTCAAACA	ATAACCCGCCCATAACACAA
9	*nrfC*	GCACCCGTAGAGACTTCGTC	GCCTTCCGGTACTTTGTTTG
10	*nrfE*	CCGTTTGAGCGTAGTTTTCC	ATTGTCCAAGGTCGAATCCA
11	*omp64*	GCGGACAGTAAGCCTGAAAC	TGTTGTCGCATTTGAACCAC
12	*ompW*	GGCGAAGTGGCAAAAGTAAA	CAACACCTAAATTCGCATCG
16	*pepD*	GGCGCAAAAGTAGCATTCTC	TTGTCGGTCCGATAGAAACC
13	*PM0741*	GGCTCGGATTCATTTACCAC	AATAGACCGCATCCAGCTTC
	tbpA	ATTGGCAACCATCGGATTTA	GCACCTAAGCGATCACGAGT
14	*tonB1*	CTCCCTTGGTGCTGGTTATG	AATTTTTGCCGGTTGATACG
15	*torA*	GAATTTCCTTGTGCCGAGAG	GCTTCGCCGTATACCAAGTC

Article 2

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Effects of Growth Conditions on Biofilm Formation by *Actinobacillus pleuropneumoniae*

Short title: Biofilm formation in *A. pleuropneumoniae*

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§ Élaboration et réalisation des expériences de profil transcriptionnel, incluant la préparations des cultures bactériennes, et participation à la rédaction du manuscrit.

ABSTRACT

Biofilm formation is an important virulence trait of many bacterial pathogens. It has been reported in the literature that only two of the reference strains of the swine pathogen *Actinobacillus pleuropneumoniae*, representing serotypes 5b and 11, were able to form biofilm *in vitro*. In this study, we compared biofilm formation by the serotype 1 reference strain S4074 of *A. pleuropneumoniae* grown in five different culture media. We observed that strain S4074 of *A. pleuropneumoniae* is able to form biofilms after growth in one of the culture conditions tested (BHI medium, supplier B). Confocal laser scanning microscopy using a fluorescent probe specific to the poly-N-acetylglucosamine polysaccharide (PGA) further confirmed biofilm formation. In accordance, biofilm formation was susceptible to dispersin B, a PGA hydrolase. Transcriptional profiles of *A. pleuropneumoniae* S4074 following growth in BHI-B, which allowed a robust biofilm formation, and in BHI-A, in which only a slight biofilm formation was observed, were compared. Genes such as *tadC, tadD,* genes with homology to autotransporter adhesins as well as genes *pgaABC* involved in PGA biosynthesis and genes involved in zinc transport were up-regulated after growth in BHI-B. Interestingly, biofilm formation was inhibited by zinc, which was found to be more present in BHI-A (no or slight biofilm) following chemical analysis. We also observed biofilm formation in reference strains representing serotypes 3, 4, 5a, 12 and 14 as well as in 20 of the 37 fresh field isolates tested. Our data indicate that *A. pleuropneumoniae* has the ability to form biofilms under appropriate growth conditions and transition from a biofilm-positive to a biofilm-negative phenotype was reversible. Further studies are needed to identify which bacterial genes are specifically involved in biofilm formation and/or regulation, and what are the mechanisms by which zinc represses this phenotype in *A. pleuropneumoniae*.

Key words: *Actinobacillus pleuropneumoniae* / biofilm / growth conditions / transcriptomics

1. **INTRODUCTION**

Actinobacillus pleuropneumoniae, a member of the *Pasteurellaceae*, is an important swine pathogen responsible for economic losses in the swine industry. To date, fifteen serotypes of *A. pleuropneumoniae* have been described based on capsular antigens [3, 10]. The virulence of the bacteria is mediated by the coordinated action of several virulence factors, namely the capsule, lipopolysaccharides (LPS), Apx toxins and outer membrane proteins involved in iron uptake [4, 11, 14, 18, 19, 28, 29].

It is widely accepted that the majority of bacteria in virtually all ecosystems (natural, engineered and pathogenic ecosystems) grow in matrix-enclosed biofilms [7]. The matrix provides biofilm cells with a protected microenvironment containing nutrients, secreted enzymes and DNA. The matrix also contributes to the increased resistance to antibiotics and host defenses exhibited by biofilm cells [15]. All members of the *Pasteurellaceae* are inhabitants of mucosal surfaces of mammals and therefore formation of a biofilm may be crucial to their persistence *in vivo*. However, biofilms have only been investigated in a few species of the *Pasteurellaceae* family [16]. In *A. pleuropneumoniae*, the formation of biofilms on polystyrene microtiter plate is dependent on the production of PGA, a linear polymer of N-acetylglucosamine residues in β(1,6) linkage [17, 21]. The production of PGA is encoded by the genes *pgaABCD* [21]. A novel insertion element, ISApl1, was recently identified in an A/T rich region of the *pgaC* gene of the biofilm-negative *A. pleuropneumoniae* strain HB04 [25]. PGA is a substrate for dispersin B (DspB), a biofilm-releasing glycosyl hydrolase produced by *Aggregatibacter (Actinobacillus) actinomycetemcomitans* and *A. pleuropneumoniae* [21, 22]. It has also been reported that only two of the fifteen *A. pleuropneumoniae* reference strains, representing serotypes 5b and 11, were able to form a biofilm *in vitro* and that the transition from a biofilm-positive to biofilm-negative phenotype was irreversible [20]. However, Li et al. [24] recently observed slight biomass of biofilm when the *A. pleuropneumoniae* serotype 1 reference strain S4074 was grown in serum-free TSB but not in serum-

containing TSB. In addition, an enhanced biofilm formation was observed in *luxS* [24] and *hns* [8] mutants of *A. pleuropneumoniae* strain S4074.

The aims of the present study were: (i) to re-evaluate biofilm formation by *A. pleuropneumoniae* reference strain S4074 (serotype 1) under different growth conditions using a standard microtiter plate and crystal violet staining protocol; (ii) to evaluate the ability of 16 reference strains and 37 fresh field isolates to form biofilm in the growth condition shown to allow the best biofilm formation; (iii) and to determine the transcriptomic profile of *A. pleuropneumoniae* strain S4074 when grown in that culture condition.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

Bacterial strains used in the present study are listed in Table 1. Bacteria were grown on Brain Heart Infusion agar plates (BHI; Difco Laboratories, Detroit, MI, USA) supplemented with 15 µg/ml nicotinamide adenine dinucleotide (NAD). A colony was transferred into 5 ml of Luria-Bertani broth (LB; Difco Laboratories, Detroit, MI, USA), Tryptic Soy Broth (TSB; Difco), Mueller Hinton broth (MH; Difco) or BHI (BHI-A; Difco or BHI-B; Oxoid Ltd, Basingstoke, Hampshire, England) with 5 µg/ml NAD and incubated at 37˚C overnight with agitation. This culture was used for the biofilm assays.

2.2. Biofilm assay in microtiter plates

The microtiter plate biofilm assay is a static assay particularly useful for examining early events in biofilm formation [27]. The wells of a sterile 96-well microtiter plate (Costar® 3599, Corning, NY, USA) were filled in triplicate with a dilution (1/100) of an overnight bacterial culture. Following an incubation of 6 or 24h at 37˚C, the wells were washed by immersion in water and excess water was removed by inverting plates onto a paper towel. The wells were then filled with 100 µl of crystal violet (0,1%) and the plate was incubated for 2 minutes at room temperature. After removal

of the crystal violet solution, the plate was washed and dried in a 37˚C incubator for 30 minutes and 100 µl of ethanol (70%) were added to the wells. Absorbance was measured at 590 nm using a spectrophotometer (Powerwave, BioTek Instruments, Winooski, VT, USA).

2.3. Scanning laser confocal microscopy

The same biofilm assay protocol was used as described previously. After the 6 or 24h incubation, the wells were filled with 100 µl of Wheat Germ Agglutinin (WGA)–Oregon Green 488 (Molecular Probes, Eugene, OR, USA) diluted 1/100 in PBS and the plate was incubated for 30 minutes at room temperature in the dark. The plate was then washed with water and filled with PBS. The plate was observed with a confocal microscope (Olympus FV1000 IX81). WGA was excited at 488 nm and detected using 520 nm filters. The images were processed using Fluoview software (Olympus)

2.4. Transcriptomic microarray experiments

2.4.1. RNA extractions

For the microarray experiments, BHI-A or BHI-B broths were inoculated with 500 µl of an overnight culture of *A. pleuropneumoniae* serotype 1 strain S4074 and grown at 37°C in an orbital shaker until an optical density of 0.6 was reached. Ice-cold RNA degradation stop solution (95% ethanol, 5% buffer-saturated phenol), shown to effectively prevent RNA degradation and therefore preserve the integrity of the transcriptome [2], was added to the bacterial culture at a ratio of 1:10 (vol/vol). The sample was mixed by inversion, incubated on ice for 5 min, and then spun at 5000 g for 10 min to pellet the cells. Bacterial RNA isolation was then carried out using the QIAGEN RNeasy MiniKit (QIAGEN, Mississauga, ON, Canada), as prescribed by the manufacturer. During the extraction, samples were subjected to an on-column DNase treatment, as suggested by the manufacturer and then treated with Turbo DNase (Ambion, Austin, TX, USA) to ensure that all DNA contaminants were eliminated. The RNA concentration, quality and integrity were assessed spectrophotometrically and on gel.

2.4.2. Microarray construction and design

For the construction of AppChip2, 2033 ORFs from the complete genome sequence of *A. pleuropneumoniae* serotype 5b strain L20, representing more than 95% of all ORFs with a length greater than 160 nt., were amplified and spotted in duplicate on the chip. Spotted sheared genomic DNA from *A. pleuropneumoniae* L20 and porcine DNA are used as controls (GEO: GPL6658). Additional information concerning chip production is described by Gouré et al. [13].

2.4.3. Microarray hybridizations

cDNA synthesis and microarray hybridizations were performed as described [6]. Briefly, equal amounts (15 μg) of test RNA and control RNA were used to set up a standard reverse transcription reaction using random octamers (BioCorp, Montreal, QC, Canada), SuperScript II (Invitrogen, Carlsbad, CA, USA) and aminoallyl-dUTP (Sigma, St.Louis, MO, USA), and the resulting cDNA was indirectly labelled using a monofunctional NHS-ester Cy3 or Cy5 dye (Amersham, Buckinghamshire, UK). The labelling efficiency was assessed spectrophotometrically. Labelled samples were then combined and added to the AppChip2 for overnight hybridization. Five hybridizations were performed for the serotype 1 strain S4074 BHI-A vs BHI-B experiments. All slides were scanned using a Perkin-Elmer ScanArray Express scanner.

2.4.4. Microarray analysis and bioinformatics

Microarray data analysis was conducted with the TM4 Suite of software from the J. Craig Venture Institute [30] as described by Deslandes et al. [9]. Briefly, raw data was first generated using SpotFinder v.3.1.1. Locally weighted linear regression (lowess) was then performed in the Microarray Data Analysis System (MIDAS) in order to normalize the data. The Significance Analysis of Microarray (SAM) algorithm [33], which is implemented in TIGR microarray expression viewer (TMEV), was used to generate a list of differentially expressed genes. During SAM analysis, a false discovery rate (FDR) of 0% was estimated for the serotype 1 strain S4074 BHI-A vs BHI-B experiments.

2.5. Effects of dispersin B and zinc on biofilm formation

Biofilms were grown for 6 or 24h in BHI-B as described above. The wells were washed with water and then filled with 100 µl of PBS containing 0.2, 2.0 or 20 µg/ml of dispersin B (Kane Biotech Inc, Winnipeg, MB, Canada) as described by Izano et al. [17]. After incubation at 37˚C for 5 min, the wells were rinsed with water and stained with crystal violet. To monitor the effect of zinc on biofilm formation, bacteria were grown for 6 or 24h in BHI-B supplemented with 50-250 µg/ml of $ZnCl_2$.

2.6 Statistical analysis

The statistical significance (P value) of differences in biofilm phenotypes (mean optical density values) was determined by a paired, one-tailed t test using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA).

3. RESULTS

3.1. Biofilm formation and growth conditions

The ability of the *A. pleuropneumoniae* serotype 1 reference strain S4074 to form biofilms was evaluated using different growth media (Figure 1). No biofilm was present in the wells containing bacterial cells grown in LB broth while only a slight biofilm was observed in wells containing cells grown in TSB, MH or BHI-A broths after 24h of incubation. However a pronounced biofilm ($P < 0.01$) was formed when strain S4074 was grown in BHI-B broth. This was not due to an increased growth in BHI-B compared to BHI-A as similar growth curves were observed in both media.

We then evaluated biofilm formation by all the reference strains of *A. pleuropneumoniae* after growth for 6 or 24h in BHI-B. Similarly to what was observed with the serotype 1, we found that growth in BHI-B, but not BHI-A, allows biofilm formation in reference strains representing serotypes 4, 5a and 14. In addition to the already reported biofilm formation in serotypes 5b and 11, we also observed biofilms for serotype 3 and 12 reference strains. Moreover, biofilm formation

(OD_{590nm} > 0.1) was observed in 20 (54%) of the 37 fresh field isolates of serotypes 1, 5, 7 and 15 that were tested (Figure 2). In general, serotypes 5a, 5b and 7 field isolates tend to form more biofilms (mean OD of 1.15, 1.47 and 1.47 after 24h) than isolates from serotypes 1 and 15 (mean OD of 0.36 and 0.80 after 24h).

When *A. pleuropneumoniae* strain S4074 grown in BHI-A (no or slight biofilm) was transferred to BHI-B we observed the formation of a pronounced biofilm ($P < 0.05$). When these cells were then transferred back to BHI-A, the phenotype returned to a slight biofilm ($P < 0.05$). This was also observed with field isolates representing different serotypes (data not shown).

3.2. Scanning laser confocal microscopy

We observed that for many reference strains, including strain S4074, and field isolates, pronounced biofilms were present after a short incubation period of only 6h (Figure 2). The biofilm was visualized by confocal laser scanning microscopy using a fluorescent probe (WGA-Oregon Green) specific to the poly-N-acetylglucosamine matrix polysaccharide (Figure 3). It is evident from these micrographs that *A. pleuropneumoniae* strain S4074 does not form biofilm when grown in BHI-A while a thick poly-N-acetylglucosamine matrix is formed by *A. pleuropneumoniae* serotype 5b strain L20 grown in the same condition. However, both strains showed a pronounced biofilm when grown in BHI-B. In the case of strain S4074, the biofilm is even more important after 6h than 24h of incubation (Figure 3). Because scanning laser confocal microscopy allows optical sectioning of the biofilm either in the horizontal or the vertical dimension it is possible to evaluate the thickness of the biofilm. We evaluated the thickness of *A. pleuropneumoniae* strain S4074 biofilm to be of ~25 μm after growth in BHI-B for 6h (Figure 3C) and even greater (~65 μm) for *A. pleuropneumoniae* strain L20.

3.3. Transcriptomic profiling under different growth conditions

To assess the transcriptional response of *A. pleuropneumoniae* S4074 after growth in BHI-B compared to BHI-A, transcript profiling experiments using DNA microarrays

were performed. Overall, 232 genes were significantly differentially expressed during growth in BHI-B; 152 being up-regulated and 80 being down-regulated (Table II). The genes that showed the highest level of up-regulation after growth in BHI-B belonged to the "Amino Acid Biosynthesis", "Energy Metabolism", "Transport and Binding Proteins", "Cell Envelope" and "Hypothetical/Unknown/Unclassified" functional classes (Figure 4). Genes such as *tadC* and *tadD* (tight adherence proteins C and D), genes with homology to autotransporter adhesins (APL_0443 and APL_0104) as well as genes *pgaABC* involved in PGA biosynthesis were up-regulated after growth in BHI-B. A cluster of genes involved in dipeptide transport (*dppABCDF*) and genes involved in the synthesis of an urease (*ureAEFG*) were also up-regulated. Down-regulated genes after growth in BHI-B mostly belonged to the "Transport and Binding Proteins", "Cell Envelope", "Protein Synthesis" and "Hypothetical/Unknown/Unclassified" functional classes. Most notably, *cys* genes involved in sulphate transport systems were down-regulated, as well as a gene (APL_1096) sharing 59% identity with the dispersin B gene of *A. actinomycetemcomitans*.

3.4. Effect of dispersin B on biofilm formation

Enzymatic treatment with dispersin B of biofilms of *A. pleuropneumoniae* strains S4074 and L20 grown for 6 or 24h almost completely dispersed them ($P < 0.05$) confirming the presence of PGA in the biofilm matrix.

3.5. Effect of zinc on biofilm formation

Chemical analysis showed differences in some divalent cations concentration between BHI-A (Fe <0.10 ppm, Zn 2.03 ppm) and BHI-B (Fe 0.10 ppm, Zn 1.75 ppm) while no differences were observed for others (Ca, Cu, Mg, Mn). We therefore hypothesized that the difference in biofilm formation observed after growth in BHI-B compared to BHI-A might be due to cations concentration. Since the concentration of zinc was found to be higher in BHI-A (no or slight biofilm) we tested a possible inhibitory effect of this cation on biofilm formation. The addition of $ZnCl_2$ to BHI-B inhibited, in a dose-dependent manner, the formation of biofilms by *A.*

pleuropneumoniae strains S4074 and L20 (Figure 5). A complete inhibition ($P < 0.01$) was observed when 100 µg/ml of $ZnCl_2$ was added to BHI-B, a concentration which did not affect growth after 24h (data not shown). A similar inhibition was also observed with the addition of $ZnSO_4$, ZnO, and $Zn_3(PO_4)_2$ but not with $MgCl_2$ or $CaCl_2$ thus confirming that the inhibition was due to the addition of zinc. Biofilm formation in *A. actinomycetemcomitans* was also inhibited by zinc (data not shown). Interestingly, genes potentially involved in zinc transport (*znuA* and APL_0096) were up-regulated after growth in BHI-B (Table II).

4. DISCUSSION

Biofilm formation is an important virulence trait of many bacterial pathogens including *A. pleuropneumoniae*. It has been previously reported that only two of the fifteen *A. pleuropneumoniae* reference strains, representing serotypes 5b and 11, were able to form a biofilm *in vitro* [20]. We observed however an increased stickiness of colonies when strain *A. pleuropneumoniae* S4074 was grown on plates made of BHI from one of two different suppliers. In addition, Li et al. [24] recently observed slight biomass of biofilm when the *A. pleuropneumoniae* serotype 1 reference strain S4074 was grown in serum-free TSB and that an enhanced biofilm formation was observed in *luxS* [24] and *hns* [8] mutants of *A. pleuropneumoniae* S4074. These observations brought us to re-evaluate biofilm formation by strain *A. pleuropneumoniae* S4074 under different growth conditions using a standard microtiter plate and crystal violet staining protocol. Our data indicate that strain S4074 has the ability to form a pronounced biofilm when grown in the appropriate conditions, and that the biofilm was sensitive to dispersin B treatment and can be inhibited by zinc. Transition from a biofilm-positive to a biofilm-negative phenotype is not irreversible in contrast to what was reported by Kaplan and Mulks [20] under different conditions.

Transcript profiling experiments using DNA microarrays indicated that overall, 232 genes were significantly differentially expressed during growth in BHI-B. Genes such as *tadC, tadD,* genes with homology to autotransporter adhesins as well as genes *pgaABC* involved in PGA biosynthesis were up-regulated after growth in BHI-B. While we can hypothesize that these genes might be important for the formation of the biofilm itself, it is also interesting to note that many of the same genes (*tadB, rcpA*, gene APL_0443 with high homology to the Hsf autotransporter adhesin of *Haemophilus influenzae* as well as genes *pgaBC* involved in biofilm biosynthesis) were up-regulated, when the transcriptomic profile of *A. pleuropneumoniae* was determined after contact with porcine lung epithelial cells [1], thus emphasizing the possible importance of biofilm formation for the establishment of the infection.

Initial steps in biofilm development require the transcription, early on, of genes involved in reversible attachment and motility, before a subsequent switch towards the transcription of genes involved in the irreversible attachment of bacteria [35]. This second irreversible attachment might require the synthesis of adhesive organelles, such as the curli fibers (*csg* genes). Interestingly, gene APL_0220 is a putative lipoprotein of the CsgG family, responsible for the transport and assembly of curli fibers. The up-regulation of other genes possibly involved in adhesion processes (*tadC*, *tadD*, Hsf homolog APL_0443) might indicate that bacterial cells were entering or in the middle of this irreversible attachment phase. In *A. actinomycetemcomitans*, the Tad locus is essential for biofilm formation [32]. The fact that the transcription of a zinc-specific transporter (*znuA*) was increased, combined with the decrease in transcription of an hypothetical Zn-dependant protease (APL_1898) and lower concentration of this metal in BHI-B lead us to believe that Zn restriction might be a signal leading to increase biofilm formation.

It is tempting to speculate that growth in BHI-B affected the expression of regulators which in turn affected PGA expression and biofilm formation. Indeed, it has been recently shown that an enhanced biofilm formation was observed in a *hns* mutant of *A. pleuropneumoniae* strain S4074 [8] and that over-expression of RpoE in a *rseA*

mutant is sufficient to alleviate repression of biofilm formation by H-NS[1]. However, other genes have been shown to affect biofilm formation in *A. pleuropneumoniae*. An enhanced biofilm formation was observed in a quorum sensing (*luxS*) mutant [24] while a mutant in the ArcAB two-component system facilitating metabolic adaptation to anaerobicity (*arcA*) [5] and an autotransporter serine protease (AasP) mutant were deficient in biofilm formation [31]. It is interesting to note that many genes involved in branched-chain amino acid biosynthesis (*ilv* genes) were up-regulated after growth in BHI-B. Limitation of branched-chain amino acids was shown to be a cue for expression of a subset of *in vivo* induced genes in *A. pleuropneumoniae*, including not only genes involved in the biosynthesis of branched-chain amino acids, but also other genes that are induced during infection of the natural host [34].

Our data indicate that many strains of *A. pleuropneumoniae* have the ability to form biofilms under appropriate growth conditions. This is an important observation considering that *A. pleuropneumoniae* biofilm cells exhibit increased resistance to antibiotics compared to planktonic cells [17] and may also exhibit increased resistance to biocides [12]. Biofilms are often associated with chronic infections but the fact that *A. pleuropneumoniae* can form an important biofilm after only 6 hours of incubation suggests that biofilm formation might also play a role in acute infections.

We have undertaken the screen of a large library of mini-*Tn10* isogenic mutants of *A. pleuropneumoniae* S4074 in order to identify other genes that are involved in biofilm formation and/or regulation. A better understanding of biofilm formation in *A. pleuropneumoniae* might lead to the development of molecules or strategies to

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[1]Bossé J.T., Sinha S., O'Dwyer C.A., Rycroft A.N., Kroll J.S., Langford, P.R., H-NS is a specific regulator of biofilm formation in *Actinobacillus pleuropneumoniae*, Proceedings of the International *Pasteurellaceae* Society meeting, Sorrento, Italy, 2008, P110.

interfere with biofilm formation and prevent infection in pigs. In that respect, we made an important, and unexpected, observation that zinc could completely inhibit biofilm formation in *A. pleuropneumoniae* and *A. actinomycetemcomitans,* which also synthesizes PGA [21]. We do not know at this time how zinc interferes with PGA biosynthesis and biofilm formation but some glycosyltransferases have been shown to be inhibited by zinc [23]. Hypozincemia which occurs during infection and inflammation [26] might therefore favour biofilm formation by *A. pleuropneumoniae*. Knowing that PGA functions as a biofilm matrix polysaccharide in phylogenetically diverse bacterial species such as *Staphylococcus aureus*, *S. epidermidis*, and *Escherichia coli* [21], it would be worth investigating whether zinc can also interfere with PGA biosynthesis in these other bacterial pathogens.

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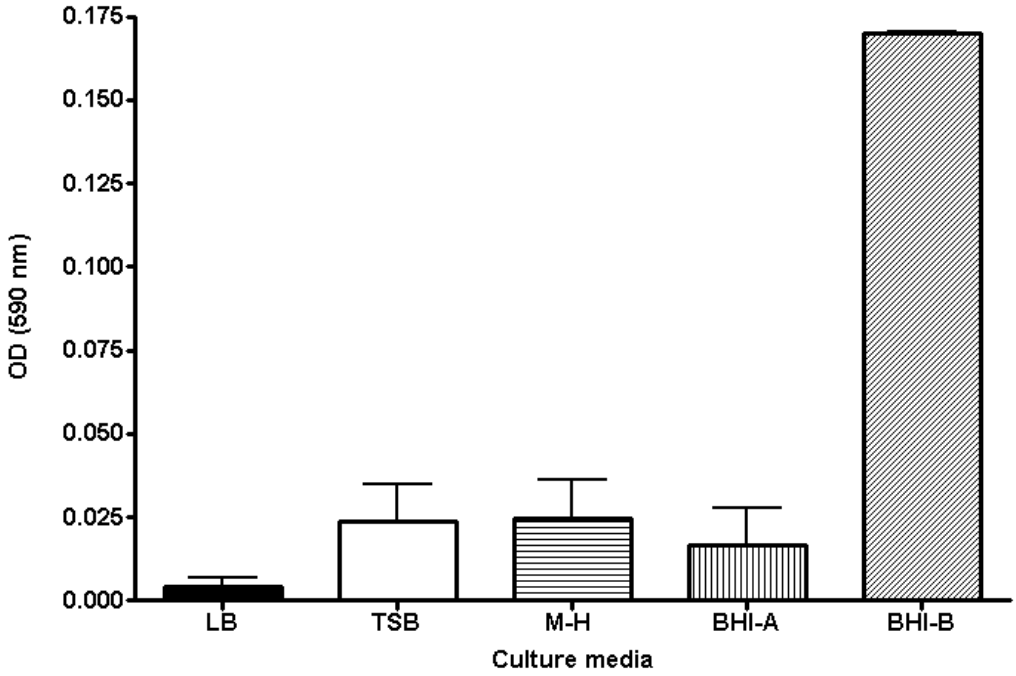
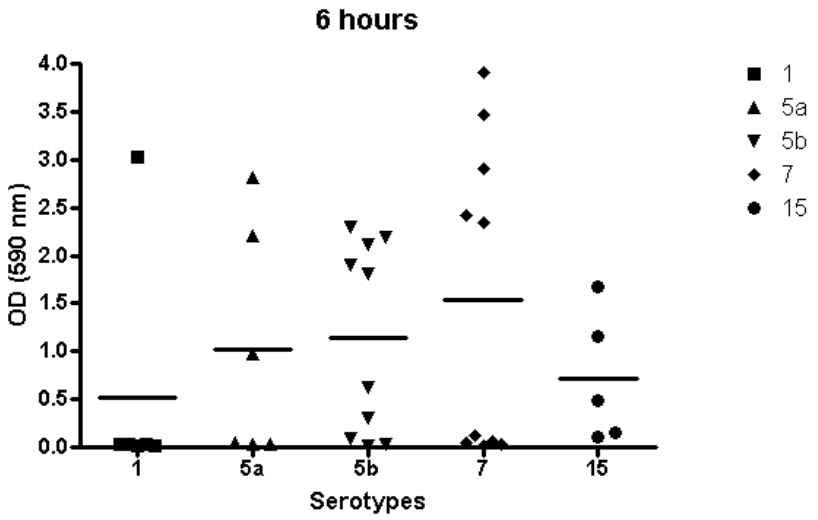


Figure 1. Biofilm formation by *A. pleuropneumoniae* serotype 1 reference strain S4074 grown in different culture media using the crystal violet staining protocol described in Materials and Methods. LB: Luria-Bertani; TSB: Tryptic Soy Broth; M-H: Mueller Hinton; BHI: Brain Heart Infusion.

A



B

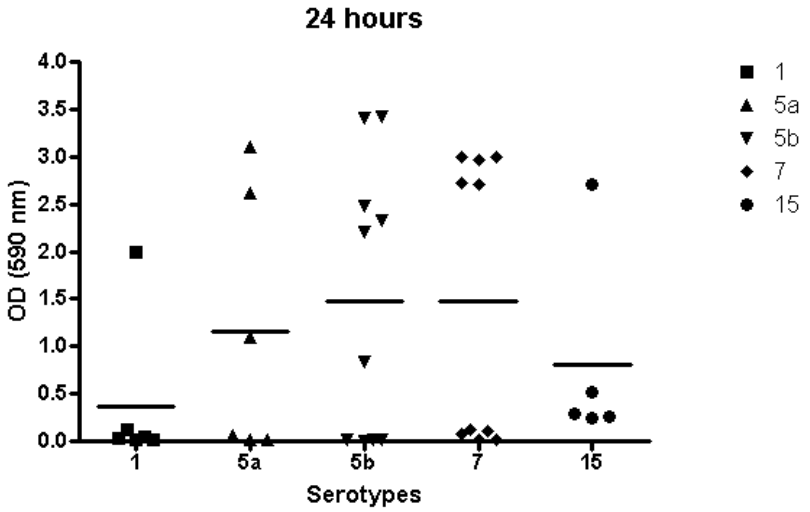


Figure 2. Thirty-seven independent fresh field isolates of *A. pleuropneumoniae* (representing serotypes 1, 5, 7 and 15) were tested for their ability to form biofilms when grown for 6 (A) and 24h (B) in BHI-B using the microtiter plate assay.

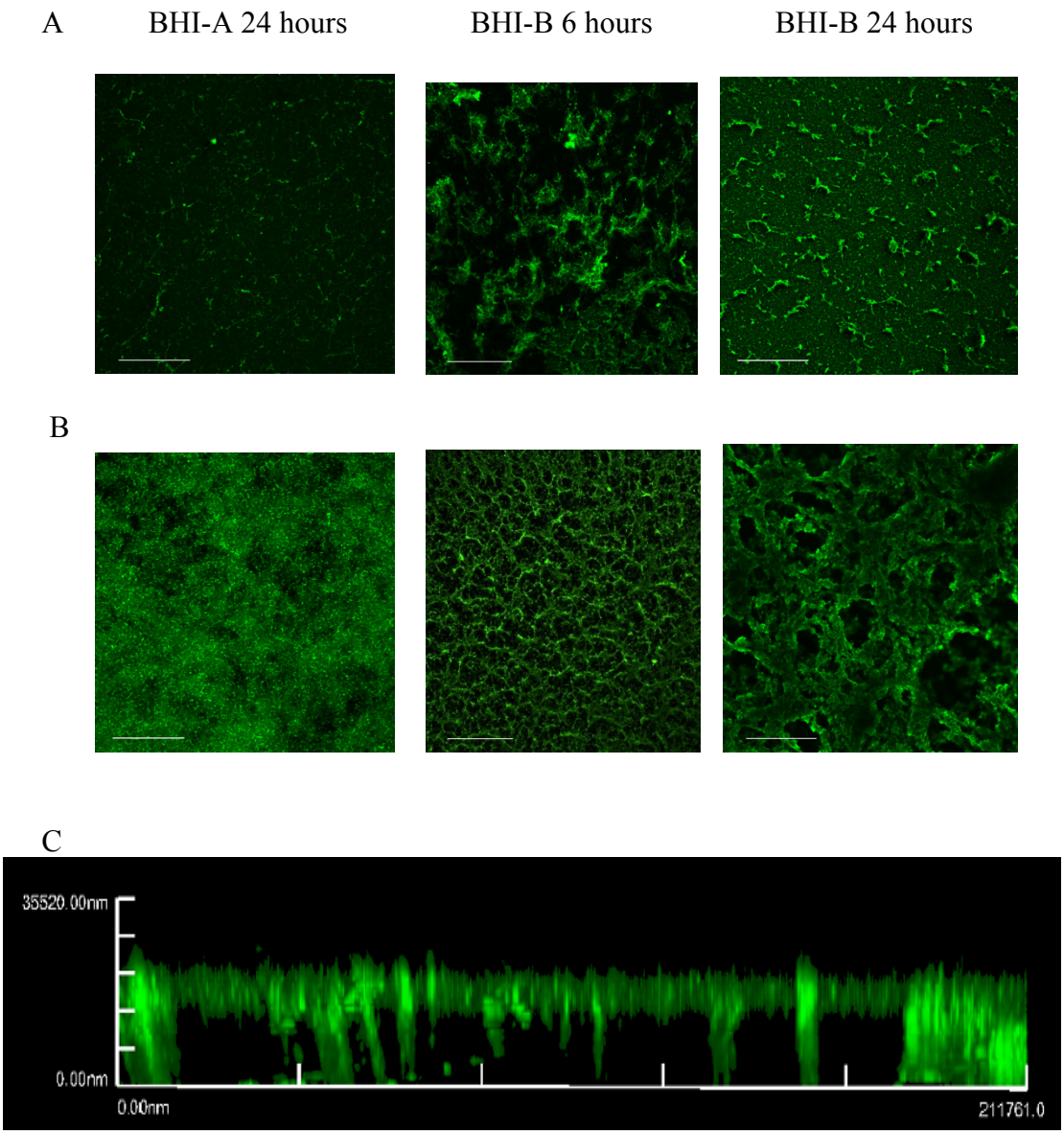


Figure 3. Confocal scanning laser microscopic images of *A. pleuropneumoniae* serotype 1 strain S4074 (A and C) and serotype 5b strain L20 (B) biofilms stained with WGA-Oregon Green 488. (C) Stack of sections through the X-Z plane of a biofilm formed after 6 hours in BHI-B. Bars = 50 µm.

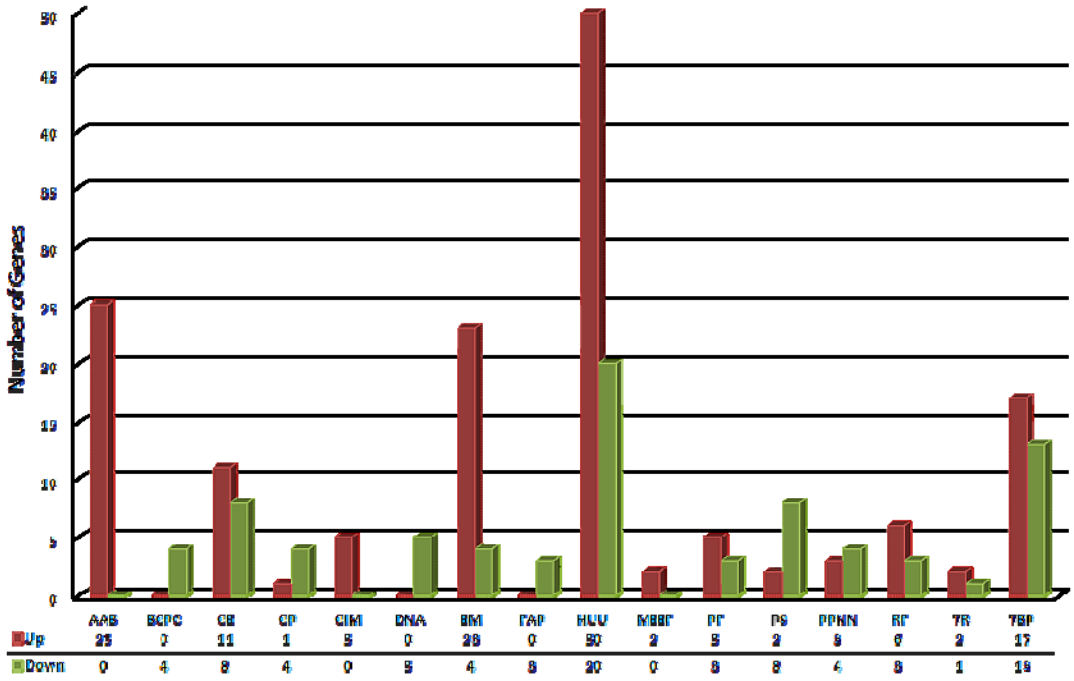


Figure 4. Functional classification of the differentially expressed genes during growth of *A. pleuropneumoniae* S4074 in BHI-B according to TIGRFAMs. AAB: amino acids biosynthesis; BCPC: biosynthesis of cofactors, prosthetic groups and carriers; CE: cell envelope; CP: cellular processes; CIM: central intermediary metabolism; DNA: DNA metabolism; EM: energy metabolism; FAPM: fatty acid and phospholipid metabolism; HUU: hypothetical proteins/unclassified/unknown; MEEF: mobile and extrachromosomal element functions; PF: protein fate; PS: protein synthesis; PPNN: purines, pyrimidines, nucleosides and nucleotides; RF: regulatory functions; TR: transcription; TBP: transport and binding proteins.

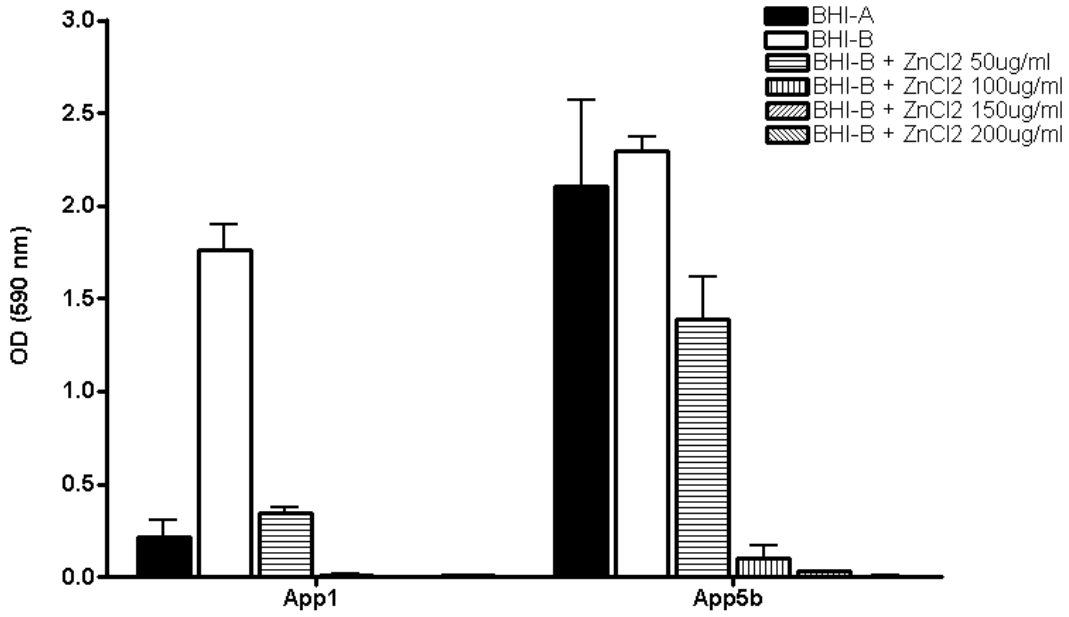


Figure 5. Effect of the addition of $ZnCl_2$ on biofilm formation by *A. pleuropneumoniae* serotype 1 strain S4074 (App1) and serotype 5b strain L20 (App5b) grown for 6 hours.

Table I. *A. pleuropneumoniae* strains used in the present study.

Strains	Relevant traits	Source
Reference strains		
S4074	Serotype 1	K.R.Mittal[1]
4226	Serotype 2	K.R.Mittal[1]
1421	Serotype 3	K.R.Mittal[1]
1462	Serotype 4	K.R.Mittal[1]
K17	Serotype 5a	K.R.Mittal[1]
L20	Serotype 5b	K.R.Mittal[1]
FEMO	Serotype 6	K.R.Mittal[1]
WF.83	Serotype 7	K.R.Mittal[1]
405	Serotype 8	K.R.Mittal[1]
13261	Serotype 9	K.R.Mittal[1]
13039	Serotype 10	K.R.Mittal[1]
56153	Serotype 11	K.R.Mittal[1]
832985	Serotype 12	K.R.Mittal[1]
N273[4]	Serotype 13	M. Gottschalk[1]
3906[4]	Serotype 14	M. Gottschalk[1]
HS143	Serotype 15	M. Gottschalk[1]
Field strains		
05-7430, 05-7431	Serotype 1	M. Ngeleka [2]
111A, 719, 2398, 2521	Serotype 1	D. Slavic [3]
05-4817, 05-C996, 06-996	Serotype 5a	S. Messier [1]
04-37943, 04-3128, 05-508	Serotype 5a	M. Ngeleka [2]
05-6501, 06-4091	Serotype 5b	S. Messier [1]
03-14796, 03-22382, 03-22383, 05-4832	Serotype 5b	M. Ngeleka [2]
366A, 400, 564D, 888	Serotype 5b	D. Slavic [3]
05-3695, 06-3008, 06-3060, 06-4108	Serotype 7	S. Messier [1]
04-37257, 05-14401	Serotype 7	M. Ngeleka [2]
881, 986, 1951, 4648	Serotype 7	D. Slavic [3]
05-13146, 05-14657, 05-20080, 05-20081, 05-2983	Serotype 15	M. Ngeleka [2]

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[4] These strains are NAD-independent and belong to biotype II.

Table II. *A. pleuropneumoniae* strain S4074 genes which are up- or down-regulated after growth in BHI-B compared to growth in BHI-A.

Locus Tag	Gene	Description	Fold Change
Amino Acid Biosynthesis			
APL_0728	*ilvH*	Acetolactate synthase small subunit	5.707
APL_0662	*aspC*	Putative aspartate aminotransferase	5.324
APL_0427	*gdhA*	NADP-specific glutamate dehydrogenase	4.943
APL_0727	*ilvI*	Acetolactate synthase large subunit	4.204
APL_0099	*ilvG*	Acetolactate synthase isozyme II large subunit (AHAS-II)	3.915
APL_1499	*thrC*	Threonine synthase	3.198
APL_0097	*ilvD*	Dihydroxy-acid dehydratase	3.142
APL_0393	*leuA*	2-isopropylmalate synthase	3.000
APL_0098	*ilvM*	Acetolactate synthase isozyme II small subunit (AHAS-II)	2.934
APL_2027	*hisF*	Imidazole glycerol phosphate synthase subunit hisF	2.833
APL_0702	*serC*	Phosphoserine aminotransferase	2.788
APL_0432	*leuB*	3-isopropylmalate dehydrogenase	2.643
APL_0899	*dapA*	Dihydrodipicolinate synthase	2.401
APL_0211	*glyA*	Glycine/serine hydroxymethyltransferase	2.398
APL_0133	*cysB*	HTH-type transcriptional regulator CysB	2.340
APL_1853	*ilvC*	Ketol-acid reductoisomerase	2.313
APL_0072	*ilvE*	Branched-chain-amino-acid aminotransferase	2.001
APL_0859	*trpCF*	Tryptophan biosynthesis protein trpCF	1.883
APL_2025	*hisH*	Imidazole glycerol phosphate synthase subunit hisH	1.777
APL_2026	*hisA*	Phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	1.739
APL_1198	*APL_1198*	Putative NAD(P)H nitroreductase	1.708
APL_0139	*leuC*	3-isopropylmalate dehydratase large subunit 2	1.605
APL_1230	*serB*	Phosphoserine phosphatase	1.438
APL_0620	*aroG*	Phospho-2-dehydro-3-deoxyheptonate aldolase	1.428
APL_1873	*dapE*	Succinyl-diaminopimelate desuccinylase	1.380
Biosynthesis of cofactors, prosthetic groups, and carriers			
APL_0207	*Dxs*	1-deoxy-D-xylulose-5-phosphate synthase (DXPS)	-1.555
APL_1461	*menA*	1,4-dihydroxy-2-naphthoateoctaprenyltransferase	-1.631
APL_0382	*ribD*	Riboflavin biosynthesis protein	-1.726
APL_1408	*gshA*	Glutathione biosynthesis bifunctional protein GshAB	-1.789
Cell Envelope			
APL_1494	*ftpA*	Fine tangled pili major subunit	5.705
APL_1921	*pgaA*	Biofilm PGA synthesis protein PgaA precursor	5.308
APL_0460	*plpD*	Lipoprotein Plp4	3.801
APL_1923	*pgaC*	Biofilm PGA synthesis N-glycosyltransferase PgaC	3.591
APL_1922	*pgaB*	Biofilm PGA synthesis lipoprotein PgaB precursor	3.093
APL_0006	*ompP2A*	Outer membrane protein P2	2.515
APL_0550	*tadC*	Tight adherence protein C	1.985
APL_0442	*sanA*	SanA protein	1.776
APL_0549	*tadD*	Tight adherence protein D	1.749
APL_0332	*hlpB*	Lipoprotein HlpB	1.627
APL_1364	*gmhA*	Putative phosphoheptose isomerase	1.386
APL_0873	*rlpB*	Putative rare lipoprotein B	-1.391
APL_1028	*APL_1028*	Possible lipooligosaccharide N-acetylglucosamine glycosyltransferase	-1.445

APL_0747	*mepA*	Penicillin-insensitive murein endopeptidase precursor	-1.446
APL_0436	*mreC*	Rod shape-determining protein MreC	-1.585
APL_1086	*ompW*	Outer membrane protein W precursor	-1.606
APL_1029	*APL_1029*	Hypothetical protein	-1.650
APL_1424	*oxaA*	Inner membrane protein OxaA	-1.772
APL_0933	*ompP1*	Putative outer membrane protein precursor	-2.808
Cellular Processes			
APL_1489	*Tpx*	Putative thiol peroxidase	2.252
APL_0988	*hktE*	Catalase	-1.461
APL_0669	*APL_0669*	Putative iron dependent peroxidase	-1.483
APL_1442	*apxID*	RTX-I toxin secretion component	-1.506
APL_1346	*ftsY*	Cell division protein FtsY-like protein	-1.530
Central Intermediary Metabolism			
APL_1615	*Gst*	Putative glutathione S-transferase	3.269
APL_1614	*ureE*	Urease accessory protein UreE	2.601
APL_1613	*ureF*	Urease accessory protein UreF	2.478
APL_1612	*ureG*	Urease accessory protein UreG	2.165
APL_1618	*ureA*	Urease gamma subunit UreA	1.653
DNA Metabolism			
APL_1931	*tagI*	3-methyladenine-DNA glycosidase	-1.500
APL_1474	*dnaG*	DNA primase	-1.551
APL_1282	*dnaQ*	DNA polymerase III subunit	-1.579
APL_1255	*parE*	DNA topoisomerase IV subunit	-1.630
APL_1505	*holC*	DNA polymerase III subunit	-1.663
Energy Metabolism			
APL_1197	*APL_1197*	3-hydroxyacid dehydrogenase	3.100
APL_0841	*pntB*	NAD(P) transhydrogenase subunit beta	2.726
APL_1908	*xylA*	Xylose isomerase	2.243
APL_0894	*fdxH*	Formate dehydrogenase, iron-sulfur subunit	2.161
APL_1425	*napC*	Cytochrome c-type protein NapC	2.159
APL_1799	*torC*	Pentahemic c-type cytochrome	2.156
APL_0892	*fdxG*	Formate dehydrogenase, nitrate-inducible, major subunit	2.116
APL_1798	*torA*	Trimethylamine-N-oxide reductase precursor	1.977
APL_0381	*glpC*	Anaerobic glycerol-3-phosphate dehydrogenase subunit C	1.919
APL_0842	*pntA*	NAD(P) transhydrogenase subunit alpha	1.903
APL_0895	*fdnI*	Formate dehydrogenase, cytochrome b556 subunit	1.816
APL_1208	*adhC*	Putative alcohol dehydrogenase class 3	1.801
APL_0971	*APL_0971*	Putative acyl CoA thioester hydrolase	1.796
APL_0652	*manB*	Phosphomannomutase	1.677
APL_0483	*APL_0483*	Predicted nitroreductase	1.668
APL_0142	*glxK*	Glycerate kinase	1.564
APL_0452	*sucC*	Succinyl-CoA synthetase beta chain	1.515
APL_0461	*APL_0461*	Predicted hydrolases of the HAD superfamily	1.456
APL_0687	*Dld*	D-lactate dehydrogenase	1.439
APL_1510	*gpsA*	Glycerol-3-phosphate dehydrogenase (NAD(P)+)	1.414
APL_1427	*napH*	Ferredoxin-type protein NapH-like protein	1.360
APL_0789	*APL_0789*	Dioxygenase	1.253
APL_0983	*tktA*	Transketolase 2	1.233
APL_1036	*pflB*	Formate acetyltransferase	-1.653

APL_1498	*mgsA*	Methylglyoxal synthase	-1.790
APL_1840	*ubiC*	4-hydroxybenzoate synthetase (chorismate lyase)	-1.952
APL_0857	*sdaA*	L-serine dehydratase	-3.016
Fatty Acid and Phospholipid Metabolism			
APL_1407	*Psd*	Phosphatidylserine decarboxylase	-1.419
APL_1384	*fabH*	3-oxoacyl-[acyl-carrier-protein] synthase 3	-1.826
APL_1385	*plsX*	Fatty acid/phospholipid synthesis protein PlsX	-2.706
Mobile and extrachromosomal element functions			
APL_1056	*APL_1056*	Transposase	1.560
APL_0985	*APL_0985*	Transposase	1.271
Protein Fate			
APL_0871	*pepE*	Peptidase E	2.551
APL_1101	*pepA*	Putative cytosol aminopeptidase	1.913
APL_0254	*pepD*	Aminoacyl-histidine dipeptidase	1.903
APL_1883	*ptrA*	Protease 3 precursor	1.680
APL_0928	*hscB*	Co-chaperone protein HscB-like protein	1.377
APL_1068	*secF*	Protein-export membrane protein SecF	-1.496
APL_0321	*dsbB*	Disulfide bond formation protein B	-1.557
APL_1035	*pflA*	Pyruvate formate-lyase 1-activating enzyme	-1.774
Protein Synthesis			
APL_1821	*rpmE*	50S ribosomal protein L31	2.211
APL_0484	*rimK*	Ribosomal protein S6 modification protein	1.533
APL_1781	*rpsM*	30S ribosomal protein S13	-1.401
APL_0205	*APL_0205*	Predicted rRNA methyltransferase	-1.538
APL_0399	*ksgA*	Dimethyladenosine transferase	-1.578
APL_0679	*glnS*	Glutaminyl-tRNA synthetase	-1.584
APL_0641	*truB*	tRNA pseudouridine synthase B	-1.742
APL_1383	*trmB*	tRNA (guanine-N(7)-)-methyltransferase	-1.756
APL_0574	*APL_0574*	tRNA-specific adenosine deaminase	-1.778
APL_0723	*Tgt*	Queuine tRNA-ribosyltransferase	-1.937
Purines, pyrimidines, nucleosides, and nucleotides			
APL_0958	*purH*	Bifunctional purine biosynthesis protein PurH	1.856
APL_0593	*guaB*	Inosine-5'-monophosphate dehydrogenase	1.485
APL_1343	*Cdd*	Cytidine deaminase	1.278
APL_1014	*deoD*	Purine nucleoside phosphorylase DeoD-like protein	-1.430
APL_0351	*Ndk*	Nucleoside diphosphate kinase	-1.531
APL_1839	*Udp*	Uridine phosphorylase	-1.617
APL_1075	*purA*	Adenylosuccinate synthetase	-1.762
Regulatory Functions			
APL_0059	*narP*	Nitrate/nitrite response regulator protein	2.552
APL_0823	*glpR*	Glycerol-3-phosphate regulon repressor	1.908
APL_1295	*argR*	Arginine repressor	1.896
APL_0126	*APL_0126*	HIT-like protein	1.580
APL_0395	*rseA*	Putative sigma-E factor negative regulatory protein	1.524
APL_1668	*rbsR*	Ribose operon repressor	1.302
APL_1270	*sprT*	Putative SprT-like protein	-1.483
APL_1233	*malT*	HTH-type transcriptional regulator MalT	-1.484
APL_1540	*tldD*	TldD-like protein	-1.578

Transcription			
APL_0560	*rhlB*	ATP-dependent RNA helicase RhlB	1.409
APL_0423	*rnhA*	Ribonuclease HI	1.345
APL_0201	*nusB*	Transcription antitermination protein NusB	-1.457
Transport and Binding Proteins			
APL_0967	*gltS*	Sodium/glutamate symport carrier protein	4.155
APL_0377	*glpT*	Glycerol-3-phosphate transporter	3.247
APL_0064	*dppA*	Periplasmic dipeptide transport protein	3.168
APL_0869	*abgB*	Aminobenzoyl-glutamate utilization-like protein	3.004
APL_1857	*merP*	Copper chaperone MerP	2.911
APL_0068	*dppF*	Dipeptide transport ATP-binding protein DppF	2.860
APL_1665	*gntP_1*	Gluconate permease	2.723
APL_0066	*dppC*	Dipeptide transport system permease protein DppC	2.640
APL_1440	*znuA*	High-affinity zinc uptake system protein ZnuA precursor	2.600
APL_0065	*dppB*	Dipeptide transport system permease protein DppB	2.229
APL_0067	*dppD*	Dipeptide transport ATP-binding protein DppD	2.036
APL_1448	*afuC*	Ferric ABC transporter ATP-binding protein	1.855
APL_1319	*ptsB*	PTS system sucrose-specific EIIBC component	1.744
APL_1320	*thiQ*	Thiamine transport ATP-binding protein ThiQ	1.569
APL_1622	*cbiM*	Predicted ABC transport permease protein CbiM	1.433
APL_1620	*cbiO*	Predicted ABC transport ATP-binding protein CbiO	1.417
APL_1173	*pnuC*	Nicotinamide mononucleotide transporter	1.408
APL_0749	*APL_0749*	Potassium efflux system KefA	-1.436
APL_1212	*tehA*	Tellurite resistance protein TehA	-1.543
APL_0716	*APL_0716*	Iron(III) ABC transporter, permease protein	-1.547
APL_1253	*APL_1253*	Putative sodium/sulphate transporter	-1.598
APL_1846	*cysT*	Sulfate transport system permease protein cysT	-1.684
APL_0191	*APL_0191*	Predicted Na+-dependent transporter of the SNF family	-1.751
APL_1083	*arcD*	Putative arginine/ornithine antiporter	-1.786
APL_2016	*fhuA*	Ferrichrome-iron receptor FhuA	-2.031
APL_1847	*cysW*	Sulfate transport system permease protein cysW	-2.195
APL_1844	*cysN*	Sulphate adenylate transferase subunit 1	-2.375
APL_1848	*cysA*	Sulfate/thiosulfate import ATP-binding protein cysA	-2.401
APL_1843	*cysJ*	Sulfite reductase [NADPH] flavoprotein alpha-component	-2.757
APL_1127	*APL_1127*	Predicted Na+/alanine symporter	-3.402
Hypothetical/Unknown/Unclassified			
APL_1100	*APL_1100*	Hypothetical protein	3.395
APL_0920	*APL_0920*	Hypothetical protein	2.835
APL_1882	*APL_1882*	Hypothetical protein	2.776
APL_1856	*APL_1856*	Hypothetical protein	2.775
APL_1855	*APL_1855*	Hypothetical protein	2.763
APL_0443	*APL_0443*	Autotransporter adhesin	2.762
APL_1252	*APL_1252*	Hypothetical protein	2.739
APL_0134	*APL_0134*	Hypothetical protein	2.681
APL_0836	*APL_0836*	Putative transcriptional regulator	2.661
APL_1588	*APL_1588*	Predicted TRAP transporter solute receptor	2.464
APL_1491	*APL_1491*	Hypothetical protein	2.282
APL_0104	*APL_0104*	Autotransporter adhesin	2.231
APL_1069	*ftnA*	Ferritin-like protein 1	2.194
APL_1059	*APL_1059*	Hypothetical transposase-like protein	2.172

APL_1690	*APL_1690*	Inner membrane protein	2.168
APL_0245	*APL_0245*	Transferrin binding protein-like solute binding protein	2.097
APL_1191	*namA*	NADPH dehydrogenase	2.078
APL_1948	*APL_1948*	Hypothetical protein	2.061
APL_0870	*APL_0870*	Putative C4-dicarboxylate transporter	2.034
APL_0643	*APL_0643*	Hypothetical protein	2.029
APL_1743	*APL_1743*	Ser/Thr protein phosphatase family protein	1.999
APL_0426	*APL_0426*	Hypothetical protein	1.994
APL_1791	*APL_1791*	Putative periplasmic iron/siderophore binding protein	1.944
APL_0970	*APL_0970*	Hypothetical protein	1.908
APL_1070	*ftnB*	Ferritin-like protein 2	1.907
APL_1894	*APL_1894*	Hypothetical protein	1.907
APL_1374	*APL_1374*	Hypothetical protein	1.803
APL_1206	*APL_1206*	Plasmid stability-like protein	1.794
APL_1881	*APL_1881*	Hypothetical protein	1.792
APL_0038	*APL_0038*	Hypothetical protein	1.730
APL_1355	*APL_1355*	Hypothetical protein	1.716
APL_0471	*APL_0471*	Hypothetical protein	1.707
APL_1438	*APL_1438*	Hypothetical protein	1.689
APL_1437	*APL_1437*	Hypothetical protein	1.643
APL_1423	*APL_1423*	Hypothetical protein	1.612
APL_0125	*APL_0125*	Hypothetical protein	1.608
APL_0096	*APL_0096*	Zinc transporter family protein ZIP	1.592
APL_0220	*APL_0220*	Putative lipoprotein	1.583
APL_1934	*APL_1934*	Hypothetical protein	1.570
APL_1574	*APL_1574*	Hypothetical protein	1.543
APL_0036	*APL_0036*	Hypothetical protein	1.533
APL_0222	*APL_0222*	Putative lipoprotein	1.518
APL_1088	*APL_1088*	Hypothetical protein	1.512
APL_1207	*APL_1207*	Hypothetical protein	1.510
APL_0463	*APL_0463*	Predicted sortase and related acyltransferases	1.448
APL_1859	*APL_1859*	Probable NADH-dependent butanol dehydrogenase 1	1.448
APL_1828	*APL_1828*	PilT protein-like protein	1.447
APL_0433	*msrB*	Methionine sulfoxide reductase B	1.415
APL_1189	*APL_1189*	Hypothetical protein	1.393
APL_0090	*APL_0090*	Hypothetical protein	1.360
APL_1709	*APL_1709*	Hypothetical protein	-1.307
APL_0357	*APL_0357*	Hypothetical protein	-1.328
APL_1380	*APL_1380*	Hypothetical protein	-1.394
APL_1729	*APL_1729*	Hypothetical protein	-1.401
APL_1062	*APL_1062*	Hypothetical protein	-1.468
APL_0179	*APL_0179*	Hypothetical protein	-1.481
APL_0940	*APL_0940*	Hypothetical protein	-1.482
APL_1273	*APL_1273*	Putative fimbrial biogenesis and twitching motility protein PilF-like protein	-1.488
APL_1131	*APL_1131*	Hypothetical protein	-1.540
APL_0583	*APL_0583*	Hypothetical protein	-1.585
APL_1096	*APL_1096*	Hypothetical protein (59% ID dispersine B)	-1.594
APL_0936	*APL_0936*	Hypothetical protein	-1.616
APL_1115	*APL_1115*	Hypothetical protein	-1.639
APL_0811	*APL_0811*	Hypothetical protein	-1.682
APL_1898	*ap2029*	Hypothetical protein	-1.798
APL_1654	*gidB*	Methyltransferase GidB	-1.816
APL_0340	*APL_0340*	Hypothetical protein	-1.893

APL_1381	*APL_1381*	Hypothetical protein	-1.926
APL_0053	*typA*	GTP-binding protein	-2.043
APL_1681	*APL_1681*	Hypothetical protein	-2.233

Article 3

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Modulation of Gene Expression in *Actinobacillus pleuropneumoniae* Exposed to Bronchoalveolar Fluid

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§ Élaboration et réalisation des expériences de profil transcriptionnel, et participation à l'analyse des résultats et à la rédaction du manuscript.

Abstract

Background

Actinobacillus pleuropneumoniae, the causative agent of porcine contagious pleuropneumonia, is an important pathogen of swine throughout the world. It must rapidly overcome the innate pulmonary immune defenses of the pig to cause disease. To better understand this process, the objective of this study was to identify genes that are differentially expressed in a medium that mimics the lung environment early in infection process.

Methods and principal findings

Since bronchoalveolar lavage fluid (BALF) contains innate immune and other components found in the lungs, we examined the gene expression of a virulent serovar 1 strain of *A. pleuropneumoniae* after a 30 min exposure to BALF using DNA microarrays and real-time PCR. The functional classes of genes found to be up-regulated most often in BALF were those encoding proteins involved in energy metabolism, especially anaerobic metabolism, and in cell envelope, DNA, and protein biosynthesis. As well, transcription of a number of known virulence genes including *apxIVA* and the *g*ene for SapF, a protein which is involved in resistance to antimicrobial peptides, was also up-regulated in BALF. Seventy-nine percent of the genes that were up-regulated in BALF encoded a known protein product, and of these, 44% had been reported to be either expressed *in vivo* and/or involved in virulence.

Conclusions

The results of this study suggest that in early stages of infection, *A. pleuropneumoniae* may modulate expression of genes involved in anaerobic energy generation and in the synthesis of proteins involved in cell wall biogenesis, as well as established virulence factors. Given that many of these genes are thought to be expressed *in vivo* or involved in virulence, incubation in BALF appears, at least partially, to simulate *in vivo* conditions and may provide a useful medium for the discovery of novel vaccine or therapeutic targets.

Introduction

Actinobacillus pleuropneumoniae is a species-specific, swine pathogen that causes a necrotizing, fibrinohaemorrhagic pneumonia with pleurisy [1]. Depending upon the immune status of the animal, the disease can range in severity from peracute to chronic [2,3]. Although a protective immune response is usually acquired through the adaptive immune system following acute infection, vaccines offer only partial protection against this organism.

The lungs, which are the primary site of infection by *A. pleuropneumoniae*, have a large surface area that is directly in contact with the external environment. There are no published data for swine, but in the human lung, there is an average of 480 million alveoli [4] with an area of 120 to 140 m^2 [5]. The lungs are protected by both innate and adaptive immune systems. Two major components are involved in the innate immune system: a cellular component comprised of leukocytes as well as airways and alveolar epithelial cells, and a humoral component which includes surfactant lipids and proteins, collectins, defensins, cathelicidins, lysozyme, and lactoferrin [6]. Most of these innate immune components reside in a thin layer of fluid lining the lung epithelial cell layer and some have been shown to directly kill bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* [7-9]. Inhaled particles, including pathogens, first encounter the resident immune system in this fluid layer, which forms the first line of defense [10,11]. Little work has been done to examine bacterial gene expression in BALF, but in a recent study Schwab et al. [12] found that *Myobacterium tuberculosis* genes encoding proteins which enabled the organism to use surfactant lipids as a substrate and those for synthesis of phthiolocerol dimycocerosate (PDIM), a protective cell wall component, were up-regulated in the presence of a whole lung surfactant preparation.

A. pleuropneumoniae is capable of overcoming innate pulmonary immune mechanisms of the pig. It can rapidly multiply and spread in naive herds, with some pigs dying within 24 h of infection without showing any clinical signs. Several virulence factors have been described in *A. pleuropneumoniae* to explain its

pathogenesis; factors for colonization, nutrient acquisition, immune evasion and tissue destruction have all been implicated in the disease process [13,14]. Although some aspects of the pathogenesis can be explained by the production of tissue-damaging RTX toxins and the ability of the pathogen to acquire nutrients such as iron in the host, the factors involved in bacterial survival and rapid multiplication in the host are largely unknown.

To identify the genes that may be involved in the survival and pathogenesis of *A. pleuropneumoniae* in the host we used porcine bronchoalveolar fluid as a medium to simulate, in part, the lung environment. By analogy with other species, BALF collected from swine likely contains plasma proteins and proteins with unknown functions [15] as well as proteins with diverse functions including anti-oxidation, lipid-metabolism, and tissue repair and proliferation in addition to innate immune components and dissolved minerals. Because BALF contains components that perform diverse functions in the lungs, *A. pleuropneumoniae* gene expression in this fluid could mimic gene expression in the host. Therefore, the objective of this study was to identify *A. pleuropneumoniae* genes that are differentially expressed in BALF to better understand the survival and pathogenesis of this important swine pathogen early in the disease process.

Results and discussion

Differential gene expression in BALF

The survival of *A. pleuropneumoniae* CM5 was assessed in BALF before carrying out the experiments to identify differentially expressed genes, since this fluid contains many antibacterial substances [6,16]. No significant decrease was observed in *A. pleuropneumoniae* CM5 cell numbers following incubation for 30 min in BALF, while 70% of the *E. coli* DH5α cells were killed at this time.

The genes that were differentially expressed by *A. pleuropneumoniae* after 30 min of incubation in BALF were identified with DNA microarrays by hybridization of Cy3-labeled cDNA from the BALF-incubated bacteria (target sample) and Cy5-labelled

cDNA from the BHI-incubated bacteria (reference sample). One hundred and fifty-six genes were differentially expressed in BALF at a false discovery rate (FDR) of 1.07 %; 52 genes were down-regulated while 104 were up-regulated. Forty-one (26%) of these genes encode hypothetical proteins (Table 1).

The differential expression of selected genes representing various biological functional classes of interest was confirmed by real-time PCR analysis. Although fold change in gene expression measured by real-time PCR was generally higher, there was a good correlation between the two data sets, and no genes that were deemed up-regulated with the microarrays were demonstrated to be down-regulated by qRT-PCR, and vice-versa (Table 2). The reason that the three *nqr* genes tested appeared to be overestimated in the microarray analysis is not clear, but these slightly divergent results were not likely due to dynamic range or % G+C considerations.

The genes found to be most frequently up-regulated in BALF were those encoding proteins involved in energy metabolism and in cell envelope, DNA, and protein biosynthesis (Table S1). Genes encoding proteins for co-factor biosynthesis, toxin production and secretion and trafficking of ions and biomolecules were also up-regulated. Representative genes belonging to these functional classes are described below.

Modulation of gene expression for enhanced protein synthesis and energy generation in BALF

Incubation of *A. pleuropneumoniae* CM5 in BALF for 30 min resulted in the increased expression of genes encoding 30S and 50S ribosomal subunits proteins and tRNA modification enzymes (Table S1). Such up-regulation of ribosomal genes could play a role in the synthesis of proteins described below.

Genes encoding proteins involved in energy metabolism were also up-regulated in BALF, with some showing an increase of more than 6-fold. Most of these genes encoded enzymes involved in anaerobic respiration, including those that were part of

the dimethyl sulfoxide reductase (*dms*) operon), periplasmic nitrate reductase (*nap*) operon, nitrite reductase (*nrf*) operon and a primary dehydrogenase, hydrogenase 2 (*hya*) operon (Table S1). Dimethyl sulfoxide (DMSO) reductase catalyzes the transfer of electrons to dimethyl sulfoxide and other substrates; the periplasmic-nitrate and nitrite reductases are involved in transfer of electrons to nitrate and nitrite respectively [17]. Hydrogenase 2, a primary dehydrogenase, uses the hydrogen produced by formate hydrogen lyase from formate as a substrate [18] for energy production [19, 20]. A putative formate transporter, *focA,* was also up-regulated in BALF.

Previous studies have shown that *A. pleuropneumoniae* up-regulates the transcription of genes encoding the enzymes involved in anaerobic metabolism in porcine lungs and lung washings [21-23]. *A. pleuropneumoniae* recovered from BALF following infection have increased expression of hydrogenase 2 [21], aspartate ammonia lyase (Asp) [24] and DMSO reductase [25], with the DMSO reductase levels being higher in cells recovered from both the acute and chronic infections [22,23].

The components present in the BALF that could lead to the up-regulation of anaerobic energy-metabolism genes in *A. pleuropneumoniae* are largely unknown; however, glutathione in the airway epithelium might be an activator of HlyX, which is the *A. pleuropneumoniae* equivalent of FNR in *E. coli* [23]. For example, it has been reported HlyX up-regulates DMSO reductase (*dms*) and aspartate ammonia lyase (*asp*), which breaks down aspartate to fumarate and ammonia.. Fumarate is used as an electron acceptor under anaerobic conditions in *A. pleuropneumoniae* [27-30]. The fact that a significant change of expression of *hlyX* was not observed may be because differences in the level of expression of this gene tend to be small. Moreover, like *fnr*, regulation of the *hlyX* gene product is likely affected by a multitude of factors including protein stability, growth phase and nutrient availability [31,32]

The up-regulation of the genes encoding the periplasmic nitrate (Nap) reductase in the BALF suggests a role for nitrate metabolism in the energy production of *A.*

pleuropneumoniae in the host. Nap uses nitrate as an electron acceptor. As nitrate has a higher redox potential than most of the other electron acceptors under anaerobic conditions [17,33] it is a preferred electron acceptor. Nitrate is formed from nitric oxide in the body and is present in various body fluids [34-37] where it can serve as a cue for the up-regulation of the nitrate-responsive genes in *A. pleuropneumoniae*. Nap has a higher affinity for nitrate than the membrane-bound nitrate reductase (Nar) [38], and it can be used for nitrate utilization in the body fluids with low nitrate concentrations such as are found in the respiratory tract.

Nitrite reductase (Nrf) is another nitrate metabolism-related enzyme whose genes were up-regulated in BALF. This enzyme converts nitrite, a potential bacterial cell-damaging substance, produced by the nitrate reductase, to ammonia. Nrf can also convert nitric oxide to ammonium [39], thus antagonizing one of the important defense molecules of the host.

Given that *A. pleuropneumoniae* is a host-associated pathogen which resides in oxygen-deprived environments in both the acute and carrier states of the disease, the major production of energy is potentially through anaerobic metabolism. The absence of three main TCA-cycle enzymes (citrate synthase, aconitase and isocitrate dehydrogenase) in the genomes of serotype 3 and serotype 5 *A. pleuropneumoniae* again points to the importance of the anaerobic metabolism in the survival of the organism [40]. In addition, many upper respiratory tract pathogens including *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Pasteurella multocida, Neisseria meningitidis,* carry genes for anaerobic energy generation, consistent with the notion that anaerobic metabolism might have an important role in the survival and virulence in the respiratory tract.

Some of the genes encoding enzymes involved in anaerobic energy production in *A. pleuropneumoniae* have been shown to be essential for virulence. For example, knockout mutants of *hlyX* are unable to survive in lung epithelium, sequestered lungs or tonsils [29]; *dmsA* mutants are attenuated in acute disease [25]; and *asp* mutants cause less severe lung lesions than the wild type organism [24]. Similarly, in

*Bordetella pertussi*s, another respiratory tract pathogen, the FNR homolog, Btr [41] is essential for the survival of this pathogen in the mice [42].

The role of the nitrate-inducible energy metabolism genes, *nap* and *nrf*, is unknown in *A. pleuropneumoniae*. Nitrate metabolism has, however, been shown to be essential for the entry and replication of *Salmonella* Typhi in epithelial cells [43] and for the survival and virulence of *Mycobacterium bovis* in mice [44,45].

In addition to the genes encoding the enzymes of energy metabolism discussed above, the transcription of Na^+ -translocating NADH-quinone reductase (NQR) was also enhanced in the BALF (Table 3 and S1). NQR is a primary Na^+ pump that translocates Na^+ ions outside the cytoplasmic membrane to generate a sodium motive force, instead of a proton motive force, for energy production [46,47]. The NQR enzyme is a complex of six subunits encoded by the *nqrABCDEF* operon [48,49]; all six genes are present in all of the genomes of *A. pleuropneumoniae* reported to date. Another Na^+-cycling gene, *nhaB* (an Na^+ / H^+ antiporter), which, like NQR, could be involved in energy generation or in sodium homeostasis [41], was also up-regulated in the BALF.

In previous studies, the *nqrB* [23] and *nhaB* [22] genes and the NqrA (AopA) protein [50], which are all involved in Na^+-cycling, have been reported to be up-regulated in *A. pleuropneumoniae* when it is grown *in vivo*. The importance of NQR, the major Na^+-cycling enzyme, in survival and pathogenesis of *A. pleuropneumoniae* is unknown. However, BHI containing 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide (HQNO), an inhibitor of NQR, does not allow the growth of *A. pleuropneumoniae* CM5, while *E. coli* DH5α grows well in the medium in the presence of HQNO (unpublished data). Further, in signature tagged mutagenesis studies, the *nqrB* gene was found to be essential for persistence *A. pleuropneumoniae* in the host [51].

Although genome sequencing has revealed that many bacterial pathogens possess homologues of *nqr* and other primary and secondary sodium pumps [46], the role of Na^+ -cycling in pathogenesis is largely unknown, except in *Vibrio cholerae*. In *V.*

cholerae, mutation of *nqr* results in the increased expression of *toxT*, a positive regulator of virulence factors including cholera toxin and toxin co-regulated pilus [52,53]. NQR is best known for its involvement in energy transduction, cytoplasmic pH regulation and ion homeostasis in marine and halophilic bacteria [54,55].

Other BALF-up-regulated *A. pleuropneumoniae* genes encoding enzymes of energy metabolism included the heme exporter gene (*ccmC),* ATP synthase epsilon chain *(atpC)*, deoxyribosephosphate aldolase (*deoC)* and 1-phosphofructokinase (*fruK).* The *ccmC* gene is a part of the *ccmABCDEFGH* operon which encodes proteins required for the maturation of cytochrome C [56], an essential component of the electron-transfer chain [57]; whereas AtpC is a part of the F1 complex of ATP synthase [58]; DeoC cleaves deoxyribose 5-phosphate to acetaldehyde and glyceraldehyde 3-phosphate for the central carbon metabolism [59]; and FruK regulates the flow of glucose through glycolysis [60]. Thus, *A. pleuropneumoniae* enhances the transcription of the genes encoding both the central carbon metabolism and the energy transduction proteins in the BALF.

Modulation of gene expression for survival and virulence in BALF

Following incubation in the BALF, *A. pleuropneumoniae* CM5 up-regulates genes required for cell wall synthesis, repair and recombination of DNA, and secretion and trafficking of ions and biomolecules (Table S1).

Several genes encoding cell wall biosynthesis proteins were up-regulated in BALF, including those required for the synthesis of peptidoglycan, LPS and integral membrane proteins (Table S1).

The genes encoding peptidoglycan biosynthesis enzymes included phosphoglucosamine mutase (*mrsA)*, alanine racemase *(alr*), and D-alanyl D-alanine carboxypeptidase fraction A *(dacA*). MrsA converts glucosamine-6-phosphate to glucosamine-1-phophate which finally yields UDP-N-acetyl glucosamine for both peptidoglycan and LPS biosynthesis [61,63] while Alr catalyses the isomerization of L-alanine into D-alanine which is essential in bacteria for peptidoglycan biosynthesis

[61,64], and DacA catalyzes transpeptidation between neighboring peptide chains of N-acetylmuramyl-N-acetylglucosyl polysaccharides to produce cross-links in the cell wall. DacA can also act as a carboxypeptidase to control the amount of cross-linking in peptidoglycan [65,66].

A semi-rough LPS is present in *A. pleuropneumoniae* serotype 1 [67], and the BALF-up-regulated genes encoding LPS biosynthesis proteins included tetraacyldisaccharide 4'kinase (*lpxK)* required for lipid-A biosynthesis, and the bifunctional protein (*hldE*) and UTP glucose-1-phosphate uridylyltransferase (*galU*) required for LPS biosynthesis. The genes encoding capsular export proteins, *cpxA* (ATP binding protein) and *cpxC* (capsule polysaccharide export inner membrane protein) were also up-regulated in BALF. While the genes encoding peptidoglycan and LPS biosynthesis proteins described above are assumed to be essential for the survival of *A. pleuropneumoniae*, a clear role for capsular polysaccharides in the virulence of the bacterium has been demonstrated [68,69]. In addition to the cell surface polysaccharides, the genes encoding outer membrane protein OmpW (outer membrane protein W precursor) and a lipoprotein (outer membrane antigenic lipoprotein B precursor) were also up-regulated in BALF. The up-regulation of the genes encoding proteins of cell wall biosynthesis may perhaps help the organism to overcome the cell surface-damaging components of the BALF.

The transcription of the genes encoding proteins involved in replication, and recombination and repair was enhanced in BALF. Genes representing subunits of DNA polymerase III, various recombination proteins of the RecF machine, and an exonuclease (*uvrA of uvrABC)* were all up-regulated in BALF. Replication and recombination are two intertwined processes [70]; enhancement of transcription of genes involved in these two processes is consistent with active replication of *A. pleuropneumoniae* in BALF.

For survival in the host, bacteria require nutrients for biosynthesis of various biomolecules. In BALF, *A. pleuropneumoniae* increased the transcription of the genes encoding proteins required for the transport of various nutrients. For example,

complex-carbohydrate transport genes *malF* and *malG*, involved in maltose and maltodextrin transport, were up-regulated in BALF as were amino acid, serine (*sdaC*) and branched chain amino acid (*brnQ*), transport genes. As in *A. pleuropneumoniae,* Group A *Streptococcus* also enhances transcription of genes encoding proteins required for the acquisition and metabolism of complex carbohydrates in saliva by increasing the expression of SptR/S, a bacterial two component regulatory system [71].

A. pleuropneumoniae also increased the transcription of the genes encoding proteins required for the transport of iron and potassium in the BALF. The genes for cell membrane biopolymer-transport proteins, ExbD and ExbD2, and FbpB (iron (III) ABC transporter, ATP-binding protein), which are involved in energy-coupled transport of the iron-containing compound, transferrin [14] were up-regulated in BALF, as was the gene for iron efflux, *fieF*. The cation efflux pump, FieF, probably protects the bacterium from ferrous iron toxicity [72]. The gene encoding PtsN (PTS system, nitrogen regulatory IIA like protein), which regulates K^+ transport in cells, was also up-regulated in BALF. PtsN has recently been shown to regulate transport of K^+ through its interaction with a K^+ transporter in *E. coli* [73].

BALF also led to the increased expression of the *A. pleuropneumoniae* CM5 genes encoding toxin synthesis and antimicrobial-resistance compounds. The ApxIV RTX toxin is thought to be expressed only *in vivo* [74,75]. Following exposure to BALF we have shown for the first time, that *apxIVA* can be expressed *in vitro*. ApxIVA is a homolog of FrpC in *Neisseria meningitidis*. FrpC is involved in the tissue invasion of *N. meningitidis* [76]. The role of ApxIV in the pathogenesis of *A. pleuropneumoniae* is, however, unknown.

The *sapF* gene is a part of the *sapABCDF* operon which is involved in resistance to antimicrobial peptides in *Vibrio fischeri* [77], and in non-typable *Haemophilus influenzae* [78]. Also, *sapD* mutants of non-typable *H. influenzae* have been shown to be attenuated in a chinchilla model of otitis media [79]. *A. pleuropneumoniae*

possesses a complete *sap* operon, which could have significant role in the survival of the pathogen in the host.

Glyoxylase II (*gloB*) is an enzyme involved in conversion of dicarbonyl compounds to less reactive hydroxy acids [80]. The enzyme has been shown to be essential for *in vivo* survival of *A. pleuropneumoniae* [51]; it probably protects the organism against the harmful dicarbonyl compounds of the host.

The *ostA* gene was up-regulated in *A. pleuropneumoniae* CM5 after incubation in BALF. The role for OstA in *A. pleuropneumoniae* is not known at this time, but in *Helicobacter pylori,* OstA confers protection against organic solvents and antibiotics [81], and in *E. coli*, it is essential for survival and has a direct role in membrane biogenesis and effects the lipid:protein ratio of the cell membrane [82]. In *N. meningitidis*, OstA is required for LPS biosynthesis [82].

The transcription of *secB*, which is a part of the Sec machinery, was also enhanced in BALF. The Sec machinery is a major route for translocation of protein across and integration of some proteins into the cytoplasmic membrane of bacteria [83]. In *A. pleuropneumoniae*, *secA* and *secB*, another protein of the Sec machinery, are expressed *in vivo* [23, 84, and 85].

Down-regulated genes

No up-regulated genes were detected in five functional classes. Genes for amino acid (9) and nucleotide (3) biosynthesis were down-regulated suggesting that some or all amino acids and nucleotides were either directly or indirectly, freely available in BALF. Consistent with this notion, amino acid transporters such as BrnQ (for branched chain amino acid) and SdaC (for serine transport) were up-regulated. Genes for transcriptional regulators (3), protein stabilization and folding (4), and transposon functions (3) were also down-regulated. It is difficult to speculate about the precise role of the various down-regulated transcriptional regulators, but FadR (a member of the GntR family of regulators) is an activator of unsaturated fatty acid acid synthesis in *E. coli*, although the authors do note that the FadR regulon in other

gammaprotobacteria such as *Haemophilus influenzae* is much smaller [86]. Nevertheless, it is reasonable to assume that fatty acids would be freely available in BALF and their synthesis would not be required [87] Similarly, it is difficult to know the precise role of protein folding and stabilization, but proteins such as HtpG and HtpX are usually up-regulated during stress such as nutrient deprivation and their down-regulation is consistent with BALF being a comparatively non-stressful environment [88,89]. The fact that the expression of transposases is reported to be associated with starvation and other stressful conditions is also consistent with BALF being a favorable environment for *A. pleuropneumonie* [90]

In summary, incubation in BALF appears to partially simulate *in vivo* conditions and may provide a useful medium for the discovery of novel vaccine or therapeutic targets. In this environment*, A. pleuropneumoniae* is actively involved in protein and cell envelope biosynthesis and in general, BALF appears to provide a comparatively favorable and nutrient replete environment. Although more than 40% of the genes that were up-regulated following a 30 min exposure to BALF had been reported in earlier studies (Table 3), we have described an additional 70 genes whose precise role in survival and virulence of *A. pleuropneumoniae* is unknown and merit further study.

Materials and methods

Collection and concentration of bronchoalveolar fluid (BALF)

BALF was obtained from ten specific pathogen free pigs, each weighing about 15 kg. The pigs were euthanized, and the lungs were lavaged in situ using a catheter passed through a bronchoscope to instill 100 ml of sterile PBS into the trachea. After ~ 1 min, the lung washings were collected and centrifuged to remove cell debris. The cell-free lavage was concentrated with a 5 kDa molecular weight cut off ultrafiltration device, Vivacell 70 (Vivascience Ltd., Stonehouse, Gloucestershire, UK). A total of about 100 ml of BALF was collected from each pig and concentrated to a final volume of 5 ml. The concentrated BALF from each pig was pooled and sterilized by passage through a 0.22 μm membrane filter. From collection to

concentration, BALF was kept at 4°C; for long-term storage, the concentrated BALF was stored at -80°C. Molecules less than 5 kDa in molecular weight were not concentrated by this method; nevertheless, the fluid still contained these substances in the concentrations found before ultrafiltration and the concentrated BALF represents the alveolar epithelial fluid better than unprocessed BALF. The procedure used for BALF collection received approval from the Animal Care Committee of the University of Guelph and was consistent with the Guidelines of the Canadian Council on Animal Care.

Assessment of the bacterial survival in BALF

Exponential growth phase cultures of *A. pleuropneumoniae* CM5 and *Escherichia coli* DH5α were incubated in 2 ml of concentrated BALF at 37°C. As a control, the bacteria were also incubated in phosphate-buffered saline (PBS). A 50-µl aliquot was taken from each of the cultures after 15 and 30 min of incubation in BALF and PBS and plated onto brain heart infusion (BHI; Becton, Dickinson and Company, Sparks, MD, USA) agar supplemented with 0.01% (wt/vol) β-nicotinamide adenine dinucleotide (NAD). The number of colony-forming units (CFU) was counted after incubation overnight at 37°C. The number of bacteria surviving in BALF at each time point was expressed as the percent of number of bacteria surviving in PBS.

The data were analyzed using one-way analysis of variance (ANOVA); the means were compared using Tukey's method.

Culture conditions for the identification of differentially expressed genes in BALF

The virulent *A. pleuropneumoniae* serotype 1 strain CM5 was grown in BHI (Becton, Dickinson and Company) broth supplemented with 0.01% (wt/vol) NAD, at 37°C to an OD_{600} of 0.7 (approximately 10^7 CFU/ml). The cell suspension was split into two equal parts and centrifuged at 10,000 x *g* for 1 min to pellet the cells. One pellet was suspended in pre-warmed concentrated BALF and the other in fresh pre-warmed BHI broth supplemented with NAD. The volume of BALF and BHI broth used to suspend

the cell pellets was equal to that of the culture from which the pellets were obtained, so that the resulting cell suspension contained approximately 10^7 CFU/ml. The cell suspensions were incubated with constant agitation at 37°C for 30 min and harvested by centrifugation for RNA extraction.

RNA extraction

RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer. RNA quantity and quality was determined using an RNA 6000 Nano LabChip read in a Bioanalyzer 2100 instrument (Agilent Technologies, Santa Clara, CA, USA). The RNA was treated with Turbo DNA-free (Ambion, Austin, TX, USA) to remove the traces of contaminating DNA. For hybridization in microarray experiments, RNA was extracted from 3 independent biological replicates.

Labeling of cDNA and microarray hybridizations

cDNA synthesis was carried out as described previously [91]. Briefly, RNA (15 µg per reaction) from the target (BALF-incubated bacteria) and the reference (BHI-incubated bacteria) samples was used to synthesize cDNA in the presence of amino-allyl-dUTP (Sigma-Aldrich, St. Louis MO, US), random octamer primers (Biocorps, Montreal, QC, Canada), and the SuperScript II transcriptase (Invitrogen, Carlsbad, CA, US). The cDNAs were labeled indirectly with mono-functional NHS-ester Cy3 or Cy5 dye (GE Healthcare, Buckinghamshire, UK) and the efficiency of the labeling reactions was determined spectrophotometrically. RNA from three independent biological replicates was used in the labeling reaction. Four hybridizations, including the dye-swap experiment, were carried out between the target and the reference samples. The microarray data from this study were submitted to the Gene Expression Omnibus repository at NCBI and assigned accession number GEO: GSE13006.

Microarray chip design

The AppChip2 microarray chip used in this study is an evolved version of the AppChip1 chip, and like its predecessor, was a part of the *A. pleuropneumoniae* 5b L20 genome sequencing project [92]. For the construction of AppChip2, open-reading-frame (ORF) PCR fragments of 160-nucleotide length and above were spotted in duplicate on the microarray slides. The spots represent 2033 ORFs, covering 95% of the total ORFs, from the complete genome sequence of the organism. Spotted sheared genomic DNA from *A. pleuropneumoniae* L20 and porcine DNA are used as controls (http://ibs-isb.nrc-cnrc.gc.ca/glycobiology/appchips_e.html). Other details concerning chip production are described elsewhere [93].

Microarray data analysis

Microarray image and data analysis were carried out using the TM4 Suite [94] of software. Briefly, images were analyzed with Spotfinder v3.1.1. Background intensity was subtracted from the integrated intensity of each spot, and the spots that were less than one standard deviation above background intensity were eliminated, as were the ones with total intensity less than 10,000. Replicate spots were analyzed subsequent to LOWESS (locally weighted linear regression) normalization of the data. Genes that were represented by good quality spots on a minimum of three replicate slides were considered for the downstream analysis using SAM (significance analysis of microarray) to identify differentially expressed genes. A median false discovery rate (FDR = expected rate of falsely identified up- or down-regulated genes [95]) of 1.07% was used to generate a list of differentially expressed genes, which were classified into various functional classes using the JCVI Comprehensive Microbial Resource [96] tool.

Quantitative real-time PCR

RNA capacity (maximum RNA concentration that can be used without affecting the efficiency of reverse transcription), optimum primer concentration (list of primers is given in Table 4), and the gene dynamic ranges were determined before carrying out the real-time PCR for the relative quantification of the target genes. The synthesis of template cDNA was carried out in a 20-µl reaction mixture containing 500 ng RNA, using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Streetsville, ON, Canada). SYBR-Green-dye-based real-time PCR methodology was carried out using the MicroAmp Optical 96- well plates (Applied Biosystems) in a StepOnePlus thermocycler (Applied Biosystems) for the relative quantification of the target genes. The 20-µl PCR reactions mixture contained 10 µl of 2 x Power SYBR Green PCR Master Mix (Applied Biosystems), 100 nM of forward and reverse primer, and 5 µl of the template cDNA. The real-time PCR thermal profile included the heat-activation of AmpliTaq Gold DNA Polymerase at 95°C for 10 min, and the three-step 40-cycle PCR of denaturation at 95°C for 15 sec, primer annealing and extension at 60°C for 1 min.

The Comparative C_T (or $\Delta\Delta C_T$) method [97] where $\Delta\Delta C_T = (C_{T,\,target} - C_{T,\,syp})_{BALF} - (C_{T,\,target} - C_{T,\,syp})_{BHI}$ was used to determine the relative gene expression of the target genes in BALF. As an endogenous control, the level of prolyl-tRNA-synthetase gene expression was used to normalize the target gene expression levels, since this gene exhibited the least variation in expression across various conditions in both the microarray and real-time PCR experiments. Three independent biological replicates were tested in triplicates in the PCR experiments for the relative quantification of the target genes.

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Tables

Table 1. Functional classes of differentially expressed genes.

Functional class	No. up-regulated	No. down-regulated
Protein biosynthesis	16	1
Amino acid biosynthesis	0	9
Cofactor biosynthesis (heme and vitamins)	6	0
Nucleotide biosynthesis	0	3
Lipid biosynthesis	1	0
Cell envelope biosynthesis	11	0
Detoxification and toxin production	5	1
DNA metabolism	8	0
Energy metabolism	22	4
Protein folding and stabilization	0	4
Transcriptional regulators	0	3
Secretion and trafficking	13	5
Transposon functions	0	3
Unclassified and unknowns	22	19
Total	104	52

Table 2. Verification of the microarray data by real-time PCR

Gene	Gene name	Fold change by real-time PCR	Fold change by microarray
dmsA	Anaerobic dimethyl sulfoxide reductase chain A precursor	17.90 ± 6.52	5.74
dmsB	Anaerobic dimethyl sulfoxide reductase chain B	10.12 ± 3.34	2.78
nqrB	Na^+-translocating NADH quinone reductase subunit B	4.62 ± 1.63	7.65
nqrC	Na^+-translocating NADH quinone reductase subunit C	4.57 ± 1.3	6.35
nqrE	Na^+-translocating NADH quinone reductase subunit E	4.84 ± 1.59	6.36
napB	Nitrate reductase cytochrome ctype subunit	11.61 ± 3.94	4.69
napF	Ferredoxin-type protein NapF	15.94 ± 5.35	6.42
napD	Putative NapD protein	18.59 ± 7.25	3.93
apxIVA	RTX toxin protein	4.07 ± 2.02	1.93
dapA	Dihydrodipicolinate synthase	0.09 ± 0.02	0.20
leuC	3-isopropylmalate dehydratase large subunit 2	0.15 ± 0.14	0.28
ilvH	Acetolactate synthase small subunit	0.13 ± 0.17	0.27

Table 3. BALF up-regulated virulence-associated genes reported in other studies

Genes	Type of study	Reference no.
bioD1, *nhaB*, *apxIVA*, *rps*, *dmsA*, *hya*	SCOTS (acute infection; 7 days PI))	(22)
nqrB, *dnaG*, *rpsT*, *rplL*, *rho*, *secA*, *truD*, *nusA*, *atpD*, *sap*, *rps*, *rpl*	SCOTS (chronic infection; 21 days PI)	(23)
dmsA	Knockout mutation	(25)
nqr, *hemA*, *napB*, *atp*, *ccm*, *recR*, *tonB*, *galU*, *cpxC*	Signature tagged mutagenesis (24 h PI)	(51)
sec, *nusG*	*In vivo* expression technology (12 and 16 h PI)	(84)
exbB2, *atp*, *dnaK*	Signature tagged mutagenesis (20 h PI)	(85)
apxIVA, malF, malG, APL_0668 (predicted periplasmic lipoprotein involved in iron transport)	*In vivo* transcript profiling of *A. pleuropneumoniae* by microarray	Deslandes (personal communication)

Complete gene names are given in Table S1

Table S1. Differentially expressed genes of bronchoalveolar lavage fluid-exposed *A. pleuropneumoniae* grouped according to biological role

ORF No.	Gene	Predicted protein product	Fold change
Protein biosynthesis			
APL_1769	*rpsQ*	ribosomal protein	4.27
APL_0638	*nusA*	transcription elongation protein NusA	4.10
APL_0247	*rho*	transcription termination factor Rho	3.06
APL_1785	*rplQ*	ribosomal protein	2.76
APL_0042	*rlmB*	23S rRNA (guanosine-2'-O-)-methyltransferase	2.71
APL_1558	*rpsT*	ribosomal protein	2.26
APL_0399	*ksgA*	dimethyladenosine transferase	2.02
APL_0352	*metG*	methionyl tRNA synthetase	1.97
APL_0601	*rpsl*	ribosomal protein	1.94
APL_0641	*truB*	tRNA pseudouridine synthase B	1.89
APL_0575	*deaD*	coldshock DEAD box protein A like protein	1.88
APL_1721	*rplL*	ribosomal protein	1.87
APL_1770	*rplX*	ribosomal protein	1.74
APL_1383	*dnaX*	tRNA (guanineN(7)) methyltransferase	1.74
APL_0365	*hrpA*	ATP dependent RNA helicase	1.71
APL_1228	*infA*	translation initiation factor IF1	1.67
APL_1070	*ftnB*	ferritin-like protein 2	-2.29
		Mean	2.08
Amino acid biosynthesis			
APL_1230	*serB*	phosphoserine phosphatase	-1.61
APL_1155	*metE*	5methyltetrahydropteroyltriglutamate homocysteine methyltransferase	-1.72
APL_2023	*hisB*	histidine biosynthesis bifunctional protein HisB	-1.77
APL_0708	*serC*	phosphoserine aminotransferase	-2.11
APL_0727	*ilvI*	acetolactate synthase large subunit	-2.14
APL_2019	*hisG*	ATP phosphoribosyl transferase (ATP-PRTase) (ATP-PRT)	-2.58
APL_0139	*leuC*	3-isopropylmalate dehydratase large subunit 2	-3.49
APL_0728	*ilvH*	acetolactate synthase small subunit	-3.67
APL_0899	*dapA*	dihydrodipicolinate synthase	-4.84
		Mean	-2.66

Protein folding and stabilization			
APL_1039	*htpX*	putative protease HtpXl-like protein	-2.37
APL_1705	APL_1705	FKBP type peptidylprolyl cistrans isomerase	-2.38
APL_0987	*htpG*	chaperone protein HtpG	-2.44
APL_1034	*prlC*	oligopeptidase A	-3.07
		Mean	-2.57
Cofactor biosynthesis			
APL_1523	*chuW*	coproporphyrinogen III oxidase	2.24
APL_0963	*hemN*	oxygen independent coproporphyrinogen III oxidase	1.92
APL_0404	*hemA*	glutamyl tRNA reductase (GluTR)	1.82
APL_1988	*hemB*	delta-aminolevulinic acid dehydratase	2.54
APL_0614	*bioD1*	dethiobiotin synthetase 1	2.50
APL_1485	*pdxY*	pyridoxamine kinase	1.89
		Mean	2.15
Lipid biosynthesis			
APL_1107	*plsB*	glycerol-3-phosphate acyl transferase	1.72
Nucleotide biosynthesis			
APL_2018	*purC*	phosphoribosylaminoimidazole succinocarboxamide synthase (SAICAR synthetase)	-2.41
APL_1106	*purT*	phosphoribosylglycinamide formyl transferase 2	-3.91
APL_1172	*purD*	phosphoribosylamine glycine ligase	-8.22
		Mean	-4.85
Cell envelope			
APL_1086	*ompW*	outer membrane protein W precursor	6.20
APL_1454	*mrsA*	phosphoglucosamine mutase	3.40
APL_0186	APL_0186	ADP heptose:LPS heptosyl transferase I	2.96
APL_1930	APL_1930	outer membrane antigenic lipoprotein B precursor	2.41
APL_0402	*hldE*	bifunctional protein HldE	2.25
APL_1596	*dacA*	D-alanyl D-alanine carboxypeptidase fraction A	1.96
APL_1585	*cpxA*	ATP binding protein	1.96
APL_0252	*alr*	alanine racemase	1.90
APL_0651	*galU*	UTP glucose-1-phosphate uridylyl transferase	1.87
APL_1583	*cpxC*	capsule polysaccharide export inner membrane protein	1.65

APL_1278	*lpxK*	tetraacyl disaccharide 4' kinase	1.62
		Mean	2.56
Detoxification and toxin production			
APL_1379	*ccp*	cytochrome c peroxidase	7.51
APL_0998	*apxIVA*	RTX toxin protein	1.93
APL_0415	*gloB*	putative hydroxyacyl glutathione hydrolase	1.86
APL_1249	*sapF*	peptide transport system ATP binding protein SapF	1.74
APL_0962	*ostA*	organic solvent tolerance protein precursor	1.60
APL_0766	*rec2*	recombination protein 2	-2.42
		Mean	2.04
DNA metabolism			
APL_0074	*recR*	recombination protein RecR	2.22
APL_1602	*mutS*	DNA mismatch repair protein MutS	2.18
APL_1282	*dnaQ*	DNA polymerase III subunit epsilon	2.15
APL_0874	*holA*	DNA polymerase III subunit delta	1.79
APL_0545	*recO*	DNA repair protein RecO	1.70
APL_1474	*dnaG*	DNA primase	1.62
APL_0962	*recQ*	ATP dependent DNA helicase RecQ	1.60
APL_0782	*uvrA*	UvrABC system protein A	1.49
		Mean	1.84
Mobile elements			
APL_0612	APL_0612	putative transposase	-2.39
APL_1057	APL_1057	transposase	-2.74
APL_1058	APL_1058	transposase	-3.11
		Mean	-2.75
Energy metabolism			
APL_1428	*napG*	ferredoxin type protein NapG like protein	6.56
APL_1431	*napF*	ferredoxin type protein NapF	6.42
APL_1426	*napB*	nitrate reductase cytochrome ctype subunit	4.69
APL_1430	*napD*	putative NapD protein	3.93
APL_1427	*napH*	ferredoxin type protein NapH like protein	3.88
APL_0101	*nrfB*	cytochrome c type protein NrfB precursor	3.61
APL_0151	*nqrB*	Na+-translocating NADH-quinone reductase subunit B	7.65
APL_0154	*nqrE*	Na+-translocating NADH-quinone reductase subunit E	6.36
APL_0152	*nqrC*	Na+-translocating NADH-quinone reductase	6.35

		subunit C	
APL_0102	*nrfC*	nitrate reductase	5.78
APL_1674	*dmsA*	anaerobic dimethyl sulfoxide reductase chain A precursor	5.74
APL_1331	*hyaA*	hydrogenase2 small chain precursor	5.63
APL_0344	*fruK*	1-phosphofructokinase	4.70
APL_1676	*dms C*	anaerobic dimethyl sulfoxide reductase chain C	3.99
APL_1675	*dmsB*	anaerobic dimethyl sulfoxide reductase chain B	2.78
APL_1334	*hyaB*	hydrogenase2 large chain precursor	2.51
APL_1645	*atpc*	ATP synthase epsilon chain	2.38
APL_1015	*deoC*	deoxyribose phosphate aldolase	2.25
APL_1037	*focA*	putative formate transporter	2.12
APL_0322	*nhaB*	Na+/H+ antiporter 2	2.03
APL_1370	*ccmC*	heme exporter protein C	1.79
APL_0991	*nrfD*	nitrite reductase transmembrane protein	1.64
APL_1908	*xylA*	xylose isomerase	-2.07
APL_0451	*sucD*	succinylCoA ligase [ADP forming] subunit alpha	-2.31
APL_0132	*pfhB1*	putative haloacid dehalogenase like hydrolase	-3.46
APL_0339	*pepC*	phosphoenolpyruvate carboxylase	-8.36
		Mean	2.95
Secretion and trafficking			
APL_0077	*exbD2*	biopolymer transport protein ExbD2	8.21
APL_1239	*malG*	maltose transport system permease protein	6.08
APL_0078	*exbB2*	biopolymer transport protein ExbB2	4.97
APL_1238	*malF*	maltose transport system permease protein MalF	4.38
APL_1991	*brnQ*	branched chain amino acid transport system carrier protein BraB (branched chain amino acid uptake)	3.80
APL_0856	*sdaC*	serine transporter	3.70
APL_0276	*frpB*	iron regulated outer membrane protein B	2.55
APL_0717	APL_0717	iron(III) ABC transporter, ATP binding protein	2.38
APL_1509	*secB*	protein export protein SecB	2.36
APL_0335	*ptsN*	PTS system, nitrogen regulatory IIA-like protein	2.09
APL_0604	*fieF*	cation efflux pump FieF	1.80
APL_0301	*tolR*	biopolymer transport protein TolR	1.77

APL_1569	*exbD*	biopolymer transport protein ExbD	1.75
APL_1911	*xylH*	xylose transport system permease protein	-1.65
APL_1672	*rbsB*	D-ribose binding periplasmic protein precursor RbsB	-2.25
APL_1258	*pstB*	phosphate import ATP binding protein PstB	-2.93
APL_1630	*mtlA*	PTS system mannitol-specific EIICBA component	-3.37
APL_0374	*glpF*	glycerol uptake facilitator protein	-7.49
		Mean	1.56
Regulatory proteins			
APL_0932	APL_0932	putative HTH-type transcriptional regulator	-1.80
APL_0571	*gntR*	HTH-type transcriptional regulator	-3.85
APL_1262	APL_1262	transcriptional regulator MerR family,	-5.97
		Mean	-3.87
Unclassified and unknowns			
APL_0668	APL_0668	hypothetical protein	8.01
APL_1115	APL_1115	hypothetical protein	3.21
APL_0637	APL_0637	hypothetical protein	3.15
APL_0707	APL_0707	hypothetical protein	3.09
APL_1626	APL_1626	hypothetical protein	2.97
APL_0029	APL_0029	ABC transporter periplasmic protein	2.80
APL_1360	APL_1360	hypothetical protein	2.48
APL_1220	APL_1220	hypothetical protein	2.33
APL_1836	APL_1836	hypothetical protein	2.13
APL_0363	APL_0363	hypothetical protein	2.02
APL_0695	APL_0695	hypothetical protein	1.92
APL_1221	APL_1221	hypothetical protein	1.72
APL_0734	APL_0734	hypothetical protein	1.64
APL_0762	APL_0762	hypothetical protein	1.64
APL_1381	APL_1381	hypothetical protein	1.60
APL_1920	APL_1920	site-specific recombinase	1.60
APL_0334	APL_0334	hypothetical protein	1.59
APL_1722	APL_1722	hypothetical protein	1.58
APL_1722	APL_1722	hypothetical protein	1.58
APL_0576	*nlpI*	lipoprotein NlpI-like precursor	1.46
APL_1930	APL_1930	hypothetical protein	2.41
APL_1006	APL_1006	hypothetical protein	2.32
APL_0885	APL_0885	hypothetical protein	-1.56

APL_1900	APL_1900	hypothetical protein	-1.59
APL_1046	APL_1046	lysine exporter protein	-1.67
APL_1187	APL_1187	hypothetical protein	-1.73
APL_0426	APL_0426	hypothetical protein	-1.77
APL_0815	APL_0815	hypothetical protein	-1.77
APL_1415	APL_1415	hypothetical protein	-1.86
APL_0926	APL_0926	hypothetical protein	-1.91
APL_1980	APL_1980	hypothetical protein	-2.29
APL_0999	APL_0999	hypothetical protein	-2.30
APL_1017	APL_1017	hypothetical protein	-2.50
APL_1629	*mtlD*	mannitol 1 phosphate 5 dehydrogenase	-2.71
APL_1088	APL_1088	hypothetical protein	-2.79
APL_1491	APL_1491	hypothetical protein	-2.82
APL_1574	APL_1574	hypothetical protein	-2.82
APL_1495	APL_1495	putative transcriptional regulator	-3.01
APL_0137	APL_0137	hypothetical protein	-3.73
APL_1855	APL_1855	hypothetical protein	-3.84
APL_1588	APL_1588	predicted TRAP transporter solute receptor	-7.93
		Mean	0.06

Table S2. Oligonucleotide primers used in real-time PCR experiments

Gene	Forward primer	Reverse primer
dmsA	ATGTTGCCGGACAAGCACAAGATG	TCTCAATGGACAACGGCTACCACA
dmsB	AACAGGCATCGATTGCACCGTTAC	ACTTGGACGTGCGTGTTTATTGGC
nqrB	TCGGTGCGGCGATTATCGTCTTTA	AAGTTGAATACGGTTGCCGTTGCC
nqrC	ACCCGGCTGACGATAAAGCGAATA	CCACTTGGTTTACTTTGCCCGCTT
nqrE	GCGCACTTGTTGAAGGTGTGGATT	AAACGATACGCCACCGAAGATTGC
napB	GCGCATGGCAACCTAAACATTGGT	TACAGGCTTTGCAGTAGCGGAAAC
napF	ACAACCGTCTCCGCAACTTCTACA	TTGGCTACAACGGAAGAAGCATGG
napD	TCGGCTAAAGCAAGCTGTCTGTCA	TAGCGCAAGTGAAAGCGGACATTC
apxIVA	TTGGACTTCACCTGCAAACATGCC	CGGGCAAATATTCCAAAGCGCAGA
dapA	CAACCTGCAACGCCACTATTGGTT	TACGACAGGCGAATCGACCACATT
leuC	CTTTGCCGCGAACTTCCACTTTCA	TGCGTTTGGTATCGGTACATCGGA
ilvH	GAAAGTTTAACCGTTGCGCCGACT	ACGTTCAATATGCTCGGTAGGGCT
syp	AAGAAACGCCGAATGATGCACAGG	ACACCTCGATAGCACCACCTTTGT

Article 4

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Host Pathogen Interactions of *Actinobacillus pleuropneumoniae* with Porcine Lung and Tracheal Epithelial Cells

Running title: Host Pathogen Interactions of *A. pleuropneumoniae*

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§ Élaboration et réalisation des expériences de profil transcriptionnel, incluant les expériences d'adhésion en culture cellulaire et participation à la rédaction du manuscrit.

ABSTRACT

Host pathogen interactions are of great importance in understanding the pathogenesis of infectious microorganisms. We developed *in vitro* models to study the host-pathogen interactions of porcine respiratory tract pathogens using two immortalized epithelial cell lines, namely the Newborn Pig Trachea (NPTr) and the St. Jude Porcine Lung (SJPL) cell lines. We first studied the interactions of *Actinobacillus pleuropneumoniae*, an important swine pathogen, in these models. In conditions where cytotoxicity was absent or low, we showed that *A. pleuropneumoniae* adheres to both cell lines stimulating the induction of NF-κB. The NPTr cells consequently secrete IL-8 while the SJPL cells do not as they are deprived of the NF-κB p65 subunit. Cell death ultimately occurs by necrosis, not apoptosis. The transcriptomic profile of *A. pleuropneumoniae* was determined after contact with the porcine lung epithelial cells using DNA microarrays. Genes such as *tadB, rcpA,* members of a putative adhesin locus, and a gene with high homology to the Hsf autotransporter adhesin of *Haemophilus influenzae* were upregulated as well as genes *pgaBC* involved in biofilm biosynthesis, while capsular polysaccharide associated genes were down-regulated. The *in vitro* models also proved to be efficient with other swine pathogens such as *Actinobacillus suis*, *Haemophilus parasuis* and *Pasteurella multocida*. Our results demonstrate that interactions of *A. pleuropneumoniae* with host epithelial cells seems to involve complex cross-talk which results in regulation of various bacterial genes including some coding for putative adhesins. Furthermore, our data demonstrate the potential of these in vitro models in studying the host pathogen interactions of other porcine respiratory tract pathogens.

INTRODUCTION

Porcine respiratory diseases have heavily impacted the economy of the pig rearing industry worldwide. *Actinobacillus pleuropneumoniae*, exemplar of these porcine respiratory pathogens, causes porcine pleuropneumonia, a very contagious

and often fatal disease characterized by necrotic and hemorrhagic lung lesions, coughing and severe respiratory distress. Fifteen serotypes of this Gram-negative facultative anaerobic bacteria are presently known based on surface polysaccharides (56). The virulence of this pathogen is accomplished by the help of many factors including exotoxins, endotoxin, capsule polysaccharides, adhesins, and outer membrane proteins such as iron-acquisition systems.

The four pore-forming exotoxins of *A. pleuropneumoniae*, called Apx, are cytolytic and/or hemolytic (20, 51). In fact, the virulence of the different serotypes coincides greatly with the presence of the Apx toxins, particularly ApxI and ApxII. Serotypes 1, 5, 9 and 11 are known to be especially virulent and all express both ApxI and ApxII (19).

As demonstrated by Jacques *et al.,* lipopolysaccharides (LPS) are the major molecules responsible for adhesion, principally the core oligosaccharide region (45, 48). However, other putative adhesins have also been described such as type IV fimbriae expressed under specific conditions in different serotypes (63), a 60-kDa collagen-binding protein (14), as well as a 55-kDa OMP (60), and an autotransporter protein (4).

A close relative of *A. pleuropneumoniae*, *Aggregatibacter (Actinobacillus) actinomycetemcomitans*, was found to be invasive of the human KB cell line and primary gingival cells (17). The invasiveness of this strain has been shown to be related to the colonial morphology, as a switch from a rough to a smooth morphology leads to the loss of its invasive capacity. *Haemophilus parasuis* has also been shown to be invasive to porcine brain microvascular endothelial cells (43, 61). Moreover, *A. actinomycetemcomitans* has been demonstrated to induce cell death by apoptosis of numerous cell types while the cytolethal distending toxin of *Haemophilus ducreyi* has been shown to induce apoptosis of Jurkat T cells (23, 37). Adherence, invasion, toxin secretion and other mechanisms involved in the pathogenesis of *Pasteurellaceae* lead to changes in cellular processes including the induction of

nuclear factors and cytokines production. In fact, *A. pleuropneumoniae* stimulates the production of pro-inflammatory cytokines such as IL-1β, IL-8 and TNF-α which are detected in alveolar fluid and tissue lesions (56). Likewise, a study by our group demonstrated that the production of IL-6, TNF-α, IL-1β, MCP-1 and IL-8 by porcine alveolar macrophages is induced by purified serotype 1 *A. pleuropneumoniae* LPS as well as by heat-killed bacteria (48). IL-8, a neutrophil chemoattractant, is of particular interest as neutrophil accumulation at the infection site is characteristic of porcine pleuropneumonia (56).

Changes in bacterial gene expression also occur during infection. Studies have been conducted to investigate the gene expression of *A. pleuropneumoniae* in conditions mimicking that of the host. A study by our group used microarray technology to detect changes in gene expression of *A. pleuropneumoniae* serotype 1 grown under iron-restricted conditions (13). In this study, many genes involved in iron acquisition were shown to be up-regulated while genes involved mainly in energy metabolism were down-regulated. *In vivo* studies based on SCOTS, IVET or STM technology (3, 4, 22, 31, 53) have allowed the detection of adhesin and toxin genes, as well as genes involved in metabolism, stress, regulation and transport.

Epithelial cells play an important role as the interface between host mucosal surfaces and the surrounding environment, and are the initial site of colonization for most bacterial pathogens. Two porcine respiratory tract epithelial cell lines have been established and reported in the literature, namely the Newborn Pig Trachea (NPTr) (15) and the St. Jude Porcine Lung (SJPL) (52) cell lines. The NPTr cell line was established from a 2-day-old piglet from a pathogen free herd while the SJPL cell line was spontaneously established from the lung of a normal 4-week old female Yorkshire pig (15, 52).

The use of these cell lines has the possibility to generate a great amount of information on the infection mechanism of *A. pleuropneumoniae,* as well as that of other swine bacterial or viral respiratory tract pathogens. Consequently, we

developed *in vitro* models using these cell lines and investigated host-pathogen interactions including adherence, invasion, and bacterial transcriptomic profile, as well as cell death, nuclear factor expression and cytokine production by the epithelial cells. This is the first report of models using immortalized cell lines to study interactions of *A. pleuropneumoniae* with respiratory tract epithelial cells of porcine origin.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All strains used in this study are listed in Table 1. All *A. pleuropneumoniae* strains and the *Pasteurella multocida* capsular type A and D strains were grown in brain heart infusion (BHI) broth and/or agar (Gibco, Burlington, VT) supplemented with 15 µg/ml NAD at 37°C in 5% CO_2. The *Actinobacillus suis* strain was grown in the same conditions, with the addition of 25 µg/ml nalidixic acid and 5 µg/ml chloramphenicol. Both *H. parasuis* strains were grown on pleuropneumonia-like organisms medium (PPLO) broth and on chocolate agar at 37°C without CO_2.

Cell culture. The Newborn Pig Trachea (NPTr) cell line (Instituto Zooprofilattico Sperimental, Brescia, Italy) (15) was grown at 37°C in 5% CO_2 in Minimum Essential Medium (MEM) (Gibco), supplemented with 10% fetal bovine serum (FBS) (Gibco), and 1% sodium pyruvate (Gibco). The St. Jude Porcine Lung (SJPL) cell line (St. Jude Children's Hospital, Memphis, TN) (52) was grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Gibco), supplemented with 10% FBS, 1% sodium pyruvate and 1.5% MEM non-essential amino acids solution (Gibco). Both cell lines were tested by PCR using porcine specific primers, and amplicons were sequenced to ensure their origin (41).

Cytotoxicity detection assay. The cellular cytotoxicity was measured in the different assays using the LDH measurement CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) as prescribed by the manufacturer.

Non-infected cells were used as a negative control, while total lysis of cells by a treatment with 2% Triton X-100 represented the 100% cytotoxicity positive control. Optical densities at 490nm were measured with a Power Wave X340 (Biotek Instruments Inc, Winooski, VT) microplate reader and used to calculate the percentage of cytotoxicity.

Apoptosis detection assays. Apoptosis assays were performed using the cell death detection ELISA (Roche, Laval, Québec, Canada) and the caspase-3 Western detection kit (Cell Signalling Technology Inc. Beverly, MA) as prescribed by the manufacturers. A bacterial suspension at an OD_{600nm} of 0.6 was added to a confluent monolayer of cells at a MOI of 10:1 and incubated for 3 h at 37°C in 5% CO_2. Uninfected cells were used as negative controls and cells treated with 20 μg/ml camptothecin (Sigma) for 4 h at 37°C in 5% CO_2 were used as positive controls. Following the infection, the supernatant and adherent cells were recovered. Plates for the cell death detection ELISA were read at 405 nm in a Power Wave X340 (Biotek Instruments Inc) microplate reader. For the caspase-3 Westerns, the samples were loaded on a 12% (w/v) polyacrylamide gel and migrated at 100 V and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked 1h at room temperature in 2% skim milk and then incubated O/N at 4°C with polyclonal rabbit antibodies against cleaved caspase-3 and caspase-3. Membranes were washed three times in Tris-buffered saline (TBS) and incubated with mouse anti-IgG antibodies conjugated with horseradish peroxidase for 1h at ambient temperature and revealed with 3, 3`, 5, 5`-tetramethylbenzidine (TMB) (Sigma).

Microscopy. Cells were seeded to semi-confluence in 4-well LabTekII chamber microscopy slides (Nunc, Naperville, IL) and incubated O/N. One ml of a 2.5×10^6 CFU/ml suspension of *A. pleuropneumoniae* S4074 was added and slides were then incubated 2 h at 37°C, 5% CO_2. Following four washes with Dubelcco's phosphate-buffered saline (DPBS) (Gibco), cells were fixed 10 minutes in methanol and stained 30 minutes with Giemsa (Sigma). Four washes with DPBS were performed and the

slides were left to dry. Non-infected cells were also stained as controls. Observation was done at a 1000x magnification on a Leica DMR microscope.

Adherence assay. To quantify the adherence of the different strains on both cell lines, 2.5×10^5 epithelial cells were seeded into wells of 24-well tissue culture plates (Sarstedt, Numbrecht, Germany) and incubated O/N. Bacteria from an O/N culture grown at an OD_{600nm} of 0.6 were resuspended in the adequate cell culture medium to a concentration of 2.5×10^6 CFU/ml. One ml of this suspension was added to each well at a MOI of 10:1 and the plates were incubated from 1 to 3 h. Non-adherent bacteria were removed by washing four times with DPBS. Cell with adherent bacteria were released from the wells by adding 100 µl of 1X trypsin-EDTA (Gibco) and resuspended in 900 µl DPBS buffer. Serial dilutions were performed and plated on agar plates to determine the number of bacteria that adhered to the epithelial cells.

Statistical analysis. Data were log-transformed prior to analysis. A two-way analysis of variance, with cell line and bacterial strain as factors, was used at each time separately. The level of statistical significance was set at 0.05 throughout. Analyses were carried out using SAS version 9.1 (Cary, N.C.). Contrasts were used to examine differences between pairs of means.

Invasion assay. 2.5×10^5 epithelial cells were seeded into wells of 24-well tissue culture plates and incubated O/N. One ml of a 2.5×10^7 CFU/ml bacterial suspension was added to the wells (MOI of 100:1). Plates were incubated for 1 to 3 h. Non-adherent bacteria were removed by washing four times with DPBS buffer. One ml of DMEM containing 100 µg/ml of gentamicin was added to each well, followed by a 1 h incubation period at 37°C in 5% CO_2. Killed bacteria were removed by washing twice with DPBS buffer. Cells were then lysed with 100 µl of sterile dH_2O, and samples were plated on agar plates and incubated O/N.

Protein profiling of SJPL and NPTr cells in contact with *A. pleuropneumoniae.* Protein profiling of the cells was performed to detect

differentially expressed proteins. Two T175 flasks were seeded with a confluent monolayer of cells, and 25 ml of DMEM culture medium with or without 1×10^7 CFU/ml of bacteria grown at an OD_{600nm} of 0.6 was added to the flasks. Flasks were incubated 3 h at 37°C in 5% CO_2, then washed 3 times with DPBS, and 500 µl of a lysis solution (20 mM MOPS, 0.5% triton X-100 and protease inhibitors) was added. Cells were removed from the flasks and transfered to microcentrifuge tubes on ice. Sonication at ~180 joules was performed using an ultrasonic processor (Cole-Parmer, Vernon Hills, IL) in order to lyse the cells. The samples were then ultracentrifugated at 50,000 rpm for 30 minutes in a Sorvall RC M100 ultracentrifuge. The supernatant was preserved and analyzed for protein concentration using the Bradford assay (Bio-Rad). Samples were diluted to 2 mg/ml and frozen at -80°C. The samples were then analyzed using the Kinexus antibody microarray, which tracks changes in protein expression of 608 different cell signaling proteins in duplicate, including phosphorylation sites and kinases (Kinex Bioinformatics Inc., Vancouver, BC, Canada). Fifty µg of proteins from both the untreated (control) and treated cells were labeled with the same proprietary fluorescent dye. Each sample was separately applied to opposite sides of the antibody microarray that contains a dam to prevent mixing of the samples. Following incubation of samples with the Kinex™ chip, the unbound proteins were washed away, and the chips were scanned with a Perkin-Elmer ScanArray Express Reader. Image analysis of the TIF files that were produced were performed with ImaGene 7.0 software from BioDiscovery (El Segundo, CA). Qualitative and semi-quantitative analyses of the expression and phosphorylation states of the cell signalling proteins were performed.

Electrophoretic mobility shift assay (EMSA) for the detection of NF-κB and AP-1. Cells were infected at a MOI of 1:10 for 30 minutes, 1 h and 3 h with *A. pleuropneumoniae* grown at an OD_{600nm} of 0.6. Uninfected cells were used as control. Cell stimulation was terminated by the addition of cold PBS. Six µg of nuclear proteins extracted as described by Blanchette *et al* (9) were incubated 20 min at room temperature in 1 µl binding buffer (100 mM HEPES (pH 7.9), 40% v/v glycerol, 10% w/v Ficoll, 250 mM KCl, 10 mM DTT, 5mM EDTA, 250 mM NaCl,

and 10mg/ml BSA) and 200ng/µl poly (dI-dC), 0.02% bromophenol blue with 1 µl of the labeled oligonucleotide containing a consensus sequence of NF-κB/c-Rel homodimeric and heterodimeric complexes (5'AGTTGAGGGGACTTTCCCAGGC-3'; Santa Cruz Biotechnology, Santa Cruz, CA) or of AP-1complexes (5'CGCTTGATGACTCAGCCGGAA-3'; Santa Cruz Biotechnology) which were previously labelled using T4 polynucleotide kinase and γ-^{32}P-dATP (GE Healthcare, Piscataway, NJ). After incubation, DNA-protein complexes were resolved by electrophoresis in 5% (w/v) non-denaturing polyacrylamide gel. Subsequently gels were dried and autoradiographed. The non-specific probes (SP-1 (5'ATTCGATCGGGGCGGGGCGAG-3') used to confirm the specificity of the DNA/nuclear protein reactions were synthesized in our laboratory. Cold competitor assays were conducted by adding a 100-fold molar excess of homologous unlabeled oligonucleotide for NF-κB or AP-1 and non-competitor SP-1. For supershift assays, 2 µg of nuclear proteins were incubated with binding buffer, poly (dI-dC), 0.02% bromophenol blue, labeled ologonucleotide and 4 µg specific antibody (αp50 and αp65N both from Santa Cruz Biotechnology) at room temperature 1 hr, and complexes resolved on standard non-denaturing 5% (w/v) polyacrylamide gel. For the IRAK inhibition assays, the cells were pre-incubated for 1 hr with 50 µM IRAK 1/4 inhibitor (Calbiochem, Darmstadt, Germany), before addition of the bacteria for an incubation of 3 h. Nuclear proteins and EMSA assay was then performed as mentioned above.

Stimulation of cytokine production. Induction assays were performed with both cell lines as described by Ramjeet *et al.* (48). Briefly, 1 ml culture medium containing 1 x 10^9 CFU *A. pleuropneumoniae* S4074, heat-killed at 60°C for 45 min, was added to wells of a 24-well tissue culture plates containing a monolayer of epithelial cells. The plates were incubated from 30 min to 48 h at 37°C in 5% CO_2. The supernatant was then collected and analyzed by ELISA to detect the amount of IL-1β, TNF-α, IL-6 and IL-8 produced by the stimulated epithelial cells as described by Ramjeet *et al.*(48). The stimulation tests were also performed using 35 to 3500 endotoxin units/ml of *A. pleuropneumoniae* serotype 1 S4074 LPS. These LPS

concentrations were shown to induce a response in porcine alveolar macrophages (48). As a control, NF-κB inhibitions were performed where cells were pre-incubated for 1 hr at 37°C in 5% CO_2 with 25 μg/ml caffeic acid phenethyl ester (CAPE) (Sigma) before addition of the bacteria for an additional incubation of 12 h. The supernatant was collected and tested by ELISA for IL-8 concentration.

RNA extractions for microarray experiments. Monolayers of SJPL cells in T175 flasks were infected for 3h with 100 μl of an OD_{600} of 0.6 culture of *A. pleuropneumoniae* (MOI of 10:1). Planktonic bacteria were harvested from the culture supernatant while adherent bacteria were harvested with the epithelial cells following two washes in PBS buffer. Ice cold RNA degradation stop solution (95% ethanol, 5% buffer-saturated phenol) was added to all samples at a 1:10 (v/v) ratio, and samples were then frozen at -80ºC after a 5 min centrifugation at 4000 g. The isolation of bacterial RNA was carried out using the QIAGEN RNeasy MiniKit with an in-column DNAse treatment, as prescribed by the manufacturer. RNA was further treated with Turbo DNase (Ambion, Tx) as prescribed by the manufacturer

Transcriptomic microarray experiments. The *AppChip1* design was part of the *A. pleuropneumoniae* 5b L20 genome sequencing project led by the team of Dr. John Nash (NRC-IBS, Ottawa, Canada). The microarrays used in this study contain PCR amplicons representing all the ORFs that were identified in the *A. pleuropneumoniae* 5b L20 genome (13, 18). RNA was reverse transcribed to cDNA using Invitrogen Superscript II. cDNA was indirectly labelled with monofunctionnal Cy3 or Cy5 NHS-ester (Amersham Biosciences, Piscataway, NJ). Samples from planktonic growth or adhesion versus growth in DMEM medium, were combined and co-hybridized on the microarray. Four hybridizations were conducted for each condition including a pair of microarray for which Cy3 and Cy5 dyes were swapped. Microarrays analysis was carried out using the TM4 Suite of softwares (TIGR) and the SAM algorithm, using a false discovery rate (FDR) value of 0% (50). For the planktonic and adhesion experiments, Cy5 signal was compared to Cy3 signal in order to obtain a list of significantly differentially expressed genes. In order to obtain the list of

differentially expressed genes between planktonic growth and adhesion, log2 ratios were compared in TM4 also using the SAM algorithm. Functional classification was performed using TIGR's Comprehensive Microbial Resource (47). Data files were submitted to the Gene Expression Omnibus (GEO accession number GSE12009).

RESULTS

Effect of bacterial infection on viability of epithelial cells. *A. pleuropneumoniae* expresses potent exotoxins. To ensure cell viability in our experiments, cell death assays were performed at different MOIs (10:1, 100:1 and 1000:1) and incubation periods (0.5 h, 1 h, 2 h, 3 h and 4 h) with *A. pleuropneumoniae* strain S4074 representing serotype 1. LDH cytotoxicity assays to detect necrosis were first performed. Important cytotoxicity was observed after an incubaction of 4 h (up to 80% at an MOI of 10:1) or with an MOI of 100:1 (up to 40% at 3 h) (data not shown). An MOI of 10:1 and incubation times not surpassing 3 h were chosen for subsequent tests as a result of low cytotoxicity in these conditions (Figure 1). Since cell death by apoptosis cannot be detected by the LDH test, apoptosis assays were also preformed. An ELISA assay detecting DNA degradation and a Western blot assay detecting caspase-3 activation were carried out and demonstrated that neither cell lines undergo apoptosis after 3 h of bacterial infection at an MOI of 10:1 (data not shown).

Adherence of *A. pleuropneumoniae.* Standardization of adherence models was performed using both cell lines and the *A. pleuropneumoniae* reference strain S4074. Microscopy assays visually demonstrated the adhesion of the bacteria to the cells (Figure 2). The increase of adherence over time is well demonstrated in the adherence assay, with an increase of about 1 log every hour (Figure 3).

Protein profiling of SJPL and NPTr cells incubated with *A. pleuropneumoniae.* A protein profiling of SJPL and NPTr cells incubated with *A. pleuropneumoniae* was performed as a rapid screening method using the Kinexus

antibody microarray. 608 cell signalling proteins, including 250 phospho-sites, 240 protein kinases and 110 cell signalling proteins that regulate cell proliferation, stress and apoptosis were represented on the microarray. Only proteins with a fold change of ±1 and higher on a $\log_2$ scale were deemed differentially expressed. Twenty proteins were up-regulated for the SJPL cells in contrast to 21 for the NPTr cells, while 25 proteins were down-regulated for both the SJPL and NPTr cells (Supplemental material: Table I and II). Amongst the up-regulated proteins, most were implicated in stress response. Mostly proteins implicated in cell growth and proliferation were observed to be down-regulated. Amongst the proteins differentially expressed, IKKα, IKKβ, IRAK4 and 3 different MEKK proteins were detected and directed our focus towards the NF-κB and AP-1 pathway, and ultimately to an examination of the production of cytokines by the epithelial cells.

NF-κB induction and cytokine production. EMSA detecting the induction of NF-κB and AP-1 were performed on both cell lines following incubation with *A. pleuropneumoniae* S4074 since proteins involved in these pathways were observed to be differentially expressed in our previous experiment. In comparison to the basal level of uninfected cells, a clear induction of NF-κB was noticed for the NPTr cells as soon as at 1 h post-infection which is represented by the appearance of bands of higher density in the upper part of the gel, corresponding to a band shift (Figure 4A). Only a slight increase in density was observed for the SJPL cells at 3 h post-infection. In order to assess the specificity of the NF-κB induction, a supershift assay using specific antibodies was carried out for the detection of 2 subunits of NF-κB, p50 and p65. The p50 subunit was found to be induced for the SJPL cells but not the NPTr cells after 3 h of incubation with *A. pleuropneumoniae* S4074 and inversely, the p65 subunit was induced in the NPTr cells only (Figure 4B). No induction of the AP-1 transcription factor was detected for both cell lines in comparison to the uninfected cells (data not shown).

To further investigate the bacterial-based induction of the NF-κB transcription factor, we evaluated the cytokine production by both cell lines in stimulated

conditions. Incubations for up to 48 h of the SJPL and NPTr cells with heat-killed *A. pleuropneumoniae* were then performed to quantify the production of IL-1β, IL-6, IL-8 and TNF-α, proinflammatory cytokines involved in innate immunity, by the epithelial cells. ELISAs performed on the supernatant samples showed that, in these conditions, the NPTr cells, but not the SJPL cells, secrete IL-8. Production of IL-8 by the NPTr cells increased over time to reach 2500 pg/ml at 48 h (Figure 5) in comparison to 800 pg/ml with purified LPS. However, no IL-1β, IL-6 or TNF-α were detected in the samples from both cell lines (data not shown). Following NF-κB inhibition in the NPTr cells by CAPE, IL-8 concentrations decreased to basal levels (data not shown). This demonstrates that the production of IL-8 observed for the NPTr cells is indeed due to the induction of NF-κB.

To further investigate the mechanism of NF-κB induction in both cell lines, we performed an EMSA on cells pre-treated with an IRAK 1/4 inhibitor. IRAK1/4 is recruited by MyD88 protein immediately after Toll receptor activation at the beginning of the NF-κB pathway (27). The level of NF-κB induction in comparison to non inhibited cells consequently demonstrated indirectly if the activation of Toll-like receptors by the bacteria is responsible for this induction. Our EMSA results indicate that for the SJPL cells, NF-κB induction occurs through the Toll receptor pathway (data not shown) but for the NPTr cells, NF-κB induction occurs through a different pathway.

***A. pleuropneumoniae* transcriptomic profiling.** To assess the transcriptional response of *A. pleuropneumoniae* to both planktonic life over and adherence to SJPL cells, transcript profiling experiments using DNA microarrays were performed after an incubation time of 3 h. Overall, 170 genes were significantly differentially expressed during planktonic growth (Tables 2 and 3), this number dropping to 131 during adhesion to SJPL cells (Tables 4 and 5). While some genes showed similar patterns of expression during both conditions, 150 were differentially expressed between both conditions (supplemental material: Table III).

The genes that showed the highest level of up-regulation during planktonic growth belonged to the "Energy Metabolism" functional class, and this class was also the most affected with 24 out of the 82 up-regulated genes. Surprisingly, most of these genes are involved in anaerobic respiration. Various enzymes involved in anaerobic respiration using alternative electron acceptors were up-regulated : genes encoding subunits of the formate dehydrogenase (*fdxG, fdnHI*) and nitrate reductase (*nrfABC*), which are essential for anaerobic respiration on nitrate (35, 62), and subunits of the fumarate reductase (*frdACD*), which allows fumarate to serve as a terminal electron acceptor (10), were all up-regulated. Furthermore, genes *pgi*, *fbp* and *pykA*, which encode three enzymes involved at various steps of glycolysis, respectively glucose-6-phosphate isomerase, fructose-1,6-bisphosphatase and pyruvate kinase, showed an increase in transcription. Two dehydrogenases, alcohol dehydrogenase I (*adhI*) and malate:quinone oxidoreductase (*mqo*), are also involved in anaerobic respiration, the latter being controlled by the ArcA-ArcB two component system (59). Genes *aspA* and *dmsA* were also up-regulated. The "Transport and Binding Proteins" class was the second most affected with 12 up-regulated genes. Genes involved in the transport of l-lactate (*lctP*), formate and nitrite (*yrhG*), sucrose (*ptsB*) and glycerol (*glpT*) were all induced, as well as gene *modA*, which encodes a periplasmic protein involved in the ABC-transport of molybdate (25). Gene *APL_0443*, coding for a putative autotransporter adhesin, showed a 1.9 fold induction.

Down-regulated genes during planktonic growth mostly belonged to the "Protein synthesis" and the "Transport and Binding Proteins: cations and iron" functional classes. The *hgbA* hemoglobin receptor genes, as well as its *hugZ* heme utilization protein co-transcript were down-regulated, and other well-established iron-acquisition related genes included *tonB1* and *exbD2*. These genes are co-transcribed with other members of the TonB1 (*exbB1*, *exbD1*, *tbpA*, *tbpB*) and TonB2 (*exbB2*, *exbD2*) energy transduction system (7), but these were not identified in our study. At this time, genes *exbB1*, *exbD1* and *tbpB* are not present on AppChip1, and gene *tbpA* was not down-regulated. Genes *exbB2* and *tonB2*, however, exhibit a 2 fold average

down-regulation, but variations between chips might have caused these to be ignored by our very stringent analysis parameter (FDR=0%). A high number of genes that were identified for the first time in *A. pleuropneumoniae* in our previous transcript profiling experiment under iron restriction (13) were also down-regulated. ORFs APL_1952 – APL_1955, which code for a putative second hemoglobin receptor system and are likely transcriptionally linked, were repressed, as well as genes *APL_0714-APL_0715-APL_0717*, encoding a putative ABC-type siderophore transport system, and genes *yfeABD*, likely responsible for the ABC-like periplasmic binding protein-dependent transport of chelated iron and possibly manganese. Genes *cpxABC*, coding for the capsule polysaccharide ABC-type export system, were also all down-regulated, along with gene *ssa1*, encoding a putative autotransporter serine protease.

Interestingly, some genes potentially involved in adhesion and biofilm biosynthesis were up-regulated during adherence to SJPL cells. Genes *rcpA* and *tadB*, which belong to a large operon of 14 genes, were up-regulated, as well as genes *pgaBC*, involved in poly-β-1,6-N-acetyl-D-glucosamine biofilm biosynthesis. A small number of genes involved in iron acquisition were also up-regulated, the most notable being *fecE* and *APL_1955*. Once again, genes involved in anaerobic respiration were shown to be up-regulated. Other enzymes coding for hydrogenases (*hyaA, hybB*) and dehydrogenases (*lldD*, *fdhE*) involved in energy metabolisms also showed up-regulation. Gene *fucO,* essential for the anaerobic degradation of fucose, and genes *fucI* and *fucK*, (11), involved in the general fucose degradation pathway, were also up-regulated.

Only 52 genes were identified as down-regulated, and most of them belong to the "Energy Metabolism" functional class. The six enzymes which catalyze the first six steps of glycolysis (*gapA*, *pgk*, *fbp*, *tpiA*, *pgi* and *fba*) were down-regulated, as well as gene *maeA*, responsible for the first step of gluconeogenesis, and gene *tktA*, which links glycolysis to the pentose-phosphate pathway. *hlyX*, coding for the *A. pleuropneumoniae* FNR anaerobic global regulator homolog, was repressed 2.72

fold. Toxin genes *apxIC* and *apxIIA* also showed down-regulation during adhesion to SJPL cells.

Adherence and invasion of *A. pleuropneumoniae* and other *Pasteurellaceae.* Other serotypes of *A. pleuropneumoniae* as well as different swine *Pasteurellaceae* were tested in the adherence models. Differences in adherence were observed between strains for a given cell line as well as between the cell lines for a given strain (Figure 6). We noticed that the field strains of *A. pleuropneumoniae* adhered significantly more to the cell lines than the reference strain of the same serotype. We also noticed that the adherence level to a given cell line is strain dependent. Following the observation that all *Pasteurellaceae* tested adhered to the cell lines, invasion tests were performed. *A. pleuropneumoniae* S4074 did not invade either cell lines in our infection model while the other *Pasteurellaceae* tested showed invasion. *H. parasuis* showed the highest level of invasion, although at a reduced level compared to invasion seen with endothelial cells (Figure 7) (61).

DISCUSSION

Using immortalized porcine lung and tracheal epithelial cells, we were able to study the host pathogen interactions of *A. pleuropneumoniae.* In our models, *A. pleuropneumoniae* provoked cell death very rapidly through necrosis and not apoptosis. The presence of this bacterium causes many changes in the protein profile of both epithelial cell lines. Indeed, using an antibody microarray as a rapid screening for differential protein expression, we were able to direct our efforts toward the NF-κB pathway as numerous differentially expressed proteins were implicated in the NF-κB pathway including IKKα, and IKKβ. NF-κB consists of homo- or heterodimer composed of the five mammalian Rel proteins, p65, c-Rel, p50, p52 and RelB (24), the p50/p65 heterodimer being the most abundant and active of the NF-κB complexes (2). Out of the 5 subunits, only p65 (RelA), RelB and c-Rel were found to contain the C terminal transactivation domains (TDs) essential for gene activation. In contrast, p50 and p52 do not possess the TDs, and therefore

cannot act as transcriptional activators by themselves (39). Additionally, p50 and p52 are synthesized as precursor proteins that belong to the IκB family known as inhibitors of NF-κB and homo- or heterodimers of p50 and p52 were also reported to repress κB site-dependent transcription *in vivo* (39). Interestingly the p50 subunit was found to be induced in the SJPL cells but not in the NPTr cells after 3 h of incubation with *A. pleuropneumoniae* S4074 and inversely, the p65 subunit was induced in the NPTr cells only. It should be noted that the absence of detection of either p50 in the NPTr cells or p65 in the SJPL cells is not due to a weak bacterial-induced expression, but is most probably due to the incapacity of the cell line to express the protein since no basal level of expression was observed in unstimulated conditions for the subunits p50 in the NPTr cells and p65 in the SJPL cells. Those results suggest that in the absence of p65, inactive p50/p50 homodimers are more likely to form in the SJPL cells. The absence of IL-8 production by the SJPL cells might be explained by the weak NF-κB induction observed in the EMSAs, but certainly correlates with the absence of the p65 subunit necessary for attachment to the IL-8 promoter. Previous studies have also shown that the binding affinity of p50 to the human IL-8 promoter is weak compared to the binding of the p65 subunit (38). Different pathways can activate NF-κB, the most frequent in Gram-negative bacterial infection, being the classical pathway through Toll-like receptors activation by LPS (44). We demonstrated that this is the case for the SJPL cells, but not for the NPTr cells. A possibility is that an alternative pathway for NF-κB activation was used in the NPTr cells where IKKα homodimers are activated instead of the IKKβ in the classical pathway leading to NF-κB2/p100 phosphorylation. This is a possibility, as this modification creates the production of p52 (44), a subunit which seems to be present in the stimulated NPTr cells as seen in the EMSA where a band slightly higher than p50 was detected and as IKKα was up-regulated in the NPTr antibody microarray. This pathway is generally triggered by TNF receptor family members, such as LTβR, BAFF-R, CD40 and CD30 (44). Additional experiments are however necessary to confirm this theory.

The presence of the epithelial cells stimulated differential expression of many *A. pleuropneumoniae* genes. Although it was shown previously, with the evidence of a putative involvement in virulence of genes *dmsA* and *aspA*, that genes involved in anaerobic respiration might in fact be essential for full virulence of *A. pleuropneumoniae* in the host (3, 5, 30), it is still unclear why genes involved in anaerobic respiration are up-regulated in our experimental conditions. While such a metabolic switch might be important *in vivo* to adapt to the lack of oxygen in the deep lung tissues, it should not be necessary in our experimental setup unless this apparent aerobic/anaerobic shift is controled by a host cell-associated factor rather than by oxygen sensors. In fact, it is worth noting that this metabolic shift does not seem to be complete since genes involved in aerobic respiration are not down-regulated. The upregulation of gene *sodA*, coding for a cytoplasmic Mn superoxide dismutase (21) also seems to indicate that aerobic respiration is not stopped, as these cytoplasmic superoxide dismutases are specifically useful in removing superoxide anions generated during the course of aerobic respiration (54).

A gene with possible involvement in virulence was also identified. Gene *APL_0443* is described as an autotransporter adhesin. This protein shows region of high homology with the *A. actinomycetemcomitans* extracellular matrix protein adhesin A (EmaA), an oligomeric autotransporter with a YadA domain (57), and the putative *Mannheimia haemolytica* Hsf protein (40). In *Haemophilus influenzae* serotype b, Hsf (*Haemophilus* surface fibrils) is considered as the major nonpilus adhesin (55), and was found to be associated with adherence to human epithelial cells (6, 26). Whether this putative *A. pleuropneumoniae* Hsf has these properties remains to be seen, but the up-regulation of this gene during planktonic life over SJPL cells might hint to a possible role in the initial steps of *A. pleuropneumoniae* adhesion during infection.

The fact that iron, in DMEM culture media, is available only in the form of ferric nitrate (12) might explain why iron acquisition systems are more expressed in cell-free DMEM than during planktonic growth. Experiments conducted in our lab

have shown that ferric nitrate cannot support growth of *A. pleuropneumoniae* in an EDDHA iron-restricted medium (13). Lysis of SJPL cells, leading to the release of the intra-cellular content of those cells in the medium, supplies the planktonic bacteria with more readily available sources of iron, for example iron-sulfur clusters in the catalytic core of enzymes and ferritin-bound ferric iron.

Some genes with possible involvement in virulence also came up as down-regulated during planktonic growth. Down-regulation of the *cpxABC* operon during planktonic growth over SJPL cells might indicate that, when in contact with host cells, *A. pleuropneumoniae* might wear a thinner polysaccharide layer in order to unmask some adhesins. Surprisingly, this down-regulation of the *cpx* operon was not seen during adhesion to SJPL cells. Upon verification of fold changes during adhesion, only cpxC showed a low level of down-regulation (-1.24), although not statistically significant. Repression of the gene *ssa1* was surprising since this gene, also termed *aasP*, was shown to be expressed *in vivo* during the chronic stage of the disease (3). However, this gene was also shown to be iron-responsive, as indicated by its up-regulation during iron-restriction (13). It might therefore simply follow the same trend as other iron-responsive genes which were down-regulated during planktonic growth.

Our main focus, when looking at over-expressed genes during adherence to porcine lung epithelial cells, was to search for new potential adhesins. Genes *tadB* and *rcpA* are part of a large operon which, in *A. actinomycetemcomitans*, is composed of 14 genes (36) and mediates non-specific adhesion to solid surfaces, whether they are biological surfaces or not (16). The genetic organization of the *A. pleuropneumoniae tad* locus is identical to that of *A. actinomycetemcomitans* (58). Although it is suspected that the *tad* genes might be transcribed as an operon, only two genes were identified as up-regulated in our study. The 12 other genes are present on the microarray, but are not significantly induced. Expression of the *tad* genes is responsible for the rough colony phenotype of *A. actinomycetemcomitans*, but smooth variants often arise after continued passage on rich medium (49) as

mutations often appear in the promoter region of gene *flp-1* (58). We suspect that this might also be the case for *A. pleuropneumoniae* since most field isolates exhibit this rough colony phenotype while the reference strains are often smooth colony variants. As is the case for *A. actinomycetemcomitans*, the Tad proteins might play an important role for the colonization of the respiratory tract by *A. pleuropneumoniae*, but this will have to be further investigated. Other genes possibly involved in adhesion were also up-regulated during adhesion to SJPL cells. Genes *pgaB* and *pgaC* are both involved in PGA (poly-β-1,6-N-acetyl-D-glucosamine) biofilm formation. A *pgaABCD* cluster is present in the App5b L20 genome, and gene *pgaC* has been shown to be present in 15 reference strains (29). These results are interesting since the only components that have been clearly shown to be involved in *A. pleuropneumoniae* adhesion to lung surfaces to date are LPS (1, 8, 34, 45, 46).

Gene *hlyX* was down-regulated during adhesion to SJPL cells. This gene, which encodes the *A. pleuropneumoniae* Fnr anaerobic global regulator homolog, was shown to be important for the colonization and persistence of *A. pleuropneumoniae* in the respiratory tract of swine (5). The repression of *hlyX* probably explains the repression of *aspA*, which is presumably regulated by HlyX, as well as the down-regulation of a few other genes linked with anaerobic respiration (*fdxG*, *torZ*, *nrfB*, *frdD*). Genes putatively regulated by HlyX have been shown to be induced by bronchoalveolar lavage fluid from infected pigs (30), and it is possible that *hlyX* expression follows the same pattern. Also, putative HlyX-regulated genes were up-regulated during planktonic growth over SJPL cells.

ApxI and ApxII have been shown to be major virulence factors in *A. pleuropneumoniae*. Not much is known about transcriptional regulation of those toxins in *A. pleuropneumoniae.* Studies have shown that levels of oxygen do not influence the level of ApxI and ApxII (33), and that the iron response regulator, Fur, seems to have variable effects depending on calcium concentration in the culture medium (28). Under high calcium concentration, Fur seemed to act as an activator

of the *apxI* operon, while it seemed to act as a repressor under low calcium concentration. A previous microarray study conducted under iron restriction showed that Fur does have an effect on ApxI transcription (13). One would normally expect these toxins to be induced in conditions mimicking the *in vivo* environment, mostly after contact with epithelial cells. Down-regulation of genes *apxIC* and *apxIIA* was therefore intriguing. Perhaps smaller concentrations of RTX toxins are required when the bacteria are in close proximity of host cells, leading to the downregulation of the toxins following adherence.

Adherence is seen in both models for all *A. pleuropneumonaie* strains and serotypes tested. It is interesting to note that field strains adhere more to the cell lines than the reference strain of the same serotype. No invasion is noticed for *A. pleuropneumoniae*, even though close relatives, such as *A. actinomycetemcomitans* and *H. parasuis*, are known to be invasive (17, 42, 61).

Overall these results showed the efficacy of the models and allowed us to gain a great amount of knowledge on *A. pleuropneumoniae* host-pathogen interactions. Indeed, interaction of *A. pleuropneumoniae* with host epithelial cells seems to involve complex cross-talk which results in the regulation of various bacterial genes. Many virulence genes were up-regulated including genes coding for the putative adhesins Hsf and PGA, while capsular polysaccharide associated genes were down regulated possibly exposing adhesins usually hidden by a thick capsule. Incubation with *A. pleuropneumoniae* then lead, for both cell lines, to the induction of NF-κB. This is done through the activation of a Toll receptor for the SJPL cells but through an alternative pathway for the NPTr cells. The NPTr cells then secrete IL-8, which is known to attract neutrophils to the infection site, while the SJPL cells do not due to the absence of the p65 subunit of NF-κB. These models are a biologically relevant tool to study porcine respiratory tract pathogen which could be further used, in the future, to evaluate the effect of a pre-infection with agents such as mycoplasmas and viruses often present with bacterial pathogens in the field conditions.

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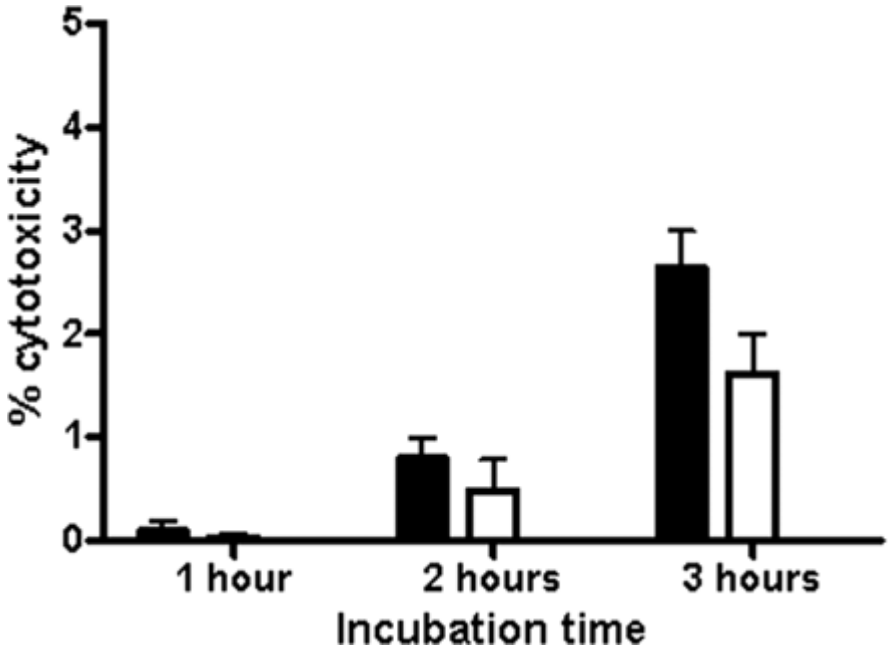

Figure 1. SJPL (filled bars) and NPTr (empty bars) cells were assessed for cytotoxicity following an infection with *A. pleuropneumoniae* strain S4074 at an MOI of 10:1.

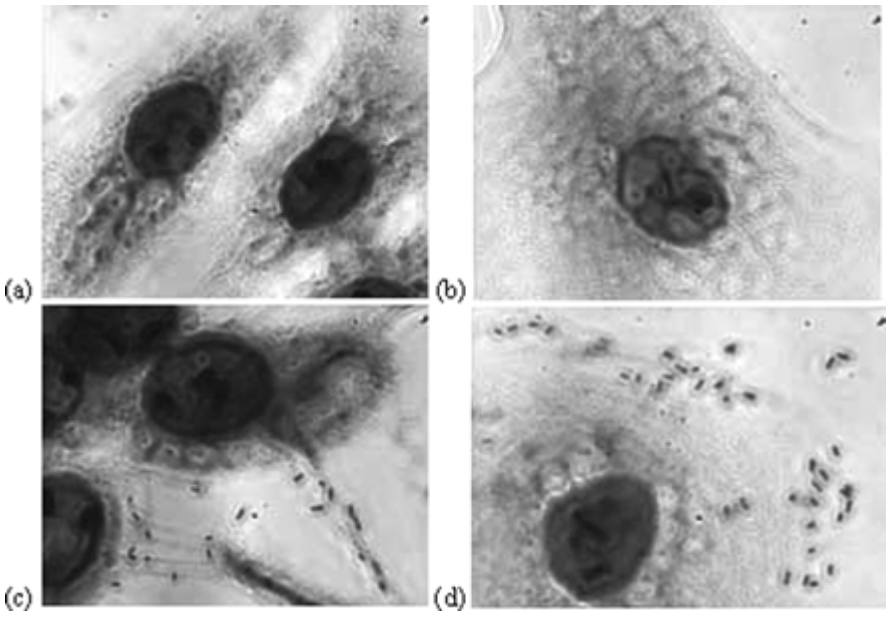

Figure 2. NPTr (a and c) and SJPL (b and d) cells stained with Giemsa in the presence (c and d) or absence (a and b) of *A. pleuropneumoniae* S4074 seen through a Leica DMR microscope at a magnification of 1000X.

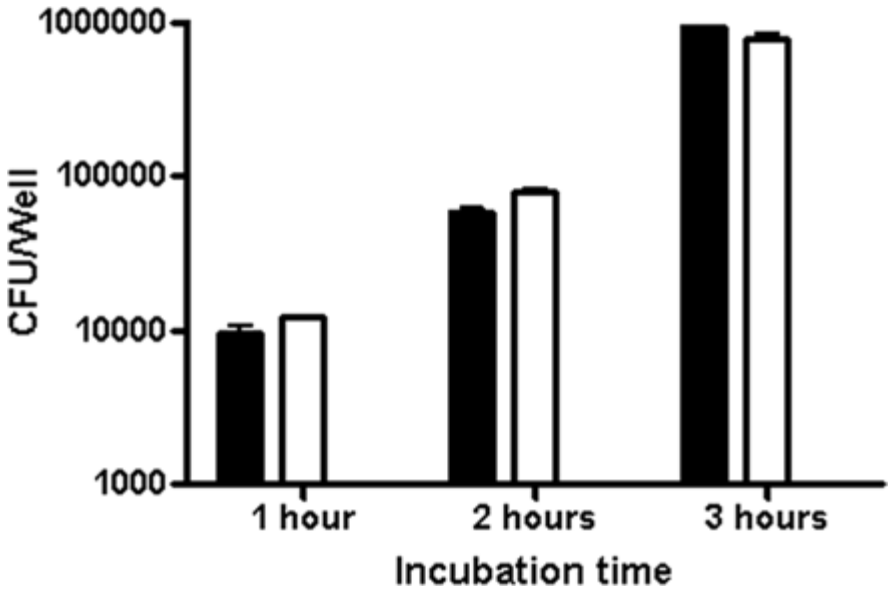

Figure 3. Adherence of *A. pleuropneumoniae* S4074 to SJPL (filled bars) and NPTr (empty bars) cells from 1 to 3 h.

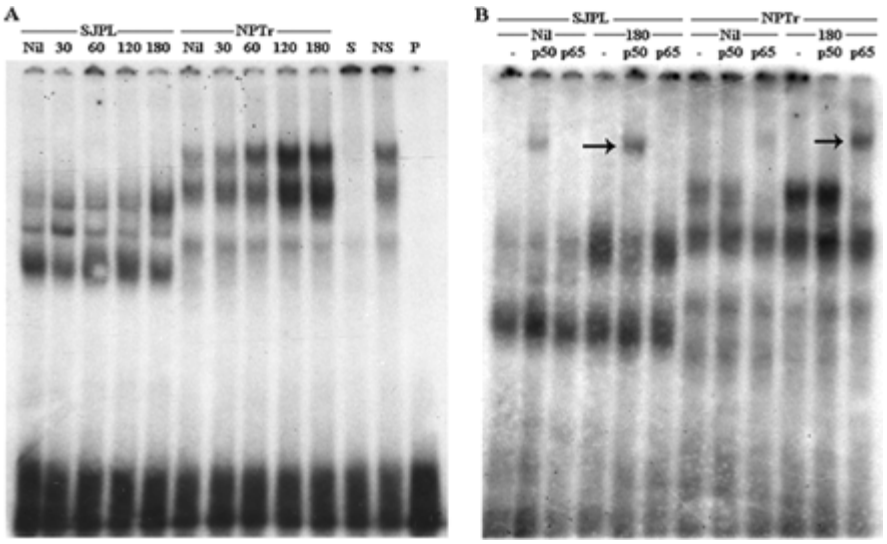

Figure 4. EMSA (A) and supershift assay (B) performed on nuclear proteins of SJPL and NPTr cells following an incubation (30 – 180 minutes) with *A. pleuropneumoniae* S4074 or not treated for control (Nil). For controls, proteins were incubated with specific oligos (S) and non-specific oligos (NS). The probe alone was also loaded on the gel (P). For the supershift assay (B), proteins where incubated with p50 antibodies (p50), p65 antibodies (p65), or no antibodies (-). Arrows demonstrate the subunit band shifts (B).

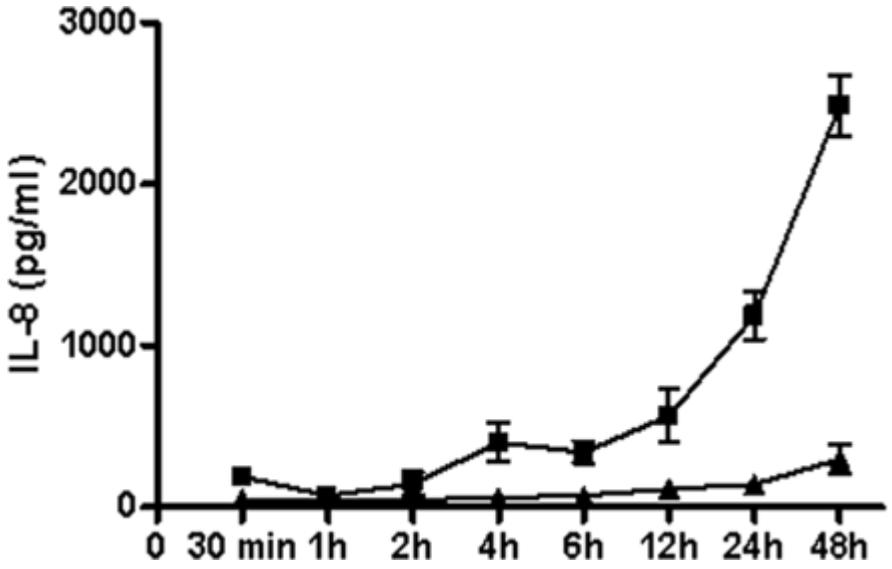

Figure 5. Production of IL-8 by NPTr cells following an induction with heat-killed *A. pleuropneumoniae* S4074 (■), and when not stimulated (▲).

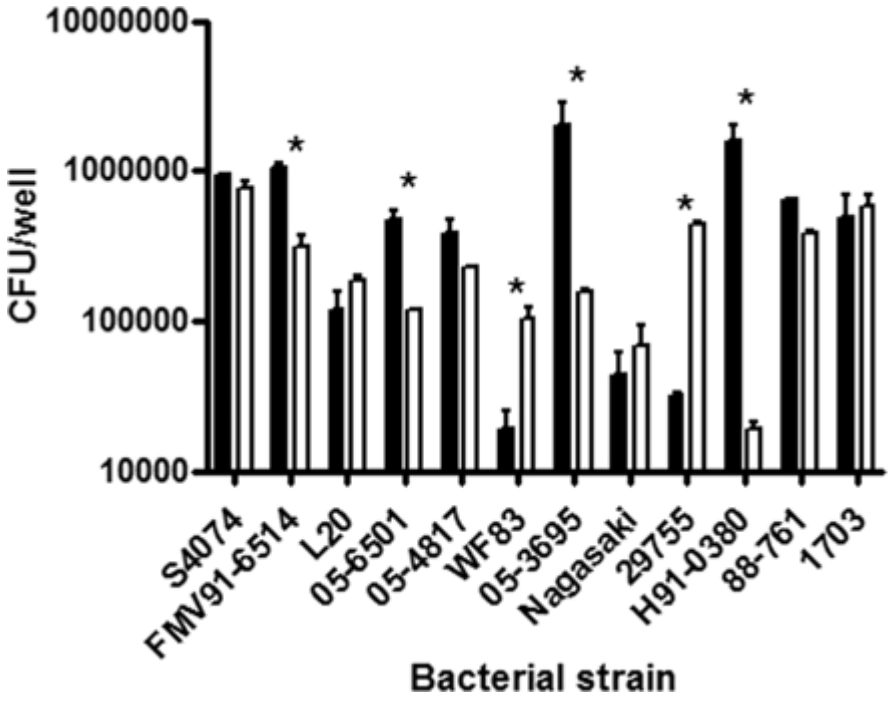

Figure 6. Adherence of twelve *Pasteurellaceae* to the SJPL (filled bars) and NPTr (empty bars) cell line after 3h of incubation. The strains include *A. pleuropneumoniae* serotype 1 S4074 and FMV91-6514, *A. pleuropneumoniae* serotype 5b L20 and 05-6501, *A. pleuropneumoniae serotype* 5a 05-4817, *A. pleuropneumoniae* serotype 7 WF83 and 05-3695, *H. parasuis* serotype 5 Nagasaki and 29755, *A. suis* serotype O2/K2 H91-0380 and *P. multocida* capsular type A 88-761 and capsular type D 1703. Asterisks represent statistical differences ($P<0.05$) in adherence of the given strain between the two cell lines.

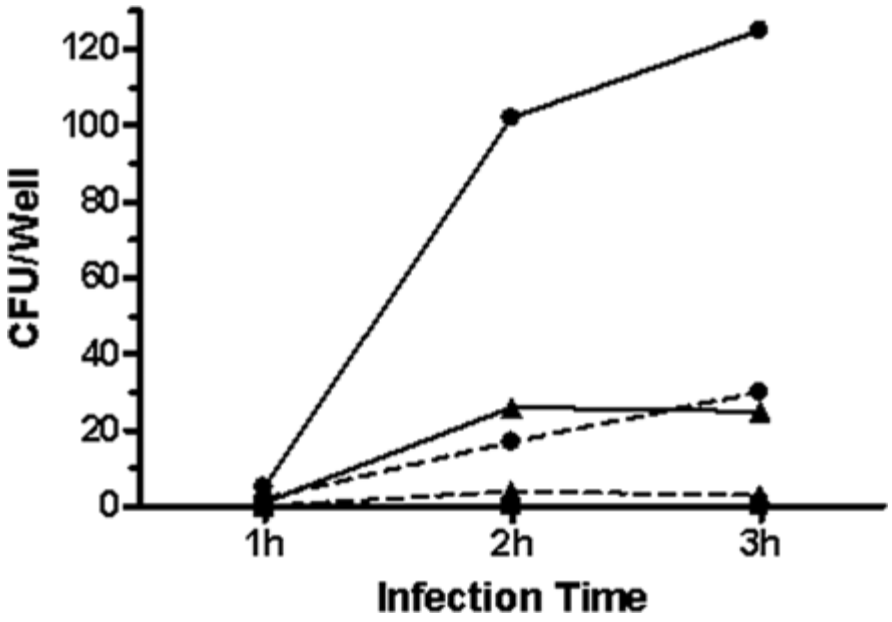

Figure 7. Invasion by *A. pleuropneumoniae* S4074 (■), *H. parasuis* Nagasaki (▲) and *H. parasuis* 29755 (●) of SJPL (full line) and NPTr (dash line) cells from 1 h to 3 h.

Tables

Table 1. Bacterial strains used in the present study

Strains	Serotype	Source or reference
A. pleuropneumoniae S4074	1	K.R. Mittal[a]
A. pleuropneumoniae L20	5b	K.R. Mittal[a]
A. pleuropneumoniae WF83	7	K.R. Mittal[a]
A. pleuropneumoniae FMV91-6514	1 (Rough field strain)	(32)
A. pleuropneumoniae 05-4817	5a (field strain)	K.R. Mittal[a]
A. pleuropneumoniae 05-6501	5b (field strain)	K.R. Mittal[a]
A. pleuropneumoniae 05-3695	7 (field strain)	K.R. Mittal[a]
H. parasuis Nagasaki	5	M. Gottschalk[a]
H. parasuis 29755	5	E. Thacker[b]
A. suis H91-0380	O2/K2	J. MacInnes[c]
P. multocida 88-761	A	K.R. Mittal[a]
P. multocida 1703	D	K.R. Mittal[a]

[a] Faculté de médecine vétérinaire, Université de Montréal.
[b] Faculty of Veterinary Medicine, Iowa State University.
[c] Departement of Pathobiology, Ontario Veterinary College, University of Guelph.

Table 2. *A. pleuropneumoniae* genes which are up-regulated during planktonic life over SJPL cells (82 genes)

Locus Tag	Gene	Description	Fold Change
Hypothetical/Unclassified/Unknown			
APL_1839	*udp*	COG2820: Uridine phosphorylase, probable outer membrane protein, possible efflux protein	3.937
APL_1833	*APL_1833*	COG2717: Predicted membrane protein, conserved hypothetical protein	3.388
APL_1435	*APL_1435*	Unassigned protein	3.214
APL_1285	*APL_1285*	Hypothetical protein	2.894
APL_0145	*APL_0145*	COG1611: Predicted Rossmann fold nucleotide-binding protein	2.726
APL_1976	*yedF*	COG0425: Predicted redox protein, regulator of disulfide bond formation	2.669
APL_0116	*APL_0116*	Hypothetical protein	2.523
APL_0093	*APL_0093*	DUF1260 domain containing protein	2.020
APL_1374	*APL_1374*	Unassigned protein	1.946
APL_0465	*APL_0465*	DUF74 domain containing protein	1.943
APL_1919	*APL_1919*	predicted enzyme related to aldose 1-epimerase	1.939
APL_1138	*APL_1138*	DUF526 domain containing protein	1.933
APL_0443	*APL_0443*	COG5295: Autotransporter adhesin	1.918
APL_1656	*APL_1656*	COG0561: Predicted hydrolases of the HAD superfamily	1.842
APL_1203	*APL_1203*	DUF479 domain containing protein, hypothetical protein	1.811
APL_1609	*APL_1609*	DUF533 domain containing protein	1.724
APL_1881	*APL_1881*	Putative carbamoylphosphate synthase large subunit	1.703
APL_1244	*APL_1244*	Hypothetical protein	1.555
Biosynthesis of cofactors			
APL_1622	*cbiM*	Predicted ABC cobalt transport permease protein CbiM	2.419
APL_0931	*iscS*	Cysteine desulfurase, iron-sulfur cluster assembly	2.374
APL_0930	*nifU*	NifU-like protein, involved in Fe-S cluster formation	2.011
APL_1555	*hemL*	Glutamate-1-semialdehyde 2,1-aminomutase	1.619
Energy metabolism			
APL_1832	*APL_1832*	COG2041: Sulfite oxidase and related enzymes	13.073
APL_0892	*fdxG*	Formate dehydrogenase, nitrate-inducible, major subunit	12.558
APL_0895	*fdnI*	Formate dehydrogenase, cytochrome b556 subunit	10.946
APL_0894	*fdxH*	Formate dehydrogenase, iron-sulfur subunit	6.0988
APL_0687	*dld*	D-lactate dehydrogenase	5.442
APL_0100	*nrfA*	Ammonia-forming cytochrome c-552 nitrite reductase	5.039
APL_0106	*putA*	Bifunctional protein PutA	4.634
APL_0101	*nrfB*	Nitrate reductase, cytochrome-C type protein	4.295
APL_1091	*aspA*	Aspartate ammonia-lyase	4.254
APL_1528	*frdC*	Fumarate reductase subunit C	3.713
APL_1137	*pgi*	Glucose-6-phosphate isomerase	3.136
APL_1959	*adhI*	Alcohol dehydrogenases 1	2.992
APL_1414	*mqo*	Putative malate:quinone oxidoreductase	2.802

APL_1379	*ccp*	Cytochrome c peroxidase	2.796
APL_1450	*fbp*	Fructose-1,6-bisphosphatase	2.528
APL_1529	*frdA*	Fumarate reductase, flavoprotein subunit	2.420
APL_0187	*pykA*	Pyruvate kinase	2.322
APL_1197	*APL_1197*	3-hydroxyacid dehydrogenase	2.270
APL_0102	*nrfC*	Nitrate reductase	2.250
APL_1526	*frdD*	Fumarate reductase subunit D	2.178
APL_0486	*maeA*	Malic enzyme (NADP-dependent)	2.074
APL_1674	*dmsA*	Anaerobic dimethyl sulfoxide reductase chain A precursor	1.934
APL_0483	*APL_0483*	Predicted nitroreductase	1.912
APL_0084	*trxB*	Thioredoxin reductase	1.486
Transport and binding proteins: cations and iron			
APL_1265	*copA*	Copper-transporting P-type ATPase	1.518
Transport and binding proteins : others			
APL_0107	*putP*	Na+/proline symporter	6.218
APL_1173	*pnuC*	Nicotinamide mononucleotide transporter	2.448
APL_0377	*glpT*	Glycerol-3-phosphate transporter	2.331
APL_1254	*APL_!254*	COG0471: Di- and tricarboxylate transporters	2.237
APL_1319	*ptsB*	PTS system sucrose-specific EIIBC component	2.206
APL_0447	*lctP*	L-lactate permease	2.204
APL_0262	*modA*	Molybdate-binding periplasmic protein precursor	2.049
APL_1620	*cbiO*	Predicted ABC-type cobalt transport system, ATPase component	1.902
APL_1627	*APL_1627*	Putative di- and tricarboxylate transporters	1.876
APL_1624	*cbiK*	Putative periplasmic binding protein CbiK	1.823
APL_1902	*yrhG*	COG2116: Formate/nitrite family of transporters	1.718
APL_1371	*ccmB*	Heme exporter protein B, cytochrome c-type biogenesis protein	1.596
Regulatory functions			
APL_108	*iclR*	Putative HTH-type transcriptional regulator	2.832
APL_0395	*rseA*	Putative sigma-E factor negative regulatory protein	2.260
APL_0823	*glpR*	Glycerol-3-phosphate regulon repressor	2.175
APL_0997	*lacZ*	Beta-galactosidase	1.654
Transcription			
APL_1475	*rpoD*	RNA polymerase sigma-70 factor	2.135
APL_0560	*rhlB*	ATP-dependent RNA helicase RhlB	1.911
Purines, pyrimidines, nucleosides, and nucleotides			
APL_0646	*cpdB*	2',3'-cyclic-nucleotide 2'-phosphodiesterase precursor	2.087
Protein fate			
APL_0742	*degS*	Protease DegS precursor	1.768
APL_0871	*pepE*	Peptidase E	1.686
Protein synthesis			
APL_0484	*rimK*	Ribosomal protein S6 modification enzyme	3.093
APL_0146	*dusA*	tRNA-dihydrouridine synthase A	2.456
Cellular processes			
APL_0004	*sodC*	Cu/Zn superoxide dismutase precursor	3.003

APL_0251	*sodA*	Manganese superoxide dismutase	2.356
APL_1241	*APL_1241*	Probable carbon starvation protein A, predicted membrane protein	2.098
APL_1405	*oapA*	Cell envelope opacity-associated protein A	1.793
Cell envelope			
APL_1494	*ftpA*	DNA-binding ferritin-like protein (oxidative damage protectant), fine tangled pili major subunit (24 kDa surface protein)	6.378
Fatty acids and phospholipids metabolism			
APL_0397	*lcfA*	Acyl-CoA synthetases (AMP-forming)/AMP-acid ligases II	1.746
Mobile and extrachromosomal element functions			
APL_1501	*APL_1501*	Transposase	2.078
APL_0990	*APL_0990*	Transposase	1.814
APL_0985	*APL_0985*	Transposase	1.680
DNA metabolism			
APL_1143	*recA*	RecA recombinase	1.491
Central intermediary metabolism			
APL_0109	*APL_0109*	Possible 5-formyltetrahydrofolate cyclo-ligase	2.607
APL_0375	*glpK*	Glycerol kinase	2.316

Table 3. *A. pleuropneumoniae* genes which are down-regulated during planktonic life over SJPL cells (88 genes)

Locus Tag	Gene	Description	Fold Change
Hypothetical/Unclassified/Unknown			
APL_1387	*APL_1387*	Predicted metal-binding, possibly nucleic acid-binding protein	-2.431
APL_0053	*typA*	Predicted membrane GTPase involved in stress response	-2.386
APL_0173	*APL_0173*	Hypothetical cytosine deaminase and related metal-dependent hydrolases	-2.056
APL_1863	*APL_1863*	Predicted glycosyltransferase	-2.027
APL_1956	*APL_1956*	Hypothetical protein	-2.000
APL_0936	*APL_0936*	Putative integral membrane protein	-1.829
Energy metabolism			
APL_1849	*lldD*	L-lactate dehydrogenase (FMN-dependent) and related alpha-hydroxy acid dehydrogenases	-3.284
APL_0592	*guaA*	GMP synthase	-2.629
APL_1219	*fldA*	Flavodoxin	-2.276
Transport and binding proteins: cations and iron			
APL_1047	*hgbA*	Hemoglobin-binding protein A precursor	-9.346
APL_1571	*tonB1*	Periplasmic energy transducing protein TonB1	-5.319
APL_1952	*APL_1952*	Outer membrane receptor protein, mostly Fe transport	-3.517
APL_0077	*exbD2*	Energy transducing protein ExbD2	-3.499
APL_1953	*APL_1953*	Outer membrane receptor protein, mostly Fe transport	-2.874
APL_0670	*APL_0670*	Putative Fe2+/Pb2+ permease	-2.406
APL_1048	*hugZ*	Heme utilization protein	-2.387
APL_0272	*yfeA*	Iron (chelated) ABC transporter, periplasmic-binding protein	-2.339
APL_0271	*yfeB*	putative chelated iron transport system ATP-binding protein	-2.251
APL_0714	*APL_0714*	Putative ABC-type enterochelin transport system, periplasmic component	-2.178
APL_1954	*APL_1954*	Outer membrane receptor proteins, mostly Fe transport	-2.163
APL_0715	*APL_0715*	COG4606: ABC-type enterochelin transport system, permease component	-1.993
APL_1955	*APL_1955*	Outer membrane receptor proteins, mostly Fe transport	-1.891
APL_0717	*APL_0717*	COG4604: ABC-type enterochelin transport system, ATPase component	-1.760
APL_0127	*yfeD*	Putative iron transport system membrane protein	-1.718
Transport and binding proteins : others			
APL_0300	*tolQ*	Colicin transport protein	-2.584
APL_1392	*ptnC*	Mannose permease component IIC	-2.135
APL_1393	*ptnD*	Mannose permease component IID	-2.043
APL_1584	*cpxB*	Capsule polysaccharide export inner-membrane	-1.863

		protein	
APL_1585	*cpxA*	Capsule polysaccharide export ATP-binding protein	-1.572
APL_1583	*cpxC*	Capsule polysaccharide export inner-membrane protein	-1.549
Transcription			
APL_0638	*nusA*	Transcription elongation factor	-3.997
APL_0577	*pnp*	Polyribonucleotide nucleotidyltransferase	-2.375
APL_0757	*rnb*	Exoribonuclease II	-1.665
APL_0543	*rnc*	Ribonuclease III	-1.539
Purines, pyrimidines, nucleosides, and nucleotides			
APL_0148	*rnr1*	Ribonucleotide reductase, alpha subunit	-2.556
APL_0593	*guaB*	Inosine-5'-monophosphate dehydrogenase	-2.474
APL_0147	*APL_0147*	Ribonucleotide reductase, beta subunit	-2.438
APL_0255	*gpt*	Xanthine phosphoribosyltransferase	-2.316
APL_0661	*purK*	Phosphoribosylaminoimidazole carboxylase ATPase subunit	-2.091
APL_1172	*purD*	Phosphoribosylamine-glycine ligase	-1.883
APL_2018	*purC*	Phosphoribosylaminoimidazole-succinocarboxamide synthase	-1.717
APL_0834	*udk*	Uridine kinase	-1.396
Protein fate			
APL_1507	*tig*	FKBP-type peptidyl-prolyl cis-trans isomerase (trigger factor)	-3.849
APL_0364	*Ssa1*	Autotransporter serine protease	-2.279
Protein synthesis			
APL_1718	*rplK*	50S ribosomal protein L11	-5.061
APL_0639	*infB*	Translation initiation factor 2 (IF-2)	-4.243
APL_1774	*rplF*	Ribosomal protein L6	-3.954
APL_1720	*rplJ*	Ribosomal protein L10	-3.833
APL_1558	*rpsT*	30S ribosomal protein S20	-3.720
APL_1765	*rplV*	50S ribosomal protein L22	-3.039
APL_0566	*rpsB*	30S ribosomal protein S2	-2.842
APL_1400	*rpsG*	30S ribosomal protein S7	-2.714
APL_0487	*rplY*	50S ribosomal protein L25 (general stress protein Ctc)	-2.364
APL_1762	*rplW*	50S ribosomal protein L23	-2.330
APL_1763	*rplB*	50S ribosomal protein L2	-2.265
APL_1761	*rplD*	50S ribosomal protein L4	-2.254
APL_0223	*infC*	Translation initiation factor 3 (IF-3)	-2.192
APL_1773	*rpsH*	30S ribosomal protein S8	-2.100
APL_1775	*rplR*	50S ribosomal protein L18	-2.020
APL_0399	*ksgA*	Dimethyladenosine transferase (rRNA methylation)	-1.950
APL_1401	*rpsL*	30S ribosomal protein S12	-1.914
APL_1972	*rpmG*	50S ribosomal protein L33	-1.902
APL_0040	*yhbZ*	Hypothetical GTP-binding protein	-1.721
APL_1228	*infA*	Translation initiation factor 1 (IF-1)	-1.716
APL_0678	*efp*	Translation elongation factor P (EF-P)	-1.708
APL_1781	*rpsM*	30S ribosomal protein S13	-1.705
APL_1383	*APL_1383*	tRNA (guanine-N(7)-)-methyltransferase	-1.688
APL_0641	*truB*	tRNA pseudouridine synthase B	-1.660
APL_1476	*tyrS*	Tyrosyl-tRNA synthetase	-1.646

APL_1789	*rplS*	50S ribosomal protein L19	-1.521
Cellular processes			
APL_0669	*APL_0669*	Predicted iron-dependent peroxidase	-2.630
APL_1443	*apxIB*	Toxin RTX-I translocation ATP-binding protein	-1.753
Cell envelope			
APL_0933	*ompP1*	Putative long-chain fatty acid transport protein precursor	-8.199
APL_0651	*galU*	UTP-glucose-1-phosphate uridylyltransferase	-2.114
APL_1989	*APL_1989*	Predicted membrane protein	-1.951
APL_1071	*APL_1071*	putative xanthine/uracil permease	-1.817
Fatty acids and phospholipids metabolism			
APL_1864	*accB*	Biotin carboxyl carrier protein of acetyl-CoA carboxylase	-2.570
APL_1865	*accC*	Biotin carboxylase	-2.444
APL_1889	*fabA*	3-hydroxydecanoyl-(acyl carrier protein) dehydratase	-2.426
APL_1385	*plsX*	Fatty acid/phospholipid biosynthesis enzyme PlsX	-1.922
Amino acid biosynthesis			
APL_0194	*aroK*	Shikimate kinase	-2.401
APL_1499	*thrC*	Threonine synthase	-2.083
DNA metabolism			
APL_0190	*fis*	Factor for inversion stimulation Fis, transcriptional activator	-2.209
APL_0074	*recR*	Recombinational DNA repair protein (RecF pathway)	-1.634
Central intermediary metabolism			
APL_1508	*APL_1508*	Putative rhodanese-related sulfurtransferase	-2.860
APL_0175	*dksA*	DnaK suppressor protein	-1.845
APL_0349	*glgA*	Glycogen synthase	-1.505

Table 4. *A. pleuropneumoniae* genes which are up-regulated during adherence to SJPL cells (79 genes)

Locus Tag	Gene	Description	Fold Change
Hypothetical/Unclassified/Unknown			
APL_0568	*APL_0568*	Hypothetical membrane protein	3.083
APL_1459	*APL_1459*	Hypothetical protein	2.882
APL_1690	*APL_1690*	Predicted periplasmic/secreted protein	2.609
APL_1471	*APL_1471*	Putative sugar transferase	2.450
APL_1380	*APL_1380*	Uncharacterized conserved protein	2.310
APL_2002	*APL_2002*	Hypothetical protein	2.301
APL_0750	*APL_0750*	MscS family protein	2.181
APL_1575	*APL_1575*	Hypothetical protein	1.940
APL_1103	*APL_1103*	Predicted inner membrane protein	1.844
APL_1854	*pqiA*	Uncharacterized paraquat-inducible protein A	1.746
APL_0217	*APL_0217*	Hypothetical protein	1.640
Biosynthesis of cofactors			
APL_0776	*ispE*	4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate synthase	2.913
Energy metabolism			
APL_1849	*lldD*	L-lactate dehydrogenase	6.756
APL_1820	*rpe*	D-ribulose-phosphate-3 epimerase	6.687
APL_1191	*namA*	NADPH dehydrogenase	4.524
APL_1331	*hyaA*	Hydrogenase-2 small chain precursor	3.366
APL_1685	*fucK*	L-fuculokinase	3.195
APL_1689	*fucO*	Probable alcohol dehydrogenase, class IV	2.887
APL_1684	*fucI*	L-fucose isomerase	2.812
APL_1333	*hybB*	Putative Ni/Fe hydrogenase 2 b-type cytochrome component	2.796
APL_1019	*kdgK*	2-dehydro-3-deoxygluconokinase	2.334
APL_0896	*fdhE*	Formate dehydrogenase accessory protein FdhE	2.007
Transport and binding proteins: cations and iron			
APL_1793	*fecE*	Fe(III) dicitrate ABC transporter, ATP-binding protein	6.113
APL_1955	*APL_1955*	Outer membrane receptor proteins, mostly Fe transport	3.271
APL_1981	*corA*	Magnesium transport protein CorA	3.001
APL_0077	*exbD2*	Energy transducing protein ExbD2	2.346
Transport and binding proteins : others			
APL_0066	*dppC*	Dipeptide transport system, permease components	12.073
APL_0870	*APL_0870*	Putative C4-dicarboxylate transporter	6.308
APL_1713	*APL_1713*	Putative oligopeptide transporter	4.639
APL_0191	*APL_0191*	Predicted Na+-dependent transporters of the SNF family	4.638
APL_0309	*yheS*	Putative ABC transporter ATP-binding protein	2.331

		YheS	
APL_1848	*cysA*	Sulfate/thiosulfate import ATP-binding protein cysA	1.938
APL_1249	*sapF*	Peptide transport system ATP-binding protein SapF	1.858
APL_0260	*modC*	Molybdenum import ATP-binding protein ModC	1.799
APL_0582	*sotB*	Putative efflux transporter	1.773
Regulatory functions			
APL_1099	*APL_1099*	Organic radical activating enzymes	7.175
APL_1962	*hflX*	GTP-binding protein hflX	2.005
Transcription			
APL_0176	*pcnB*	Putative poly(A) polymerase	2.566
Purines, pyrimidines, nucleosides, and nucleotides			
APL_0775	*prsA*	Ribose-phosphate pyrophosphokinase	8.463
APL_0425	*purF*	Amidophosphoribosyltransferase	1.812
Protein fate			
APL_1742	*srp54*	Signal recognition particle protein (sigma-54 like)	6.861
APL_1905	*dnaJ*	Chaperone protein dnaJ	3.383
APL_1330	*hypF*	Carbamoyltransferase HypF	2.080
Protein synthesis			
APL_0034	*engD*	GTP-dependent nucleic acid-binding protein EngD	17.924
APL_0575	*deaD*	Superfamily II DNA and RNA helicases	6.254
APL_0487	*rplY*	50S ribosomal protein L25 (general stress protein Ctc)	3.429
APL_1325	*APL_1325*	Putative 2-methylthioadenine synthetase	2.357
APL_1112	*rumA*	23S rRNA (uracil-5-)-methyltransferase RumB	2.145
APL_0853	*APL_0853*	Methionine synthase II (cobalamin-independent)	2.078
Cellular processes			
APL_1922	*pgaB*	Biofilm PGA synthesis lipoprotein PgaB precursor	7.257
APL_0011	*ftsL*	Cell division protein FtsL	3.349
APL_1923	*pgaC*	Biofilm PGA synthesis N-glycosyltransferase PgaC	2.454
Cell envelope			
APL_0551	*tadB*	Tight adherence protein B	2.453
APL_1841	*murI*	Glutamate racemase	2.340
APL_0016	*murD*	UDP-N-acetylmuramoy-L-alanine-D-glutamate synthetase	2.113
APL_1598	*mrdB*	Rod-shape determining protein	2.096
APL_0419	*lgtF*	UDP-glucose-lipooligosaccharide glucosyltransferase	1.929
APL_1554	*wecA*	Putative undecaprenyl-phosphate α-N-acetylglucosaminyl 1-phosphate transferase	1.577
APL_0555	*rcpA*	Rough colony protein A	1.559
Fatty acids and phospholipids metabolism			
APL_1864	*accB*	Biotin carboxyl carrier protein of acetyl-CoA carboxylase	8.665
APL_1865	*accC*	Biotin carboxylase	4.016
APL_0887	*fadI*	3-ketoacyl-CoA thiolase	3.675

Amino acids biosynthesis			
APL_0320	*metC*	Cystathionine beta-lyase	9.278
APL_0099	*ilvG*	Acetolactate synthase isozyme II large subunit	6.897
APL_1452	*serA*	D-3-phosphoglycerate dehydrogenase	3.896
APL_0469	*trpB*	Tryptophan synthase beta chain	3.274
APL_0139	*leuC*	3-isopropylmalate dehydratase large subunit 2	3.253
APL_1951	*proA*	Gamma-glutamyl phosphate reductase	2.846
APL_1147	*trpG*	Putative anthranilate synthase component II	2.723
APL_0249	*thrB*	Homoserine kinase	2.459
APL_1125	*APL_1125*	Putative cysteine desulfurase	2.068
DNA metabolism			
APL_1196	*APL_1196*	Type I site-specific restriction-modification system, R subunit	3.251
APL_1194	*APL_1194*	Type I restriction-modification system methyltransferase subunit	2.816
APL_1146	*rmuC*	DNA recombination protein RmuC homolog	2.462
APL_0874	*holA*	DNA polymerase III, delta subunit	2.022
APL_0287	*hsdM*	Putative type I restriction-modification system methyltransferase subunit	2.005
Central intermediary metabolism			
APL_2045	*sseA*	Probable thiosulfate sulfurtransferase	1.942
APL_1843	*cysJ*	Sulfite reductase [NADPH] flavoprotein alpha-component	1.780

Table 5. *A. pleuropneumoniae* genes which are down-regulated during adherence to SJPL cells (52 genes)

Locus Tag	Gene	Description	Fold Change
Hypothetical/Unclassified/Unknown			
APL_1887	*APL_1887*	Esterase domain containing protein	-6.273
APL_1284	*APL_1284*	Putative DNA-binding protein	-3.076
APL_0049	*APL_0049*	Hypothetical protein	-2.982
APL_0970	*APL_0970*	Hypothetical protein	-2.980
APL_1100	*APL_1100*	Hypothetical protein	-2.928
APL_1437	*APL_1437*	Hypothetical protein	-2.751
APL_0116	*APL_0116*	Hypothetical protein	-2.566
APL_0389	*APL_0389*	Lipoprotein E precursor, predicted secreted acid phosphatase	-2.509
APL_0704	*APL_0704*	Potential type III restriction enzyme	-2.293
APL_1365	*hly*	Hypothetical protein	-2.174
APL_0110	*APL_0110*	Hypothetical protein	-2.144
APL_1396	*APL_1396*	Hypothetical protein	-2.125
APL_0756	*APL_0756*	Hypothetical protein	-2.098
APL_0889	*APL_0889*	Hypothetical protein	-1.757
Energy metabolism			
APL_0434	*gapA*	Glyceraldehyde-3-phosphate dehydrogenase	-4.981
APL_0892	*fdxG*	Formate dehydrogenase, nitrate-inducible, major subunit	-4.853
APL_0771	*lpdA*	Dihydrolipoyl dehydrogenase	-4.765
APL_1379	*ccp*	Cytochrome c peroxidase	-4.447
APL_0983	*tktA*	Transketolase 2	-3.790
APL_0894	*fdxH*	Formate dehydrogenase, iron-sulfur subunit	-3.627
APL_1251	*pgk*	3-phosphoglycerate kinase	-3.625
APL_1450	*fbp*	Fructose-1,6-bisphosphatase	-3.315
APL_0181	*gloA*	Lactoylglutathione lyase	-3.046
APL_1925	*tpiA*	Triosephosphate isomerase	-3.023
APL_1091	*aspA*	Aspartate ammonia-lyase	-2.722
APL_0688	*torZ*	Trimethylamine-N-oxide reductase precursor	-2.687
APL_1011	*adh2*	Aldehyde-alcohol dehydrogenase 2	-2.642
APL_0772	*aceF*	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex (E2)	-2.620
APL_1137	*pgi*	Glucose-6-phosphate isomerase	-2.595
APL_0486	*maeA*	NADP-dependent malic enzyme	-2.416
APL_1526	*frdD*	Fumarate reductase subunit D	-2.267
APL_0101	*nrfB*	Nitrate reductase, cytochrome-C type protein	-2.231
APL_1250	*fba*	Fructose bisphosphate aldolase	-2.124
Transport and binding proteins : others			
APL_1620	*cbiO*	Predicted ABC-type cobalt transport, ATPase component	-2.108
APL_0447	*lctP*	Putative L-lactate permease	-2.068
APL_0719	*APL_0719*	Putative phosphate permeases	-1.816
APL_0791	*APL_0791*	Transmembrane transport protein-permease	-1.702

Regulatory functions			
APL_0656	*hlyX*	Regulatory protein HlyX	-2.727
APL_0629	*cpxR*	Transcriptional regulatory protein CpxR	-2.367
Purines, pyrimidines, nucleosides, and nucleotides			
APL_0769	*ushA*	UshA precursor	-3.470
APL_0646	*cpdB*	2',3'-cyclic-nucleotide 2'-phosphodiesterase precursor	-2.778
APL_1014	*deoD*	Purine-nucleoside phosphorylase	-2.247
Protein fate			
APL_1154	*APL_1154*	Putative zinc protease	-2.401
APL_1456	*slyD*	FkbP-type peptidyl-prolyl cis-trans isomerase	-2.313
Protein synthesis			
APL_0740	*rpsA*	30s ribosomal protein S1	-1.608
Cellular processes			
APL_1445	*apxIC*	RTX-I toxin-activating lysine-acyltransferase ApxIC	-4.117
APL_0956	*apxIIA*	RTX-II toxin determinant A	-4.052
APL_0004	*sodC*	Superoxide dismutase (Cu/Zn) precursor	-3.193
Cell envelope			
APL_1494	*ftpA*	COG0783: DNA-binding ferritin-like protein (oxidative damage protectant)	-4.223
APL_0652	*manB*	Phosphomannomutase	-3.375
Central intermediary metabolism			
APL_0645	*ackA*	Acetate kinase	-3.190
APL_1899	*ppa*	Inorganic pyrophosphatase	-2.207

Table S.1 Protein profiling of SJPL cells after 3h of incubation with *A. pleuropneumoniae* S4074 using the Kinexus antibody microarray

Fold Change (Log2)	Protein name	Swiss-prot number
2,31	Mnk2	Q9HBH9
2,04	ZIPK	O43293
1,83	Hpk1	Q92918
1,75	GSK 3a/b	P49841
1,62	Jun	P05412
1,56	KAP	Q16667
1,38	Tau	P10636
1,26	PyDK2	Q15119
1,24	STAT6	P42226
1,24	PKA R2b	P31323
1,20	PKR1	P19525
1,15	Hsp60 (Myobact-Hps65)	P10809
1,11	AcCoA carboxylase	Q05397
1,07	Synapsin 1	P17600
1,07	FAK	Q05397
1,04	Synapsin 1	P17600
1,03	Mcl1	Q07820
1,02	MEK1+B23 (NPM)	Q02750
1,02	MAPKAPK2	P49137
1,00	CASP8	Q14790
-3,18	Abl	P00519
-2,98	IKKb	O14920
-2,34	Jun	P05412
-2,01	PDK2	Q15119
-1,99	PTP-PEST	Q05209
-1,92	eIF2Be	Q13144
-1,86	JAK3	P52333
-1,76	S6Ka	P23443
-1.51	EGFR	P00533
-1,49	GSK3a/b	P49841
-1,45	AMPKa1/2	Q13131
-1,41	PKCe	Q02156
-1,40	PI3K p110 delta	NA
-1,39	PKCa/b2	P17252
-1,25	Caveolin 2	P51636
-1,14	Ksr1	Q8IVT5
-1,13	Integrin b1	P05556
-1,12	PDGFRb	P09619
-1,11	MEK1	Q02750
-1,08	PKD (PK Cm)	Q15139
-1,07	eEF2K	O00418
-1,06	Smac/DIABLO	Q9NR28
-1,06	JNK	P45983
-1,02	ERP57	P30101
-1,02	PKCb2	P05771

Table S2. Protein profiling of NPTr cells after 3h of incubation with *A. pleuropneumoniae* S4074 using the Kinexus antibody microarray

Fold Change (Log2)	Protein name	Swiss-prot number
5,35	IKKa/b	O15111
4,13	Hsp40	P25685
2,13	PKD (PKCm)	Q15139
1,82	Raf1	P04049
1,48	Lyn	P07948
1,28	PKCl	P41743
1,26	MEK2[MAP2K2]	P36507
1,25	Hsp27	P04792
1,23	Msk1	O75582
1,21	TrkB	Q16620
1,20	P38aMAPK	P33981
1,19	TTK	P33981
1,18	S6	P62753
1,15	CDK1/2	P06493
1,10	Tlk1	Q9UKI8
1,09	Tau	P10636
1,06	PKCb2	P05771
1,06	PKR1	P19525
1,04	Vimentin	P08670
1,03	Vinculin	P18206
1,01	Erk1/2	P27361
-3,85	PTEN	P60484
-2,95	RSK1/2	Q15428
-2,60	Tyrosine Hydroxylase	P07101
-1,89	GAP-43	P17677
-1,65	Tlk1	Q9UKI8
-1,53	CASP12	O08736
-1,44	PRKAB1	Q9Y478
-1,43	Histone H3	P84243
-1,42	FLT4	P35916
-1,40	Jun	P05412
-1,37	CASP4	P49662
-1,36	CaMK1g	Q96NX5
-1,35	DFF45	O00273
-1,34	CAMK2d	Q13557
-1,34	PKD (PKCm)	Q15139
-1,27	P53	P04637
-1,24	Hsp70	P08107
-1,24	CK1g2	P78368
-1,23	Racl/cdc42	P60953
-1,16	JNK	P45983
-1,14	CASP7	P55210
-1,08	Histone H2A.X	P16104
-1,06	Grp94	P14625
-1,02	IRAK4	Q9NWZ3
-1,01	Rb	P06400

Table S3. Indirect comparison between *A. pleuropneumoniae* transcript profiling during adherence to SJPL cells and *A. pleuropneumoniae* transcript profiling during planktonic growth over SJPL cells (148 genes)

Locus Tag	Gene	Description	Fold Change
APL_0034	*engD*	COG0012: Predicted GTPase, probable translation factor	42.512
APL_1047	*hgbA*	Hemoglobin-binding protein A precursor	33.534
APL_1849	*lldD*	L-lactate dehydrogenase	25.876
APL_1864	*accB*	Biotin carboxyl carrier protein of acetyl-CoA carboxylase	21.417
APL_0933	*ompP1*	Putative outer membrane protein precursor	19.307
APL_0066	*dppC*	Dipeptide transport system permease protein DppC	15.857
APL_0320	*metC*	Cystathionine beta-lyase	13.237
APL_0575	*deaD*	Cold-shock DEAD box protein A-like	12.421
APL_1865	*accC*	Biotin carboxylase	10.091
APL_1922	*pgaB*	Biofilm PGA synthesis lipoprotein PgaB precursor	9.511
APL_0191	*APL_0191*	COG0733: Na+-dependent transporters of the SNF family	8.985
APL_1742	*srp54*	Signal recognition particle protein (sigma-54 like)	8.555
APL_0487	*rplY*	50S ribosomal protein L25	8.428
APL_1099	*APL_1099*	COG0602: Organic radical activating enzymes	7.955
APL_1571	*tonB1*	Periplasmic protein TonB, links inner and outer membranes	7.678
APL_0870	*APL_0870*	COG3069: Putative C4-dicarboxylate transporter	7.158
APL_1400	*rpsG*	Ribosomal protein S7	6.978
APL_0077	*exbD2*	biopolymer transport protein	6.410
APL_0099	*ilvG*	Acetolactate synthase isozyme II large subunit (AHAS-II)	6.259
APL_1820	*rpe*	Ribulose-phosphate 3-epimerase	6.130
APL_1955	*APL_1955*	COG1629: Outer membrane receptor proteins, mostly Fe transport	5.987
APL_0668	*APL_0668*	Hypothetical protein	5.379
APL_1558	*rpsT*	30S ribosomal protein S20	4.914
APL_1952	*APL_1952*	COG1629: Outer membrane receptor proteins, mostly Fe transport	4.879
APL_1452	*serA*	D-3-phosphoglycerate dehydrogenase	4.664
APL_0857	*sdaA*	L-serine dehydratase	4.428
APL_0669	*APL_0669*	Putative iron dependent peroxidase	3.868
APL_0076	*tonB2*	Periplasmic protein TonB, links inner and outer membranes	3.834
APL_1508	*APL_1508*	Putative Rhodanese-related sulfurtransferase	3.825
APL_0271	*yfeB*	Putative chelated iron transport system ATP-binding protein	3.810
APL_0040	*yhbZ*	Hypothetical GTP-binding protein	3.717
APL_0272	*yfeA*	Iron (chelated) ABC transporter, periplasmic-binding protein	3.702
APL_0249	*thrB*	Homoserine kinase	3.677
APL_1905	*dnaJ*	Chaperone protein dnaJ	3.665
APL_1951	*proA*	Gamma-glutamyl phosphate reductase	3.345

APL_0470	*trpA*	Tryptophan synthase alpha chain	3.343
APL_1048	*hugZ*	Heme utilization protein	3.266
APL_1380	*APL_1380*	Hypothetical protein	3.256
APL_0714	*APL_0714*	ABC transport system periplasmic protein	3.064
APL_0148	*rnr1*	Ribonucleoside-diphosphate reductase large chain	3.022
APL_1896	*APL_1896*	COG0730: Predicted permeases	2.932
APL_1071	*APL_1071*	COG2252: Permeases	2.719
APL_1019	*kdgK*	2-dehydro-3-deoxygluconokinase	2.616
APL_0192	*dam*	DNA adenine methylase	2.590
APL_1824	*menB*	Naphthoate synthase	2.468
APL_1407	*psd*	Phosphatidylserine decarboxylase	2.434
APL_0287	*hsdM2*	Putative type I restriction-modification system M-protein	2.414
APL_0159	*hcaT*	Putative 3-phenylpropionic acid transporter	2.308
APL_1525	*lysA*	Diaminopimelate decarboxylase	2.070
APL_1103	*APL_1103*	COG0795: Predicted permeases	2.006
APL_1788	*trmD*	tRNA-(guanine-N1)-methyltransferase	1.844
APL_0133	*cysB*	HTH-type transcriptional regulator CysB	1.792
APL_1563	*mioC*	Flavodoxin	-1.735
APL_1004	*nhaP*	Na(+)/H(+) exchanger beta-like protein	-1.772
APL_0782	*uvrA*	UvrABC system protein A	-1.803
APL_0704	*APL_0704*	Hypothetical	-2.176
APL_0791	*APL_0791*	COG0477: Permeases of the major facilitator superfamily	-2.206
APL_0481	*APL_0481*	Hypothetical protein	-2.277
APL_0792	*APL_0792*	Hypothetical protein	-2.307
APL_0278	*APL_0278*	Putative Mg-dependent deoxyribonuclease	-2.312
APL_1283	*APL_1283*	ABC transporter ATP-binding protein	-2.392
APL_0889	*APL_0889*	Hypothetial protein	-2.416
APL_0756	*APL_0756*	Hypothetical protein	-2.501
APL_1396	*APL_1396*	Hypothetical protein	-2.513
APL_1244	*APL_1244*	Hypothetical protein	-2.530
APL_1555	*hemL*	Glutamate-1-semialdehyde 2,1-aminomutase	-2.583
APL_1444	*apxIA*	RTX-I toxin determinant A	-2.594
APL_0656	*hlyX*	Regulatory protein HlyX	-2.615
APL_1501	*APL_1501*	COG2801: Transposase and inactivated derivatives	-2.622
APL_0235	*bolA*	Putative transcriptional regulator, BolA	-2.759
APL_1656	*APL_1656*	Hypothetical protein	-2.762
APL_0149	*APL_0149*	Hypothetical protein	-2.768
APL_1355	*APL_1355*	Hypothetical protein	-2.849
APL_0924	*fdx*	2Fe-2S ferredoxin	-3.013
APL_0110	*APL_0110*	Hypothetical protein	-3.085
APL_0146	*dusA*	tRNA-dihydrouridine synthase A	-3.095
APL_0930	*nifu*	NifU-like protein	-3.214
APL_2001	*APL_2001*	Hypothetical protein	-3.228
APL_1203	*APL_1203*	Hypothetical protein	-3.241
APL_1008	*hemX*	putative uroporphyrinogen III C-methyltransferase	-3.261
APL_1529	*frdA*	Fumarate reductase flavoprotein subunit	-3.280
APL_1489	*tpx*	Putative thiol peroxidase	-3.286
APL_0397	*lcfA*	Long-chain-fatty-acid--CoA ligase	-3.336
APL_1456	*slyD*	FkbP-type peptidyl-prolyl cis-trans isomerase	-3.348
APL_1426	*napB*	Nitrate reductase cytochrome c-type subunit	-3.420
APL_1365	*hly*	21 kDa hemolysin precursor	-3.435
APL_0983	*tktA*	Transketolase	-3.520

APL_0645	*ackA*	Acetate kinase	-3.525
APL_1355	*APL_1355*	Hypothetical protein	-3.533
APL_0483	*APL_0483*	COG0778: Nitroreductase	-3.594
APL_1251	*pgk*	3-phosphoglycerate kinase	-3.610
APL_1382	*APL_1382*	Hypothetical protein	-3.719
APL_1319	*ptsB*	PTS system sucrose-specific EIIBC component	-3.744
APL_1100	*APL_1100*	Hypothetical protein	-3.784
APL_1622	*cbiM*	predicted ABC transport permease protein CbiM	-3.883
APL_0443	*APL_0443*	COG5295: Autotransporter adhesin	-3.898
APL_1254	*APL_1254*	Hypothetical protein	-3.936
APL_0719	*APL_0719*	Putative phosphate permease	-3.986
APL_1405	*oapA*	Opacity associated protein A	-3.989
APL_0688	*torZ*	Trimethylamine-N-oxide reductase precursor	-4.060
APL_1620	*cbiO*	Predicted ABC transport ATP-binding protein CbiO	-4.064
APL_1012	*groEL*	60 kDa chaperonin	-4.137
APL_0465	*APL_0465*	Hypothetical protein	-4.176
APL_0447	*lctP*	Putative L-lactate permease	-4.187
APL_0484	*rimK*	Ribosomal protein S6 modification protein	-4.192
APL_0377	*glpT*	Glycerol-3-phosphate transporter	-4.257
APL_1138	*APL_1138*	Hypothetical protein	-4.302
APL_0108	*iclR*	Putative HTH-type transcriptional regulator	-4.329
APL_0145	*APL_0145*	Hypothetical protein	-4.379
APL_1526	*frdD*	Fumarate reductase subunit D	-4.627
APL_1976	*yedF*	Hypothetical protein	-4.665
APL_0486	*maeB*	NADP-dependent malic enzyme (NADP-ME)	-4.743
APL_0446	*ykgE*	Putative dehydrogenase subunit	-4.891
APL_0769	*ushA*	UshA precursor	-4.920
APL_1173	*pnuC*	Nicotinamide mononucleotide transporter	-5.061
APL_0371	*Lnt*	COG0815: Apolipoprotein N-acyltransferase	-5.074
APL_0187	*pykA*	Pyruvate kinase	-5.134
APL_0646	*cpdB*	2',3'-cyclic-nucleotide 2'-phosphodiesterase precursor	-5.264
APL_0049	*APL_0049*	Hypothetical protein	-5.304
APL_1113	*eno*	Enolase	-5.533
APL_0931	*iscS*	Cysteine desulfurase	-5.848
APL_1435	*APL_1435*	Hypothetical protein	-6.035
APL_0771	*lpdA*	Dihydrolipoyl dehydrogenase	-6.253
APL_0718	*APL_0718*	Hypothetical protein	-6.300
APL_0772	*aceF*	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex (E2)	-6.453
APL_0109	*APL_0109*	Hypothetical protein	-6.505
APL_0116	*APL_0116*	Hypothetical protein	-6.726
APL_0251	*sodA*	Manganese superoxide dismutase	-6.761
APL_0956	*apxIIA*	RTX-II toxin determinant A	-6.818
APL_0181	*gloA*	Lactoylglutathione lyase	-7.031
APL_1839	*udp*	Uridine phosphorylase	-7.087
APL_0106	*putA*	Bifunctional protein PutA	-7.148
APL_0970	*APL_0970*	Hypothetical protein	-7.192
APL_1137	*pgi*	Glucose-6-phosphate isomerase	-7.204
APL_1450	*fbp*	Fructose-1,6-bisphosphatase	-8.280
APL_1527	*frdC*	Fumarate reductase subunit C	-8.376
APL_0004	*sodC*	Superoxide dismutase [Cu-Zn] precursor	-8.629
APL_1087	*clpB*	Chaperone ClpB	-9.154

APL_0100	*nrfA*	Cytochrome c-552 precursor	-9.800
APL_0101	*nrfB*	Cytochrome c-type protein NrfB precursor	-9.809
APL_1882	*APL_1882*	Hypothetical protein	-15.462
APL_1285	*APL_1285*	Hypothetical protein	-15.518
APL_1379	*ccp*	Cytochrome c peroxidase	-16.073
APL_1494	*ftpA*	Fine tangled pili major subunit	-19.604
APL_0895	*fdnI*	Formate dehydrogenase, cytochrome b556 subunit	-20.914
APL_1832	*APL_1832*	Hypothetical protein	-21.875
APL_0894	*fdxH*	Formate dehydrogenase, iron-sulfur subunit	-25.335
APL_0892	*fdxG*	Formate dehydrogenase, nitrate-inducible, major subunit	-39.375

Article 5

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Transcriptional Profiling of *Actinobacillus pleuropneumoniae* During the Acute Phase of a Natural Infection in Pigs

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§ Échantillonage sur le terrain, élaboration et réalisation des expériences de profil transcriptionnel, analyse des résultats, colorations pour la microscopie et rédaction du manuscrit.

Abstract

Background: *Actinobacillus pleuropneumoniae* is the etiological agent of porcine pleuropneumonia, a respiratory disease which causes great economic losses worldwide. Many virulence factors are involved in the pathogenesis, namely capsular polysaccharides, RTX toxins, LPS and many iron acquisition systems. In order to identify genes that are expressed *in vivo* during a natural infection, we undertook transcript profiling experiments with an *A. pleuropneumoniae* DNA microarray, after recovery of bacterial mRNAs from serotype 5b-infected porcine lungs. AppChip2 contains 2033 PCR amplicons based on the genomic sequence of *App* serotype 5b strain L20, representing more than 95% of ORFs greater than 160 bp in length.

Results: Transcriptional profiling of *A. pleuropneumoniae* recovered from the lung of a pig suffering from a natural infection or following growth of the bacterial isolate in BHI medium was performed. An RNA extraction protocol combining beadbeating and hot-acid-phenol was developed in order to maximize bacterial mRNA yields and quality following total RNA extraction from lung lesions. Nearly all *A. pleuropneumoniae* transcripts could be detected on our microarrays, and 150 genes were deemed differentially expressed *in vivo* during the acute phase of the infection. Our results indicate that, for example, gene *apxIVA* from an operon coding for RTX toxin ApxIV is highly up-regulated *in vivo*, and that two genes from the operon coding for type IV fimbriae (APL_0878 and APL_0879) were also up-regulated. These transcriptional profiling data, combined with previous comparative genomic hybridizations performed by our group, revealed that 66 out of the 72 up-regulated genes are conserved amongst all serotypes and that 3 of them code for products that are predicted outer membrane proteins (genes *irp* and *APL_0959*, predicted to code

for a TonB-dependent receptor and a filamentous hemagglutinin/adhesin respectively) or lipoproteins (gene *APL_0920*). Only 4 of 72 up-regulated genes had previously been identified in controled experimental infections.

Conclusions: These genes that we have identified as up-regulated in *vivo*, conserved across serotypes and coding for potential outer membrane proteins represent potential candidates for the development of a cross-protective vaccine against porcine pleuropneumonia.

Background

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a highly contagious respiratory disease that causes great economic losses worldwide [1]. Transmission occurs through aerosol or close contact with infected animals or asymptomatic carriers, and can affect pigs of all ages [2]. In the acute phase, the disease is often lethal in 24 to 48h, and leads to the formation of extensive fibrinohemorrhagic lung lesions. Animals that survive the infection often become healthy carriers, and develop localized necrotizing lesions associated with pleuritis [3]. Fifteen different serotypes can be identified based on differences in capsular polysaccharides. While serotypes 1 to 12 and 15 usually belong to biotype 1, which contains strains that are NAD-dependent, serotypes 13 and 14 are usually NAD-independent and belong to biotype 2 [4]. However, biotype 2 variants of serotypes 2, 4, 7 and 9 have been reported [5-7]. Despite years of research, mechanisms involved in *A. pleuropneumoniae* pathogenicity are still not fully understood. While many virulence factors have been identified, such as lipopolysaccharides, capsular

polysaccharides, Apx toxins and iron acquisition systems [4], or suggested, like biofilm formation [8], autotransporter adhesin [9] and autotransporter protease synthesis [10], very little is known about the overall contribution of each component to the infection process.

In order to gain new insight into the disease, researchers have relied on techniques that allow for the identification of genes expressed in bacteria during infection of the host [11], namely *In Vivo* Expression Technology (IVET), Signature Tagged Mutagenesis (STM) and Selective Capture of Transcribed Sequences (SCOTS). First, an IVET experiment with *A. pleuropneumoniae* led to the identification of 10 loci (termed *ivi*) transcribed *in vivo* [12] and highlighted the importance of genes involved in branched-chain amino acid (BCAA) synthesis. [13], [14]. Later, over the course of two STM experiments conducted with *A. pleuropneumoniae*, nearly 3000 mutants were screened *in vivo* [15, 16]. Both experiments showed that iron acquisition was highly important for the virulence of *A. pleuropneumoniae*, with the identification of mutants impaired for the ExbB-ExbD-TonB energy-transducing system. The study by Sheehan et al [16], lead to the identification of a second set of *exbB-exbD-tonB* genes, and it was determined that inactivation of *tonB2* and not *tonB1* lead to attenuation *in vivo* [17]. More recently, SCOTS experiments were conducted with *A. pleuropneumoniae*, at two different stages of infection : at the end of the acute stage, in necrotic lung tissues (7 days post-infection) [18], and in the chronic stage (21 days post-infection) [10]. These studies demonstrated the importance of anaerobic metabolism and HlyX-regulated mechanisms during *A. pleuropneumoniae* infection in the lungs, and led to the identification and

characterisation of a maturation autotransporter protease, AasP, responsible for cleavage and release of fragments of OmlA from the cell surface [19]. The HlyX regulon was characterized recently, and iron-regulated protein B (FrpB) was identified as a potentially important virulence factor up-regulated when *A. pleuropneumoniae* is grown in oxygen-deprived environments [20]. Although these *in vivo* studies have been very valuable in gaining a better understanding of the mechanisms involved in *A. pleuropneumoniae* pathogenesis, limitations associated with the techniques used lead only to a partial overview of the transcriptional events taking place *in vivo*.

DNA microarrays have been used to determine the complete transcriptomic profile of microorganisms *in vitro*, but technical drawbacks that are associated with the use of this technique *in vivo*, such as the qualitative and quantitative recovery of bacterial RNA from host tissues, explain why the use of this technique to monitor bacterial infections has been limited [21]. While various cell culture, *ex-vivo* or mouse infection models have been used, *in vivo* experiments can provide more accurate information on bacterial adaptation and virulence when the natural host is studied. To the best of our knowledge, only two genome-wide transcriptional profiling experiments using DNA microarrays have been conducted with bacterial pathogens in their natural host, during a natural infection. LaRocque et al. have evaluated the transcriptome of *Vibrio cholerae* in the early and late stages of the disease by collecting stools of naturally infected patients [22]. Son et al. used fresh sputum samples from a patient with cystic fibrosis to study the *in vivo* transcriptome of *Pseudomonas aeruginosa* [23]. Additionally, Agarwal et al. used a custom-made Fur

and iron regulon microarray to investigate the expression of these genes during gonococcal infection in women [24].

In this study, we have conducted *in vivo* transcript profiling with DNA microarrays of *A. pleuropneumoniae* during the acute phase of a natural infection in pigs. Our results indicate that 150 genes are differentially expressed during the acute phase of infection in the host. Of these, 72 were up-regulated, and three of these encode lipoproteins or OMPs that are well-conserved among strains of *A. pleuropneumoniae*.

Results and discussion

Animal samples

While working with samples from commercial herds means that we are working with disease cases that are representative of what is really happening in the field, it also means that limited amounts of material is available. During the course of our experiments, we were able to collect samples from three different animals from the same herd, before antibiotherapy was undertaken to eradicate the infection. All the animals that were sampled had presented symptoms of the disease for less than 24h. Two animals died as a result of the infection, and were sampled a few hours after death. Those samples were not used for the microarray experiments since significant transcriptomic changes might have occurred between the time of death and the time of sampling. However, preliminary microscopic observations were made and our RNA extraction protocol was tested with those samples. The last animal was euthanized on site by a veterinarian, and sampled less than 5 minutes after death. The infectious field strain was isolated from this animal. While it can be argued that

different animals might react differently to an infection by *A. pleuropneumoniae*, and therefore elicit different transcriptional changes in the pathogen, we are confident that the results that were obtained reflect accurately the conditions encountered by *A. pleuropneumoniae* in its natural host following acute infection.

Macroscopic and microscopic observations

Clinical signs and macroscopic and microscopic examinations were consistent with acute porcine pleuropneumonia. Clinical signs ranged from depression to respiratory distress, while macroscopic observation of deceased pigs revealed lobar fibrinohemorrhagic pleuropneumonia, along with necrotic lesions and the accumulation of bloody fluid in the thorax. On microscopic examination, multiple pulmonary necrotic areas surrounded by or containing oat cells were present, as well as fibrin accumulation in the alveolar lumen, interlobular septa and pleura (Figure 1). The infectious field strain that was isolated from the lung tissues was named 896-07, and serotyping analysis showed that it belongs to serotype 5b, one of the most prevalent in North America [4].

Comparative genomic hybridizations

CGH experiments conducted previously in our laboratory have shown that the majority of genes in the reference 5b L20 strain were conserved in reference strains and fresh field isolates. Conversely, 205 genes were identified as divergent in at least one strain, including 39 that are either phage or transposon related out of the 2033 ORFs represented on the microarray, which represents 10.1% of divergent genes [25]. As anticipated, clusters of genes associated with LPS/capsule biosynthesis and toxin production showed variations.

AppChip2 was designed using the complete genome sequence from the serotype 5b reference strain L20. In order to validate its use to assess transcriptomic events taking place with our infectious field strain during the infection, we first used it to analyze the gene content of the 896-07 field strain . The CGH data obtained with strain 896-07 was added to that obtained with other field and reference strains [25] in order to generate a strain dendogram (Fig.2). Serotyping data correlates well with genetic data since strain 896-07 forms a cluster with the serotype 5b reference strain L20. The same clusters as those observed previously could be found in our dendrogram [25].

Only 28 genes were identified as divergent/absent in our field strain, when compared to the reference serotype 5b L20 strain (Table 1). This represents only 1.43% of all the genes (1954) that are represented on AppChip2 [25]. Out of these 28 genes, 20 can be found in two major regions of the genome: 14 putative ORFs code for phage structures, while 6 are located in the *A. pleuropneumoniae* tight adherence (*tad*) locus. In *Aggregatibacter actinomycetemcomitans*, a close relative of *A. pleuropneumoniae*, the *tad* locus is involved in adherence to biological and abiological surfaces [26]. In *A. pleuropneumoniae*, genes from this locus were shown to be up-regulated during adhesion to lung epithelial cells *in vitro* [9]. The other important locus for gene divergence/absence was composed of phage-related features, and these were shown to be divergent during inter and intra-serotype comparisons in previous experiments [25]. Some genes might also be present in the genome of strain 896-07 and absent in reference strain L20, and therefore absent on AppChip2.

RNA extraction

Tissue samples were taken where lung lesions were present to ensure that bacterial yields would be optimal. A beadbeating step was performed prior to a standard hot acid-phenol-chloroform RNA extraction protocol to extract bacterial RNA from infected lung tissues. Tissues were homogenized with 1.0 mm zirconia/silica beads to release but not lyse bacterial cells from the tissue. This size of beads is too large to lyse bacterial cells (Biospec, personal communication). Following homogenization, serial dilutions of the resulting supernatant were plated in order to determine an approximate number of CFU per gram of tissue. Our results indicated that *A. pleuropneumoniae* could easily be isolated in pure culture from lung tissues of all animals, and that the number of CFU varied from 1 X 10^6 to 1 X 10^7 per gram of infected tissue. Centrifugation of homogenized samples enabled the removal of most cellular debris and the recovery of bacteria. Approximately 20 to 45 μg of total RNA could routinely be extracted from approximately 2 g of necrotic lung tissue, with no apparent contamination by eukaryotic RNA, as observed on agarose gel when using bacterial RNA extracted from BHI broth and eukaryotic RNA recovered from porcine lung epithelial cells (SJPL cell line) [27] as on-gel controls (data not shown). Nevertheless, the MicrobENRICH kit was still used to remove any possible trace of remaining eukaryotic RNA, and treatments with TurboDNase removed traces of contaminating DNA. No trace of bacterial or host DNA could be detected following PCR amplification with primers for gene *ompW* and for pig mitochondrial DNA. We propose that this combination of techniques could prove useful in isolating pathogens from other tissues as well.

In vivo transcript profiling

Before investigating the *in vivo* transcript profile of *A. pleuropneumoniae*, we used self-self hybridizations in order to quantifiy the dye bias effect after hybridization on AppChip2. Duplicate experiments showed a R^2 correlation factor of 0.996 between the intensity detected for the Cy3 labelled and Cy5 labelled probes, and only two genes seemed to qualify as obvious outliers (data not shown). Both genes, APL_1141 and APL_0484, were not identified as differentially expressed during the course of a natural infection.

While designing the microarray experiments, growth in BHI medium to an optical density of 0.3 at a 600 nm wavelenght was chosen as our reference condition. This condition was not only chosen for practical reasons, but also to fulfill two precise objectives: first, to allow for the identification of genes that, while important to the infection process, have not been identified previously *in vitro* in rich medium, and second, to allow comparison of the results obtained *in vivo* to those obtained in our laboratory in other growth conditions by keeping the same reference [28-30]. Working with a limited amount of biological material meant that only one time-point in the infection could be investigated. In the acute stage of porcine pleuropneumonia, bacteria are likely in the exponential phase of growth. This is also the case in the control condition. The transcriptome of *A. pleuropneumonia* is certainly very different when the infection is in the chronic stage. Therefore, while our results are an accurate representation of the transcriptomic events that occur *in vivo* during the acute phase of the infection process as compared to those occuring *in vitro* in a rich

culture medium, events occuring earlier of later in the infection process cannot be inferred.

In vivo transcript profiling experiments were performed on samples from three different lesion sites on the euthanized pig. Since this pig was euthanized on site by a veterinarian, the time laps between the death of the animal and tissue sampling was kept to a minimum. Following microarray hybridization and analysis, 86.7% of all coding sequences included on AppChip2 could be detected during our experiments, and 150 genes were significantly differentially expressed *in vivo* when their level of expression was compared to that seen *in vitro*, with a FDR of 4.25% over the course of three hybridizations: 72 of these were up-regulated, while 78 were down-regulated. Functional classification of these genes revealed that up-regulated genes mostly belonged to the "Transport and Binding Proteins" and "Energy Metabolism" classes, with respectively 17 and 14 genes (see Additional file 1 and Figure 3). Repressed genes mostly belonged to the "Protein Synthesis", "Energy Metabolism" and "Cell Envelope" functional classes. A large number of genes encoding "Hypothetical/Unknown /Unclassified" products were differentially expressed. Only ten out of the 150 differentially expressed genes (8.2%) could be found in the list of divergent or highly divergent/absent genes in at least one reference strain of *A. pleuropneumoniae*, as established by Gouré et al. [25]. Three of these are involved in surface structures, namely LPS and capsule, biosynthesis (*genes kpsF*, *cps5b* and *cpxC*), and two others are found in phage-associated regions (APL_0512 and APL_0524). Of the last five genes, four are divergent in only one strain (*hktE*, *fur*, *phoR* and *hisH*, respectively in reference strains from serotypes 8, 2, 15 and 7), and

the APL_0999 gene codes for a hypothetical protein that is divergent in serotype 2, 7-11 and 14 reference strains.

a) Validation of microarray results by qRT-PCR

In order to confirm results obtained using the AppChip2 *A. pleuropneumoniae* microarray, eleven genes were selected for qRT-PCR analysis. Five of these were up-regulated *in vivo* (*sohB*, *hbpA*, *kpsF*, *apxIVA*, *phoR*) and six were down-regulated (*nlpI*, *visC*, *proQ*, *APL_1456*, *APL_1135* and *nusG*). These genes represented a large range of $\log_2$ ratio values. In all cases, microarray results were supported by those obtained by qRT-PCR: all genes that were identified as up-regulated using the AppChip2 also showed up-regulation following qRT-PCR analysis, and the same was true for selected down-regulated genes (Figure 4).

b) Comparison with other in vivo studies

Among previous *in vivo* experiments that were conducted with *A. pleuropneumoniae*, the one that was closest to ours in terms of experimental conditions and results was by Baltes et al.[18]. In their experiment, Selective Capture of Transcribed Sequences (SCOTS) was performed on lung tissues after a 7 day infection with *A. pleuropneumoniae* (end of acute phase), and 46 genes were identified [18]. Of the 150 differentially expressed genes that we have identified (see Additional file 1), 15 were also identified in other *in vivo* studies conducted with *A. pleuropneumoniae* (Table 2): only 4 of these were up-regulated following transcript profiling *in vivo*. These include 3 genes already identified by Baltes et al. using SCOTS at the end of the acute stage of the disease [18]: *fucI*, encoding the L-fucose isomerase, *nrdD*, encoding an anaerobic ribonucleoside triphosphate reductase, and *apxIVA*, encoding

the ApxIVA toxin structural protein. The latter, the gene encoding the recently discovered fourth *A. pleuropneumoniae* Apx toxin [31], had only been detected *in vivo* [32-34] until it was discovered recently that it is also up-regulated after contact with broncho-alveolar lavage fluids [30]. It is also the only *apx* gene that was up-regulated in our experiment, but it is worth noting that the *apxIBCD* and *apxIIAB* genes, although not differentially expressed, were all actively transcribed *in vivo*. Finally, the last gene that was up-regulated in our study and identified in another *in vivo* study is *nanE*, which codes for a putative N-acetylmannosamine-6-phosphate 2-epimerase, an enzyme involved in the use of *N*-acetylneuraminate and *N*-acetylmannosamine as carbon sources [35]. The differences between our results and those obtained in other *in vivo* studies can be easily explained: while the second SCOTS experiment was conducted with animals suffering from chronic infections [10], a condition which differs greatly from our field case, the STM and IVET techniques do not provide a "snap-shot" of the transcriptome at a precise time point during the infection process. While STM and IVET can theoretically provide a global overview of transcriptomic events, the experiments conducted with *A. pleuropneumoniae* are not likely to have achieved this goal. Only 800 mutants were screened by Fuller et al. with IVET [12], well short of the approximately 2000 genes of the *A. pleuropneumoniae* genome, and although Sheehan et al. screened 2064 mutants using STM [16], the authors noted that both their STM screen and the previous STM experiment by Fuller et al. [15] were likely not saturating. STM relies on transposon mutagenesis, and there are some insertional hot spots in the *A. pleuropneumoniae* genome [16].

We also compared our results to the transcriptional profile of a virulent strain of *Pseudomonas aeruginosa*, another lung pathogen, isolated from a chronically infected cystic fibrosis patient [23]. There were no striking similarities when comparing the functions of differentially expressed genes in both studies. Since *P. aeruginosa* is well-known for the versatility of nutrients it can use for growth, and since both conditions (chronic infection vs acute infection) were highly different, this was to be expected. However, one interesting aspect of both cases was the list of genes that weren't up-regulated as predicted. As with Son et al., we noticed that few of the landmark virulence genes in *A. pleuropneumoniae* were up-regulated *in vivo*. Out of the three Apx toxins present in serotype 5b, only the gene encoding the ApxIV toxin was up-regulated. The Hsf autotransporter homolog, thought to be important in adhesion processes, as well as *pga* genes necessary for biofilm formation, were not identified either.

Well-characterized iron acquisition genes such as those coding for the hemoglobin receptor HgbA (despite the important hemorrhage noticed in the infected lungs) and the genes coding for transferrin binding proteins TbpA and TbpB did no show differential expression, nor did genes involved in anaerobic respiration. While in the latter case it can be argued that the environmental conditions encountered during the acute infection might explain why differential expression of these gene transcripts was not observed, it could also be hypothesized that years of evolution of the field strain in nature might have led to the loss of some regulatory mechanisms that could still be present in well-characterized laboratory strains, thereby leading to the constitutive expression of some important virulence genes. In the case of the *P.*

aeruginosa clinical isolate, it was shown that more than 300 genes had higher levels of constitutive expression in the clinical isolate than in a well-characterised reference strain, therefore showing the deregulation of several pathways [23]. Whether this is also the case for *A. pleuropneumoniae* will have to be assessed.

c) Down-regulated genes

Interestingly, genes involved in protein synthesis were down-regulated *in vivo* towards the end of the acute phase of the disease. A total of 8 different 30S or 50S ribosomal genes showed levels of down-regulation ranging from -1.59 to -3.97 fold. These genes are scattered in the L20 genome, but all their respective operonic structures are well conserved when compared to those observed in *E. coli* [36]. Not much is known, however, about the regulation of these transcriptional units. While some experimental evidence seems to indicate that *rpsJ* could be activated by FNR [37] and repressed by ArcA [38], *rpsT* seems to be repressed by FNR [37]. Both *rpsK* and *rpsU* are transcribed from σ70 promoters, which is the main sigma factor during the exponential growth phase [39]. This would probably indicate that *A. pleuropneumoniae* has a slower metabolism and replication rate *in vivo* than when it is growing in a rich culture medium. Further strengthening this hypothesis is the fact that two subunits from DNA polymerase III (*dnaN*, *dnaX*), which is the primary enzyme for replicative DNA functions in *E. coli*, are down-regulated, as well the genes coding for the DNA primase (*dnaG*) and the cell division protein FtsX.

With regards to their possible involvement in virulence processes, some genes that were down-regulated were of particular interest. The Fur transcriptional regulator, which represses transcription of genes associated with iron acquisition [40], showed

a 2.6 fold down-regulation in transcription. In bacteria, Fur down-regulates the transcription of genes involved in iron-acquisition and iron homeostasis in bacterial cells, and its action is directly linked to the amount of available iron, as it requires Fe^{2+} as a cofactor [41]. In *E. coli*, Fur also regulates its own level of expression. Iron restriction if often thought to be an important signal for bacteria, and has often been linked with the expression of virulence factors such as toxins [42]. Therefore, it would seem that the *in vivo* environment towards the end of the acute phase of pleuropneumonia is not iron-restricted for *A. pleuropneumoniae*. This is not surprising considering that there is extensive hemorrhage and tissue destruction, caused most probably by the secretion of Apx toxins. However, a few genes involved in iron acquisition, namely *APL_0096*, *APL_0668*, *hbpA* and *irp*, were up-regulated. Since these genes were not identified in a previous transcript profiling experiment under iron-restricted conditions [28], this might imply that they are not regulated by Fur. The *hfq* gene, which codes for a protein involved in RNA molecule stability and RNA-RNA interactions [43] and that therefore has an important regulatory function, was also down-regulated. Hfq is an RNA chaperone that acts as a post-transcriptional riboregulator [44]. The role of Hfq, a Sm-like protein, has been investigated with greater interest over the last decade since it was shown that it is involved in the function of small RNAs (sRNAs) [45]. It is believed to form a complex with sRNAs and RNAse E, thereby targeting mRNAs that are recognized by sRNAs for degradation. The fact that the *hfq* gene is down-regulated *in vivo* at the end of the acute phase of the disease could lead to a decline of the activity of sRNAs that require Hfq to function properly. No sRNAs have been identified in *A.*

pleuropneumoniae yet, but they have been found in numerous bacterial pathogens [46].

Also of interest was the down-regulation of the *cps5b* (-2.21 fold) and *cpxC* (-1.82 fold) genes, involved respectively in the biosynthesis of capsular polysaccharide and in the export of these polysaccharides. Down-regulation of *cpxABC*, involved in capsule biosynthesis, has also been observed during planktonic life in liquid medium in contact with porcine lung epithelial cells [9]. The down-regulation of genes involved in capsule synthesis could potentially lead to the production of a thinner capsule on the surface of bacteria, thereby unmasking potential adhesins. Indeed, an acapsular mutant of *A. pleuropneumoniae* that was generated in our laboratory was shown to adhere more strongly to frozen porcine tracheal sections than the capsulated wild-type strain [47].

d) Up-regulated genes

Numerous genes that could be involved in the progression of the disease were identified as up-regulated. Gene *sohB* has the highest observed fold change (5.39X) and codes for a putative secreted serine protease that seems to be well conserved in other pathogenic bacteria, such as *Haemophilus parasuis*, *Neisseria meningitidis* and *N. gonorrhoeae*. In the lungs, *A. pleuropneumoniae* causes extensive tissue damage that could result from the combined action of Apx toxins and secreted proteases [48].

Adhesion processes are essential for the establishment of bacterial infections. The *apf* promoter was shown to be active *in vitro* during adhesion experiments to primary epithelial cells, and *in vivo* early in the infection process [49]. Here, we report that genes *apfB* and *apfC*, coding for the type IV pilin biogenesis proteins ApfB and

ApfC, were up-regulated *in vivo* during natural infection by an *A. pleuropneumoniae* isolate. Type IV fimbriae, although primarily involved in promoting the attachment to biotic or abiotic surfaces, are also involved in many other processes, including DNA uptake, biofilm formation and twitching mobility [50]. Interestingly, another gene that could share similar functions was also up-regulated. *APL_0220* codes for a member of the CscG superfamily of proteins involved in the assembly of curli fibers. Curli fibers are important for biofilm formation in numerous pathogens, including *E. coli* and *Salmonella enteritidis*, and are involved in the first steps of attachment. Curli-deficient strains tend to form flat biofilms [51]. CscG is the outer membrane protein that is responsible for the secretion of the curli structural subunits, and the *cscG* gene is generally found within an operon composed of the genes *cscDEFG*. In *A. pleuropneumoniae* L20, this organization is not respected: *APL_0220* seems to form an operon with the genes *APL_0221* and *APL_0222*. Neither of these two other genes shares homology with known *csc* genes. Finally, the *kpsF* gene, coding for an arabinose-5-phosphate isomerase, should also be mentioned since it is involved in the pathway responsible for the synthesis of 2-keto-3deozyoctulosonic acid (Kdo), which is present in the *A. pleuropneumoniae* LPS core oligosaccharide [52] and capsule of serotype 5 [53]. Studies in our laboratory have shown that it is the core oligosaccharide that is responsible for the previously observed LPS-associated adhesion mechanism [54, 55].

Two operon structures that were up-regulated *in vivo* during the acute phase of the disease could give some insight into the environment the bacteria encounters in the host, and are the main reason why the “Transport and Binding Protein” functional

class is so prominently represented in our list of up-regulated genes. Genes from the maltose operon, involved in the uptake and catabolism of maltose [56], were up-regulated: the genes *malP*, *malK*, *malF*, *malG*, *malQ* and a *malM* homolog (*APL_1234*) were actively transcribed in the host (average fold increase of 3.1), and gene *lamB1*, which codes for a maltoporin, showed a 5.14 fold change in the only hybridization in which the reporter wasn't manually flagged because of signal saturation. In *E. coli*, the *mal* genes are essential for the transport and utilization of maltose and maltodextrin [56]. Although it is not known whether maltose or maltodextrin are readily available as carbohydrate sources in mammalian lungs, it was previously shown that maltodextrin utilization is important for the colonization of the oro-pharynx in Group A *Streptococcus* [57, 58]. It was also recently shown that catabolism of maltose provides a competitive advantage to pathogenic and commensal *E. coli* strains in the gut [59]. Meanwhile, genes from the *ula* operon (*ulaDCAG*), coding for a PTS transport system for ascorbate, showed an average fold change of 2.76. In *E. coli*, these genes are responsible for the transport and utilization of ascorbate in anaerobic conditions [60]. While ascorbate use has not been linked to bacterial virulence before, it has been shown on multiple occasions that genes involved in anaerobic metabolism are important for the virulence of *A. pleuropneumoniae* [10, 61-63]. In pigs lungs, following extensive infection and tissue destruction, the bacteria are likely to face oxygen deprivation. A few other genes that are involved in anaerobic metabolism were up-regulated. The *hybB* gene codes for hydrogenase 2, a nickel-containing enzyme thought to be responsible for H_2-dependent reduction of quinone under anaerobic conditions [64]. The

phosphoenolpyruvate carboxylase and fumarate reductase subunit C, respectively encoded by genes *pepC* and *frdC*, are also required during anaerobic growth. However, other genes related to anaerobic metabolism and that were previously shown to be important *in vivo* [61-63], namely *dmsA*, *aspA* and *hlyX*, were not found in our list of differentially expressed genes. The *dmsA* gene did show a mean fold change of 1.60 over two successful hybridizations, but *hlyX* had a mean fold change of -1.66 over three successful hybridizations. However, neither of these changes were deemed statistically significant. This suggests that HlyX is probably not highly expressed during the acute phase of the disease caused by *A. pleuropneumoniae*. Accordingly, of 17 genes in our list that were previously shown to be up-regulated directly or indirectly by HlyX under anaerobiosis, 12 (*dnaX, visC, dsbC, mazG, amiB, cpxC, dacA, rpoZ, APL_0086, APL_1597, APL_1802* and *APL_2043*) were down-regulated *in vivo*, while only 5 (*sohB, mglB, hybB, APL_0096* and *APL_0920*) were up-regulated.

Two genes coding for proteins with regulatory functions were found to be up-regulated. The *phoR* gene codes for the phosphate regulon PhoR sensing protein, located in the inner membrane. In *E. coli*, this protein is part of a two-component regulatory system that responds to periplasmic orthophosphate concentration variations [65]. The phosphate regulon in *E. coli* controls the expression of at least 47 genes, and has been shown to be involved in virulence. Numerous *phoR*, *phoB* (the other component of the two-component system) and *pts* (the phosphate transport genes genetically and functionally linked to *phoBR*) mutants were found to be less virulent or avirulent when compared to wild-type strains, with phenotypes such as

increased sensitivity to serum, acidity and cationic antimicrobial peptides [66] and reduced colonization [67]. *A. pleuropneumoniae* strain L20 harbours an operon composed of the genes *ptsSCAB-phoBR*. Although only *phoR* was deemed significantly up-regulated, all other genes showed levels of variations ranging from a 1.5- to 2-fold increase. Since it seems unlikely that porcine lungs are devoid of phosphate, the *A. pleuropneumoniae* PhoBR system might be important in order to adapt correctly to changing conditions inside the host. Another gene coding for a member of a two-component system was up-regulated: gene *APL_0628* codes for a *cpxA* homolog. In different EPEC and UPEC strains, the CpxAR system was found to important for the correct folding and assembly of pili subunits [68-70]. However, the L20 *cpxA* homolog seems to either be truncated, or found on two separate gene loci. This seems to be the case since *cpxA* is found down-stream of *cpxR* (*APL_0629*), and gene *APL_0627* is a hypothetical two-component sensor protein with a histidine-kinase domain.

e) Up-regulated genes which are conserved and predicted to code for OMPs or lipoproteins

The main goal of our experiment was to identify genes that were expressed *in vivo* during infection of the natural host. We were also interested by genes showing high-level of sequence conservation among different field and reference strains [25], and coding for proteins predicted to localize to the outer membrane [71]. Three genes satisfied those criteria: two that are coding for outer membrane proteins (*APL_0959* and *irp*), and one coding for a lipoprotein (*APL_0920*) (see Additional file 1).

The *irp* (iron responsive protein) gene codes for a predicted TonB-dependant receptor, possibly involved in hemin transport. However, since this was deduced from sequence homologies, the real function of *irp* in *A. pleuropneumoniae* cannot be reliably inferred, but it is conserved in various bacterial pathogens. Interestingly, the *irp* gene is located directly next to *APL_0920*, a conserved lipoprotein in the L20 genome although they are transcribed in opposite directions. The protein sequence deduced from the *APL_0920* gene sequence is present in all sequenced *A. pleuropneumoniae* strains and in *Mannheimia haemolytica* PHL213, but no other strong homologies can be found in other bacterial species.

The *APL_0959* gene codes for a hypothetical hemagglutinin/hemolysin-like outer membrane protein. The protein sequence deduced from the gene sequence shares approximately 30% identity with different large sections of the filamentous hemagglitinin/adhesin (FhaB) from bacteria of the *Bordetella* genus, which also colonize the respiratory tract of various mammalian hosts. While it was first identified for its hemagglutination properties [72], FHA can bind carbohydrates, heparan sulphate and integrin [73]. It is the most important adhesin in *B. pertussis*, and deletion of FHA in *B. bronchiseptica*, another important swine respiratory pathogen, caused lower colonization than the wild-type strain at all respiratory tract sites tested with was unable to cause disease[74]. The *A. pleuropneumoniae* FHA homolog is conserved amongst all field and reference strains previously tested [25], and was predicted to locate to the outer membrane [71]. In *B. bronchiseptica*, FhaB is believed to be important early in the infection process in order to allow colonization of the ciliated epithelial cells of the upper respiratory tract. In

Histophilus somni, the FhaB homolog is thought to also have an important role in biofilm formation in cases of myocarditis caused by this micro-organism [75]. Up-regulation of the *A. pleuropneumoniae fhaB* homolog during the acute phase of the disease hints at a similar role for this adhesin in the establishment of pleuropneumonia. Surprisingly, this gene was not identified as up-regulated in previous adhesion experiments performed in our lab [9], and it even showed a slight, although not significant, down-regulation when *A. pleuropneumoniae* adhered to immortalized lung epithelial cells. This can either reflect differences in regulatory events between a freshly isolated field strain and a well-conserved and characterized laboratory strain, or simply that *in vitro* experiments, however carefully designed they may be, can never really completely reproduce complex *in vivo* environments.

Despite years of research, an efficient cross-serotype vaccine that can prevent porcine pleuropneumonia has yet to be successfully produced. To generate a vaccine that provides good cross-serotype protection against porcine pleuropneumonia, one would most probably have to include antigens that are conserved amongst all serotypes that the host could encounter and be surface-exposed. The three proteins that are encoded by genes *fhaB*, *irp* and *APL_0920* have all these features, and might therefore represent good candidates to include in future cross-serotype vaccines against *A. pleuropneumoniae*. FHA of *B. pertussis* is present in various combined diphteria-tetanus-pertussis (dTpa) acellular vaccines. These vaccines have been successfully used to prevent whopping cough and have contributed to a considerable decrease in the number of cases registered per year [76]. Thus the *A. pleuropneumonie* FhaB homolog represents an excellent candidate to include in a

future pleuropneumonia vaccine that could actually lead to efficient protection against this important swine pathogen.

Conclusions

To the best of our knowledge, this study represents the first and only genome-wide *in vivo* gene expression experiment to be conducted with *A. pleuropneumoniae* in its natural host, the pig, following a natural infection. Of the 150 genes that we could identify as differentially expressed, 72 showed greater levels of expression *in vivo* at the end of the acute phase of the disease than in a rich culture medium, while 78 were repressed. By comparing our results to those obtained following comparative genomics and proteomics experiments, we were able to identify three genes that were conserved in tested reference strains and in fresh field isolates of *A. pleuropneumoniae*, and these were also predicted to code for outer membrane proteins or lipoproteins. These three genes, namely *fhaB* (APL_0959), *irp* (APL_0919) and *APL_0920*, could all represent excellent candidates for the development of a protective cross-serotype subunit vaccine against *A. pleuropneumoniae*.

While our results can help decipher some of the adaptations *A. pleuropneumoniae* has to make once it infects the host, much more research is necessary in order to understand the whole picture of the disease. The acute stage and the chronic stage of the disease are probably highly different conditions, and it would be interesting in future works to compare our present data with the transcriptomic profile of *A. pleuropneumoniae* during a chronic infection.

Methods

Animals

Animals were cared for accordingly to the Canadian Council on Animal Care (CCAC) guidelines on the care and use of farm animals in research, testing and teaching [77]. Clinical cases of pleuropneumonia were diagnosed in finishing pigs of a conventional farrow to finish herd. Lung samples were recovered from commercial pigs (Landrace x Yorkshire x Duroc) weighting more than 90 kg that were showing severe clinical signs and were either euthanized on site by a veterinarian (one case) or died from the disease (two cases). Some lung samples were stored in PBS containing 10% v/v of a solution preventing RNA degradation (95% ethanol, 5% buffer-saturated phenol)[78] and frozen at -80°C for RNA extraction, while others were sent to the Diagnostic Laboratory of the Faculté de médecine vétérinaire (Université de Montréal) for bacterial culture and serotyping. Samples from all animals were paraffin-embedded, and 4 μm sections were HPS-stained for microscopic observations.

Bacterial strains and growth conditions

The *A. pleuropneumoniae* field strain 896-07 was isolated directly from the infected lung tissues and was routinely grown in BHI medium (Difco) supplemented with either 15 μg/ml (agar) or 5 μg/ml (broth) of NAD. For the transcript profiling experiment, this strain was grown in BHI-NAD broth at 37°C in an orbital shaker until an optical density of 0.3 at 600 nm was reached in order to obtain our reference condition. For the comparative genomic hybridization experiments, *A. pleuropneumoniae* serotype 5b strain L20 gDNA was used as a reference.

RNA extractions

Bacterial RNA from the *A. pleuropneumoniae* field strain was extracted directly from three samples of frozen necrotic porcine lung tissues from the pig that was euthanised on site using an acid-phenol-chloroform extraction protocol. Briefly, tissue samples of approximately 1 g were cut in small pieces for homogenization with 1.0 mm zirconia/silica beads with a Mini-BeadBeater (Biospec, OK). Supernatant was recovered and subjected to two centrifugation steps: a first one at 1000 X g for 5 min at 4°C to remove cellular debris, and another step at 5000 X g for 5 min in order to pellet bacteria and remaining debris. Pellets were resuspended in PBS and pooled, and 0.5 volume of a SDS lysis solution (2% SDS and 16 mM EDTA) pre-heated to 100°C was added. Samples were incubated at 100°C for 5 min. before being subjected to two hot-acid-phenol-chloroform (Ambion, Tx) extractions, followed by two chloroform/isoamyl alcohol (Fluka) extractions. RNA was precipitated in 0.6 volumes of isopropylic alcohol and 10% sodium acetate 3M pH 5.2 overnight, and further treated with the MicrobENRICH kit and Turbo DNase (Ambion, Tx) as prescribed by the manufacturer to ensure that contaminating eukaryotic RNA and eukaryotic and bacterial DNA were eliminated from the samples. PCR reaction using primers specific for *A. pleuropneumoniae* gene *ompW* and for porcine mitochondrial DNA were carried out to ensure that no bacterial or host DNA was left in the sample. RNA quality was verified on gel and also with a Nanodrop spectrophotometer.

Genomic DNA extraction

Genomic DNA was extracted from the field strain 896-07 and the L20 reference strain as previously described [25]. Briefly, bacteria were suspended in TE buffer

and treated with lysozyme (2 mg/ml) and RNase A for 10 minutes at room temperature, followed by digestion with proteinase K (0.1 mg/ml) in the presence of 0.1% SDS (wt/vol) for 1 hour at 37°C, until complete lysis was achieved. Genomic DNA was then isolated by extracting twice with phenol-chloroform-isoamyl alcohol (25:24:1), and twice with chloroform, and was finally precipitated in ethanol.

Microarray hybridization

For the comparative genomic hybridization (CGH) experiments, samples were labeled and hybridized to the AppChip2 microarrays as previously described [25]. Briefly, genomic DNA from the reference L20 strain and from the field strain 896-07 was nebulized to 0.4 – 1.2 kb by passing nitrogen gas through an AeroMist Nebulizer chamber (IPI Medical Products, Chicago, IL) at 15 psi for 1 min. A total of 5 μg of fragmented DNA was fluorescently labeled using direct chemical coupling with the Label-IT (Mirus Corp., Madison, WI) cyanine dyes Cy3 (reference strain) and Cy5 (field strain) as recommended by the manufacturer. Labeling efficiency was assessed spectrophotometrically, and samples were then combined and hybridized overnight to the AppChip2. For a complete description of AppChip2, see Gouré et al. 2009 [25].

Transcript profiling experiments were conducted as previously described [28]. Briefly, we proceeded to indirectly label cDNA synthesized from 15 μg of RNA from our reference (field strain grown in BHI broth) and experimental conditions (bacterial mRNAs isolated from necrotic lung tissues of the euthanized pig) using a monofunctional NHS-ester Cy3 or Cy5 dye (Amersham). Labelling efficiency was assessed spectrophotometrically, and labelled samples were then combined and

added to the *A. pleuropneumoniae* 5b strain L20 microarrays for overnight hybridization. All microarray slides were scanned using a Perkin-Elmer ScanArray Express scanner. Our microarray data was submitted to the Gene Expression Omnibus [79] [GEO:GSE15911]. Experiments were conducted on three different days, using different *in vivo* and *in vitro* samples. In order to ensure that the dye bias effect was minimal, two self-self hybridization assays using amplified RNA (MessageAmp, Ambion, Tx, USA) extracted from lung samples were conducted.

Bioinformatics

All bioinformatics analyses were performed with the TM4 Microarray Software Suite [80] as previously described [28]. Raw data was generated using Spotfinder v.3.1.1. The integrated intensities of each spot, equivalent to the sum of unsaturated pixels in a spot, were quantified and the integrated intensity of the local background was subtracted for each spot. The same operation was performed with the median spot intensities. Spots with bad morphology, high local background and signal saturation were manually flaged and excluded from the data set. Data was normalized with the MIDAS software tool using cross-channel Loess normalization. Spots with median intensities lower than 1000 were removed from the normalized data set. Intensities for duplicate spots were averaged to generate the final normalized data set [25, 28].

For the CGH experiments, a threshold of ±0.9 on a $\log_2$ scale was used to identify genes that are likely divergent in the field strain, or were deemed absent from the field strain if the $\log_2$ ratio values was less than -3 [25]. A total of two hybridizations were conducted. For the transcript profiling experiments. Brefly, the Significance

Analysis of Microarray (SAM) algorithm [81], which is included in the MeV software, was used to generate a list of differentially expressed genes following three distinct hybridizations. Using a false discovery rate (FDR) of 4.25%, a list of 150 differentially expressed genes was generated; this value estimates the proportion of genes likely to have been identified by chance. Functional classification of these genes was conducted using TIGR's Comprehensive Microbial Resource (CMR) [82]. Proteins were assigned to their corresponding pathways using the MetaCyc Metabolic Pathway Database [36]. Homologies were assessed using Blast tools [83] hosted on the NCBI and TIGR servers. The strain dendrogram was generated using Dendroscope v2.2.2 built 9 [84], adding results from field strain 896-07 to data previously obtained [25].

Real-Time quantitative RT-PCR

Microarray results for the transcript profiling experiments were verified by real-time quantitative RT-PCR (qRT-PCR), using the QuantiTect® SYBR® Green RT-PCR Kit (Qiagen) on the same RNA samples that were used for the transcript profiling experiments. Reactions were performed in triplicate with a 16-place Cepheid Smart Cycler® System in a total volume of 25 µl. Oligonucleotide primers (see Additional file 2) were designed using PrimerBlast [85] and tested on genomic DNA extracted from the *A. pleuropneumoniae* 896-07 field strain. To ensure that amplification with these primers resulted in single amplicon of the anticipated size, they were PCR tested before proceeding to qRT-PCR analysis. Primer pairs which amplified fragments of 195 to 205 bp with a melting temperature of 60°C were selected. Eleven genes (5 up-regulated, 6 down-regulated) were selected for analysis. Relative

expression of each gene as determined by qRT-PCR was normalized to that of the *rluC* gene which showed a stable level of expression throughout the different microarray experiments (data not shown). Quantitative measures were obtained using the $2^{-\Delta\Delta C_T}$ method [86].

Authors's contributions

VD designed the transcript profiling experiments, carried out downstream data analysis, and drafted the manuscript. MD participated in the study design, supplied the infected lung tissues and revised the manuscript. CG conducted the microscopy work and revised the manuscript. JHEN designed AppChip2 and helped with the downstream data analysis. JH participated in the study design and revised the manuscript. MJ participated in the conception and supervised the design of the study and revised the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1: HPS staining of paraffin-embedded infected lung tissue sections, at 100X magnification. Severe fibrinohemorragic pneumonia with infiltration of oat cells.

Figure 2: Hierarchical clustering of reference and field strains of *A. pleuropneumoniae*, including strain 896-07, based on CGH data obtained with AppChip2. Strains are identified according to "serotype-strain number". Field strains are the same as those used by Gouré et al.[25]. R: reference strain. NT: Non-typeable.

Figure 3: Functional classification of *A. pleuropneumoniae* genes that are differentially expressed in infected pig lungs. AAB, amino acid biosynthesis. BCPGC, biosynthesis of cofactors, prosthetic groups and carriers. CE, cell enveloppe. CP, cellular process. CIM, central intermediary metabolism. DM, DNA metabolism. EM, energy metabolism. FAPM, fatty acid and phospholipid metabolism. HUU, hypothetical/unclassified/unknown. MEE, mobile extrachromosomal element functions. PF, protein fate. PS, protein synthesis. PPNN, purines, pyrimidins, nucleosides and nucleotides. RF, regulatory functions. ST, signal transduction. Tr, transcription. TBP, transport and binding proteins.

Figure 4: Validation of microarray results by qRT-PCR. Results from five up-regulated and six down-regulated genes are presented. Mean $\log_2$ ratios obtained during qRT-PCR experiments are plotted against the mean $\log_2$ ratios obtained with the microarrays. Numbers on the graph refer to the gene numbers in Additional file 2.

Tables

Table 1: Genes that are divergent or absent in the *A. pleuropneumoniae* 896-07 field strain after comparison with the genome of serotype 5b reference strain L20.

R	Locus Tag	Gene	Description	Log_2 ratio
P	APL_0495	*APL_0495*	Putative DNA-methyltransferase	-1.135
P	APL_0496	*APL_0496*	Hypothetical protein	-2.324
P	APL_0499	*APL_0499*	Hypothetical protein	-1.166
P	APL_0500	*APL_0500*	Hypothetical protein	-1.071
P	APL_0501	*APL_0501*	Possible DNA methylase	-1.418
P	APL_0502	*APL_0502*	Hypothetical protein	-1.794
P	APL_0503	*rusA*	Predicted Endodeoxyribonuclease RusA	-1.065
P	APL_0505	*APL_0505*	Putative endolysin	-1.306
P	APL_0509	*APL_0509*	Bacteriophage capsid protein	-1.825
P	APL_0518	*APL_0518*	Hypothetical protein	-3.227
P	APL_0520	*APL_0520*	Hypothetical protein	-1.101
P	APL_0522	*APL_0522*	Phage-related tail assembly protein K	-1.635
P	APL_0523	*APL_0523*	Putative phage tail assembly protein	-1.533
P	APL_0524	*APL_0524*	Predicted phage tail protein	-1.097
T	APL_0547	*tadF*	Tight adherence protein F	-0.909
T	APL_0550	*tadC*	Tight adherence protein C	-1.528
T	APL_0551	*tadB*	Tight adherence protein B	-1.917
T	APL_0552	*tadA*	Tight adherence protein A	-1.781
T	APL_0553	*tadZ*	Flp operon protein D	-1.991
T	APL_0556	*rcpC*	Flp operon protein C	-1.815
O	APL_0427	*adhA*	NADP-specific glutamate dehydrogenase	1.081
O	APL_0445	*ykgF*	Putative electron transport protein	-1.144
O	APL_0560	*rhlB*	ATP-dependent RNA helicase RhlB	-2.063
O	APL_0672	*APL_0672*	Hypothetical protein	0.927
O	APL_1959	*adhI*	Alcohol dehydrogenase 1	1.015
O	APL_2001	*APL_2001*	Hypothetical protein	-1.618
O	APL_2011	*aldA*	Putative aldehyde dehydrogenase AldA	1.813
O	APL_2038	*lolC*	Lipoprotein-releasing system transmembrane protein LolC	-0.915

Genes are ordered according to their location in the *A. pleuropneumoniae* genome (locus tag). Two important genomic regions (R) were found in this list : a phage region (P), and the *tad* locus (T).

R : genomic region. P : phage region. T : *tad* locus. O : other region

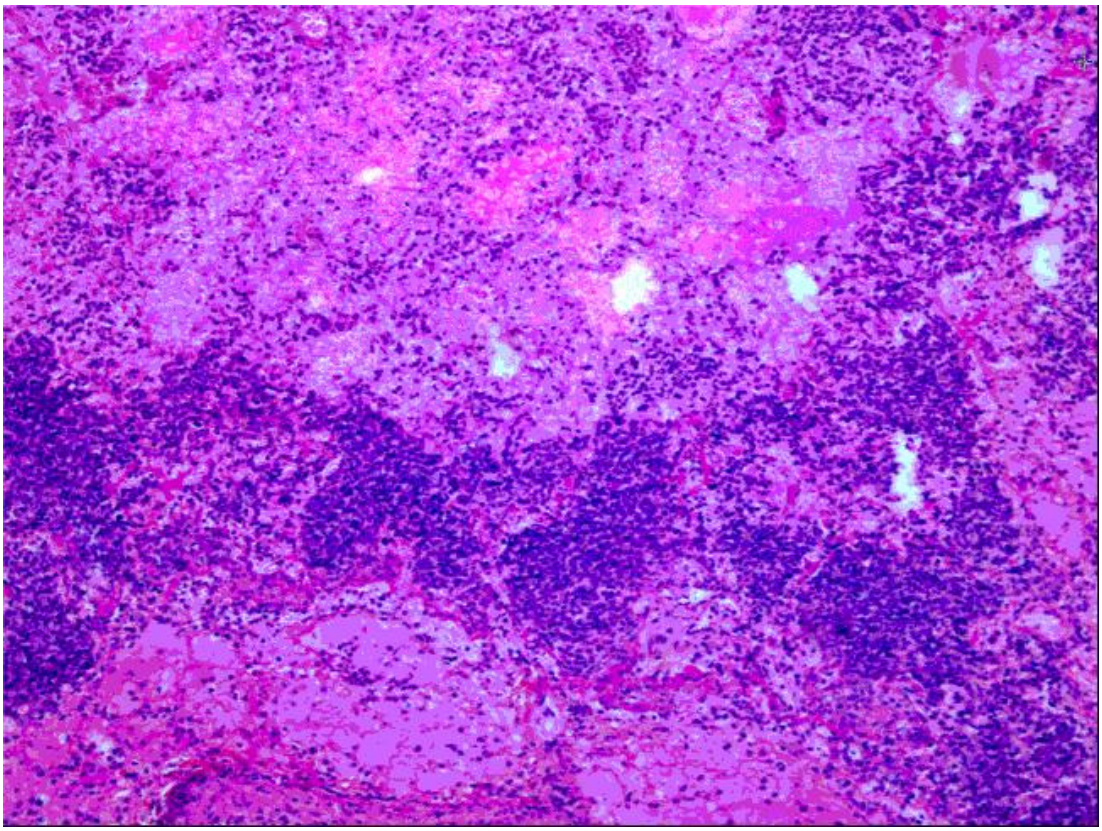
Table 2: Comparison between genes that were identified by transcript profiling *in vivo* (this study) and other *in vivo* techniques in *A. pleuropneumoniae*

Locus tag	Gene	TP	Other
APL_0030	*prfC*	↓	IVET (Fuller et al. 1999) STM (Sheehan et al. 2003)
APL_0771	*lpdA*	↓	STM (Fuller et al. 2000)
APL_1583	*cpxC*	↓	STM (Sheehan et al. 2003)
APL_1580	*cps5b*	↓	STM (Sheehan et al. 2003)
APL_0333	*visC*	↓	STM (Sheehan et al. 2003)
APL_1218	*fur*	↓	STM (Sheehan et al. 2003)
APL_1388	*APL_1388*	↓	STM (Sheehan et al. 2003)
APL_0998	*apxIVA*	↑	SCOTS (Baltes et al. 2004)
APL_1684	*fucI*	↑	SCOTS (Baltes et al. 2004)
APL_0163	*nrdD*	↑	SCOTS (Baltes et al. 2004)
APL_1292	*APL_1292*	↓	SCOTS (Baltes et al. 2007)
APL_0282	*potC*	↓	SCOTS (Baltes et al. 2007)
APL_1474	*dnaG*	↓	SCOTS (Baltes et al. 2007)
APL_1401	*rpsL*	↓	SCOTS (Baltes et al. 2007)
APL_1752	*nanE*	↑	SCOTS (Baltes et al. 2007)

TP : Results obtained during transcript profiling

↑: Up-regulated

↓: Down-regulated



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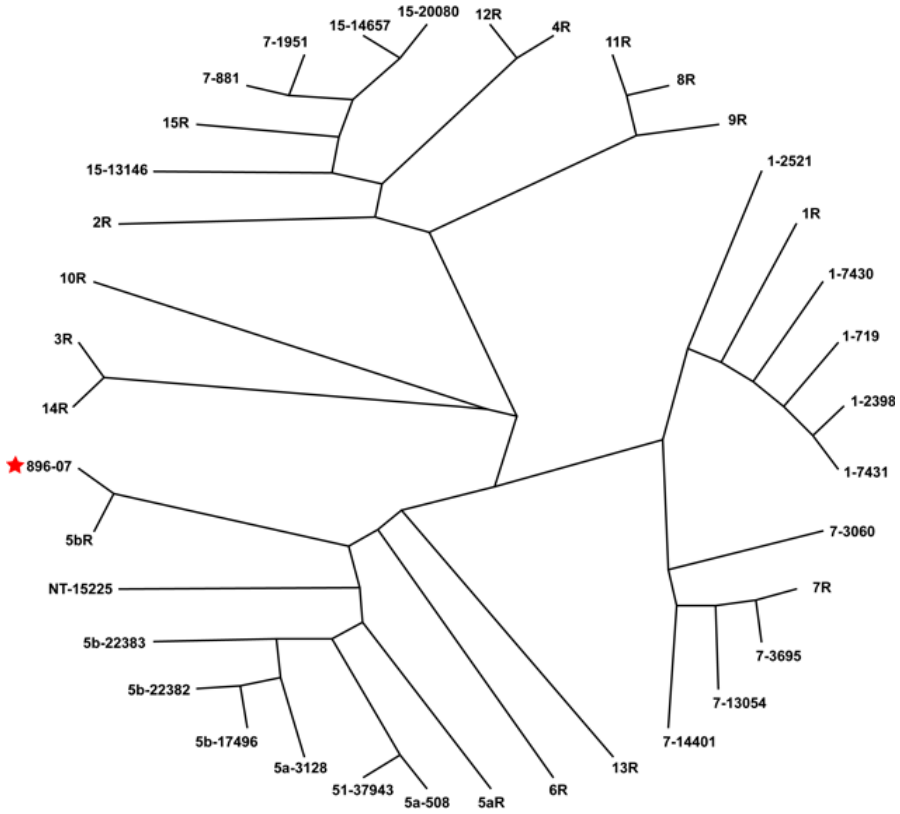

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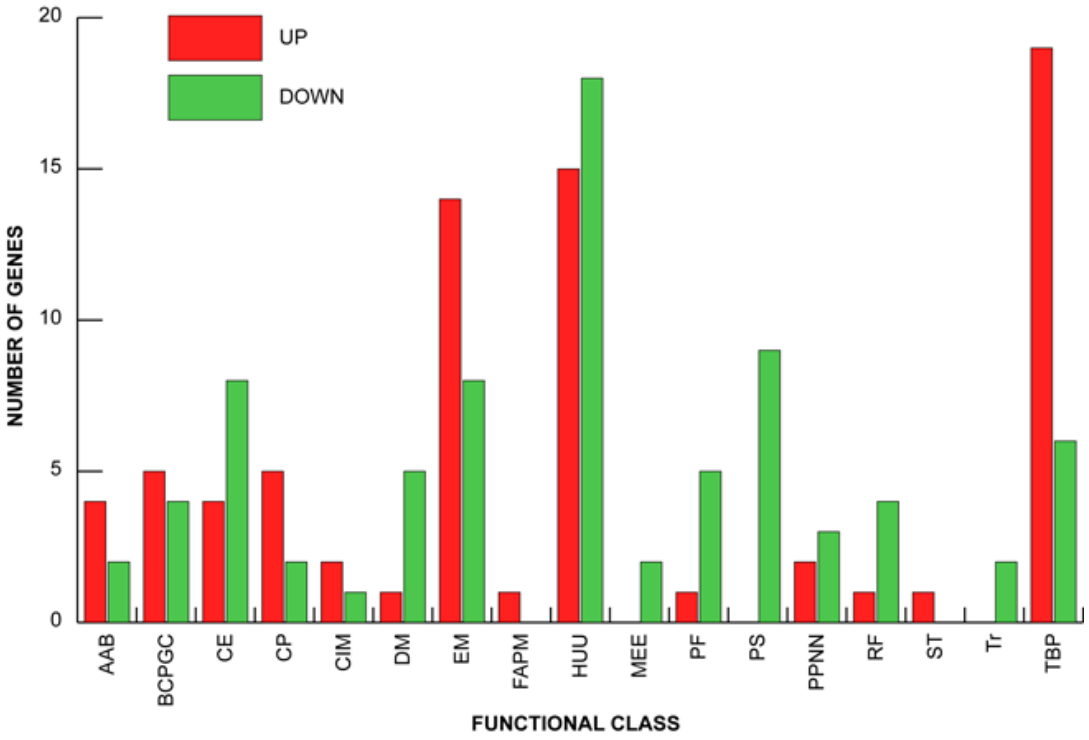

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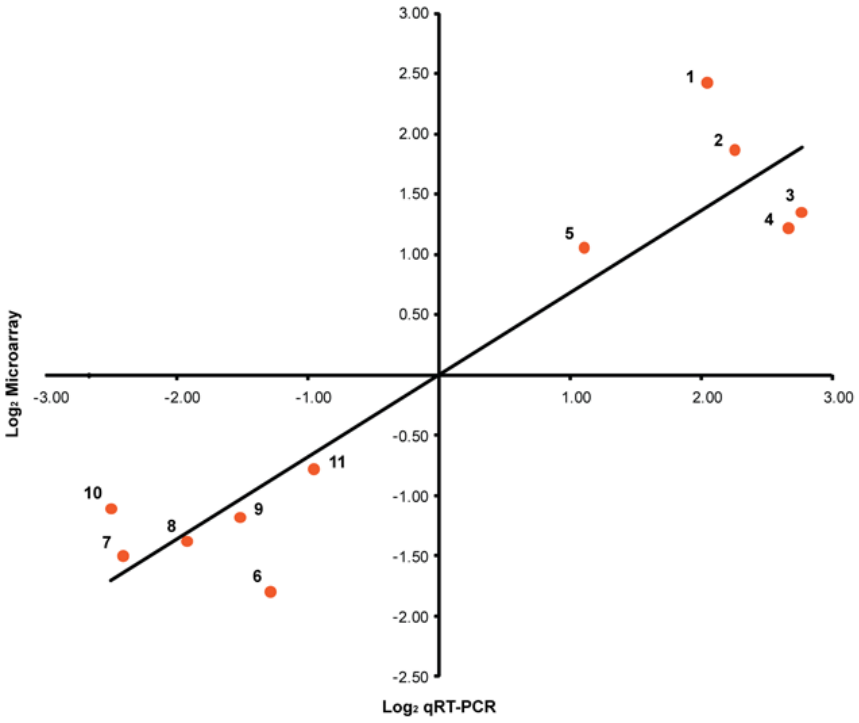

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Additional file 1: *A. pleuropneumoniae* genes which are differentially expressed in infected pig lungs (150 genes). Individual genes and their corresponding locus tag are sorted according to their functional class and fold change. *q*-values as calculated by SAM are indicated in %.

Locus Tag	Gene	Description	Fold	*q* (%)
Amino Acid Biosynthesis				
APL_2025	*hisH*	Imidazole glycerol phosphate synthase subunit HisH	4.230	1.31
APL_0139	*leuC*	3-isopropylmalate dehydratase large subunit 2	3.077	0.00
APL_2026	*hisA*	Phosphoribosylformimino-5-aminoimidazole	2.976	0.00
APL_1043	*APL_1043*	Probable aminotransferase	2.949	0.00
APL_1862	*aroQ*	3-dehydroquinate dehydratase	-2.680	3.65
APL_0319	*metE*	Cobalamin-independant homocysteine transmethylase	-3.478	3.74
Biosynthesis of cofactors				
APL_0572	*pdxS*	Pyridoxal biosynthesis lyase PdxS	2.825	0.78
APL_0776	*ispE*	4-diphosphocytidyl-2-C-methyl-D-erythritolkinase	2.612	0.00
APL_0822	*ubiA*	4-hydroxybenzoate octaprenyltransferase	2.401	0.78
APL_0535	*thiG*	Thiazole biosynthesis protein ThiG	2.280	0.78
APL_0310	*moeA*	Molybdopterin biosynthesis protein MoeA	1.739	2.06
APL_0903	*folE*	GTP cyclohydrolase I	-1.626	3.65
APL_1513	*coaA*	Pantothenate kinase	-1.750	4.00
APL_1008	*hemX*	Putative uroporphyrin-III C-methyltransferase	-1.841	3.69
APL_0333	*visC*	Putative monooxygenase family protein	-2.823	0.00
Cell Envelope				
APL_1494	*ftpA*	Fine tangled pili major subunit; DNA-binding ferritin-like protein (oxidative damage protectant)	3.428	3.95
APL_0387	*kpsF*	Arabinose-5-phosphate isomerase; polysialic acid capsule expression protein	2.548	0.78
APL_1599	*mrdA*	Penicillin-binding protein 2	2.478	3.30
APL_0878	*apfBC*	Fimbrial biogenesis protein	2.205	3.94
APL_1136	*amiB*	Putative N-acetylmuramoyl-L-alanine amidase AmiB	-1.616	4.06
APL_1583	*cpxC*	Capsule polysaccharide export inner-membrane protein	-1.819	4.06
APL_1863	*APL_1863*	Putative mannosyltransferase	-2.169	4.06
APL_1580	*cps5b*	Region 2 capsular polysaccharide biosynthesis protein	-2.208	3.79
APL_1596	*dacA*	D-alanyl-D-alanine carboxypeptidase fraction A; penicillin binding protein 5 precursor	-2.217	4.00
APL_0873^{+}	*rlpB*	Putative rare lipoprotein B	-2.243	1.13
APL_0681	*APL_0681*	Putative soluble lytic murein transglycosylase precursor	-2.523	1.13
APL_1597	*APL_1597*	Possible rare lipoprotein A RlpA-like protein	-2.571	4.11
Cellular Processes				
APL_0766	*rec2*	Recombination protein 2	2.909	3.30
APL_0303	*tolB*	Translocation protein TolB precursor	2.883	0.00
APL_0998	*apxIVA*	RTX toxin protein ApxIV structural component	2.121	1.22
APL_0988	*hktE*	Catalase	1.735	3.70

	APL_1344	*ftsX*	Cell division protein FtsX-like protein	-2.029	1.13
	APL_0118	*cspC*	Cold shock-like protein CspC	-4.413	4.00
Central intermediary metabolism					
	APL_1752	*nanE*	Putative N-acetylmannosamine-6-phosphate 2-	2.830	2.42
	APL_1755	*nagB*	Glucosamine-6-phosphate deaminase	1.708	0.78
	APL_1508	*APL_1508*	Putative Rhodanese-related sulfurtransferase	-2.788	4.00
DNA metabolism					
	APL_0370	*recB*	Exodeoxyribonuclease V beta chain	1.537	2.17
	APL_1474	*dnaG*	DNA primase	-1.565	4.00
	APL_0265	*dnaX*	DNA polymerase III subunit γ/τ	-1.595	3.74
	APL_1142	*recX*	Regulatory protein RecX	-1.698	3.74
	APL_1170	*priB*	Primosomal replication protein	-1.761	4.25
	APL_0002	*dnaN*	DNA polymerase III subunit β	-1.763	4.25
Energy metabolism					
	APL_1240	*malQ*	4-alpha-glucanotransferase	3.293	0.78
	APL_1333	*hybB*	Pative Ni/Fe-hydrogenase 2 b-type cytochrome subunit	3.123	0.00
	APL_1698	*ulaD*	Probable 3-keto-L-gulonate-6-phosphate decarboxylase	3.054	3.94
	APL_0869	*abgB*	Aminobenzoyl-glutamate utilization-like protein	2.942	0.00
	APL_1232	*malP*	Maltodextrin phosphorylase	2.879	0.00
	APL_1701	*ulaG*	L-ascorbate-6-phosphate lactonase UlaG-like protein	2.452	1.31
	APL_1684	*fucI*	L-fucose isomerase	2.342	0.00
	APL_1019	*kdgK*	2-dehydro-3-deoxygluconokinase	2.290	1.31
	APL_2011	*aldA*	Putative aldehyde dehydrogenase AldA	2.215	2.42
	APL_0452	*sucC*	Succinyl-CoA synthetase beta chain	2.209	2.35
	APL_0969	*glnE*	Glutamate-ammonia-ligase adenylyltransferase	2.003	2.14
	APL_0339	*pepC*	Phosphoenolpyruvate carboxylase	1.760	3.95
	APL_1527	*frdC*	Fumarate reductase subunit C	1.573	2.09
	APL_0375	*glpK*	Glycerol kinase	1.506	4.11
	APL_1450	*fbp*	Fructose-1,6-bisphosphatase	-1.607	3.65
	APL_0771	*lpdA*	Dihydrolipoyl dehydrogenase	-1.617	4.00
	APL_0607	*nfnB*	Putative NAD(P)H nitroreductase	-1.661	3.69
	APL_1638	*dsbA2*	Thiol disulfide oxidoreductase	-1.944	3.74
	APL_0755	*fabI*	Enoyl-[acyl-carrier-protein] reductase (NADH)	-1.949	3.65
	APL_1652	*atpB*	ATP synthase A chain	-1.959	4.00
	APL_1479	*APL_1479*	Thioredoxin-like protein	-2.018	3.79
	APL_0644	*pta*	Phosphate acetyltransferase	-2.995	4.13
Fatty acid and phospholipid metabolism					
	APL_1689	*APL_1689*	Probable alcohol dehydrogenase	1.898	0.78
Mobile and extrachromosomal element functions					
	APL_0524	*APL_0524*	Predicted phage tail protein	-2.435	3.74
	APL_0984	*APL_0984*	Putative transposase	-4.773	3.79
Protein fate					
	APL_0008	*sohB*	Putative secreted serine protease SohB	5.390	2.02
	APL_1962	*hflX*	GTP-binding protein hflX	-1.680	3.74
	APL_0458	*dsbC*	Thiol:disulfide interchange protein DsbC precursor	-1.853	1.13

	APL_1509	*secB*	Protein-export protein SecB	-2.115	4.30
	APL_1456	*slyD*	FKBP-type peptidyl-prolyl cis-trans isomerase SlyD	-2.268	4.25
	APL_0743	*secG*	Protein-export membrane protein	-4.155	3.79
Protein Synthesis					
	APL_1759	*rpsJ*	30S ribosomal protein S10	-1.592	3.74
	APL_0030	*prfC*	Peptide chain release factor 3	-1.734	3.74
	APL_0982	*rpmE*	50S ribosomal protein L31	-2.172	2.49
	APL_1169	*rplI*	50S ribosomal protein L9	-2.266	3.79
	APL_1473	*rpsU*	30S ribosomal protein S21	-2.355	1.13
	APL_1401	*rpsL*	30S ribosomal protein S12	-2.850	1.13
	APL_1972	*rpmG*	50S ribosomal protein L33	-3.096	1.98
	APL_1558	*rpsT*	30S ribosomal protein S20	-3.559	3.79
	APL_1782	*rpsK*	30S ribosomal protein S11	-3.967	4.00
Purines, pyrimidines, nucleosides, and nucleotides					
	APL_0775	*prsA*	Ribose-phosphate pyrophosphokinase	2.560	2.09
	APL_0163	*nrdD*	Anaerobic ribonucleoside triphosphate reductase	1.517	3.05
	APL_0646	*cpdB*	2',3'-cyclic-nucleotide 2'-phosphodiesterase precursor	-2.043	4.00
	APL_0682	*hpt*	Hypoxanthine-guanine phosphoribosyltransferase	-2.293	3.79
	APL_0256	*gmk*	Guanylate kinase	-2.569	4.13
Regulatory functions					
	APL_0628	*cpxA*	Putative sensor kinase CpxA	1.997	4.30
	APL_0657	*sspB*	Stringent starvation protein B; ClpXP protease specificity-enhancing factor	-1.598	3.97
	APL_0615	*mlc*	NagC-like transcriptional regulator	-2.028	3.79
	APL_1961	*hfq*	RNA-binding protein Hfq	-2.181	3.65
	APL_1218	*fur*	Ferric uptake regulation protein	-2.612	4.25
Signal Transduction					
	APL_1256	*phoR*	Phosphate regulon sensor protein PhoR	2.079	1.31
Transcription					
	APL_1717	*nusG*	Transcription antitermination protein NusG	-1.716	4.13
	APL_1826	*rpoZ*	DNA-directed RNA polymerase omega subunit	-2.432	3.74
Transport and Binding Proteins					
	APL_0855	*hbpA*	Heme-binding lipoprotein A precursor	3.649	0.00
	APL_0870	*APL_0870*	Putative C4-dicarboxylate transporter	3.414	0.00
	APL_1234	*malM*	Maltose regulon periplasmic protein	3.382	1.31
	APL_1700	*ulaA*	Predicted ascorbate-specific permease IIC component	3.155	4.11
	APL_1238	*malF*	Maltose transport system permease protein MalF	3.119	1.18
	APL_1239	*malG*	Maltose transport system permease protein MalG	3.073	2.17
	APL_1236	*malK*	Maltose/maltodextrin import ATP-binding protein	2.936	0.00
	APL_1847	*cysW*	Sulfate transport system permease protein cysW	2.529	0.00
	APL_0848	*APL_0848*	Putative ABC transporter periplasmic binding protein	2.527	0.78
	APL_1699	*ulaC*	Ascorbate-specific phosphotransferase enzyme IIA	2.404	3.95
	APL_0919*	*irp*	Iron-regulated outer membrane protein, TonB-	2.255	3.13
	APL_0220	*csgG*	Putative lipoprotein CsgG	2.156	3.83
	APL_0369	*norM*	Putative multidrug efflux protein, Na+/drug antiporter	2.134	2.21

	APL_1665	*gntP1*	Gluconate permease	1.835	2.17
	APL_0096	*APL_0096*	Zinc/iron transporter family protein ZIP	1.815	3.05
	APL_0167	*rnfC*	Electron transport complex protein RnfC	1.699	1.31
	APL_0450	*mglB*	D-galactose-binding periplasmic protein precursor	1.659	4.11
	APL_1292	*APL_1292*	Predicted ABC transporter ATP-binding protein	-1.549	3.69
	APL_0967	*gltS*	Sodium/glutamate symport carrier protein	-1.672	3.65
	APL_1388	*APL_1388*	Predicted ABC-transport permease	-1.829	3.65
	APL_1880	*mscS*	Small-conductance mechanosensitive channel	-1.931	2.49
	APL_1457	*aqpZ*	Aquaporin Z	-2.073	4.00
	APL_0282	*potC*	Spermidine/putrescine transport system permease	-2.089	4.00
Hypothetical/Unknown/Unclassified					
	APL_0920^{+}	*APL_0920*	Hypothetical protein	4.469	0.00
	APL_0966	*APL_0966*	Putative transport protein	2.604	0.00
	APL_0668	*APL_0668*	Predicted periplasmic lipoprotein involved in iron	2.416	1.31
	APL_1188	*APL_1188*	Hypothetical protein	2.367	0.78
	APL_1934	*APL_1934*	Hypothetical protein, conserved inner membrane	2.302	0.00
	APL_0959*	*APL_0959*	Hemagglutinin/hemolysin-like protein; filamentous haemagglutinin outer membrane protein	2.237	0.78
	APL_0999	*APL_0999*	Hypothetical protein	2.027	3.83
	APL_0141	*APL_0141*	Hypothetical protein; possible H+/gluconate symporter	1.965	3.83
	APL_1002	*APL_1002*	Hypothetical protein	1.920	0.00
	APL_1044	*APL_1044*	Hypothetical protein; predicted permease rarD	1.864	3.83
	APL_0815	*APL_0815*	Hypothetical protein	1.856	3.05
	APL_1082	*arcD*	Putative arginine/ornithine antiporter	1.818	4.09
	APL_0512	*APL_0512*	Hypothetical protein	1.777	0.78
	APL_0904	*kdkA*	3-deoxy-D-manno-octulosonic acid kinase	1.745	3.01
	APL_1751	*APL_1751*	Hypothetical protein; putative lipase or esterase	1.548	3.83
	APL_0828	*APL_0828*	Hypothetical protein, uncharacterized conserved protein	-1.631	3.97
	APL_1404^{+}	*oapB*	Opacity associated protein B	-1.771	1.13
	APL_2043	*APL_2043*	Hypothetical protein	-1.882	3.74
	APL_0049*	*APL_0049*	Hypothetical protein	-2.001	4.00
	APL_0119	*proQ*	proQ-like protein	-2.067	4.01
	APL_1135	*APL_1135*	Hypothetical protein; putative ATPase	-2.164	3.74
	APL_1802	*APL_1802*	Hypothetical protein	-2.181	3.97
	APL_0630	*mazG*	Predicted pyrophosphatase	-2.235	3.79
	APL_1458	*APL_1458*	Hypothetical protein; putative periplasmic/secreted	-2.249	4.00
	APL_1382	*APL_1382*	Hypothetical protein	-2.374	3.94
	APL_0226	*APL_0226*	Hypothetical protein; predicted kinase	-2.834	3.79
	APL_0428^{+}	*smpA*	Small protein A	-3.072	0.00
	APL_0359	*nlpC*	Putative lipoprotein	-3.115	0.00
	APL_1639	*slyX*	SlyX-like protein	-3.344	3.79
	APL_0653	*csrA*	Carbon storage regulator CsrA	-3.444	3.65
	APL_0576	*nlpI*	Lipoprotein NlpI-like precursor	-3.488	4.00
	APL_0086	*APL_0086*	Hypothetical protein	-4.126	0.00

* Conserved outer membrane protein, as predicted by Gouré & al. and Chung & al.

[+] Conserved lipoprotein, as predicted by Gouré & al. and Chung & al.

Additional file 2: Oligonucleotide primers used for microarray result validation by qRT-PCR

#	*Gene*	Forward Primer	Reverse Primer
1	*sohB*	ATGCGGTCATTTCTTTAGCG	ATTTTATTCGCTACGCACGC
2	*hbpA*	CACGCTATGCCAAACTGAAA	TCGGTAGCGTGTTGTAGTGC
3	*kpsF*	GTAAATTGCTCAATCGCGTG	AACTGTCCGTACCGAATTGC
4	*apxIVA*	CTGAATAAACCGGACGGAAA	CATGGTCGAATAACGCTCCT
5	*phoR*	GCATTTACCGCATTTAACCG	GTTTCACCAAGCAGCGATTT
6	*nlpI*	ATTAAATCCGGACGAACGTG	TAAAGGCATCAATCGCACTG
7	*visC*	GATTACGAGCAAACCGCATT	GCTCCCGATTAAATTGTTGC
8	*proQ*	AGAAGCCGGTATTGTGGATG	GAAGCTTCTTTCGCAACTCG
9	*APL 1456*	GGAAGAAGGTTACGGCGAAT	ACTCTTGCCCGGCTAACATA
10	*APL 1135*	TTAAACGGTGAACTTGGTGC	CGAATCCCCATAAACTCCAA
11	*nusG*	ACCGACCGAAGAAGTTGTTG	AAAATACGATCCGCTTCACG
Ref	*rluC*	TACAGAACAGTTGCGGAAA	CATTCGCATAACGCTCTTCA
	Pig mito.	GGCCACATTAGCACTACTCAACATC	AGATCCGATGATTACGTGCAAC
	ompW	GGCGAAGTGGCAAAAGTAAA	CAACACCTAAATTCGCAATCG

: refers to the annotation of the gene on Fig. 4

DISCUSSION

La régulation fine et précise de l'expression des gènes liés à la virulence est vitale chez les bactéries pathogènes. Chez *A. pleuropneumoniae*, les stimuli régulant l'expression des facteurs de virulence ainsi que les phénomènes métaboliques impliqués lors de l'infection sont largement inconnus. Dans le but de mieux comprendre le comportement et la réponse transcriptionnelle d'*Actinobacillus pleuropneumoniae* lorsque la bactérie se retrouve chez son hôte naturel, nous avons établi le profil transcriptomique du pathogène dans différentes conditions simulant l'infection ainsi que lors de la phase aiguë de l'infection *in vivo*.

Utilisation de la souche de référence S4074 du sérotype 1 plutôt que la souche L20 du sérotype 5b

Avant même de débuter les expériences de profil transcriptomique, il est rapidement devenu clair que l'utilisation de la souche 5b L20, ayant servi à l'élaboration de la puce, dans le cadre des expériences de profil transcriptomique allait être problématique. L'utlisation de plusieurs protocoles d'extraction différents, allant d'ensembles commerciaux (QIAGEN, RNeasy, ou Invitrogen, Trizol) à l'utilisation de protocoles traditionnels d'extraction au phénol-chloroforme, a démontré qu'il est très ardu de réussir à obtenir de bons rendements d'ARN pour la souche L20 du sérotype 5b. Sur gélose, les colonies de cette souche sont d'aspect rugueux et adhèrent à la surface, et il y a formation d'aggrégats lors de la croissance en milieu liquide. Dans les faits, la souche de référence L20 du sérotype 5b est l'une des deux souches de référence, avec la souche du sérotype 11, à pouvoir former des biofilms *in vitro* (Kaplan et al. 2005), ce qui explique fort probablement les difficultés rencontrées lors des expériences d'extractions d'ARN. Nous avons donc eu recours à la souche de référence S4074 de sérotype 1 pour les expériences de profil transcriptomique. Des expériences de génomique comparative ont montré que seulement 85 gènes présents sur la biopuce AppChip2 (donc 4.2% des gènes) divergent entre les souches L20 et S4074 (Gouré et al. 2009). L'utilisation de la souche de référence S4074 semblait donc plus appropriée techniquement, malgré la possibilité de pertes de certaines données potentiellement intéressantes.

Croissance d'*A. pleuropneumoniae* en présence d'une concentration limitante en fer

Il a été démontré à plusieurs reprises que les bactéries possèdent des mécanismes élaborés permettant de détecter les variations de concentration en ions ferriques, et que ces variations entraînent des changements dans la régulation de gènes liés, entre autre, à la virulence (Braun 2001). La protéine régulatrice Fur, qui est retrouvée chez une très vaste variétée de bactéries et qui est aussi présente chez *A. pleuropneumoniae*, cause la répression de la transcription des gènes liés à l'acquisition du fer lorsque les niveaux intra-cellulaires d'ions ferriques sont suffisants (Escolar et al. 1999; Hsu et al. 2003). Chez *A. pleuropneumoniae*, il a été démontré que Fur joue un rôle, en collaboration avec le calcium, dans la régulation positive de la transcription de l'opéron codant pour la toxine ApxI (Hsu et al. 2003).

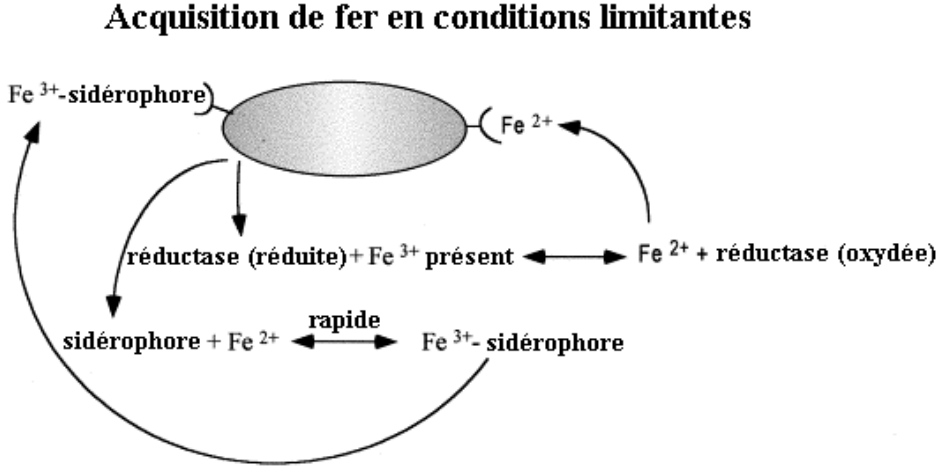
Tel qu'attendu, cette étude (Article 1) a permis de mettre en évidence que la majorité des gènes associés à l'acquisition du fer ont été surexprimés lorsque *A. pleuropneumoniae* a été mise en croissance en présence de 50 μg/ml de EDDHA, un agent chélateur spécifique aux ions ferriques. Ainsi, les gènes codant pour les deux protéines TonB d'*A. pleuropneumonie* ont été surexprimés, de même que celui codant pour le récepteur spécifique pour l'hémoglobine porcine (HgbA). Bien qu'absents de la première version de la biopuce (AppChip1) de *A. pleuropneumoniae* 5b L20, le niveau d'expression des gènes *exbB1*, *exbD1* et *tbpA* a été mesuré par qRT-PCR, et tous ces gènes se sont avérés être surexprimés en conditions limitantes en fer. Ces gènes sont tous liés transcriptionnellement au gène *tonB1* (Tonpitak et al. 2000) et codent respectivement pour les deux autres composantes du système de transduction d'énergie TonB1-ExbB1-ExbD1 ainsi que pour la protéine transmembranaire TbpA nécessaire à l'acquisition du fer à partir de la transferrine porcine. Puisqu'il était déjà connu que le fer ne joue aucun rôle dans la régulation du récepteur FhuA (Mikael et al. 2003), le système Afu est donc le seul autre système impliqué dans l'acquisition du fer connu chez *A. pleuropneumonia* qui n'est pas surexprimé dans notre étude, et ce malgré la présence de nombreux rapporteurs spécifiques aux gènes *afuABC* sur la biopuce AppChip1. Néanmoins, le gène *apxIC*,

qui code pour la protéine activatrice de la toxine ApxI et qui est voisin du gène *afuA* dans le génome d'*A. pleuropneumoniae*, bien que transcrit en sens opposé, a quant à lui subi une légère hausse d'expression en présence d'EDDHA. Ces deux observations sont contraires à ce qui a été observé précédemment par Hsu et al. 2003, à savoir que la combinaison Fur et calcium (donc un milieu riche en cations divalents Fe^{2+} et Ca^{2+}) stimulait la production des transcrits de l'opéron *apxICABD*, et que Fur agit comme répresseur sur l'opéron *afuABC*. Cependant, étant donné que les toxines Apx sont fort probablement impliquées dans l'acquisition du fer via la destruction des globules rouges porcins et la lyse des cellules de l'hôte (Bossé et al. 2002), il semble logique que leur synthèse soit affectée, d'une façon ou d'une autre, par le niveau de fer environnant.

Fait intéressant, un système d'acquisition du fer reposant sur une composante dont la sécrétion est possiblement effectuée par la voie TAT (« Twin-arginin-translocation) semble montrer un fort niveau de surexpression, et une composante de ce système a par la suite montré une surexpression similaire en présence de liquide de lavages broncho-alvéolaires ainsi qu'*in vivo* lors d'une infection naturelle aiguë. En présence d'EDDHA, le gène APL_0668 ainsi que deux autres gènes environnants (voir Article 1, Figure 3d) ont tous été surexprimés. La voie de translocation TAT permet, chez les bactéries, l'export de protéines dans le cytoplasme dans leur conformation finale, repliée (De Buck et al. 2008). Alors qu'au départ, lors de l'identification du système TAT au début des années 1990, on croyait que le système était surtout impliqué dans l'export vers le cytoplasme de protéines impliquées dans le métabolisme énergétiques et devant lier un cofacteur, il a ensuite été démontré que plusieurs protéines ayant des rôles autres sont aussi exportées par TAT, et que certaines sont impliquées dans la virulence (Ochsner et al. 2002). Le système putatif composé des gènes APL_0668-APL_0669-APL_0670 est homologue à un système baptisé FepABC chez *Staphylococcus aureus* (Biswas et al. 2009). Chez *S. aureus*, *fepA* code pour une lipoprotéine périplasmique putative impliquée dans le transport du fer (APL_0668), *fepB* pour une péroxidase dépendante du fer (APL_0669) et *fepC* pour une perméase à haute affinité pour le fer ferreux (APL_0670). Des expériences ont

montré que la péxidase est exportée par le système Tat, et que le système FepABC est impliqué dans l'internalisation de fer par la bactérie (Biswas et al. 2009). Ce transport est dépendant de FepABC, mais est aussi ralenti lorsqu'une mutation affecte Tat. Il est à noter que la peroxidase APL_0669 comporte bel et bien, au début de sa séquence en acides aminés, une séquence signal d'export via le système Tat (S/TRRxFLK, acides aminés 6 à 12 dans la séquence APL_0669), et que *A. pleuropneumoniae* possède aussi un système putatif d'export Tat (TatABC, APL_1984 – APL_1986). On peut alors poser l'hypothèse que, au sein du système de transport du fer encodé par les gènes APL_0668 à APL_0670, la peroxidase APL_0669 est exportée par Tat et permet, possiblement, de rendre disponible des ions ferreux (Fe^{2+}) pouvant être pris en charge par la perméase APL_0670 et, ensuite, par la lipoprotéine périplasmique APL_0668. Récemment, il a été mis en évidence que la réduction de fer ferrique, libre ou sur un ligand, en fer ferreux par des réductases bactériennes sécrétées constitutivement se révèlent plus rapide et efficace pour générer des concentrations de fer libre satisfaisantes pour la bactérie que, par exemple, la sécrétion de sidérophores (Cowart 2002) (Figure 1). La péroxidase APL_0669, couplée à la perméase APL_0670 et à la lipoprotéine périplasmique APL_0668, pourrait accomplir un rôle similaire chez *A. pleuropneumoniae*.

Figure 1 : mécanisme proposé pour l'acquisition du fer par les bactéries en conditions limitantes. Un rôle prépondérant est accordé à la sécrétion de réductases ferriques. Tiré et modifié de Cowart et al. 2002



Dans la même lignée, un autre récepteur putatif (ap0300/APL_0276) qui est situé dans la même région génomique que des homologues des gènes *yfeAB* (APL_0272 et APL_0271), impliqués dans l'acquisition du fer chélaté, et dont la spécificité est inconnue, a été identifié. Ce récepteur potentiel a ultérieurement été renommé FrpB, et des études ont montré que le niveau de production de ce récepteur était fortement stimulé par le régulateur HlyX, l'homologue du régulateur FNR activant la transcription des gènes liés au métabolisme anaérobie chez *E. coli* (Buettner et al. 2009). Dans le même ordre d'idée, le gène *ssa1*, codant pour une protéase autotransporteur putative de type subtilysine, et plus tard rebaptisé *aasP* (Baltes et al. 2007), est surexprimé en présence de quantités limitées en fer et semble lui aussi être régulé positivement par HlyX (Buettner et al. 2009).

Sur le plan métabolique, les modifications majeures proviennent surtout de la répression d'enzymes portant des ions ferriques au sein de leurs sites actifs. Alors que plusieurs études ont démontré que, *in vivo*, *A. pleuropneumoniae* a recours à des enzymes liées au métabolisme anaérobique (Baltes et al. 2004a; Baltes et al. 2005; Baltes et al. 2007), tel ne fut pas le cas en présence d'une concentration limitante en fer alors que la bactérie semble plutôt se tourner vers un métabolisme fermentatif (surexpression de *lldD*, codant pour une lactate déhydrogénase). Ce résultat est observé malgré la hausse d'expression du gène *hlyX*. Rétroactivement, il semble possible de conclure que la présence accrue de HlyX ait probablement causé la surexpression des gènes APL_0276/*frpB* et *ssa1/aasP*. Ce phénomène, à savoir la surexpression de certains gènes par HlyX en conditions limitantes en fer, est probablement rencontré à plus grande échelle dans nos données, et explique probablement les difficultés rencontrées lors de la recherche d'un site spécifique pour la liaison de Fur. En cherchant dans les séquences situées en amont des gènes surexprimés en présence d'EDDHA, et donc possiblement réprimés par Fur, il nous a été impossible de trouver un motif conservé présentant une bonne homologie avec les séquences de boîtes Fur déjà connues. La recherche de motifs spécifiques à HlyX se serait peut-être avérée plus fructueuse à ce chapitre. Néanmoins, ces premières expériences de profil transcriptomique réalisées sur *A. pleuropneumoniae* ont permis

d'identifier de nouvelles structures putatives dont l'importance *in vivo* a par la suite été démontrée.

Croissance d'*A. pleuropneumoniae* dans des conditions stimulant la formation de biofilms

Il est de plus en plus accepté, au sein de la communauté scientifique, que la formation de biofilms est un facteur important pour la pathogénicité de nombreuses bactéries (Costerton et al. 1999; Ghigo 2003). Chez *A. pleuropneumoniae*, des expériences ont démontré que deux des souches de référence pour les 15 sérotypes possèdent ce phénotype, soit les souches de référence du sérotype 5b et du sérotype 11, alors que plus de la moitié des souches de champ de sérotypes 1, 5 et 7 présentent cette caractéristique (Kaplan et al. 2005). Suite à ces expériences, les auteurs suggèrent que la capacité de former des biofilms, qui peut être maintenue chez des isolats frais lorsque les bactéries sont cultivées sur milieu solide, est cependant perdue de manière irréversible lorsque la bactérie est cultivée en milieux liquides, phénomène qui est également observé chez *Aggregatibacter (Actinobacillus) actinomycetemcomitans* (Fine et al. 1999).

C'est une observation tout à fait fortuite qui a conduit aux études transcriptomiques sur des cultures d'*A. pleuropneumoniae* (Article 2). En comparant la croissance de la bactérie dans deux milieux de culture BHI liquides provenant de deux fournisseurs différents (BHI-A : Difco, BHI-B : Oxoid), il est apparu que la souche de référence S4074 de sérotype 1 pouvait former des aggrégats. Des expériences subséquentes ont ensuite démontré que ce phénomène pouvait aussi être observé chez les souches de référence des sérotypes 3, 4, 5a, 12 et 14, en plus des souches de référence des sérotypes 5b et 11. De plus, ces biofilms pouvaient être éliminés par un traitement à la dispersine B, enzyme d'*A. actinomycetemcomitans* responsable de la dégradation des biofilms (Kaplan et al. 2004b). Afin de déterminer quel signal, présent dans l'un des milieux de culture, permettait de réacquérir la capacité de former des biofilms, nous avons comparé les transcriptomes de la souche de référence S4074 après une

croissance similaire dans les deux milieux, tout en essayant d'établir un lien avec les analyses chimiques effectuées sur les milieux.

La première constatation importante est que le milieu BHI-B induit la transcription de nombreux gènes codant pour des molécules liées à l'adhésion : les gènes de l'opéron *pgaABC*, impliqués dans la formation de la matrice extracellulaire du biofilm, les gènes *tadCD* de l'opéron *tad*, impliqué dans l'adhérence aux surfaces biotiques et abiotiques chez *A. actinomycetemcomitans* (Tomich et al. 2007), de même que le gène codant pour l'adhésine autotransporteur Hsf ont tous été surexprimés dans ce milieu. Des gènes des opérons *pga* et *tad*, de même que le gène *hsf*, ont par la suite été identifiés comme étant surexprimés suite à la mise en contact d'*A. pleuropneumoniae* avec des cellules épithéliales pulmonaires (Article 3). Parallèlement, un gène codant pour une possible dispersine endogène de *A. pleuropneumoniae* (APL_1096 : 59% d'identité au niveau des séquences d'acides aminés avec la dispersine retrouvée chez *A. actinomycetemcomitans*) a un niveau de transcription diminué dans le milieu BHI-B. L'apparition de biofilms dans ce milieu semble donc être due à l'effet combiné de la hausse de l'expression de molécules d'adhésion et de la baisse d'expression de la dispersine endogène d'*A. pleuropneumoniae*.

À la lumière des autres résultats obtenus, deux suspects pouvant potentiellement stimuler la formation de biofilm ont été identifiés. Dans un premier temps, puisque les études de composition chimique ont révélé que le milieu BHI-B contient moins de zinc que le milieu BHI-A, et puisqu'un gène codant pour la protéine périsplasmique à haute affinité pour le zinc ZnuA (APL_1440) ainsi qu'un autre codant pour un transporteur de zinc (APL_0096) étaient surexprimés dans le milieu B, l'effet du zinc sur la formation de biofilms a été évalué. Il a été établi que l'ajout de zinc au milieu BHI-B entraînait la diminution de la formation de biofilms de manière dose-dépendante. Une étude récente a aussi mis en évidence qu'un mutant d'*E. coli* pour le gène *znuB*, impliqué dans le même système de transport de zinc que le gène *znuA*, entraînait une diminution de la formation de biofilms (Gunasekera et

al. 2009). Deuxièmement, plusieurs changements dans l'expression de gènes liés à la biosynthèse d'acides aminés, et plus particulièrement ceux liés à la biosynthèse de la leucine et des acides aminés à chaînes ramifiés (« Branched-Chain Amino Acids », BCAA), ont pu être identifiés. Ces gènes (l*euABC*, *ilvCDEGHIM*) sont parmi les plus surexprimés dans le milieu BHI-B. De plus, des études récentes effectuées avec *A. pleuropneumoniae* ont démontré que la croissance de la bactérie dans un milieu pauvre en BCAA induit l'expression de gènes liés à la virulence (Wagner et al. 2006), et qu'une mutation dans le gène *ilvI* entraîne l'atténuation de la bactérie lors d'infections expérimentales chez le porc (Subashchandrabose et al. 2009).

Fait à noter, les gènes impliqués dans la synthèse de l'uréase (*ureAEFG*), ainsi que d'autres impliqués dans le transport du nickel nécessaire au fonctionnement de l'enzyme (*cbiMO*) ont aussi été surexprimés en milieu BHI-B. L'importance de l'uréase au sein de biofilm a été démontrée en médecine dentaire, alors qu'il est de plus en plus clair que la présence de bactéries sécrétant de l'uréase inhibe le développement de caries (Burne et al. 2000). La sécrétion d'uréase par ces bactéries permet de compenser pour l'acidification du milieu causée par la grande utilisation de carbohydrates, et empêche l'établissement de bactéries acidophiles. Bien qu'aucune publication ne fasse état de la découverte d'*A. pleuropneumoniae* au sein de biofilms mixtes, l'acidification de biofilms suite à l'utilisation de carbohydrates est un phénomène qui n'est pas limité aux biofilms mixtes. Le fait qu'une mutation au sein d'un gène nécessaire au fonctionnement de l'uréase entraîne une diminution de la persistance d'*A. pleuropneumoniae* au niveau des poumons de porc lors d'infections expérimentales (Baltes et al. 2001) pourrait donc refléter le mode de vie en biofilm de la bactérie dans le cas d'infections chroniques.

Réponse transcriptomique d'*A. pleuropneumoniae* suite à la mise en contact avec du liquide de lavages broncho-alvéolaires

Suite à son entrée chez l'hôte, un des premiers éléments rencontrés par *A. pleuropneumoniae* au niveau du tractus respiratoire est la mince couche de liquide qui recouvre les cellules respiratoires épithéliales, ainsi que toutes les composantes

que ce liquide contient. Chez l'homme, le liquide de lavage broncho-alvéolaire (BALF) contient, entre autres, des peptides antimicrobiens comme les défensines, des anticorps et des dérivés d'oxide nitrique (Diamond et al. 2000; Magi et al. 2002), et l'expression de plusieurs de ces composantes est induite lors d'infections (Russell et al. 1996). Chez le porc, la présence d'au moins un peptide cationique antimicrobien a été démontré au sein du BALF lors de l'infection par *A. pleuropneumoniae* (Hennig-Pauka et al. 2006; Hennig-Pauka et al. 2007). La mise en contact d'*A. pleuropneumoniae* avec du BALF *in vitro* devrait donc permettre d'identifier des composantes exprimées spécifiquement *in vivo* (Article 3).

Afin de contrer l'effet des composantes de l'immunité innée et acquises présentent au sein du BALF, il est probable que la capsule polysaccharidique présente à la surface d'*A. pleuropneumoniae* joue un rôle prépondérant. Il est donc peu surprenant de constater que deux des gènes de l'opéron nécessaires à l'export des polysaccharides qui composent la capsule, soit les gènes *cpxA* et *cpxC*, sont surexprimés en présence de BALF. Dans le même ordre d'idée, le gène *sapF* fait partie d'un opéron, chez certaines souches de *Haemophilus influenzae*, qui code pour les composantes d'un système de résistance aux peptides antimicrobiens (Mason et al. 2006). Bien qu'*A. pleuropneumoniae* possède également les gènes *sapABCDE*, ceux-ci sont présents au sein d'un même opéron situé a environ 520 kb du gène *sapF* à l'intérieur du génome de la souche L20. Bien que *sapF* ne code que pour une composante de l'ATPase produisant l'énergie nécessaire au fonctionnement du système Sap, l'importance de ce système chez *A. pleuropneumoniae* n'a pas encore été vérifiée. Ce système jouerait aussi potentiellement un rôle dans l'acquisition du potassium (Mason et al. 2006).

Au niveau des gènes de virulence, la surexpression du gène *apxIVA* codant pour la toxine ApxIV a été observée pour la première fois *in vitro*. Identifiée auparavant *in vivo*, l'expression de cette toxine semble être stimulée par certaines substances retrouvées chez l'hôte, et qui sont probablement présentes dans le liquide de lavage broncho-alvéolaire. Alors que les gènes codant pour les récepteurs les plus connus

pour l'acquisition du fer chez *A. pleuropneumoniae*, soit les récepteurs TbpAB et HgbA, ne sont pas surexprimés en présence de BALF, il en va autrement pour le gène APL_0276/*frpB* précédemment retrouvé en présence d'une concentration limitante en fer. La séquence protéique de FrpB démontre de fortes similarités avec une famille de protéines impliquées dans l'utilisation de protéines de l'hôte liées à des ions ferriques, comme par exemple l'hémoglobine, la transferrine et la lactoferrine (TIGRFAM 01786). Bien que la spécificité de ce récepteur soit toujours inconnue, sa surexpression en présence de molécules sécrétées au sein du BALF peut permettre certaines hypothèses. Classiquement, la lactoferrine est une des protéines retrouvées chez les mammifères qui a pour rôle de se lier au fer dans les liquides sécrétoires afin d'en diminuer la disponibilité (Braun 2001). Bien que des expériences précédentes aient démontré que plusieurs souches d'*A. pleuropneumoniae* semblent incapables d'utiliser la lactoferrine porcine (D'Silva et al. 1995), la souche CM5 du sérotype 1, utilisée pour les expériences de profil transcriptomique avec le BALF, ne faisait pas partie du lot. Une deuxième possibilité peut être posée suivant l'observation selon laquelle le BALF de la race porcine Hampshire, qui est moins susceptible à l'infection par *A. pleuropneumoniae*, présente des niveaux d'haptoglobine, une protéine liant l'hémoglobine libérée suite à la lyse des globules rouge, inférieurs à ceux retrouvés chez deux autres races plus susceptibles (Kahlisch et al. 2009). La protéine APL_0276/FrpB pourrait donc possiblement lier l'haptoglobine présente dans le BALF porcin. Finalement, dans le cadre d'une étude visant à identifier les protéines d'*A. pleuropneumoniae* permettant l'utilisation de l'hémoglobine et de l'hémine porcine, Archambault et al. ont découvert deux protéines de taille similaire pouvant lier ces deux composantes (Archambault et al. 2003). Alors que l'analyse des fragments peptidiques générés par digestion enzymatique semblait indiquer que les deux protéines d'environ 75kDa n'étaient en fait qu'une seule et unique protéine, il est aussi possible que les auteurs aient bel et bien eut affaire à deux protéines possédant certains domaines conservés.

Sur le plan métabolique, la surexpression de gènes présents au sein des opérons *nap* (nitrate réductase), *nrf* (nitrite réductase) et *nqr* (NADH-quinone réductase), soit les

gènes *napBDFGH*, *nrfBC* et *nqrBCE*, est cohérente avec l'utilisation du nitrate comme accepteur final d'électrons lors de la respiration anaérobie (Xu et al. 2008). De plus, la surexpression des gènes *malF* et *malG*, codant pour une perméase spécifique au maltose, laisse présager la disponibilité de ce sucre au niveau du tractus respiratoire porcin. Ce changement sur le plan métabolique est aussi accompagné d'une forte hausse d'expression des gènes de synthèse protéique, paradoxalement accompagnée d'une diminution de la transcription des gènes liés à la synthèse d'acides aminés. Il est donc probable que certains acides aminés soient présents en quantités suffisantes dans le BALF pour que la biosynthèse directe par la bactérie devienne moins essentielle.

Réponse transcriptomique d'*A. pleuropneumoniae* suite à la mise en contact avec des cellules épithéliales pulmonaires ou suite à l'adhérence directe à ces cellules

Sous la mince couche de liquide broncho-alvéolaire rencontrée par *A. pleuropneumoniae* lors de son entrée dans les poumons porcins se trouvent les cellules épithéliales pulmonaires, cibles potentielles de la bactérie. C'est aussi sur ces cellules que devrait normalement se faire l'adhésion menant à l'établissement d'une infection plus sérieuse (Article 4).

Un premier constat surprenant lors de l'établissement du profil transcriptomique d'*A pleuropneumoniae* lors de la croissance au-dessus des cellules épithéliales ou lors de l'adhésion directe à ces cellules : aucun gène n'est à la fois surexprimé ou réprimé dans les deux conditions, et dans plusieurs cas la relation entre les deux conditions est plutôt inverse. Alors que dans les deux cas les bactéries se trouvent en présence de composantes cellulaires, il semble que ces deux conditions de croissances soient très différentes. Tout d'abord, sur le plan métabolique, alors que la croissance planctonique au-dessus des cellules SJPL semble stimuler la production d'enzymes nécessaires à l'utilisation de plusieurs accepteurs alternatifs d'électrons (nitrates/nitrites : *nrfABC*, nitroréductase *APL_0483*; fumarate : *fdrACD*; formate : *fdxGH*, *fdnI*), l'inverse est observé lorsque la bactérie adhère directement aux

cellules SJPL avec une répression de gènes associés à ces voies (*fdxGH*, *nrfB*, *frdD*). Un phénomène semblable peut être noté au niveau des gènes liés à l'acquisition du fer à partir de différentes sources. Lors de la croissances planctonique, les gènes *hgbA*, *tonB1*, *exbD2*, *hugZ* (utilisation de l'hème) et *yfeABD* (transport du fer chélaté vers le cytoplasme) ont tous été réprimés, de même que d'autres gènes codant pour des systèmes putatifs d'acquisition de fer non-caractérisés (*APL_1952* à *APL_1955* : protéines de la membrane externe possiblement impliquées dans le transport du fer; *APL_0714* à *APL_0717* : transporteur putatif ABC pour l'entérochéline; APL_0670 : perméase putative pour le fer et le plomb). Au contraire, pendant l'adhésion aux cellules SJPL, la bactérie surexprime certains de ces gènes (*exbD2*, *APL_1955*), ainsi qu'un autre possiblement associé à l'acquisition du dicitrate ferrique (*fecE*).

L'abondance de substrats pourrait possiblement expliquer le profil transcriptomique observé au cours de la croissance de la bactérie dans le milieu de culture au-dessus des cellules épithéliales. Car même en utilisant un temps d'incubation des bactéries avec les cellules relativement court de 3h, il n'en demeure pas moins qu'un bon niveau de cytotoxicité pouvait être observé au niveau des flacons de culture cellulaire, ce qui implique que le contenu des cellules lysées s'est déversé dans le milieu de culture. La disponibilité de plusieurs substrats utilisables sur le plan métabolique peut donc expliquer la surexpression des différentes enzymes liées au métabolisme énergétique, alors que le relargage de fer sous formes accessibles explique problablement la répression des gènes liés à l'acquisition de cet élément essentiel. Le profil observé lors de l'adhérence est cependant beaucoup plus difficile à interpréter. Ainsi, nous avons constaté que les gènes codant pour les enzymes catalysant les 6 premières étapes de la glycolyse (gènes *gapA*, *pgk*, *fpb*, *tpiA*, *pgi* et *fba*) étaient tous réprimés, de même que les gènes *tktA*, codant pour l'enzyme liant la glycolyse au cycle des pentoses-phosphate, et *maeA* codant pour l'enzyme responsable de la première étape de la gluconéogénèse. La bactérie semble donc relativement inerte sur le plan métabolique lors de l'adhérence aux cellules épithéliales, si ce n'est de la surexpression d'enzymes liées à la dégradation du fucose (*fucO*, *fucI*, *fucK*).

Le processus de l'adhésion étant largement inconnu chez *A. pleuropneumonie*, une attention plus particulière a donc été apportée aux gènes encodant potentiellement des composantes impliquées dans cette étape cruciale de l'infection. À ce chapitre, aucune déception : tant lors de la croissance dans le milieu de culture que suite à l'adhésion il nous a été permis d'observer la surexpression de plusieurs gènes codant pour des composantes possiblement liées à l'adhésion. Tout d'abord, lors de la croissance planctonique, le gène *APL_0443*, codant pour l'homologue de l'adhésine autotransporteur Hsf de *Haemophilus inlfuenzae* a été surexprimé, parallèlement à une diminution de l'expression de gènes liés à l'export des composantes de la capsule polysaccharidique (*cpxAB*). Par la suite, lors de l'adhérence, ce sont les gènes *pgaBC* nécessaires à la formation de biofilms, ainsi que deux gènes de l'opéron tad (*rcpA*, *tadB*) qui ont été surexprimés. Alors que l'adhésine autotransporteur Hsf permet, chez *H. influenzae* sérotype B, l'adhésion spécifique aux cellules épithéliales (Barenkamp et al. 1996; Hallstrom et al. 2006), l'expression de biofilms de même que l'aggrégation suivant l'entrée en fonction des protéines codées par l'opéron *tad* mènent à des évènements d'adhésion beaucoup moins spécifiques. Chez divers bactéries, la formation de biofilms permet l'adhésion autant à des surfaces biotiques qu'à des surfaces abiotiques. Chez *Aggregatibacter actinomycetemcomitans*, l'opéron *tad* est aussi responsable de l'adhésion non-spécifique à de multiples surfaces (Planet et al. 2003; Tomich et al. 2007). Nous posons donc l'hypothèse que chez *A. pleuropneumoniae*, l'adhésion de la bactérie à ses cellules cibles au niveau du tractus respiratoire porcin est un processus séquentiel impliquant, dans un premier temps, des évènements d'adhésion spécifiques médiés par Hsf et possiblement les LPS, suivi par la suite d'une adhésion non-spécifique via l'autoaggrégation et la formation de biofilms.

Croissance de la bactérie à l'intérieur de son hôte naturel au cours d'une infection naturelle aiguë

La dernière étape du projet, plus ambitieuse, visait à déterminer le profil transcriptomique d'*A. pleuropneumoniae* dans le cadre de l'infection de son hôte naturel (Article 5). Généralement, de telles expériences sont conduites dans des

conditions expérimentales bien contrôlées, à l'aide de groupes d'animaux soumis à des conditions d'infection bien déterminées. Ceci permet entre autres l'utilisation de groupes contrôles non-infectés, ainsi que l'utilisation de plusieurs réplicats techniques et biologiques. Malgré tout, l'utilisation d'infections expérimentales n'est jamais un reflet entièrement fidèle de la réalité, que ce soit à cause des voies d'infections privilégiées ou de la dose infectieuse choisie. Dans le cadre de nos expériences, nous avons eu la chance de pouvoir disposer d'échantillons recueillis sur des animaux naturellement infectés par une souche sauvage d'*A. pleuropneumoniae* dans un élevage porcin commercial. Néanmoins, plusieurs limites techniques sont associées à de tels échantillons. Dans un premier temps, alors que les infections expérimentales permettent généralement de recueillir un nombre non-limitant d'échantillons, tel n'est pas le cas dans le cadre d'une infection naturelle. Dans un deuxième temps, l'infection naturelle permet rarement de recueillir des échantillons sur des animaux ayant subi des infections de la même durée. Troisièmement, alors qu'il est possible d'avoir recours à une population d'animaux relativement homogène tant sur le plan génétique qu'immunologique dans le cadre d'une infection expérimentale, l'infection naturelle d'un troupeau affectera des animaux ayant une plus grande variabilité génétique ainsi que différentes populations microbiennes résidentes. Finalement, dans le cadre d'animaux d'élevages commerciaux, les éleveurs ne peuvent se permettre de laisser l'infection perdurer dans l'élevage, et les animaux à risque sont rapidement soumis à de l'antibiothérapie.

Ces limites expliquent pourquoi les expériences transcriptomiques n'ont pu être menées que sur un seul animal, soit celui qui a été euthanasié sur place par un vétérinaire. Dans ce cas précis, le temps écoulé entre la mort et la collecte des échantillons (et donc l'arrêt de la synthèse/dégradation d'ARNm) a pu être minimisé. Les deux autres animaux, décédés suite aux effets de l'infection dans le courant de la nuit, ont été échantillonnés plusieurs heures après le décès. Malgré tout, ces échantillons ont été essentiels pour la mise au point des protocoles nécessaires pour mener à bien les expériences transcriptomiques. Au final, la méthode utilisée pour l'extraction d'ARN a permis d'obtenir des rendements satisfaisants tant au niveau de

la quantité que de la qualité. L'immersion des tissus infectés dans la solution de phénol/éthanol permet de minimiser la dégradation de l'ARN. L'agitation au « beadbeater » avec des billes de 1 mm permet de libérer les bactéries présentes dans le tissu, et les étapes successives de centrifugation permettent d'éliminer un maximum de débris cellulaires. Finalement, l'extraction au phénol-acide chaud permet de minimiser les pertes en ARN, tout en obtenant du matériel de grande qualité.

Des hybridations génomiques avec de l'ADNg de la souche de champ de sérotype 5b isolée chez l'animal (896-07) sur la biopuce AppChip2 ont permis d'évaluer la variation génétique de cette souche par rapport à la souche L20. Tel que nous l'avions conclu auparavant suite à des études de génomique comparative plus complètes, les différentes souches d'*A. pleuropneumoniae* semblent être d'origine clonale et présentent une faible variation génétique (Gouré et al. 2009) : le génome de la souche 896-07 ne diverge que très légèrement de celui de la souche L20. Moins de 1.5% des gènes du génome de la souche de champ sont divergents par rapport à ceux de la souche de référence, et la grande majorité de ces gènes se retrouvent dans deux loci hautement variables : le locus *tad*, et un locus de séquences phagiques. Il est aussi possible que cette souche possède également des gènes qui sont absents du génome de la souche L20 et ne sont pas présents sur la biopuce AppChip2. Néanmoins, dans le cas des gènes qui sont présents, ces données révèlent que la biopuce AppChip2 est adéquate pour déterminer le profil transcriptomique de la souche de champ lors de l'infection.

Au niveau transcriptomique, la première surprise vient surtout des gènes qui n'ont pas été identifiés comme étant surexprimés dans les poumons des porcs infectés. Ainsi, mis à part le gène *apxIVA*, codant pour la toxine ApxIV détectée d'abord seulement *in vivo* et ensuite après la croissance dans le BALF, aucun autre gène codant pour les toxines ApxI et ApxII, aussi retrouvées chez les souches du sérotype 5, n'était surexprimé lors de l'échantillonage. Le même constat est aussi applicable dans le cas des gènes connus pour mener à l'expression des systèmes d'acquisition

de fer classique d'*A. pleuropneumoniae* : les séquences codantes des deux systèmes TonB ainsi que les gènes des récepteurs spécifiques à la transferrine (*tbpAB*) et à l'hémoglobine (*hgbA*) porcines n'ont pas montré de variation significative dans leur transcription lors de l'infection aiguë chez l'hôte comparativement à leur expression *in vitro* en milieu riche. La répression de l'expression du gène *fur* est probablement partiellement responsable de cet état. La protéine Fur, bien qu'elle réprime l'expression des gènes responsables de l'expression des systèmes d'acquisition du fer, régule aussi son propre niveau de transcription (Ratledge et al. 2000). La répression du gène *fur* signifie donc probablement que la protéine Fur est présente en quantité suffisante, et donc que la bactérie n'évolue pas en condition limitante en fer. Cependant, certains gènes codant pour d'autres systèmes d'acquisition du fer ont subi une hausse d'expression chez l'hôte : les gènes *APL_0096*, *APL_0668*, *hbpA* et *irp*, codant respectivement pour une perméase putative pour le fer ferreux, une lipoprotéine périplasmique impliquée dans le transport du fer, une possible lipoprotéine périplasmique liant l'hème et une protéine régulée par le fer, ont tous été surexprimés. De ces gènes, seul *APL_0668* avait été identifié précédemment lors d'expériences en présence d'une concentration limitante en fer, ce qui implique que les autres ne sont possiblement pas régulés par Fur.

Sur le plan métabolique, deux opérons en particulier ont montré des niveaux de surexpression importants. Les gènes *malFGKMPQ* de l'opéron *mal*, codant pour les protéines nécessaires à l'import et à l'utilisation du maltose (Boos et al. 1998), ont tous été surexprimés, ce qui semble indiquer que ce sucre soit particulièrement présent et accessible dans les voies respiratoires porcines lors de l'infection. Dans le cadre d'une étude visant à démontrer que des nanoparticules d'or pouvaient s'accumuler dans différents sites biologiques tout dépendamment des molécules utilisées pour les recouvrir, il a été démontré qu'une association de ces nanoparticules avec du maltose cause leur accumulation au niveau des poumons chez le porc (Fent et al. 2009). Ceci pourrait indiquer que le maltose peut s'accumuler au niveau des poumons. Dans le même ordre d'idée, les gènes *ulaDCAG* de l'opéron *ula*

codant pour un système de phosphotransférase impliqué dans l'acquisition et l'utilisation de l'ascorbate (Zhang et al. 2003), ont eux aussi été surexprimés.

Parmi les gènes codant pour des facteurs de virulence connus chez *A. pleuropneumoniae* on retrouve dans notre liste les gènes *cpxC* et *cps5B*, impliqués respectivement dans l'export et la synthèse des polysaccharides capsulaires, et qui sont tous deux réprimés *in vivo*. Un profil d'expression similaire était observé pour les gènes *cpx* lors de la croissance planctonique au-desssus des cellules SJPL. Il a été démontré, chez *A. pleuropneumoniae*, qu'un mutant acapsulé présentait une meilleure adhérence à des anneaux trachéaux porcins congelés (Rioux et al. 2000), et la présence d'une capsule plus mince, résultant par exemple d'une diminution de l'export et/ou de la biosynthèse des polysaccharides capsulaires, pourrait possiblement permettre d'exposer en surface des adhésines qui seraient autrement enfouies dans la capsule. D'ailleurs, malgré le fait que plusieurs gènes codant pour des structures potentiellement impliquées dans l'adhésion n'aient pas été surexprimés *in vivo* (biofilm Pga, gènes de l'opéron *tad*, adhésine autotransporteur Hsf), d'autres candidats potentiels ont néanmoins été surexprimés. Ainsi, les gènes *afpBC* codent pour des protéines impliquées dans la biogénèse de pili de type IV, des structures qui ont déjà été observées chez *A. pleuropneumoniae* (Stevenson et al. 2003; Boekema et al. 2004b), et qui sont impliquées dans divers processus allant de l'acquisition de fragments d'ADN à la formation de biofilm et à la motilité chez d'autres bactéries pathogènes (Pelicic 2008).

Le gène *APL_0959*, qui est également surexprimé, code quant à lui pour une possible protéine homologue à l'hémagglutinine filamenteuse FhaB présente chez les bactéries du genre *Bordetella*. FhaB permet l'adhésion aux carbohydrates, au sulphate d'héparane ainsi aux protéines de la famille des intégrines. FHA est l'adhésine la plus importante de *Bordetella pertussis*, et chez *Bordetella bronchiseptica*, un autre pathogène des voies respiratoires porcines, une délétion du gène *fhaB* cause une diminution de la colonisation à tous les niveaux du tractus respiratoire en plus de causer l'atténuation de la bactérie (Nicholson et al. 2009). Fait

notable, l'adhésion médiée par FHA peut, chez *B. pertussis*, mener à deux différents types d'infection : alors que l'adhésion aux cellules ciliées conduit à l'apparition de pathologie et de lésions pulmonaires, l'adhésion à l'intégrine CR3 des macrophages permet l'internalisation par les monocytes de *B. pertussis* en absence de poussée oxydative, aidant ainsi la bactérie à persister chez son hôte (Saukkonen et al. 1991). *A. pleuropneumoniae* peut aussi persister chez son hôte. La bactérie est extrêmement toxique pour les macrophages alvéolaires porcins via l'action combinée des toxines Apx et des LPS (Ramjeet et al. 2008a). Néanmoins, les macrophages alvéolaires porcins peuvent internaliser *A. pleuropneumoniae*, mais contrairement à ce qui se produit lorsque la bactérie est phagocytée par des cellules polymorphonucléaires (PMN), les macrophages porcins ne tuent pas *A. pleuropneumoniae* (Cruijsen et al. 1992), ce qui pourrait suggérer qu'un mécanisme similaire à celui retrouvé chez *B. pertussis* est utilisé. Autre fait intéressant, la maturation du pro-peptide FhaB en adhésine FHA active nécessite le clivage par une sérine protéase de type subtilysine nommé SphB1 (Coutte et al. 2001). La sérine protéase putative Ssa1/AasP, identifiée lors de l'établissement du profil transcriptomique d'*A. pleuropneumoniae* en présence d'une concentration limitante en fer (Article 1), appartiendrait aussi à cette famille de protéases.

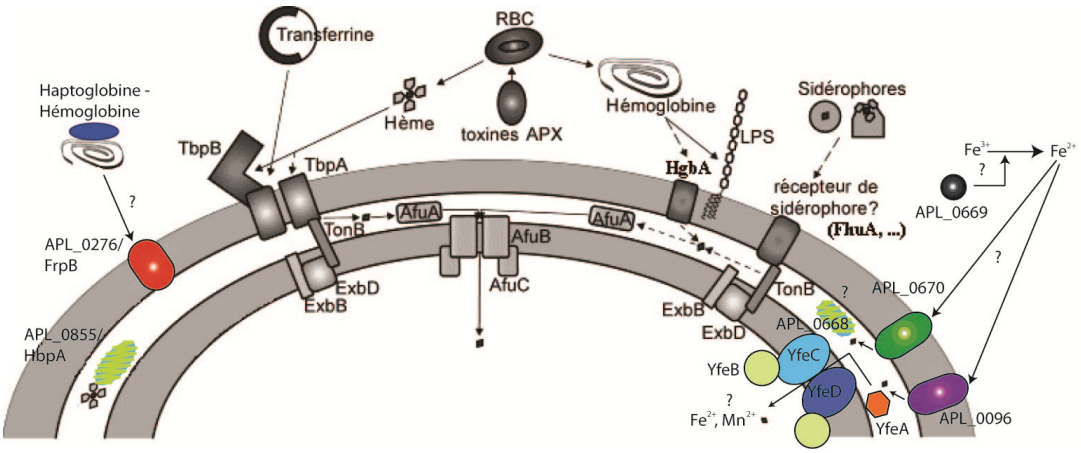
Le gène *APL_0959* partage une autre caractéristique intéressante avec deux autres gènes identifiés comme étant surexprimés lors de l'infection aiguë chez l'hôte, soit les gènes *irp* et *APL_0920* : ces trois gènes codent pour des protéines (*APL_0959* et *irp*) ou lipoprotéines (*APL_0920*) de surface d'*A. pleuropneumoniae* (Chung et al. 2007) pour lesquelles les séquences génétiques sont bien conservées entre les différents sérotypes de la bactérie (Gouré et al. 2009) (Annexe 2). Les gènes *irp* et *APL_0920* sont adjacents dans le génome d'*A. pleuropneumoniae*, bien que transcrits dans des directions opposées. Les protéines encodées par ces gènes seraient donc d'intéressantes cibles potentielles pour l'élaboration de vaccins sous-unitaires efficaces contre tous les sérotypes d'*A. pleuropneumoniae*. *B. pertussis* est la bactérie responsable de la coqueluche, et un vaccin basé sur deux protéines, FHA et la

pertactine, a amené une diminution drastique des cas de coqueluche recensés chez les enfants en bas âge (CDC 1991).

Mécanisme d'acquisition du fer chez *A. pleuropneumoniae* : mise à jour

Les travaux de profils transcriptionnels ont mené à l'identification de nombreux nouveaux facteurs de virulence potentiels chez *A. pleuropneumoniae*, et parmis ceux-ci on retrouve plusieurs mécanisme potentiels pour l'acquisition du fer. Ces nouveaux mécanismes ont été combinés à ceux déjà connus pour générer un nouveau schéma global des stratégies utilisées par *A. pleuropneumoniae* (Figure 2).

Figure 2 : Représentation schématique des différents mécanismes d'acquisition du fer connus et potentiellement identifiés suite à nos expériences. RBC : « Red Blood Cell », globules rouges. Adapté de Bossé et al. 2002.



Ont été ajoutés sur ce schéma :

- APL_0668–APL_0669–APL_0670, respectivement une lipoprotéine périplasmique impliquée dans le transport du fer, une porine specifique pour le transport du fer ferreux, et une peroxidase dépendante du fer. La peroxidase, une fois sécrétée dans le milieu externe, pourrait catalyser une réaction au cours de laquelle le fer ferrique (Fe^{3+}) serait réduit en fer ferreux (Fe^{2+}), devenant accessible pour le porine encodée par le gène APL_0669.
- APL_0096, une perméase spécifique pour le plomb et le Fe^{2+}.
- APL_0276/FrpB, une protéine de la membrane externe.

- APL_0855/HbpA, une lipoprotéine périplasmique impliquée dans le transport de l'hème.
- YfeABCD, un possible système de protéines de la membrane cytoplasmique permettant le transport vers le cytoplasme d'ions Fe^{2+} et Mn^{2+}.

Il est à noter que ces fonctions sont attribuées uniquement sur la base d'homologies de séquences avec des protéines connues, et qu'une caractérisation plus précise pourrait permettre de confirmer ou d'inférer ces rôles putatifs.

Comparaison des conditions de croissance

Au cours des différentes expériences de profil transcriptomique réalisées avec *A. pleuropneumoniae* dans le cadre de cette thèse de doctorat, la bactérie a été soumise à six conditions de croissance différentes. Alors que l'évaluation de la réponse spécifique à chaque condition permet certaines déductions ainsi que l'élaboration de certaines hypothèses quant à la réponse *in vivo* du pathogène, nous avions aussi comme hypothèse de départ que la transcription de certains gènes devrait être stimulée par plus d'une de ces conditions de croissance. Et comme notre hypothèse de départ sous-entendait aussi que les conditions testées simulaient adéquatement les conditions rencontrées *in vivo*, il en découle que nous nous attendions à pouvoir retrouver certains gènes qui soient surexprimés ou réprimés dans toutes les conditions. Néanmoins, c'est plutôt le phénomène inverse qui ressort : bien peu de gènes semblent montrer des profils similaires entre les différentes conditions testées. En effet, en comparant les différentes conditions deux par deux, on remarque que dans la très grande majorité des cas le nombre de gènes réprimés ou surexprimés qui sont communs est inférieur à 10 (Tableau 1). Des diagrammes de Venne ont aussi été élaborés afin de recenser le nombre de gènes qui sont surexprimés ou réprimés dans plus de deux conditions. Dans les meilleurs cas, certains gènes sont retrouvés dans trois conditions différentes (8 occurrences au niveau de la surexpression, 1 occurrence au niveau de la répression) (Tableau 2).

Tableau 1 : Comparaison du nombre de gènes qui sont réprimés (en vert) ou surexprimés (rouge) entre différents couples de conditions testées.

		RÉPRESSION					
		Fer	Oxoid	BALF	Planctonique	Adhérence	*In Vivo*
SUREXPRESSION	Fer		10	3	2	12	5
	Oxoid	2		0	11	1	4
	BALF	3	1		2	0	0
	Planctonique	2	19	5		0	9
	Adhérence	0	6	5	0		5
	In Vivo	1	9	4	2	8	

Figure 3 : Diagramme de Venne représentant les gènes qui sont surexprimés dans plusieurs conditions expérimentales.

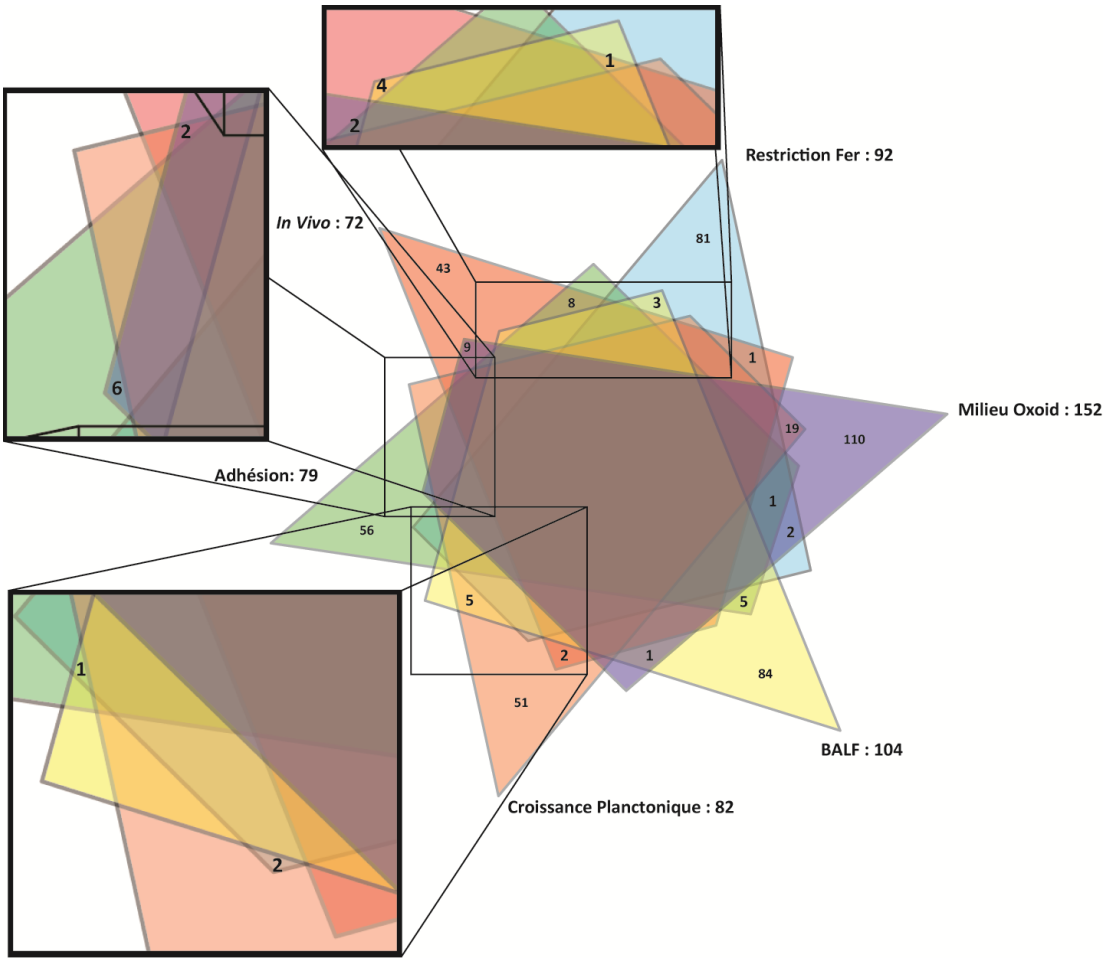


Figure 4 : Diagramme de Venne représentant les gènes qui sont réprimés dans plusieurs conditions expérimentales.

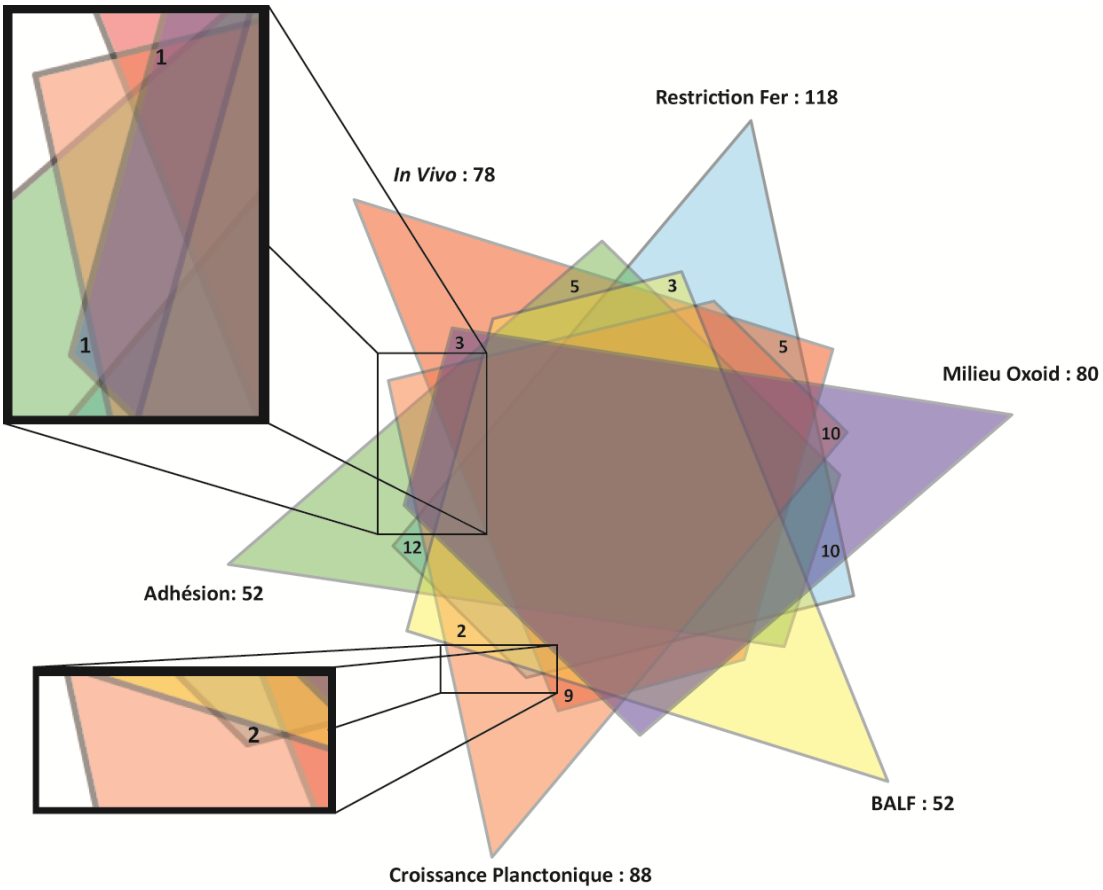


Tableau 2 : Liste des gènes surexprimés ou réprimés dans trois conditions différentes

Gènes	Fonction	Variation	Conditions
exbB2/exbD2/tonB2	Système TonB2, transfert d'énergie à des récepteurs de la membrane externe	↑	Fer, BALF, Adhérence
ilvC	Réductoisomérase kétol-acide	↑	Fer, Oxoid, Adhérence
APL_0668	Protéine hypothétique	↑	Fer, Balf, *In vivo*
ftpA	Sous-unité majeure, pili	↑	Oxoid, Planctonique, *In vivo*
glpR	Répresseur, régulon glycérol-3-phosphate	↑	Oxoid, Planctonique, *In vivo*
leuC	Isopropylmalate isomérase	↑	Oxoid, Adhérence, *In vivo*
APL_0870	Transporteur putatif pour dicarboxylate-C4	↑	Oxoid, Adhérence, *In vivo*
fdnI	Sous-unité cytochrome b556, formate déhydrogénase	↑	Oxoid, Planctonique, Adhésion
rpsM	Protéine S4, sous-unité ribosomal 30S	↓	Oxoid, Planctonique, *In vivo*

Tableau 3 : Liste des gènes surexprimés ou réprimés dans deux conditions différentes

Gènes	Fonction	Variation	Conditions
dppC	Perméase, transport de dipeptides	↑	Oxoid, Adhésion
APL_0096	Tranporteur putatif de zinc	↑	Oxoid, *In vivo*
ilvG	Acéto lactate synthase II	↑	Oxoid, Adhésion
nrfB	Nitrite réductase	↑	Balf, Planctonique
APL_0126	Protéine "HIT-like"	↑	Fer, Oxoid
APL_0220	Lipoprotéine putative	↑	Oxoid, *In vivo*
APL_0270	Protéine hypothétique	↑	Fer, Balf
glpK	Glycérol kinase	↑	Planctonique, *In vivo*
glpT	Transporteur glycérol-3-phosphate	↑	Oxoid, Planctonique
rseA	Régulateur négatif facteur sigma ε	↑	Oxoid, Planctonique
APL_0443	Adhésine autotransporteur Hsf	↑	Oxoid, Planctonique
sucC	Succinyl-CoA synthétase, chaîne β	↑	Oxoid, *In vivo*
APL_0483	Nitroréductase A	↑	Oxoid, Planctonique
rimK	Protéine ribosomale S6	↑	Oxoid, Planctonique
tadB,tadC	Locus d'adhérence *tad*	↑	Oxoid, Adhésion
rhlB	Hélicase d'ARN ATP-dépendante	↑	Oxoid, Planctonique
deaD	Protéine A, choc thermique (froid)	↑	Balf, Adhésion
dld	D-lactate déhydrogénase	↑	Oxoid, Planctonique
APL_0717	Transporteur ABC Fer(III), liaison ATP	↑	Fer, Balf
prsA	Pyrophosphokinae de ribose-phosphate	↑	Adhésion, *In vivo*
ipk	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	↑	Adhésion, *In vivo*
abgB	Protéine d'utilisation du aminobenzoyl-glutamate	↑	Oxoid, *In vivo*
pepE	peptidase E	↑	Oxoid, Planctonique
holA	ADN polymérase III, sous-unité δ	↑	Balf, Adhésion
fdxG	Formate déhydrogénase	↑	Oxoid, Planctonique
fdxH	Formate déhydrogénase, sous-unité fer-soufre	↑	Oxoid, Planctonique
APL_0920	Protéine hypothétique	↑	Oxoid, *In vivo*
apxIVA	Toxine ApxIV, sous-unité structurale	↑	Balf, *In vivo*
kdgK	Protéine hypothétique	↑	Adhésion, *In vivo*
APL_1021	α-glucosidase 2	↑	Balf, Planctonique
APL_1044	Protéine hypothétique	↑	Fer, *In vivo*
pnuC	Transporteur de mononucléotide nicotinamide	↑	Oxoid, Planctonique
namA	NADPH-déhydrogénase	↑	Oxoid, Adhésion
APL_1197	Possible NADHP-nitroréductase	↑	Oxoid, Planctonique
APL_1220	Protéine régulée par LexA	↑	Fer, Balf

malF	Transporteur de maltose	↑	Balf, *In vivo*
malG	Transporteur de maltose	↑	Balf, *In vivo*
sapF	Transporteur de peptides	↑	Balf, Adhésion
APL_1285	Protéine hypothétique	↑	Fer, Planctonique
ptsB	Système PTS sucrose-spécifique	↑	Oxoid, Planctonique
hyaA	Hydrogénase 2, petite sous-unité	↑	Balf, Adhésion
ccmB	Exporter d'héme, biosynthèse de cytochromes	↑	Balf, Planctonique
APL_1374	Protéine hypothétique	↑	Oxoid, Planctonique
ccp	Cytochrome c péroxidase	↑	Balf, Planctonique
trmB	ARNt (guanine-méthyltransférase)	↑	Balf, Adhésion
napH	Composante membranaire de la quinol déhydrogénase	↑	Oxoid, Balf
APL_1437	Protéine hypothétique	↑	Fer, Oxoid
APL_1527	Sous-unité c, fumarate réductase	↑	Planctonique, *In vivo*
mrdA	Protéine liant la pénicilline 2	↑	Adhésion, *In vivo*
cbiO	Transport ABC, protéine liant l' ATP	↑	Oxoid, Planctonique
cbiM	Protéine de transport du cobalt	↑	Oxoid, Planctonique
gntP-1	Perméase pour le gluconate	↑	Oxoid, *In vivo*
dmsA	Diméthylsulfoxide réductase anaérobique, chaîne A	↑	Balf, Planctonique
fucI	L-fucose isomérase	↑	Adhésion, *In vivo*
APL_1689	Oxidoréductase de L-1,2-propanediol	↑	Adhésion, *In vivo*
APL_1690	Protéine de la membrane interne	↑	Oxoid, Adhésion
APL_1832	Possible sulfite oxidase	↑	Fer, Planctonique
cysA	Import sulfate/thiosulfate, protéine liant l'ATP	↑	Adhésion, *In vivo*
APL_1881	Protéine hypothétique	↑	Oxoid, Planctonique
pgaB	Lipoprotéine synthétisant le PGA	↑	Oxoid, Adhésion
pgaC	N-glycosyltransférase	↑	Oxoid, Adhésion
APL_1934	Protéine hypothétique	↑	Oxoid, *In vivo*
hisH	Imidazole glycérol-phosphate synthase	↑	Oxoid, *In vivo*
hisA	imidazole-4-carboxamide isomerase	↑	Oxoid, *In vivo*
typA	Protéine liant le GTP	↓	Oxoid, Planctonique
nrfB	Nitrite réductase	↓	Fer, Adhésion
APL_0319	Protéine hypothétique	↓	Fer, In vivo
glpK	Glycérol kinase	↓	Fer, Balf
ksgA	Diméthyladénosine transférase	↓	Oxoid, Planctonique
gapA	Glycéraldéhyde-3-phosphate déhydrogénase	↓	Fer, Adhésion
maeB	Enzyme malique NADP-dépendante	↓	Fer, Adhésion
nfnB	Possible NADPH-nitroréductase	↓	Fer, In vivo

mlc	Possible répresseur transcriptionnel du métabolisme des carbohydrates	↓	Fer, In vivo
truB	ARNt, pseudouridine synthase B	↓	Oxoid, Planctonique
APL_0669	Possible péroxidase fer-dépendante	↓	Oxoid, Planctonique
torZ	Triméthylamine-N-oxide réductase	↓	Fer, Adhésion
APL_0717	Transporteur ABC Fer(III), liaison ATP	↓	Oxoid, Planctonique
APL_0718	Protéine hypothétique	↓	Fer, Adhésion
sdaA	L-sérine déhydratase	↓	Fer, Oxoid
rlpB	Possible lipoprotéine rare B	↓	Oxoid, *In vivo*
APL_0885	Protéine hypothétique	↓	Fer, Balf
fdxG	Formate déhydrogénase	↓	Fer, Adhésion
fdxH	Formate déhydrogénase, sous-unité fer-soufre	↓	Fer, Adhésion
ompP1	Précurseur protéine de la membrane externe P1	↓	Oxoid, Planctonique
APL_0936	Protéine hypothétique	↓	Oxoid, Planctonique
adh2	Alcool déhydrogénase bifonctionnelle	↓	Fer, Adhésion
deoD	Phosphorylase, nucléosides purine	↓	Oxoid, Adhésion
APL_1028	Possible glucosyltransférase	↓	Fer, Oxoid
pflB	Formate acétyltransférase	↓	Fer, Oxoid
purA	Adénylsuccinate synthase	↓	Fer, Oxoid
ompW	Protéine de la membrane externe W	↓	Fer, Oxoid
aspA	Liase d'aspartate/ammoniaque	↓	Fer, Adhésion
purT	Phosphoribosylglycinamide formyltransferase 2	↓	Fer, Balf
fur	Régulateur acquisition du fer	↓	Planctonique, *In vivo*
pgk	Phosphoglycérate kinase	↓	Fer, Adhésion
APL_1253	Transporteur sodium/sulfate	↓	Fer, Oxoid
ccp	Cytochrome c péroxidase	↓	Fer, Adhésion
APL_1381	Protéine hypothétique	↓	Fer, Oxoid
APL_1382	Protéine hypothétique	↓	Oxoid, *In vivo*
APL_1383	Protéine hypothétique	↓	Oxoid, Planctonique
trmB	ARNt (guanine-méthyltransférase)	↓	Oxoid, Planctonique
APL_1391	Système PTS mannose-spécifique	↓	Fer, Planctonique
rpsL	Protéine ribosomale S12	↓	Planctonique, *In vivo*
apxIB	Translocation de la toxine ApxI	↓	Oxoid, Planctonique
aqpZ	Aquaporine Z	↓	Fer, In vivo
menA	1,4-dihydroxy-2-naphthoate octaprényltransférase	↓	Fer, Oxoid
dnaG	ADN primase	↓	Oxoid, *In vivo*
APL_1508	Possible sulfurtransférase	↓	Planctonique, *In vivo*
APL_1526	Fumarate réductase, sous-unité d	↓	Fer, Adhésion
rpsT	Protéine ribosomale S20	↓	Planctonique, *In vivo*

cpxC	Protéine de la membrane interne, export polysaccharides capsulaires	↓	Planctonique, *In vivo*
cpxB	Protéine de la membrane interne, export polysaccharides capsulaires	↓	Planctonique, *In vivo*
rpsM	Protéine ribosomale S13	↓	Oxoid, Planctonique
rpsK	Protéine ribosomale S11	↓	Oxoid, *In vivo*
udp	Uridine phosphorylase	↓	Fer, Oxoid
ubiC	4-hydroxybenzoate synthétase	↓	Fer, Oxoid
APL_1863	Glycosyltransférase	↓	Planctonique, *In vivo*
accC	Acétyl-CoA carboxylase	↓	Fer, Planctonique
mscS	Canal méchano-sensitif à faible conductance	↓	Fer, In vivo
rpmG	Protéine ribosomale L33	↓	Planctonique, *In vivo*

Clairement, ces données valident l'affirmation selon laquelle aucune condition expérimentale ne peut parfaitement reproduire parfaitement les conditions rencontrées chez l'hôte. En fait, en se fiant uniquement aux chiffres présents dans le Tableau 1, on pourrait croire que la croissance en milieu Oxoid est aussi représentative de l'infection aiguë que l'adhésion directe aux cellules épithéliales pulmonaires avec 13 gènes communs avec la condition *in vivo* chacun. En réalité, ces données indiquent probablement qu'en plus de tenir compte des limites des conditions *in vitro* par rapport aux conditions *in vivo* lors de l'élaboration d'expériences, il faut aussi se questionner sur le moment précis du processus infectieux que l'on veut investiguer. Ainsi, lors de nos expériences *in vivo*, les animaux ont été échantillonnés après l'apparition de lésions et d'hémorragies extensives au niveau des poumons. Dans ces conditions, les bactéries présentes se trouvent probablement en présence d'une grande abondance d'éléments nutritifs, ce qui inclut le fer. Ceci signifie aussi que les bactéries ont depuis longtemps traversé la barrière créée par les cellules épithéliales pulmonaires et le liquide broncho-alvéolaire. La présence de biofilms dans le milieu Oxoid explique probablement aussi pourquoi plusieurs gènes qui sont surexprimés dans ce milieu sont aussi surexprimés lors de la croissance planctonique au-dessus des cellules épithéliales, lors de l'adhérence directe à ces cellules ainsi qu'*in vivo*. De fait, alors que nous avons pu déterminer le transcriptome de la bactérie directement *in vivo* lors de l'infection aiguë, deux autres étapes du processus infectieux ont possiblement pu être

simulées lors de nos expériences *in vitro* : alors que la culture en présence de BALF permet possiblement de bien refléter les conditions auxquelles doivent faire face les bactéries lors de leur entrée chez l'hôte, l'adhésion aux cellules épithéliales pulmonaires a probablement pu, quant à elle, reproduire en partie les évènements transcriptomiques se produisant lors de l'adhésion initiale menant à l'établissement de l'infection.

La liste des gènes présentant des variations similaires dans trois conditions est néanmoins intéressante. De cette liste, cinq des huits gènes surexprimés ont un lien confirmé avec la virulence. Ainsi, les gènes de l'opéron *exbB2-exbD2-tonB2* ainsi que le gène *APL_0668* sont tous nécessaires à l'acquisition du fer essentiel à la survie d'*A. pleuropneumoniae*. Le gène *APL_0668*, par ailleurs, bien qu'il n'ait jamais été identifié avant les expériences menées en présence d'une concentration limitante en fer (Article 1), semble être important pour *A. pleuropneumoniae* à divers stades de l'infection : sa surexpression en présence de BALF indique que ce gène pourrait possiblement être surexprimé rapidement lors de l'entrée chez l'hôte porcin. De plus, le fait qu'il soit ensuite retrouvé en phase terminale de l'infection aiguë indique que l'expression de ce gène persiste alors que l'infection progresse. Le gène *APL_0668* est conservé chez toutes les souches de référence d'*A. pleuropneumoniae* ainsi que chez de nombreuses souches de champ fraîchement isolées (Gouré et al. 2009), de même que les deux autres gènes composants ce système à l'exception de APL_0670, qui ne diverge que chez la souche de référence du sérotype 10.

Pour leur part, les gènes *ilvC* et *leuC* codent tous deux pour des enzymes impliquées dans la biosynthèse des acides aminés à chaînes ramifiés. Finalement, le gène *ftpA* code possiblement pour une sous-unité majeure de pili. Malgré ce qu'indique son annotation, la fonction réelle de la protéine codée par *ftpA* est relativement floue. D'abord identifié chez *Haemophilus ducreyi*, le peptide synthétisé par *ftpA* présente une section N-terminale dont la séquence est identique à celle de piline purifiée, mais dont la section C-terminale est plutôt semblable à la protéine *Dps* d'*E.coli* (Brentjens et al. 1996). La protéine Dps a la particularité de former des structures en anneaux

ordonnés, mais la fonction qu'elle accomplit chez *E. coli* n'a strictement rien à voir avec la fonction d'un pili classique. Chez *E. coli*, Dps séquestre le fer afin de protéger l'ADN bactérien lors de stress oxydatifs (Martinez et al. 1997). Des recherches subséquentes chez *H. ducreyi* ont démontré qu'un mutant *ftpA* n'est pas atténué lors d'infections expérimentales, mettant ainsi en doute le rôle du « pilus » FtpA dans la virulence de la bactérie (Al-Tawfiq et al. 2000). Néanmoins, la surexpression de ce « pilus » est intrigante chez *A. pleuropneumoniae*, alors qu'elle survient dans trois conditions où la bactérie ne fait pas nécessairement face à un stress oxydatif. De plus, FtpA ne fait pas partie de la liste des protéines *d'A. pleuropneumoniae* dont la localisation prédite se situe au niveau de la membrane externe (Chung et al. 2007), où sont généralement localisés les pili. Les trois autres gènes surexprimés, soit les gènes *glpR*, *fdnI* et *APL_0870*, ont surtout des rôles au niveau métabolique. Malgré que GlpR soit un régulateur, il ne semble exister aucun lien entre les gènes qu'il régule, qui sont impliqués dans le transport et l'utilisation du glycérol (Lin 1976), et la pathogénicité.

CONCLUSION

C'est souvent le propre des expériences transcriptomiques de soulever plus d'hypothèses que d'apporter des réponses précises sur les mécanismes bactériens entourant la virulence. Néanmoins, les expériences que nous avons menées ont permis d'acquérir une meilleure compréhension du mode de vie général d'*A. pleuropneumoniae* face à diverses conditions de croissance *in vitro*, ainsi que lors de la phase aiguë de l'infection chez son hôte naturel. Dans un premier temps, la croissance d'*A. pleuropneumoniae* en conditions limitantes en fer a permis l'identification de nouveaux systèmes potentiels pour l'acquisition du fer chez cette bactérie. Les expériences avec un milieu stimulant la production de biofilms ainsi que celles menées en présence de cellules épithéliales pulmonaires ont permis de mettre en évidence certains mécanismes d'adhésion non-spécifiques (*tad* et *pga*) potentiellement importants pour l'adhésion chez *A. pleuropneumoniae*, mais cette dernière condition a aussi permis d'observer la surexpression d'une adhésine autotransporteur potentiellement importante (*hsf*). La croissance de la bactérie en présence de liquides de lavages broncho-alvéolaires a probablement permis de reproduire en partie les conditions rencontrées initialement par la bactérie *in vivo*, alors que le profil transcriptomique établi suite à l'infection aiguë a permis d'observer les évènements transcriptomiques se produisant alors que l'infection évolue vers une conclusion plus dramatique.

Notre objectif principal, au début du projet, était de parvenir à établir une liste de gènes surexprimés dans plusieurs conditions simulant l'infection ainsi qu'*in vivo* chez l'animal. Ces gènes se devaient en plus d'être conservés à travers les différents sérotypes d'*A. pleuropneumoniae*, et coder pour des protéines ou lipoprotéines localisées dans la membrane externe, afin que les produits de ces gènes soient identifiés comme de bonnes cibles pour l'élaboration de vaccins sous-unitaires efficaces contre tous les sérotypes. À ce chapitre, force est de constater que, malgré que certaines conditions testées reflètent possiblement bien les conditions rencontrées *in vivo* à un moment précis, les environnements rencontrés successivement *in vivo* par la bactérie sont eux-mêmes probablement variables. Ainsi, aucun gène n'a pu être identifié comme étant surexprimé dans toutes les

conditions de croissance rencontrées. Néanmoins, certains candidats intéressants peuvent être identifiés. À la lumière des résultats obtenus, il semble que la formation de biofilm soit importante pour l'adhésion de la bactérie aux cellules épithéliales de l'hôte. Ce mécanisme d'adhésion non-spécifique est possiblement un mécanisme secondaire permettant de rendre plus forte une première interaction via, cette fois, des récepteurs spécifiques. Pour cette adhésion initiale, nous proposons que la protéine autotransporteur Hsf ainsi que l'hémagglutinine filamenteuse putative APL_0959/FhaB jouent un rôle important pour *A. pleuropneumoniae*, et que cette adhésion nécessite probablement la présence d'une capsule polysaccharidique plus mince en surface de la bactérie. Du moins, c'est ce que la baisse de transcription des gènes *cpxAB*, lors de la croissance au-dessus des cellules épithéliales, et *cpxA/cps5b*, *in vivo* lors de l'infection aiguë, semble indiquer. Bien que le gène codant pour la protéine putative FhaB n'ait pas été identifié lors des expériences d'infections des cellules épithéliales pulmonaires en culture, l'éventail des ligands qui lui sont connus chez *B. pertussis* démontre bien la versatilité de cette adhésine. Il est aussi utile de préciser que, depuis la réalisation de ces expériences, des doutes ont été soulevées quant à l'origine réelle des cellules SJPL. De plus, autant le gène *hsf* que le gène *APL_0959* sont bien conservés parmis tous les sérotypes d'A. *pleuropneumoniae* et leur surexpression respective en présence de cellules épithéliales ainsi que *in vivo* fait en sorte que les protéines codées par ces gènes deviennent attrayantes pour l'élaboration de vaccins. Les mêmes caractéristiques s'appliquent aussi aux gènes *irp* et *APL_0920*, sauf que les gènes *hsf* et *APL_0959* présentent un avantage non-négligeable puisque les produits encodées par ces gènes ciblent potentiellement une étape cruciale pour l'établissement de l'infection : la colonisation.

PERSPECTIVES

Au cours de ce projet, plusieurs gènes d'intérêt ont été identifiés et pour lesquels des recherches plus approfondies seraient de mise. Dans un premier temps, pour les gènes dont les produits sont possiblement impliqués dans l'adhésion, la génération de mutants pour ces gènes serait requise afin de vérifier si :

- la capacité de former des biofilms est altérée chez les mutants : la formation des biofilms pourrait être vérifiée après croissance en plaques 96 puits, après coloration au crystal violet, et la morphologie observée par microscopie confocale suite au marquage par la « Wheat Germ Agglutinin » (WGA) fluorescente liant les lectines le polysaccharide PGA
- l'élimination des produits codés par ces gènes entraînent un changement au niveau des interactions des bactéries avec les cellules SJPL et NPTr
- l'élimination des produits par ces gènes affectent la capacité de la bactérie à s'établir et persister chez l'animal

Plusieurs gènes pourraient ainsi être criblés, soient les gènes *pgaABC*, les gènes de l'opéron *tad*, les gènes impliqués dans la biogénèse des pili de type IV (*apfABCD*), le gène *hsf* ainsi que *APL_0959/fhaB*.

Dans le cas de ce dernier gène, son homologue chez *B. pertussis* fait partie d'un système relativement complexe nécessitant la présence de la sérine protéase autotransporteur de type subtilysine SphB1, et l'export se fait via un système de sécrétion à deux partenaires nécessitant la formation d'un pore spécifique créé par la protéine FhaC. Chez *A. pleuropneumoniae*, une sérine protéase autotransporteur a aussi été identifiée et nommée AasP. Il serait probablement intéressant de vérifier si un lien existe entre le produit du gène *APL_0959/fhaB* et l'activité de la protéine AasP. La maturation de FHA par SphB1 implique, chez *B. pertussis*, un clivage qui permet à la section C-terminale de la protéine d'être affichée en surface de la bactérie (Mazar et al. 2006). Néanmoins, une certaine quantité de la protéine est aussi relarguée dans le milieu externe (Coutte et al. 2001). Bien entendu, il faudrait avant tout s'assurer que *A. pleuropneumoniae* porte bel et bien à sa surface une protéine

homologue à FHA. Des expériences d'extraction de protéines de la membrane externe d'*A. pleuropneumoniae* sérotype 1 après culture dans différentes conditions, suivit d'une caractérisation par chromatographie en phase liquide et par spectrophotométrie de masse des protéines obtenues n'a pas permis d'identifier la protéine codée par *APL_0959* (Chung et al. 2007). Par contre, le fait que, jusqu'à récemment, la toxine ApxIV n'ait pu être isolée qu'*in vivo* tend à suggérer que *A. pleuropneumoniae* régule très strictement l'expression de certains gènes de virulence. Le clonage du gène *APL_0959* et son expression chez *E.coli* avec une étiquette hexahistidine pourrait permettre d'isoler la protéine. Un immunoblot pourrait ensuite être effectué en utilisant du sérum provenant d'un porc ayant survécu à l'infection à *A. pleuropneumoniae* afin de vérifier si des anticorps sont générés, *in vivo*, contre la protéine codée par *APL_0959*.

Chez *B. pertussis*, une certaine quantité de l'adhésine FHA est relarguée dans le milieu externe. Afin de vérifier si SphB1 était responsable du clivage nécessaire à la maturation de FHA, un mutant Δ*sphB1* a été généré, et le profil des protéines sécrétées dans le milieu externe par le mutant a été comparé a celui de la souche sauvage par migration sur gel SDS-Page coloré au bleu de Coomassie (Coutte et al. 2001). Chez le mutant Δ*sphB1*, la protéine FHA était présente dans le milieu externe en quantités beaucoup plus faibles, et avec un poids moléculaire plus important que celui de la protéine mature. La même approche expérimentale pourrait être utilisée chez *A. pleuropneumoniae* afin de vérifier si AasP interragit avec APL_0959/FhaB. Dans cette optique, il serait alors intéressant de sonder le génome de *A. pleuropneumoniae* afin de vérifier s'il est possible de trouver un homologue pour la protéine FhaC.

Les deux autres gènes surexprimés *in vivo* dont les séquences sont conservées chez tous les sérotypes d'*A. pleuropneumoniae* et dont les produits sont aussi possiblement localisés au niveau de la membrane externe devraient aussi être caractérisés de manière plus précise. Ainsi, malgré le fait que le gène *APL_0919* porte le nom de *irp* pour « iron responsive protein », le niveau d'expression de ce

gène n'a pas varié de manière significative lors des expériences en présence d'une concentration limitante en fer. Selon l'annotation du gène, *irp* coderait pour un récepteur TonB-dépendant possiblement impliqué dans le transport de l'hémine. Malgré qu'aucun récepteur spécifique à l'hémine n'ait été identifié, certaines données montrent cependant que *A. pleuropneumoniae* peut lier ce composé (Deneer et al. 1989; Gerlach et al. 1992). Il est à noter que d'autres récepteurs potentiels de composés ferriques ont pu être identifiés au cours de nos expériences. Il serait intéressant de générer des mutants pour ces gènes, et de comparer, à l'aide des disques imbibés de différentes sources de fer sur gélose, le profil des composés pouvant être utilisés par ces souches comme sources de fer.

Afin de déterminer le potentiel immunogénique des protéines codées par les gènes *APL_0959*, *irp* et *APL_0920*, des expériences d'immunisation devraient aussi être effectuées. Ces protéines pourraient être utilisées de manière individuelles ou encore en combinaisons dans des formulations de vaccins sous-unitaires. Les données obtenues lors d'infections expérimentales avec des souches mutantes pour ces gènes pourraient également permettre de déterminer lesquelles parmi ces protéines sont les plus prometteuses pour l'élaboration d'un vaccin.

Dans un autre ordre d'idée, les données recueillies lors des expériences de profil transcriptomique *in vitro* et *in vivo* ont clairement démontré que l'utilisation de conditions expérimentales ne peut jamais représenter parfaitement l'environnement *in vivo*. De plus, l'environnement *in vivo* est lui-même changeant, du point de vue de la bactérie, lorsque l'infection progresse chez l'hôte. Alors que les expériences conduites dans le cadre de projet ont permis d'observer les changements transcriptomiques se produisant chez l'hôte lors de l'infection aiguë, d'autres stades de l'infection pourraient permettre de recueillir des données importantes afin de mieux comprendre le développement de la maladie chez l'hôte. Ainsi, il serait intéressant de regarder ce qui se produit lors d'infections chroniques, alors que les bactéries sont présentes au niveau des lésions pulmonaires. Il est peu probable cependant que ces expériences puissent être conduites avec des animaux

naturellement infectés, puisqu'en général les animaux atteints par la bactérie sont traités avec des antibiotiques dans les élevages porcins.

D'autres types d'expériences peuvent également être menées avec les biopuces à ADN. Chez *A. pleuropneumoniae*, l'emphase a été mise sur deux régulateurs transcriptionnels importants : Fur et HlyX. Malgré tout, l'étendue réelle des régulons Fur et HlyX et du nombre de gènes qu'ils régulent demeure relativement peu connue. Le profil transcriptomique observé lors de la croissance en conditions limitantes en fer permet d'avoir un aperçu des gènes potentiellement régulés directement ou indirectement par Fur sans nécessairement permettre d'identifier les gènes possédant réellement des boîtes Fur au niveau de leurs promoteurs. Il serait possible d'identifier les gènes qui sont régulés directement par Fur et HlyX en procédant à des expériences de « ChIP-on-Chip » (Chromatin Immuno-Precipitation on Chip), soient des expériences d'immuno-précipitation de chromatine sur biopuce. Le principe derrière les expériences de « ChIP-on-Chip » est relativement simple : des cellules contenant un régulateur transcriptionnel actif sont fixées avec du formaldéhyde, ce qui crée un lien entre l'ADN et les protéines qui y sont attachées (Sala et al. 2009). Par immunoprécipitation, un régulateur en particulier peut être précipité, entraînant avec lui les séquences d'ADN auxquelles il est lié. En marquant l'ADN et en hybridant sur biopuce, il devient possible de visualiser les gènes qui sont directement affecté par ce régulateur. En déterminant avec précision les gènes directement sous le contrôle de HlyX et Fur, il serait possible d'identifier quels facteurs de virulence sont sous le contrôle de ces régulateurs, mais aussi d'identifier d'autres régulateurs transcriptionnels potentiels. Les expériences de « ChIP-on-Chip » nécessitent que des anticorps dirigés contre le régulateur choisi existent, ou encore que le régulateur en question soit exprimé avec une étiquette hexahistidine et ensuite précipité via l'utilisation d'un anticorps dirigé contre cette étiquette.

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ANNEXES

Annexe 1

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Actinobacillus pleuropneumoniae vaccines: from bacterins to new insights into vaccination strategies

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Abstract

With the growing emergence of antibiotic resistance and rising consumer demands concerning food safety, vaccination to prevent bacterial infections is of increasing relevance. *Actinobacillus pleuropneumoniae* is the etiological agent of porcine pleuropneumonia, a respiratory disease leading to severe economic losses in the swine industry. Despite all the research and trials that were performed with *A. pleuropneumoniae* vaccination in the past, a safe vaccine that offers complete protection against all serotypes has yet not reached the market. However, recent advances made in the identification of new potential vaccine candidates and in the targeting of specific immune responses, give encouraging vaccination perspectives. Here we review past and current knowledge on *A. pleuropneumoniae* vaccines as well as the newly available genomic tools and vaccination strategies that could be useful in the design of an efficient vaccine against *A. pleuropneumoniae* infection.

Key words: *Actinobacillus pleuropneumoniae*, vaccine, pigs, bacterins, subunit, live attenuated

Introduction

Actinobacillus pleuropneumoniae is the major cause of porcine pleuropneumonia, a highly contagious respiratory disease responsible for major economic losses in the swine industry (Taylor, 1999). The disease is characterized by hemorrhagic, fibrinous, and necrotic lung lesions and the clinical features range from acute to chronic. Pigs surviving the disease often suffer from reduced growth rates and frequently become asymptomatic carriers of the pathogen (Moller *et al.*, 1993; Sidibe *et al.*, 1993) and is the main cause of bacterial dissemination (Taylor, 1999). To date, fifteen serotypes of *A. pleuropneumoniae* based on capsular antigens have been described (Dubreuil *et al.*, 2000; Blackall *et al.*, 2002); all serotypes are capable of causing disease, although differences in virulence have been described (Frey, 1995b; Jacobsen *et al.*, 1996). Several virulence factors are known for *A. pleuropneumoniae*, such as the Apx toxins (Frey, 1995a), the lipopolysaccharides (LPS) (Jacques, 1996; Ramjeet *et al.*, 2005), the capsule and various outer membrane proteins (OMPs) (Haesebrouck *et al.*, 1997; Jacques, 2004).

The economic importance of this disease in the swine industry has stimulated intensive research in the past years in the *A. pleuropneumoniae* vaccination field. Many studies have been reported and several vaccines have been commercialized but complete satisfaction has not been obtained in the protection of pigs against *A. pleuropneumoniae* infection (Backstrom, 1999; Haesebrouck *et al.*, 2004). The purpose of this review is to summarize and present current knowledge on the achievements realized in vaccination against *A. pleuropneumoniae*. We will focus our interest on the evolution of *A. pleuropneumoniae* vaccines from the first commercialized whole-cell bacterins to more promising ones such as subunit vaccines and live attenuated vaccines. We will also discuss and provide more information on the importance of the administration route, vaccine formulation and adjuvants in the stimulation of specific immune responses in order to provide good protection. Finally, we will highlight new promising strategies and new developments in the *A. pleuropneumoniae* vaccination field.

The limits of inactivated whole-cell bacterial vaccines

The so-called "first-generation" vaccines of whole-cell bacterins were the first commercialized vaccines against *A. pleuropneumoniae* infection and consisted of heat-killed bacteria or formalin-treated whole-cells. Inactivated whole-cell vaccines have the advantage of presenting a complex array of antigenic determinants to the immune system without any concern for reversion issues raised by live attenuated vaccines. In order to enhance the expression of immunogenic and protective antigens, bacteria can be grown in specific conditions mimicking the host environment, prior to bacterin preparation. Studies have shown that bacterins obtained from *A. pleuropneumoniae* serotype 10 grown in NAD-restricted conditions induced a better protection upon challenge (Van Overbeke *et al.*, 2003). However, the use of whole-cell bacterins as an *A. pleuropneumoniae* vaccine is limited as previous immunization and challenge experiments only showed partial protection with a slight reduction in mortality (Jolie *et al.*, 1995; Furesz *et al.*, 1997). The absence of secreted proteins such as the Apx toxins which are known to be highly immunogenic and essential for protection, might explain the limited protection observed with bacterins (Haga *et al.*, 1997; Seah *et al.*, 2002). The alteration of antigenic characters of certain bacteria-associated virulence factors by heat, irradiation or chemical treatments during bacterin preparation might also affect the efficacy of the vaccine (Haesebrouck *et al.*, 1997). Moreover, bacterins offer limited cross protection (Jolie *et al.*, 1995) and do not prevent initial infection and colonization, which facilitates the emergence of healthy carriers. Indeed, one major problem encountered in using bacterins as vaccines is that they confer only partial protection against the homologous serotype and generally do not confer protection against challenge with heterologous serotypes (Higgins *et al.*, 1985; Thacker and Mulks, 1988; Fenwick and Henry, 1994). The low efficacy of bacterins might also be related to the spectrum of immune responses induced, usually limited to humoral response (Furesz *et al.*, 1997), and the blood lymphocyte subset phenotypes displayed (Appleyard *et al.*, 2002), which do not reflect natural infection. In fact,

whole inactivated bacteria display no colonization of the respiratory tract, which is important for an effective immune stimulation.

New developments in inactivated whole-cell bacterial vaccines have shown a promising strategy in *A. pleuropneumoniae* vaccination in terms of antigen immunogenicity. Genetically-inactivated ghost vaccines are empty whole cell envelopes produced by controlled expression of bacteriophage PhiX174 lysis gene E (Witte *et al.*, 1990, 1992). Expression of this gene from a plasmid in Gram-negative bacteria leads to the formation of a protein E specific tunnel which subsequently results in the outflow of cytoplasmic contents without any physical or chemical denaturation of the bacterial surface structures (Witte *et al.*, 1990, 1992). Thus, bacterial ghosts have the advantage over bacterins of sharing functional and antigenic determinants with their living counterparts. Moreover, the activating potential of bacterial ghosts in the maturation and stimulation of immune cells has also been brought to light (Felnerova *et al.*, 2004). The use of this technology could offer some promising perspectives in vaccination, as recombinant ghost bacteria can be effectively used to enhance expression and delivery of antigens (Szostak *et al.*, 1996) in order to target a specific local immune response (Lubitz *et al.*, 1999; Lubitz, 2001; Jalava *et al.*, 2003; Riedmann *et al.*, 2007). Studies have shown that immunization with *A. pleuropneumoniae* bacterial ghosts is more effective than bacterin vaccination in protecting pigs against lung colonization and infection, and could therefore prevent development of healthy carriers (Katinger *et al.*, 1999; Hensel *et al.*, 2000). Moreover, a cross protective potential in those ghost vaccines has also been suggested (Huter *et al.*, 2000). Despite the partial protection observed with bacterins and the encouraging preliminary trials with the bacterial ghost system, the use of inactivated whole-cell bacteria as vaccines is still compromised by the fact that one main concern in *A. pleuropneumoniae* vaccination is cross protection. It has been shown that a pig that survives natural or experimental infections is immunized against all serotypes of *A. pleuropneumoniae* (Nielsen, 1984). These observations suggest the presence of highly immunogenic bacterial antigens common to all serotypes which are expressed only within the host. In this context, neither bacterins

nor bacterial ghosts seem to be suitable for effective protection as the main problem associated with the use of inactivated whole-cell bacteria is the *in vivo* environment expression which cannot be completely reproduced *in vitro* (Goethe *et al.*, 2000; Van Overbeke *et al.*, 2003). Consequently, recent research in the *A. pleuropneumoniae* vaccination field has mainly focused on finding antigens highly conserved among all serotypes which could be purified and used as potential subunit vaccines, and also in the development of live attenuated mutants in order to overcome the problem of failure of cross protection.

Virulence factors of *A. pleuropneumoniae* and subunit vaccine candidates

Many virulence factors of *A. pleuropneumoniae* have been investigated for their protective potential (Table 1). In order to find candidates for the development of subunit vaccines, studies had first targeted the most accessible structures of the bacteria. Hence, components of the bacterial surface such as the capsule, LPS and several outer membrane proteins were first identified as potential vaccine candidates. An anionic fraction of a saline extact of *A. pleuropneumoniae* serotype 1 (ANEX) that contained polysaccharide, lipopolysaccharide and proteins antigens showed protective immunity in pigs when combined with an effective adjuvant (Willson *et al.*, 1995). However, the major difficulties encountered with the capsule and LPS are their high heterogeneity among the serotypes (Perry *et al.*, 1990; Dubreuil *et al.*, 2000). Thus, vaccination with these bacterial components would fail to confer good protection against heterologous serotypes. Pigs and mice immunized with LPS were previously found to be partially protected upon homologous challenge with *A. pleuropneumoniae* serotype 1 (Rioux *et al.*, 1997, 1998) while cross-serotype challenge experiments in mice vaccinated with LPS showed no protection (Rioux *et al.*, 1997). Passive immunization of mice with monoclonal antibodies directed against LPS also failed to provide protection against the heterologous serotype of *A. pleuropneumoniae* (Saze *et al.*, 1994). Moreover, pigs immunized with purified LPS or capsule were not protected against challenge with the homologous *A. pleuropneumoniae* serotype 5 (Inzana *et al.*, 1988), and other studies also showed

that neither the capsule nor the LPS seemed to be directly correlated with protection of mice in *A. pleuropneumoniae* challenge experiments (Byrd and Hooke, 1997). Consequently, research on subunit vaccines has mostly focused on finding conserved antigens such as OMPs and lipoproteins.

Although OMP profiles differ for most serotypes of *A. pleuropneumoniae* (Rapp *et al.*, 1986), a few OMPs were characterized at a molecular level and found to be present in almost all *A. pleuropneumoniae* serotypes. These include the transferrin-binding protein TfbA (or TbpB) (Gonzalez *et al.*, 1990; Gerlach *et al.*, 1992b), a 42-kDa maltose-inducible protein (Deneer and Potter, 1989), the 14-kDa peptidoglycan-associated lipoprotein PalA (Frey *et al.*, 1996), and the 50-kDa lipoprotein OmlA (Gerlach *et al.*, 1993). Many low molecular-mass OMPs of *A. pleuropneumoniae* were also detected using Surface Enhanced Laser Desorption Ionisation (SELDI) - ProteinChip™ technology. In fact, SELDI was shown to be a useful complementary approach to conventional proteomic analytical methods with *A. pleuropneumoniae*, particularly suitable for analysis of proteins in the <20 kDa mass range (Hodgetts *et al.*, 2004). Among the bacterial surface components, lipoproteins are also known to be highly immunogenic and protective. A novel method using a mild detergent treatment was developed to enhance the release of immunogenic lipoproteins from the outer membrane in culture supernatant without bacterial lysis. The main advantage of this extraction method is that the resulting cell-free supernatant (CFS) can then be used as a non-recombinant subunit vaccine (Goethe *et al.*, 2000). Thus, a subunit vaccine based on detergent-prepared CFS from *A. pleuropneumoniae* serotypes 1, 2 and 5 grown under iron-restricted conditions showed good protective activity and cross protection between serotypes 2 and 9 (Maas *et al.*, 2006b). However, experiments to assess the potential capacity of OMPs and lipoproteins to induce protective immunity were mostly restricted to immunoblot analysis with convalescent sera, while many other immunogenic OMPs were only identified by their molecular mass without any further characterization (Cruz *et al.*, 1996). For example, the outer membrane lipoprotein PalA which was previously identified as a potential vaccine candidate based on its reactivity with pig immune sera (Frey *et al.*,

1996), was later found to have a negative effect on protective immunity against *A. pleuropneumoniae* in vaccinated pigs (Van Den Bosch and Frey, 2003).

The iron acquisition systems of *A. pleuropneumoniae* include several important uptake systems such as uptake of transferrin, hemoglobin, and ferrichrome, a hydroxamate siderophore (Jacques, 2004). Not only is iron essential for survival of the bacteria but iron restriction is also an important signal controlling the expression of many genes including some coding for virulence factors (Deslandes *et al.*, 2007). Proteins involved in iron uptake are therefore potential candidates for the development of subunit vaccines and were investigated for their protective capacities. Three different transferrin-binding proteins B (TbpB) of 60, 62 and 65 kDa were identified among all *A. pleuropneumoniae* serotypes. Immunisation of pigs with the 60 kDa Tbp conferred limited protection against challenge with the homologous strain (Gerlach *et al.*, 1992a; Rossi-Campos *et al.*, 1992). An acellular pentavalent subunit vaccine (Pleurostar™ Novartis) was prepared with recombinant antigens from *A. pleuropneumoniae* and contains the transferrin-binding protein B of *A. pleuropneumoniae* serotype 7. This vaccine showed partial protection against severe challenge with *A. pleuropneumoniae* serotype 9 (Van Overbeke *et al.*, 2001). FhuA and HgbA, receptors for ferrichrome and hemoglobin, respectively, were also shown to be conserved among all serotypes and biotypes of *A. pleuropneumoniae* (Mikael *et al.*, 2002, 2003; Srikumar *et al.*, 2004; Shakarji *et al.*, 2006). Pig infection experiments have highlighted the role of HgbA as an important virulence factor which could be of interest as a potential subunit vaccine (Shakarji *et al.*, 2006).

Apx toxins are secreted toxins, members of the RTX toxins family. They represent major virulence factors of *A. pleuropneumoniae* and are known to be strongly immunogenic. The importance of Apx toxins in protective immunity against porcine pleuropneumonia was demonstrated in many studies (Inzana *et al.*, 1991). It has been shown that neutralizing antibodies directed against Apx toxins protected neutrophils from being killed and consequently allowed them to efficiently clear the ingested bacteria (Cruijsen *et al.*, 1992; Jansen, 1994). Protection of vaccinated pigs against

an aerosol challenge with *A. pleuropneumoniae* serotype 1 has been shown to be correlated with the presence of IgG1 subclass anti-hemolysin (Furesz *et al.*, 1998). A hemolysin vaccine made of purified ApxI and ApxII showed good protective activity in pigs against *A. pleuropneumoniae* serotype 1 (Haga *et al.*, 1997) while the N-terminal fragment of ApxI was shown to elicit good protection in mice against various serotypes of *A. pleuropneumoniae* (Seah *et al.*, 2002). N- and C-terminal domains as well as the activation domain of the RTX toxin ApxIII also displayed potential for further vaccination trials as pig antisera raised against those fragments expressed cytotoxin-neutralizing activities (Seah and Kwang, 2004). Immunization experiments with Apx toxins in combination with other bacterial compounds all showed that Apx toxins were essential vaccine components to confer protection against bacterial challenge (Byrd and Kadis, 1992; Van Den Bosch *et al.*, 1992; Beaudet *et al.*, 1994; Jansen, 1994; Frey, 1995a; Madsen *et al.*, 1995). Thus far, almost all commercially available *A. pleuropneumoniae* subunit vaccines known as "second-generation" vaccines contain Apx toxins (Chiers *et al.*, 1998; Van Overbeke *et al.*, 2001; Habrun *et al.*, 2002; Van Den Bosch and Frey, 2003; Tumamao *et al.*, 2004; Meeusen *et al.*, 2007).

Generally, traditional vaccine extracts are enriched for secreted or surface-exposed bacterial components, as shown above. However, internal proteins that are involved in cellular metabolism are also reported to induce a protective immunity in other systems despite their predicted periplasmic and cytoplasmic localization (Mosier *et al.*, 1998; Thomas *et al.*, 2000). The NADPH-sulfite reductase hemoprotein CysI of *A. pleuropneumoniae* was shown to be protective when tested as a subunit vaccine, as immunized pigs showed lower mortality and reduced clinical signs after challenge with virulent *A. pleuropneumoniae* (Willson *et al.*, 2001).

Many virulence factors of *A. pleuropneumoniae* alone or more often as a cocktail, have been tested as subunit vaccines for their protective capacities. Despite all the advances made in the vaccination field, none of the subunit vaccines commercialized to date provide complete protection against *A. pleuropneumoniae* infection. The

discovery of an effective subunit vaccine is also limited by the fact that many virulence factors (e.g., the toxin ApxIV) are only expressed *in vivo* (Schaller *et al.*, 1999). Thus, studies are still progressing in the finding of new *in vivo*-expressed immunogenic antigens using powerful genetic tools.

Evolution of live vaccines towards the DIVA concept

The use of live attenuated bacteria in vaccination has always been associated with the possibility of reversion to a fully virulent phenotype and the risk of development of disease in immunocompromized vaccinated animals. Indeed, live attenuated *A. pleuropneumoniae* vaccines suffer from a number of drawbacks including the risk of inoculating animals with inadequately attenuated pathogens and the possibility that the attenuated bacteria may revert to a pathogenic state resulting in disease of the inoculated animals and the possible spread of the pathogens to other animals. Despite all the disadvantages mentioned above, live attenuated vaccines, along with subunit vaccines, represent the most promising research avenues in the *A. pleuropneumoniae* vaccination field. The major reason why the use of attenuated live vaccine is a good approach in vaccination against porcine pleuropneumonia is that pigs surviving natural infection were found to be completely protected against homologous infection and partially against heterologous serotypes of *A. pleuropneumoniae* (Nielsen, 1984; Cruijsen *et al.*, 1995; Haesebrouck *et al.*, 1996). This suggests that only live bacteria can confer cross protection via *in vivo*-induced expression of protective antigens. A large number of mutants were generated and tested as live attenuated vaccines for their protective efficacy (Table 2). Intranasal immunization of mice with temperature-sensitive mutants of *A. pleuropneumoniae* serotype 1 induced protection against homologous challenge (Byrd and Hooke, 1997). An experimental streptomycin-dependent strain of *A pleuropneumoniae* was used as a live attenuated vaccine and showed protection upon homologous challenge with serotype 1 but not against serotype 15 (Tumamao *et al.*, 2004). Several mutants in metabolic genes were generated and tested in a pig infection model. Creation of a riboflavin auxotroph mutant via the partial deletion of the riboflavin biosynthesis

operon (*ribGBAH*) resulted in high attenuation in pigs (Fuller *et al.*, 1996). Another metabolic mutant *aroQ*, affected in the aromatic/chorismate biosynthesis pathway was also found to be attenuated at a similar level as the riboflavin mutant (Ingham *et al.*, 2002). Mutation in the *aroA* gene, involved in the essential aromatic biosynthetic pathway, rendered the bacteria fully avirulent with no signs of respiratory disease or lung lesions in any of the animals infected with the mutant (Garside *et al.*, 2002). However, the use of those metabolic mutants as live vaccines could be a problem since there was no or poor persistence of the bacteria in the respiratory tract of pigs after infection. In fact, to be beneficial in generating a protective immune response, the bacteria have to persist sufficiently in the host to colonize the airways.

A *dmsA* mutant, affected in the putative catalytic subunit DmsA of anaerobic dimethyl sulfoxide reductase involved in oxidative metabolism under anaerobic conditions was found to be attenuated. Interestingly, the challenge mutant strain was reisolated on days 7 and 21 post infection from the bronchoalveolar lavage fluid (BALF) from several pigs (Baltes *et al.*, 2003), suggesting a live vaccine potential for this *dmsA* mutant. A superoxide dismutase *sodC* mutant that was sensitive to *in vitro* superoxide microbicidal action failed as an attenuated live vaccine as the mutant was still virulent and caused lung lesions (Sheehan *et al.*, 2000). Although the bacteria are sensitive to superoxide mediated killing by neutrophils and alveolar macrophages, they still secrete Apx toxins which rapidly kill host cells.

In order to find potential genes that could be targeted for preparation of live attenuated vaccines, isogenic mutants of *A. pleuropneumoniae* serotype 7 were generated for two virulence genes *ureC* and *exbB*, encoding respectively the urease and the ExbBD complex involved in iron uptake. Infection experiments showed that the *ureC* mutant but not the *exbB* mutant is able to survive in pigs and is slightly attenuated (Baltes *et al.*, 2001). Urease can therefore be considered as a potential virulence factor that could be targeted in vaccination experiments.

Studies mentioned above showed that the use of attenuated live vaccines is often limited by the fact that the strain should be less virulent but must also be viable in the host and retain its colonization capabilities to induce a strong immune response. For example, an attenuated strain of *A. pleuropneumoniae* serotype 1 with a thinner capsule, strain CM5A, was able to persist in the tonsils and induce an effective protective immunity in pigs against challenge with the virulent strain CM5 (Bosse *et al.*, 1992). Several important characteristics are thus essential for a good live attenuated vaccine: (i) the strain should remain highly immunogenic; (ii) the strain has to be less virulent and cause sufficient but minimum infection and lesions to avoid substantial infection. These suggest that in the case of specific gene inactivation, the targeted genes have to be important virulence factors without being essential for the viability of the bacteria. In this regard, an *apxIA* mutant of *A. pleuropneumoniae* serotype 10 producing a C-terminal truncated ApxI toxin was constructed by insertion of a chloramphenicol resistance gene cassette. This mutant offered partial cross-protection upon challenge of vaccinated pigs with serotypes 1 and 2 (Xu *et al.*, 2006). An *apxII* mutant of *A. pleuropneumoniae* serotype 7, lacking both *apxIIA* and *apxIIC* genes coding respectively for the structural toxin ApxIIA and the post-translational activating protein ApxIIC, was constructed using site-specific mutagenesis. The HS93Tox- mutant belongs to serotype 7 and as such, also lacks the *apxIA* and *apxIC* genes coding for the ApxI toxin. This mutant strain was transformed with a plasmid containing the *apxIA* gene so that it can express the ApxI structural protein but in a non-activated form. The mutant was shown to be attenuated in a mouse model and to be capable of inducing Apx-specific antibodies (Prideaux *et al.*, 1998). Vaccination of mice with the mutant offered protection against homologous wild-type serotype 7 challenge, as well as heterologous challenge with a serotype 1 strain (Prideaux *et al.*, 1998). The same group has also used site-specific mutagenesis to generate an *apxIIC* mutant that secretes an inactivated form of ApxII toxin. Vaccination experiments showed that pigs vaccinated with this serotype 7 live mutant strain via the intranasal route were protected against a cross-serotype challenge with a virulent serotype 1 strain of *A. pleuropneumoniae* (Prideaux *et al.*, 1999). The *apx* mutants mentioned above, all

displayed non-activated forms of Apx toxins that are still immunogenic. In fact, Apx toxins were shown to be essential for immunoprotection, as previous studies showed that immunization with a non-hemolytic mutant lacking the 110 kDa hemolysin was unable to protect pigs and mice against lethal infection (Inzana *et al.*, 1991). However, the use of those attenuated mutants as live vaccine is again limited by the fact that they contain foreign DNA or antibiotic resistance genes. Indeed, licensing of mutants containing an antibiotic resistance marker for use in livestock might be difficult to obtain due to the risk of resistance transmission to other pathogens. Therefore, even if previous studies have confirmed the safety of mutants containing antibiotic resistance genes (Inzana *et al.*, 2004), the introduction of mutations without antibiotic markers might prove valuable for future *A. pleuropneumoniae* vaccine development. Another *apxIIC* mutant of *A. pleuropneumoniae* serotype 7 containing no antibiotic resistance marker was generated and showed cross protection in mice against *A. pleuropneumoniae* serotypes 1 and 3 as well as in pigs against serotype 1 (Bei *et al.*, 2005, 2007). Recently, a double *ΔapxIC/ΔapxIIC* mutant of *A. pleuropneumoniae* serotype 1 was constructed and investigated for its protective efficacy. This mutant secretes inactivated forms of both ApxI and ApxII which however retain their complete antigenicity. Upon homologous (serotype 1) and heterologous (serotype 9) challenge, intranasally vaccinated pigs were completely protected from clinical signs, showed no mortality and only few lung lesions. These results combined with the fact that the strain contains no foreign DNA suggest a significant live vaccine potential for this double mutant SLW03 (Lin *et al.*, 2007).

Another important concern in *A. pleuropneumoniae* vaccination is that bacterial vaccines currently in use do not allow the differentiation between a vaccinated animal and an infected one. Indeed, it is of major importance to discriminate between immunized and infected pigs for generating and maintaining specified pathogen-free herds which are the optimum choice with respect to long-term animal health and consumer protection. The problem is that live attenuated vaccines are not necessarily affected for surface-exposed and/or immunogenic virulence factors which are

important for mounting an antibody-based immune response. Therefore, a serology-based discrimination is not always possible between the wild type and the attenuated mutant strain. The DIVA (Differentiating Infected from Vaccinated Animals) concept can be used to allow that discrimination by introducing a negative marker in the live attenuated strain. In order to obtain a DIVA vaccine, a suitable marker has to be: (i) highly immunogenic; (ii) expressed in all serotypes; and (iii) not essential for protective immunity.

In previous studies non-capsulated mutants of *A. pleuropneumoniae* serotypes 1 and 5, obtained following chemical mutagenesis, showed attenuation and good protection upon homologous and heterologous challenge. Interestingly, infected and immunized pigs could be discriminated since production of antibodies against the capsule was not induced in the latter (Inzana *et al.*, 1993). Subsequently, Tonpitak *et al.* (2002) designed a DIVA-based vaccine against *A. pleuropneumoniae*. A double mutant *ΔureCΔapxIIA* of *A. pleuropneumoniae* serotype 2 was shown to be attenuated and protective against homologous challenge. In this mutant strain the toxin ApxII was used as a negative marker as it is highly immunogenic and is also present in 13 of the 15 serotypes of *A. pleuropneumoniae*. Thus, immunized pigs could be discriminated from infected ones by serological detection using an ApxIIA ELISA test. Starting from this double mutant prototype live negative marker vaccine, a sixfold *ΔapxIIAΔureCΔdmsAΔhybBΔaspAΔfur* mutant of *A. pleuropneumoniae* was further generated with additional mutations in three enzymes involved in anaerobic respiration and the Fur ferric uptake regulator (Maas *et al.*, 2006a). Interestingly, this mutant did not cause clinical disease in contrast to the previously described double mutant which showed some lung lesions (Tonpitak *et al.*, 2002). Moreover, although highly attenuated, the sixfold mutant was still able to colonize and persist in intact lung tissue over a period of 6 weeks in small numbers, long enough to induce a humoral immune response. From a vaccination perspective, not only was this mutant in accordance with the DIVA concept, but it also showed significant protection upon heterologous infection with an antigenically distinct *A. pleuropneumoniae* serotype 9 challenge strain (Maas *et al.*, 2006a). Despite these encouraging results, the

protective efficacy of this sixfold mutant has to be further confirmed upon challenge with other serotypes before it can be used as a live attenuated vaccine. Moreover, the short rise in body temperature observed upon vaccination is not in accordance with current licensing rules for commercial vaccines. Research in the past few years has shown a great potential of live vaccines in *A. pleuropneumoniae* vaccination in terms of safety, efficacy, stability and also production costs. However, the use of live bacteria in vaccination is usually limited to experimental trials due to ethical issues and restrictive legislation. Vaccine strains should not persist in the host until slaughter age. Hence, further studies are required to increase the safe use of live vaccines and also to improve the efficacy of subunit vaccines which would be more attractive for commercialization.

Mucosal immunity and vaccination strategies

The initial step in the pathogenesis of porcine pleuropneumonia is the colonization of the porcine respiratory tract, followed by the induction of host clearance mechanisms and damage to lung tissues (Bosse *et al.*, 2002). Thus, the epithelial lung surface constitutes the portal for entry of *A. pleuropneumoniae* via interaction with the pulmonary mucosal surface (Jacques *et al.*, 1991; Dom *et al.*, 1994; Abul-Milh *et al.*, 1999). Upon entry, the bacteria are captured by antigen presenting dendritic cells which subsequently migrate in organized mucosal lymphoid tissues such as the broncho-alveolar lymphoid tissue (BALT) to initiate the specific adaptative immune response. Activated lymphocytes are then directed back to the mucosa to mount a local immune response and produce antibodies at the site of infection (Kunkel and Butcher, 2003; Mora *et al.*, 2003a). Thus, induction of mucosal immunity suggests the activation of both humoral and cell-mediated immune responses. One important characteristic of the mucosal immune response is the local production and secretion of dimeric immunoglobulin A (sIgA). This molecule is the major immunoglobulin found in the healthy respiratory tract and is believed to be the most important immunoglobulin for defence at this site (Pilette *et al.*, 2001; Woof and Kerr, 2006). sIgA has the advantage over other antibody isotypes such as IgG to be resistant to

degradation in the protease-rich external environment of mucosal surfaces (Kilian *et al.*, 1988; Neutra and Kozlowski, 2006). The majority of polymeric IgA produced in mucosal tissues is transported across the epithelium into the luminal environment where it promotes neutralization of antigens or micro-organisms in the mucus by inhibiting the adherence to the mucosal surface. This mechanism is known as immune exclusion. Therefore, one main concern in *A. pleuropneumoniae* vaccination is to find the best vaccination strategies to stimulate an appropriate mucosal immune response which could provide an effective protection against *A. pleuropneumoniae* infection.

Several routes of administration of vaccines have been reported, such as systemic immunization via intradermal or intramuscular routes, and mucosal immunization via oral or intranasal routes (Hensel *et al.*, 1996). Most inactivated whole-cell vaccines were tested intramuscularly (Jolie *et al.*, 1995; Furesz *et al.*, 1997; Hensel *et al.*, 2000; Van Overbeke *et al.*, 2003). Indeed, bacterins were logically found to be less effective when used as mucosal vaccines (Hensel *et al.*, 1995) since killed bacteria cannot colonize the mucosal surface and therefore cannot induce an effective immune response. However, systemic immunization failed to be considered as a good vaccination method as it is associated with many disadvantages. First, the use of syringes is often associated with high risks of needle breakage and inflammatory responses at the site of injection, which could alter the quality of the product. Second, systemic immunization was generally found to be ineffective for the induction of mucosal IgA antibody response (McGhee *et al.*, 1992; Kaul and Ogra, 1998; Liu *et al.*, 1998; McCluskie *et al.*, 2002; Goonetilleke *et al.*, 2003) which is a key element in the protection against airway pathogens. The ideal vaccination strategy for respiratory pathogens should provide both humoral and cell-mediated protection, not only at the relevant mucosal surface, but also throughout the body. In this regard, the ability of mucosal immunization to prime the immune system for both systemic and mucosal responses (Kunkel and Butcher, 2003) suggests that mucosal vaccination might be a more suitable strategy to improve the efficacy of vaccines against *A. pleuropneumoniae* infection.

To date, three porcine mucosal vaccines are licensed in North America: two using the intranasal route of immunization against transmissible gastroenteritis virus and *Bordetella bronchiseptica,* and one using the oral route against rotavirus (Gerdts *et al.*, 2006). The best way to obtain an effective mucosal immune response in the upper airway is thought to be through the nasal or tonsilar immunization route. Nonetheless, based on the concept of an integrated mucosal immune system which is supported by several oral immunization studies (Pabst and Binns, 1994; Ogra *et al.*, 2001; Cox *et al.*, 2002; Bouvet *et al.*, 2002)**,** experimental oral vaccine prototypes against *A. pleuropneumoniae* infection have been developed. Oral vaccination offers many practical advantages over parenteral immunization. First, vaccine delivery is simple and does not require laborious and time-consuming procedures. Second, it eliminates the risk of inflammatory response at the injection site as well as stress to the animals. However, oral administration of antigens, especially nonreplicating ones, presents several challenges that must be overcome in order to achieve an effective protection: the immunogen must maintain its native structure and antigenicity in the acidic pH of the stomach, and it must be stable to proteolytic enzyme digestion in the gastrointestinal tract. In this regard, a variety of oral delivery systems and mucosal adjuvants have been developed to enhance the oral immunogenicity of nonreplicating antigens (Ryan *et al.*, 2001; Liao *et al.*, 2001). Recombinant DNA technology has been used to generate a *Saccharomyces cerevisiae* strain (Shin *et al.*, 2005) and a transgenic tobacco plant (Lee *et al.*, 2006), both expressing the *A. pleuropneumoniae* ApxIIA toxin (Table 1). A killed whole-cell vaccine of *A. pleuropneumoniae* serotype 1 has also been incorporated into biodegradable microspheres (Liao *et al.*, 2003) in an attempt to protect antigens from the intralumenal environment and reduce the effective dose. However each system has met with little success: both ApxIIA-based oral vaccines induced only a weak antigen-specific immune response, causing a limited protection against *A. pleuropneumoniae* in a mouse model, while the oral-vaccine microspheres induced a mucosal IgA production but a low systemic immune response (Liao *et al.*, 2003). Furthermore, oral vaccination is limited by the fact that immunogens given orally can

induce tolerance that reduces the efficacy of the vaccine. Indeed, immunogens fed daily in small doses or in a single high dose often induce oral tolerance that appears to be mediated by cellular or humoral suppressor factors (Mattingly and Waksman, 1980; Challacombe, 1987; Sosroseno, 1995).

As noted aboved, an optimal immune response in the respiratory tract could be induced by intranasal immunization. As with oral immunization, the intranasal route offers many practical advantages, except that it requires a more complex immunization protocol with full co-operation from the producer. Many live attenuated *A. pleuropneumoniae* vaccines have been tested in intranasal immunization experiments (Bosse *et al.*, 1992; Prideaux *et al.*, 1999; Tonpitak *et al.*, 2002; Maas *et al.*, 2006a), and showed a more effective protection compared to the oral vaccines previously described. Interestingly, live attenuated *A. pleuropneumoniae* intranasal vaccines were also shown to induce a protective humoral immunity (Bosse *et al.*, 1992).

One of the greatest challenges in vaccinology today is the development of novel mucosal vaccines and vaccine formulations that are safe, effective, and yet cost effective. The delivery system is a critical factor in mucosal immunization (Ryan *et al.*, 2001; Gerdts *et al.*, 2006). In general, nonreplicating antigens such as proteins and killed vaccines are poorly immunogenic when given mucosally. Hence, addition of adjuvants is particularly important in order to stimulate the mucosal immune system. However, the use of adjuvants is frequently associated with tissue damage, which is a main concern in food-producing animal. Thus, one has to choose the right combination of adjuvants in order to develop an effective vaccine that would protect against the disease, but not create unacceptable tissue reaction (Willson *et al.*, 1995). Cholera toxin and heat labile enterotoxin have been shown to be effective mucosal adjuvants for nasal delivery of numerous antigens, but their use has been restricted due to their toxicity (Takahashi *et al.*, 1996; Rappuoli *et al.*, 1999; Williams *et al.*, 1999). CpG oligonucleotides (ODN) are also known as potent adjuvants that significantly enhance cellular and humoral responses to co-administrated antigens

when given parenterally or mucosally (McCluskie and Davis, 1999; Krieg, 2000). In pigs, CpG containing a GTCGTT motif have been shown to be important for optimal stimulation of porcine lymphocytes (Rankin *et al.*, 2001). However, *in vivo* degradation of ODNs and antigens limits their uptake and their efficiency as immune stimulators. Hence, the formulation of the vaccine plays an important role in the efficiency of mucosal vaccines. Various vaccine-targeting adjuvants (VTA) formulations are suitable delivery systems for antigens and CpG ODNs by the intranasal route in pigs, notably incorporated into biphasic lipid vesicles (Alcon *et al.*, 2003, 2005). In fact, intranasal immunization of pigs with a combination of the lipoprotein OmlA and CpG ODNs in biphasic lipid vesicles induced a local immune response with significant amounts of IgG and IgA in nasal secretions (Alcon *et al.*, 2005). A recent study showed that tracheal administration of the transferrin-binding protein TbpB of *A. pleuropneumoniae* in conjunction with an adjuvant formulation containing chitosan, a cationic polysaccharide, enhances both mucosal and systemic immune responses in pigs (Kim *et al.*, 2007). In light of all the studies performed in vaccination strategies against *A. pleuropneumoniae*, intranasal administration of antigens along with appropriate vaccine formulations seems to be an effective needle-free vaccine delivery route in pigs, inducing both systemic and local immune responses.

Screening for vaccine candidates using a genome-wide approach

In vivo expression technology

Many new approaches have been used in the last decade to identify potential bacterial components to be included in subunit vaccines, or potential genes to be inactivated in live vaccine strains. Development of appropriate genetic tools has enabled the use of these new strategies in *A. pleuropneumoniae*. In many cases, researchers have tried to identify bacterial factors that are preferentially expressed *in vivo*, as these should have a role in virulence or persistence in the host. The *In Vivo Expression Technology* (IVET) (Slauch *et al.*, 1994) is a technique in which small

genomic fragments potentially containing *in vivo* active promoters are linked to a gene essential for *in vivo* growth in an auxotrophic mutant. Using IVET, Fuller *et al.* (1999) screened a library of 2400 clones, looking for promoters that were induced during an experimental infection in pigs. Ten unique genetic loci were identified and sequenced, and six of them had significant homology to known gene sequences. These genes were called *ivi* genes, for "*in vivo* induced" genes. Although these genes seemed to be mostly involved in metabolic pathways, a few of them were found to be linked with virulence. One gene contained a sequence similar to the *Haemophilus influenzae mrp* gene involved in LPS biosynthesis, and another was later identified as an *in vivo* induced organic hydroperoxide reductase that could protect *A. pleuropneumoniae* from oxidative stress encountered during the infection process (Shea and Mulks, 2002). One of the *ivi* genes, *ilvI*, encodes acetohydroxy acid synthase (AHAS) isoenzyme III, which catalyses the reaction for the first step in the biosynthesis of the branched-chain amino acids (BCAA; isoleucine, leucine and valine). Enzymes involved in this pathway have been identified as *in vivo* induced in previous studies with other pathogens (Wang *et al.*, 1996; Mei *et al.*, 1997; Sun *et al.*, 2000; Fuller *et al.*, 2000a), and it was hypothesized that BCAA biosynthesis is required for survival and virulence in lungs of mammalian hosts (Wagner and Mulks, 2006). Using a chemically defined medium, Wagner and Mulks (2006) showed that eight out of ten *ivi* genes, *iviG*, *iviI*, *iviP*, *iviS*, *iviU*, *iviX*, *iviY* and *ivi17g*, had increased activity in BCAA deprived medium. In a subsequent study, a gene with similarity to the *lrp* gene of *E.coli*, encoding the leucine-responsive regulatory protein (Lrp), was cloned, sequenced and expressed *in vitro* (Wagner and Mulks, 2007). Electrophoretic gel mobility assays showed that the *A. pleuropneumoniae* Lrp binds to the *iviG* and *iviI* promoters, and might therefore regulate the expression of these genes. The riboflavin auxotroph mutant that was generated in order to conduct the IVET experiments was then used alone as a potential live attenuated vaccine (Fuller *et al.*, 2000c). When supplied with limited amounts of riboflavin in order to permit a low level of *in vivo* replication, mortality was reduced in both homologous (serotype 1) and heterologous (serotype 5) challenges, even though there was no significant reduction in lung pathology.

Signature tagged mutagenesis

The same group also applied the Signature Tagged Mutagenesis (STM) system to *A. pleuropneumoniae* (Fuller *et al.*, 2000b). STM systems rely on the unique "tagging" of each transposon mutant with small DNA sequences. Pools of mutants are then screened *in vivo*, and mutants that are not recovered *in vivo* but still show *in vitro* growth similar to that of the wild type are further investigated. The selected mutants are thought to harbour a mutation in a gene that is essential for *in vivo* survival. Using over 800 *A. pleuropneumoniae* mini-Tn10 mutants, Fuller *et al.* (2000b) identified 110 potentially attenuated mutants representing 35 groups of unique loci. Competitive index (CI; [mutant cfu/wildtype cfu]$_{input}$/ mutant cfu/wildtype cfu]$_{output}$) determination for each mutant led to the identification of 20 mutants that were significantly attenuated *in vivo*. Seven mutants, including four mutants with relatively low *in vivo* CI (genes *yaeE*, *fkpA*, *tig*, HI0379) and three mutants for genes that had also been identified in a previous study in *Pasteurella multocida* (genes *exbB*, *atpG*, *pnp*), were selected for preliminary vaccine studies against homologous challenge. Although three out of the seven mutants caused some mortality when administered at very high dosage (10^{10} CFU, with 50% mortality in one case), all surviving animals were well protected against homologous challenge, while animals that were vaccinated with a commercial bacterin showed 37.5% mortality (Fuller *et al.*, 2000b). This system was successful in identifying genes that are known to be involved in virulence processes such as *exbB*, which is involved in various iron acquisition systems in bacteria and was one of the mutated genes in the 20 significantly attenuated mutants. The *exbB* mutant, which showed a very low *in vivo* CI, caused no mortality when administered at very high dose and surviving animals showed complete protection and low lung lesion scores (Fuller *et al.*, 2000b).

Using a genetic system similar to that of Fuller *et al.* (2000a, 2000b), Sheehan *et al.* (2003) screened a total of 2064 mini-Tn10 mutants. Whereas bacteria were recovered by lung lavage following infection in the first STM study in *A. pleuropneumoniae*, Sheehan *et al.* (2003) observed more consistent recovery of bacteria after

homogenization of the entire porcine lung. Moreover, mutants were retained for further studies only if they could be identified as potentially attenuated after two consecutive *in vivo* screening experiments. Using this protocol, 105 potentially attenuated mutants were identified, with mutations in 55 individual genes. Some of these genes, such as those involved in capsular polysaccharide export, LPS biosynthesis and iron transport, were already known virulence genes in *A. pleuropneumoniae*, and only 3 genes (genes *tig*, *pnp*, *apvD/macA*) were common to those identified by Fuller *et al.* (2000b). Eleven of the 55 attenuated mutants also showed general growth defects *in vitro*. The *in vivo* CI was determined for 14 mutants, and 8 of them showed high attenuation while the 6 other did not seem attenuated although there was consistent lack of recovery of these mutants after *in vivo* screening. This feature is common to some STM studies (Autret *et al.*, 2001; Maroncle *et al.*, 2002), and the authors hypothesized that those mutants might have very subtle effects on virulence that are not seen at higher dose or in less diverse populations.

As in the IVET study and in the first STM study by Fuller *et al.* (1999, 2000b), Sheehan *et al.* (2003) identified several new potential virulence-related genes in *A. pleuropneumoniae*. Furthermore, results from this study also helped to gain a better understanding of the diverse iron-acquisition systems of *A. pleuropneumoniae*, as a second TonB system was identified. Two mutants harbouring disrupted *tonB* genes were identified as potentially attenuated, and DNA sequencing showed two distinct copies of the gene: *tonB1*, the original *tonB* gene in *A. pleuropneumoniae*, shares homology with the *Neisseria meningitidis tonB* (Beddek *et al.*, 2004) and is located upstream of and is cotranscribed with genes *exbB, exBD* and *tbp,* coding for the transferring binding proteins (Tonpitak *et al.*, 2000). The *tonB2* gene seems to form an operon with genes *exbB2* and *exbD2*, and shares homology with *tonB* genes *from P. multocida* and *Haemophilus sp* (Sheehan *et al.*, 2003). The STM study also revealed, using *in vivo* CI experiments, that inactivation of *tonB2*, but not *tonB1*, leads to attenuation.

Selective capture of transcribed sequences

Selective Capture of Transcribed Sequences (SCOTS) is another strategy that can lead to the identification of genes transcribed *in vivo*. During SCOTS, RNA mixes comprising pathogen and host molecules are reverse-transcribed to cDNA, and pathogen-specific sequences are captured with photobiotinylated gDNA previously blocked with rRNA-coding DNA sequences (Graham and Clark-Curtiss, 1999). Enrichment of sequences specific for growth in the host is then performed by selective capture using, again, photobiotinylated gDNA, but this time previously blocked with cDNA recovered after growth of the pathogen in culture medium. The scope of SCOTS is therefore different from that of STM, which identifies only genes that are essential for *in vivo* survival, and similar to that of IVET which leads to the identification of *in vivo* induced genes. The SCOTS approach was used with *A. pleuropneumoniae*: using necrotic porcine lung tissue, Baltes and Gerlach (2004) identified 46 genes, 20 of which had previously been identified as induced *in vivo* or involved in virulence in other pathogens. Genes coding for the ApxIV toxin, the putative ABC transporter ApaA, the TbpB small subunit of the transferrin receptor, and the dimethyl sulfoxide reductase, which had all previously been detected *in vivo*, were detected by SCOTS. Other known and putative virulence factors, such as the gene coding for the HgbA hemoglobin receptor and a gene coding for a putative Hsf autotransporter adhesin were also identified (Baltes and Gerlach, 2004). In *H. influenzae*, Hsf is thought to be the major non-pilus adhesin (St Geme *et al.*, 1996; Cotter *et al.*, 2005).

The experiment was repeated using samples from chronically infected pigs (day 21 post-infection vs day 7 post-infection) (Baltes *et al.*, 2007). This time, 36 unique genes were identified, 21 of which code for proteins involved in metabolism. Three genes, coding for elongation factor EF-Tu, ubiquinone reductase, and RNA polymerase B had also been identified in the previous SCOTS study. Of particular interest were genes *hlyX*, coding for a global anaerobic regulator homologous to the *E. coli* Fnr protein, and gene *aasP*, coding for a putative autotransporter serine

protease. The HlyX protein was shown to complement anaerobic respiratory deficiencies of *fnr* mutants of *E. coli* (Green *et al.*, 1992), and also to activate a cryptic hemolytic activity that was *fnr*-independent. It has been hypothesized that, inside necrotic lung lesions, *A. pleuropneumoniae* has to rely on anaerobic metabolism to survive, and therefore the over-expression of *hlyX* does not come as a surprise. Whether or not this regulator can also enhance *in vivo* transcription of virulence genes in *A. pleuropneumoniae* has yet to be shown. Properties of the *aasP* genes were further investigated, as numerous reports over the last years have highlighted implication of autotransporters in virulence, often as Ig proteases (Mistry and Stockley, 2006; Riesbeck and Nordstrom, 2006). Both transcript and protein synthesis were shown to be increased during anaerobic growth, and a putative FNR binding site was identified in the *aasP* promoter region. The gene sequence of *aasP* is identical to that of a putative antotransporter serine protease that was identified simultaneously in a microarray experiment under iron-restriction conducted by our group, and termed Ssa1 (Deslandes *et al.*, 2007).

DNA microarrays

Although the IVET, STM and SCOTS approaches do lead to the identification of genes putatively involved in virulence, none of these techniques can give an overall knowledge of gene expression in bacteria, or are as powerful as DNA microarrays. The use of microarrays can lead to better genome coverage than IVET and STM techniques, as each and every identified ORFs of the bacterial genome is tested. As is the case for SCOTS, DNA microarrays enables the identification of genes that are overexpressed at different levels in a particular condition. Since these platforms have started to be overwhelmingly used at the end of the 1990s, scientists have taken advantage of this large genome coverage to gain an insight into genes that could potentially code for antigenic proteins. For years, scientists working with *A. pleuropneumoniae* were lacking a reliable and fully annotated genome sequence. Lately, serotype 5b strain L20 was sequenced by the team of John H. E. Nash (National Research Council, Ottawa, Canada). Using bioinformatic tools, 2170 ORFs

were identified in the complete genomic sequence of this strain (Genbank, CP000569). This information was then used to generate a DNA microarray with 2033 ORFs, corresponding to 95% of the ORFs of length greater than 160 nt in the genome sequence (http://ibs-isb.nrc-cnrc.gc.ca/glycobiology/appchips_e.html). With the genome data and microarray technology in hand, we have undertaken, with collaborators, various genomic studies in order to identify new potential vaccine candidates. Our strategy enables us to handle efficiently one of the most challenging issues encountered when working with *A. pleuropneumoniae*, i.e. the existence of fifteen distinct serotypes. Using bioinformatics and genome sequences, a list of genes that could putatively code for OMPs or lipoproteins was generated. This objective is the core of many reverse vaccinology projects (Mora *et al.*, 2003b), a strategy which relies strictly on available genomic information in order to identify *in silico* potential vaccine candidates (Serruto and Rappuoli, 2006). These candidates are then further investigated, and screened in order to satisfy existing criteria for the development of good vaccines for a particular pathogen. As an example, using this approach, approximately 600 novel vaccine candidates have been identified in the serogroup B *Neisseria meningitidis* (MenB) by the first team to attempt experiments that would later be considered as the hallmark of reverse vaccinology (Pizza *et al.*, 2000). Of these novel candidates, 28 could elicit protective immunity and could eventually induce immunity against all meningococcal isolates.

Using multiple bioinformatic algorithms to scan the *A. pleuropneumoniae* 5b L20 genome, 93 genes were identified as putative OMPs or lipoproteins, and therefore encoding potential surface-exposed antigens (Chung *et al.*, 2007). Outer membrane proteins were then enriched using various extraction protocols, which lead to the recovery of 50 of the 93 potential OMPs and lipoproteins identified *in silico* (53%) as identified with LC-MS/MS. To date, this study is the first to establish the OM proteome of *A. pleuropneumoniae*. *In silico* analyses, although powerful, have some limitations. While these analyses will enable us to generate a list of potential vaccine candidates, this list cannot be considered as entirely reprensentative of the mechanisms that are used by bacteria in their natural host. In order to monitor

interactions between bacteria and their environment, gene expression profiling with DNA microarrays can be conducted. By gathering information on the bacterial response to changes in its environment, it is likely that new genes expressed during infection conditions will be discovered. This strategy was used by researchers working on *N. meningitidis*, shortly after the first reverse-vaccinology experiments were conducted. In a study of gene expression following adhesion to human epithelial cells, approximately 350 genes showed differential expression, 189 of which were overexpressed (Grifantini *et al.*, 2002). Twelve of those overexpressed genes, 5 of which could elicit production of bactericidal antibodies, were potentially involved in adhesion, and had not been previously identified in the *in silico* mining of MenB (Serruto and Rappuoli, 2006). It is therefore clear that microarray technology can identify new potential vaccine candidates, and complement other genome mining methods.

Using DNA microarrays, we have identified genes that are expressed in conditions mimicking the *in vivo* environment (Deslandes *et al.*, 2007). Since iron-restriction has long been recognized as a condition encountered in the mammalian host, we first tested the effect of iron-restriction on *A. pleuropneumoniae* serotype 1. After supplementation of the culture medium with an iron chelator, we identified 210 genes that were differentially expressed, 92 of which were overexpressed. Logically, the major response of *A. pleuropneumoniae* to iron restriction was the induction of genes involved in iron transport. While all previously known systems were shown to be upregulated, our experiments also lead to the identification of new potential iron-acquisition systems that could also potentially be induced *in vivo*. As an example, genes showing homology with the *N. meningitidis* HmbR receptor, specific for hemoglobin, and genes showing homology with the Yfe chelated-iron acquisition system were significantly upregulated. Of particular interest was also the identification of ORFs homologous to the Ssa1 protein of *Mannheimia haemolytica*. This protein belongs to the family of subtilisin-like serine proteases, and possesses an auto-transporter domain. The gene was termed *aasP* by Baltes *et al.* (2007) shortly after. Gene *hlyX* was also upregulated under iron-restriction.

Futhermore, we also investigated the transcriptional response of *A. pleuropneumoniae* after interaction with porcine lung epithelial cells. Transcriptional response of both planktonic bacteria and adherent bacteria was assessed, and major changes were observed. Most of the genes identified were metabolism-related, but some putative components that could be involved in adhesion were also identified (*unpublished observations*). To date in *A. pleuropneumoniae*, only LPS has been shown to play a role in adhesion *in vitro* (Belanger *et al.*, 1990; Paradis *et al.*, 1994, 1999). It would be interesting to identify other genes that are expressed *in vivo* in the lungs of pigs. However, many technical limitations must be solved before a representative *in vivo* study can be conducted. Researchers who wish to perform these studies must find ways to isolate bacteria in sufficient amounts and then stabilize the transcriptome very rapidly. Furthermore, contamination with eukaryote mRNA is also a concern.

Finally, we are using DNA microarrays to perform comparative genomic hybridizations and to verify that genes of interest are highly conserved among the reference strains of the fifteen serotypes of *A. pleuropneumoniae*, as well as in field strains of those serotypes most frequently isolated in North America. Those results, combined with the ones obtained in the proteomic and transcript profiling experiments, will enable us to identify new potential vaccine targets that are both expressed *in vivo* and conserved among all serotypes and biotypes.

Discussion and perspective

The wide spectrum of research in the vaccination field has allowed great developments in *A. pleuropneumoniae* vaccines. The use of inactivated whole-cell bacterial vaccines was clearly shown to be the least promising vaccination strategy in order to obtain efficient protection against *A. pleuropneumoniae* infection. In fact, killed bacteria display no colonization of the respiratory tract. Moderate persistence and colonization of the respiratory tract is important for the development of an

effective immune response. The limited cross protection and the absence of *in vivo*-expressed antigens in non-living vaccines also account for the inefficiency of bacterins. In contrast, this review shows the great potential of subunit and live attenuated vaccines. Despite, the numerous safety and ethical drawbacks associated with the use of live bacteria, live vaccination is probably the best approach against *A. pleuropneumoniae* as it reflects natural infection and allows the *in vivo*-expression of immunogenic antigens which are crucial for effective protection. Moreover, the DIVA concept which allows the differentiation between vaccinated and infected animal is an important feature that has to be considered in order to increase the reliability of live vaccines.

Subunit vaccines are another important research avenue in *A. pleuropneumoniae* vaccination and have the advantage over live vaccines of being less restricted by legislation issues. However the development of subunit vaccines is not an easy task as it suggests not only the discovery of highly immunogenic antigens with a broad protective activity, but also the use of adjuvants and formulations which are key elements for an appropriate stimulation of the host immune system. Indeed, one main concern in vaccination strategy is to find the best way to obtain an effective immune stimulation. These include not only the use of adjuvants and formulations but also the selection of the appropriate immunization route. Thus, among the different immunization methods tested we have highlighted in this review the high potential of intranasal inoculation in the stimulation of mucosal immunity.

Another feature that has to be considered in vaccination is that the ability of a vaccine to generate an effective protection-mediating immune response can differ depending on the genetic background of pigs in a population (Magnusson *et al.*, 1997). In this way, vaccination could be allied with commercial livestock breeding strategies in order to select for more responsive pigs. Passive immunization with antibodies is also worth further investigation as an alternative method for vaccination against *A. pleuropneumoniae*. This approach has become even more attractive in terms of cost and productivity with the large scale production of IgY antibodies in

egg yolks following immunization of hens with bacterial antigens (Shin *et al.*, 2002). Despite the advances made especially for subunit and live attenuated vaccines, the incomplete knowledge on virulence factors and bacterial antigens expressed *in vivo* by *A. pleuropneumoniae* could be one of the reasons why a highly effective vaccine against *A. pleuropneumoniae* infection has not yet reached the market. Thus, the investigation for vaccine development cannot be dissociated from the new genetic tools available such as IVET, SCOTS and microarrays for the discovery of new *in vivo*-expressed antigens, and STM for the finding of essential genes for survival (Tables 3 and 4). We believe that those genetic tools in combination with trial experiments will definitely help explore new virulence pathways and subsequently allow the design of more effective vaccines against porcine pleuropneumonia.

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Table 1. Subunit vaccines against *A. pleuropneumoniae* infection

Subunit vaccine	Formulation and adjuvant	Vaccine serotype	Route of immunization	Challenge		Animal model	References
				Route	Serotype		
Anionic fraction of a saline extract (ANEX)	vegetable oils/mineral oil (Marcol-52) /aluminum hydroxide/ polyethylene glycol/ Quil-A/Amphigen/ Emulsigen-Plus	1	IM	Aerosol	1	Pig	(Willson *et al.*, 1995)
Conjugate vaccine (purified hemolysin with capsule or LPS)	Freund's complete and incomplete adjuvant	1	IM	IT	1	Pig	(Byrd and Kadis, 1992)
Capsular extracts	Aluminium hydroxide adjuvant	1	SC IP	Aerosol/ IN IP	1 1	Pig mouse	(Rosendal *et al.*, 1986)
Conjugate vaccine (capsular polysaccharide and tetanus toxoid)	Emulsigen	5b	IM	Aerosol	5	Pig	(Andresen *et al.*, 1997)
Hemopig ^{TM}, Biokema	NA	1, 2, 7, 9	SC	endobr	9	Pig	(Chiers *et al.*, 1998)

Subunit vaccine	Formulation and adjuvant	Vaccine serotype	Route of immunization	Challenge		Animal model	References
				Route	Serotype		
(capsular antigens and Apx toxins)							
Recombinant transferrin-binding protein B (TbpB)	Chitosan	5	tracheal/IM	N/A	N/A	Pig	(Kim and Lee, 2006; Kim *et al.*, 2007)
Recombinant cytolysin (CytA) and 60 kDa transferrin-binding protein (TfbA)	Amphigen	7	IM	Aerosol	1, 7	Pig	(Rossi-Campos *et al.*, 1992)
Porcilis APP, Intervet (ApxI, ApxII and ApxIII toxoids, plus 42 kDa OMP)	Alfa-tocoferol acetate	1-12	IM	Natural/ IN/ endobr/ aerosol	1, 2, 9, 15	Pig	(Chiers *et al.*, 1998; Habrun *et al.*, 2002; Van Den Bosch and Frey, 2003; Tumamao *et al.*, 2004)
Conjugate vaccines (purified outermembrane	Diluvac Forte adjuvant formulation	2, 5b	IM	Aerosol	1	Pig	(Van Den Bosch and Frey, 2003)

Subunit vaccine	Formulation and adjuvant	Vaccine serotype	Route of immunization	Challenge		Animal model	References
				Route	Serotype		
lipoprotein PalA and/or ApxI+ ApxII)							
OmlA lipoprotein	Biphasic-VTA/CpG ODN 2007/Cholera toxin/Emulsigen/VSA adjuvant	1	IN/SC	Aerosol	1	Pig	(Alcon *et al.*, 2003, 2005)
Recombinant outermembrane lipoprotein (OmlA)	Emulsigen-Plus	1	IM	Aerosol	1	Pig	(Gerlach *et al.*, 1993)
Purified OMP	Hybrid liposome ISCOM adjuvant, SAMA4	1	ID	IN	1	Mouse	(San Gil *et al.*, 1999)
Conjugate vaccines (OMP extract and/or hemolysin)	Lipid emulsion adjuvant (lecithin, peanut oil, and glycerin)	1	IM	IT	1, 5	Pig	(Madsen *et al.*, 1995)
Mixed cell-free culture	Emulsigen-Plus	2 and 9	IM	Endobr	2	Pig	(Goethe *et al.*,

Subunit vaccine	Formulation and adjuvant	Vaccine serotype	Route of immunization	Challenge		Animal model	References
				Route	Serotype		
supernatant of *A. pleuropneumoniae*							2000)
Mixed cell-free culture supernatant of *A. pleuropneumoniae ΔapxIIA* mutant	Emulsigen-Plus	1, 2, 5	IM	Aerosol	2, 9	Pig	(Maas *et al.*, 2006b)
Pleurostar™, Novartis (acellular pentavalent subunit vaccine; ApxII, OmlA1, OmlA5, CysL1, TfbA7)	NA	1, 5, 7	IM	Endobr	14	Pig	(Van Overbeke *et al.*, 2001)
Hemolysin vaccine (ApxI and ApxII)	Emulsigen	5b	IM	Aerosol	5	Pig	(Andresen *et al.*, 1997)
ApxI N-terminal domain (residues 40 to 330)	Adjuvant Montanide ISA 70	14	IP	IP	1, 5, 10, 14	Mouse	(Seah *et al.*, 2002)
Fusion proteins (ApxIA epitopes	Freund's complete	1, 10	SC	NA	NA	Mouse	(Bagdasarian *et al.*,

Subunit vaccine	Formulation and adjuvant	Vaccine serotype	Route of immunization	Challenge		Animal model	References
				Route	Serotype		
+ B subunit of the *E. coli* heat-labile enterotoxin EtxB)	adjuvant						1999)
Crude hemolysin preparation and $CaCl_2$/LiCl extracts	Aluminum phosphate/ aluminum hydroxide	1	IP IM	IP IT	1, 5	Mouse Pig	(Beaudet *et al.*, 1994)
Recombinant NADPH-sulfite reductase hemoprotein (CysI)	Emulsigen	1 and 5a	IM	Aerosol	1 and 5a	Pig	(Willson *et al.*, 2001)
Protein extract and lyophilized transgenic tobacco plant (*Nicotiana tabacum*) expressing ApxIIA	N/A	1	SC/oral	IP	1	Mouse	(Lee *et al.*, 2006)
Protein extract and lyophilized *Saccharomyces cerevisiae* expressing ApxIIA	complete and incomplete Freund's adjuvant	2	SC/oral	SC	2	Mouse	(Shin *et al.*, 2005)

Endobr = endobronchial, ID = intradermal, IM = intramuscualr, IN = intrenasal, IP = intreperitoneal, SC = subcutaneous

Table 2. Live vaccines candidates against *A. pleuropneumoniae* infection

Wild type *A. pleuropneumoniae* strain	Live vaccine	Route of immunization	Challenge		Animal model	References
			Route	Serotype		
Serotype 1 strain 4074	temperature-sensitive mutants	IN	IN	1	Mouse	(Byrd and Hooke, 1997)
Serotype 1 strain 4074	Streptomycin-dependent mutant	SC	IN	1, 15	Pig	(Tumamao *et al.*, 2004)
Serotype 1 ATCC 27088	Riboflavin-requiring mutant	Percutaneous IT	NA	NA	Pig	(Fuller *et al.*, 1996)
Serotype 1 strain HS25	*aroQ* mutant	IT	NA	NA	Pig	(Ingham *et al.*, 2002)
Serotype 1 strain 4074	*aroA* mutant	IT	N/A	N/A	Pig	(Garside *et al.*, 2002)
Serotype 7 strain AP76	Δ*dmsA* mutant	Aerosol	N/A	N/A	Pig	(Baltes *et al.*, 2003)
Serotype 1 strain 4074	[Cu,Zn]-Superoxyde dismutase mutant, *sodC*	IT	N/A	N/A	Pig	(Sheehan *et al.*, 2000)
Serotype 2 strain C5934	Δ*ureC*Δ*apxIIA* double mutant	Aerosol	Aerosol	2	Pig	(Tonpitak *et al.*, 2002)

Wild type *A. pleuropneumoniae* strain	Live vaccine	Route of immunization	Challenge		Animal model	References
			Route	Serotype		
Serotype 7 strain AP76	*ΔexbB* and *ΔureC* single mutants and *ΔexbBΔureC* double mutant	Aerosol	N/A	N/A	Pig	(Baltes *et al.*, 2001)
Serotype 1 strain CM5	attenuated strain with a thinner capsule	Aerosol	Aerosol	1	Pig	(Bosse *et al.*, 1992)
Serotype10 strain D13039	*apxIA* mutant	IN	IN	1, 2 , 10	Pig	(Xu *et al.*, 2006)
Serotype 7 strain HS93	*apxIICA* mutant secreting an inactivated ApxI toxin	IP	IP	7 and 1	Mouse	(Prideaux *et al.*, 1998)
Serotype 7 strain HS93	*apxIIC* mutant	IN	IN	1	Pig	(Prideaux *et al.*, 1999)
Serotype 5 strain J45	non-hemolytic mutant strain mIT4-H	IP	IN	5	Mouse	(Inzana *et al.*, 1991)
		SC	IT	5	Pig	
Serotype 7 strain HB04	*apxIIC* mutant, HB04C$^-$	IN/IM	IT	1, 7	Pig	(Bei *et al.*, 2007)
Serotype 7 strain HB04	*apxIIC* mutant, HB04C$^-$	IP	IP	1, 3, 7	Pig	(Bei *et al.*, 2005)

Wild type *A. pleuropneumoniae* strain	Live vaccine	Route of immunization	Challenge		Animal model	References
			Route	Serotype		
Serotype 1 strain SLW01	*ΔapxIC/ΔapxIIC* double mutant, SLW03	IN/IM	IT	1, 9	Pig	(Lin *et al.*, 2007)
Serotype 5 strain J45 and Serotype 1 strain 4074	non capsulated mutants	SC	IT	1 and 5	Pig	(Inzana *et al.*, 1993)
Serotype 2 strain C5934	*ΔapxIIΔureCΔdmsAΔhybBΔaspA Δfur* sixfold mutant	Aerosol	Aerosol	9	Pig	(Maas *et al.*, 2006a)
Serotype 1 strain AP225	STM mutants *ΔexbB, ΔatpG, Δpnp, ΔyaeE, ΔfkpA, Δtig*, ΔH10379	IN	IN	1	Pig	(Fuller *et al.*, 2000b)

IM = intramuscular, IN = intranasal, IP = intraperitoneal, SC = subcutaneous

Table 3. Proteins identified by 1D-gel and LC-MS/MS after enrichment for OMPs[a] for which the corresponding genes were also identified in gene expression experiments or by *in silico* prediction.

Protein	OrfID	Function	Method of identification	References
IlvD	ap0104	Dihydroxy-acid dehydratase	IVET	(Fuller *et al.*, 1999)
NqrABC	ap0169-ap0171	Na(+)-translocating NADH-quinone reductase	STM, SCOTS	(Sheehan *et al.*, 2003; Baltes *et al.*, 2007)
Ssa1	ap0399-ap0402	serotype-specific antigen 1 precursor	SCOTS, microarray[b]	(Baltes *et al.*, 2007; Deslandes *et al.*, 2007)
OmpP2	ap0719	outer membrane protein P2 precursor (OMP P2)	*in silico*, STM	(Sheehan *et al.*, 2003; Chung *et al.*, 2007)
APL_0829	ap0928	hypothetical protein	*in silico*, SCOTS	(Baltes and Gerlach, 2004; Chung *et al.*, 2007)
GroES	ap1134	10 kDa chaperonin	microarray	(Deslandes *et al.*, 2007)
HgbA	ap1175, ap1176	hemoglobin-binding protein A precursor	*in silico*, SCOTS, microarray	(Baltes and Gerlach, 2004; Chung *et al.*, 2007; Deslandes *et al.*, 2007)
APL_1121	ap1252	putative lipoprotein	*in silico*, microarray	(Chung *et al.*, 2007; Deslandes *et al.*, 2007)
APL_1290	ap1444	hypothetical protein	microarray	(Deslandes *et al.*, 2007)
APL_1299	ap1453	predicted TonB dependent/Ligand-Gated channel	*in silico*, microarray	(Chung *et al.*, 2007; Deslandes *et al.*, 2007)

Protein	OrfID	Function	Method of identification	References
TufB	ap1556	elongation factor Tu	SCOTS	(Baltes and Gerlach, 2004; Baltes *et al.*, 2007)
OmpA	ap1581	outer membrane protein P5 precursor	*in silico*, STM, SCOTS	(Fuller *et al.*, 2000b; Baltes and Gerlach, 2004; Chung *et al.*, 2007)
TbpA	ap1736	Transferrin-binding protein 1 Tbp1	*in silico*, SCOTS	(Baltes and Gerlach, 2004; Chung *et al.*, 2007)
CpxCD	ap1752-ap1753	capsule polysaccharide export protein	*in silico*, STM	(Sheehan *et al.*, 2003; Chung *et al.*, 2007)
AtpGAHF	ap1820-ap1823	ATP synthase	STM, SCOTS	(Fuller *et al.*, 2000b; Sheehan *et al.*, 2003; Baltes *et al.*, 2007)
APL_1694	ap1869	antigenic protein, ABC transporter-like protein	SCOTS	(Baltes and Gerlach, 2004)
APL_1748	ap1927	outer membrane lipoprotein A precursor	*in silico*, microarray	(Chung *et al.*, 2007; Deslandes *et al.*, 2007)
APL_1930	ap2118	outer membrane antigenic lipoprotein B precursor	*in silico*, STM	(Sheehan *et al.*, 2003; Chung *et al.*, 2007)
APL_2002	ap2196	hypothetical protein	*in silico*, microarray	(Chung *et al.*, 2007; Deslandes *et al.*, 2007)

[a] As identified by Chung *et al.* (Chung *et al.*, 2007). [b] Transcriptional profiling under iron-restricted conditions (Deslandes *et al.*, 2007). IVET = *in vivo* expression technology, STM = signature tagged mutagenesis, SCOTS = selective capture of transcribed sequences

Table 4. Genes identified by more than one gene expression methodology

Gene	OrfID	Function	Method of identification	References
prfC	ap0033	peptide chain release factor 3	IVET, STM	(Fuller *et al.*, 1999; Sheehan *et al.*, 2003)
typA	ap0056	GTP-binding protein	SCOTS, microarray[a]	(Baltes *et al.*, 2007; Deslandes *et al.*, 2007)
tonB2	ap0082	protein TonB2	STM, microarray	(Sheehan *et al.*, 2003; Deslandes *et al.*, 2007)
yfeB	ap0294	putative chelated iron transport system ATP-binding protein	STM, microarray	(Sheehan *et al.*, 2003; Deslandes *et al.*, 2007)
argG	ap0466	argininosuccinate synthase	STM, microarray	(Sheehan *et al.*, 2003; Deslandes *et al.*, 2007)
mreB, mreC	ap0486, ap0487	rod shape-determining protein MreB and MreC	SCOTS, microarray	(Baltes and Gerlach, 2004; Baltes *et al.*, 2007; Deslandes *et al.*, 2007)
pnp	ap0644	polyribonucleotide nucleotidyltransferase	STM	(Fuller *et al.*, 2000b; Sheehan *et al.*, 2003)
guaA	ap0659	GMP synthase (glutamine-hydrolyzing)	STM, SCOTS	(Sheehan *et al.*, 2003; Baltes and Gerlach, 2004)
hlyX	ap0726	regulatory protein HlyX	SCOTS, microarray	(Baltes *et al.*, 2007; Deslandes *et al.*, 2007)

Gene	OrfID	Function	Method of identification	References
metIN	ap1019, ap1020	D-methionine transport system permease protein MetI and ATP-binding protein MetN	STM	(Fuller *et al.*, 2000b; Sheehan *et al.*, 2003)
mrp	ap1121	Mrp-like protein	IVET, STM	(Fuller *et al.*, 1999; Sheehan *et al.*, 2003)
fur	ap1362, ap1363	ferric uptake regulation protein	STM, microarray	(Sheehan *et al.*, 2003; Deslandes *et al.*, 2007)
accA	ap1649	acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	SCOTS, microarray	(Baltes *et al.*, 2007; Deslandes *et al.*, 2007)
tig	ap1671	trigger factor	STM	(Fuller *et al.*, 2000b; Sheehan *et al.*, 2003)
tonB1	ap1740	periplasmic protein	STM, microarray	(Sheehan *et al.*, 2003; Deslandes *et al.*, 2007)
dnaJ, dnaK	ap2091, ap2092	chaperone proteins DnaJ and DnaK	STM, SCOTS	(Fuller *et al.*, 2000b; Sheehan *et al.*, 2003; Baltes and Gerlach, 2004; Baltes *et al.*, 2007)

[a] Transcriptional profiling under iron-restricted conditions (Deslandes *et al.*, 2007).

Annexe 2

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Microarray-based comparative genomic profiling of reference strains and selected Canadian field isolates of *Actinobacillus pleuropneumoniae*

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Abstract

Background:

Actinobacillus pleuropneumoniae, the causative agent of porcine pleuropneumonia, is a highly contagious respiratory pathogen that causes severe losses to the swine industry worldwide. Current commercially-available vaccines are of limited value because they do not induce cross-serovar immunity and do not prevent development of the carrier state. Microarray-based comparative genomic hybridizations (M-CGH) were used to estimate whole genomic diversity of representative *Actinobacillus pleuropneumoniae* strains. Our goal was to identify conserved genes, especially those predicted to encode outer membrane proteins and lipoproteins because of their potential for the development of more effective vaccines.

Results:

Using hierarchical clustering, our M-CGH results showed that the majority of the genes in the genome of the serovar 5 *A. pleuropneumoniae* L20 strain were conserved in the reference strains of all 15 serovars and in representative field isolates. Fifty-eight conserved genes predicted to encode for outer membrane proteins or lipoproteins were identified. As well, there were several clusters of diverged or absent genes including those associated with capsule biosynthesis, toxin production as well as genes typically associated with mobile elements.

Conclusion:

Although *A. pleuropneumoniae* strains are essentially clonal, M-CGH analysis of the reference strains of the fifteen serovars and representative field isolates revealed several classes of genes that were divergent or absent. Not surprisingly, these included genes associated with capsule biosynthesis as the capsule is associated with sero-specificity. Several of the conserved genes were identified as candidates for vaccine development, and we conclude that M-CGH is a valuable tool for reverse vaccinology.

Background

Actinobacillus pleuropneumoniae is a Gram-negative bacterium belonging to the family *Pasteurellaceae.* It is the etiological agent of porcine pleuropneumonia, a highly contagious respiratory infection that causes severe economic losses to the swine industry worldwide. The disease, transmitted by the aerosol route or direct contact with an infected pig, is characterized by hemorrhagic, fibrinous and necrotic lung lesions [1-3]. The clinical picture may range from peracute to chronic and asymptomatic carrier pigs can transmit the disease when introduced into uninfected herds. Two different biovars are recognised within the species: biovar 1 strains are nicotinamide adenine dinucleotide (NAD) dependent while biovar 2 strains are NAD-independent [4, 5]. Based on capsular polysaccharides and lipopolysaccharide (LPS) O-chain components, 15 serovars have been described. Serovars 1 to 12 and 15 usually belong to biovar 1; whereas serovars 13 and 14 isolates are typically biovar 2 [5-8], however, biovar 2 variants of serovar 2, 4, 7 and 9 have been reported [5, 9, 10].

Serotyping and other genetic typing methods for *A. pleuropneumoniae* have contributed greatly to surveillance and epidemiological studies. These tools provide important information for decision making in control programs aimed at eradication of virulent types of the bacterium. Nevertheless, serological cross-reactivity between *A. pleuropneumoniae* serovars 1, 9 and 11 [11], between serovars 3, 6 and 8 [12], and between 4 and 7 [13] has been described. In North America, serovars 1, 5 and 7 are reported to be the most prevalent, while serovars 2 and 9 are most commonly isolated in Europe, and serovar 15 is the predominant isolate from Australian pigs [14-16].

The virulence factors described for *A. pleuropneumoniae* include LPS, capsular polysaccharides, Apx toxins (I-IV), outer membrane proteins (OMPs) and various iron acquisition systems. However, the overall contribution of each component to the infection process remains unclear, as do the mechanisms of pathogenesis of this organism [16-18]. All serovars are capable of causing disease; however, some

serovars such as serovars 1, 2, 5a, 5b, 9 and 11 are more frequently involved in severe outbreaks with high mortality and pulmonary lesions. Several reports have suggested that the differences in virulence among the serovars can mainly be attributed to different combinations of Apx toxins and the amount of capsular polysaccharides [17, 19-21]. Almost all of the currently available vaccines against *A. pleuropneumoniae* are either inactivated whole-cell bacterins or subunit combinations of Apx toxins and proteins or OMPs [22]. Experimental challenge and field usage data indicate that these vaccines neither induce cross-serovar immunity nor prevent development of the carrier state and have little impact on morbidity [23-26].

Molecular techniques, including Multilocus Enzyme Electrophoresis (MLEE) [27, 28], Pulsed-Field Gel Electrophoresis (PFGE) [29] and Amplified Fragment Length Polymorphism (AFLP) analysis [30] have been used to study different strains of *A. pleuropneumoniae*. These investigations have shown that genetic diversity among isolates of the same serovar may be almost equivalent to that in the species as a whole, suggesting that *A. pleuropneumoniae* strains are genetically very similar and would seem to have a clonal population structure. However, restriction analysis fingerprinting [31, 32] studies revealed that with the exception of serovars 1 and 9, the reference strains of *A. pleuropneumoniae* are clearly different. Similarly, work of Chevallier *et al.* and Møller *et al.* revealed a more pronounced heterogeneity in the chromosomal structure among strains of serovars 1, 5a, 5b, 7, 8 and 12 [27, 29]. The notion of heterogeneity amongst serovars is also supported by early free-solution DNA-DNA hybridization studies that showed that representative strains of 12 serovars of *A. pleuropneumoniae* shared 74 to 90% sequence homology with *A. pleuropneumoniae* serovar 1 [32].

We are using a reverse vaccinology approach to identify new candidates for the development of cross-protective vaccines against *A. pleuropneumoniae*. Using the complete and annotated *A. pleuropneumoniae* L20 genome [33], Chung and coworkers published a list of 93 predicted OMPs or lipoproteins of *A.*

pleuropneumoniae obtained by using five genome scanning programs [34]. The availability of the genome sequence of *A. pleuropneumoniae* also enables us to study diversity of *A. pleuropneumoniae* on a genome-wide scale. To date, two additional complete genomic sequences have become available, *A. pleuropneumoniae* serovar 3 JL03 [GenBank:CP000687] [35], and *A. pleuropneumoniae* serovar 7 AP76 [GenBank:CP001091].

M-CGH is a powerful tool to estimate whole genomic diversity and to study the gene content and locate genomic islands in closely related strains of bacteria [36-42]. In the present study, this method was applied for the first time to study genetic relationships among reference strains of the 15 serovars of *A. pleuropneumoniae* and representative field isolates. Our goal was to identify conserved genes with particular emphasis on those predicted to encode outer membrane proteins and lipoproteins because of their potential for the development of improved vaccines.

Results and Discussion

The first microarray-based study of *A. pleupneumoniae*, which used a full-genome microarray based upon a draft version of the genome sequence of strain L20, evaluated the effects of iron limitation [43]. From the recently completed genome sequence of *A. pleuropneumoniae* L20 [33], a full genome *A. pleuropneumoniae* microarray (AppChip2; GEO Accession Number GPL6658), which takes into account corrections from the draft sequence and comprises reporters matching the sequence of more than 1800 genes, was developed and used in this study. Experiments with closely related bacterium *Actinobacillus suis* showed only weak hybridization to the AppChip2 microarray, thereby confirming its specificity (data not shown). With microarray hybridization, the presence of a specific gene in a test strain is based on comparison of the intensity of the hybridization signal obtained with the genomic DNA of the tester strain to that obtained with the genomic DNA of the control strain for the corresponding reporter. The ratio of intensity of tester signal to control signal is usually expressed on a log2 scale and we used a threshold of -1 to

define genes likely to be divergent in sequence from the strain L20 genome. We have shown previously that genes absent from the tested strain usually have log2 ratio values less than -3 [37]. The overall genomic variability of the 15 *A. pleuropneumoniae* serovars is shown in Figure 1 where the number of serovars in which a gene is variable is plotted for each gene with the gene order corresponding to the strain L20 genome sequence. Although most genes are conserved across the 15 serovars, we observed a number of distinct clusters of absent/divergent genes. A total of 205 genes were identified as either divergent or highly divergent/absent in the 15 reference strains tested (additional file : Table 3). In the largest cluster (APL_0488 to APL_0525), many genes are annotated as potential phage or prophage genes, suggesting that this cluster might correspond to a phage. A smaller cluster around APL_0947 to APL_0952 corresponds to genes annotated as transposon-related. Several of the other clusters correspond to genes annotated as components of DNA restriction and modification systems. Clusters of variable genes involved in toxin production, and in capsule and LPS biosythesis were observed as expected. Several other clusters containing unannotated genes may warrant further investigation.

We used hierarchical clustering based on the M-CGH results to examine the relationship between *A. pleuropneumoniae* serovars based on genomic content. The dendrogram of the data excluding phage and transposon-related genes is shown in Figure 2. This tree has very similar structure to the tree based on data from all 1857 genes on AppChip2 (data not shown), and both show serovars 5a and 5b forming a distinct subclade. Also, the antigenically related serovars 1, 11, and 9 cluster together as do cross-reactive serovars 4 and 7. Nevertheless we do not observe clustering of serovars 1, 5 and 7 which are the most common ones found in North America or of serovars 13 and 14 which represent biovar 2.

The M-CGH patterns for capsule biosynthesis genes (Figure 3A) show a high degree of variability for the various serovars compared to serovar 5b. This is to be expected as the capsule is a major determinant of the *A. pleuropneumoniae* serovar [6, 44]. Much less variation across serovars is observed with the *cpx* genes which are

involved in capsule export than with the *cps* and *kds* genes which are involved in capsule biosynthesis.

Serotyping of *A. pleuropneumoniae* is based mainly on the capsular polysaccharide (CPS) and the lipopolysaccharide antigenic O-chain component [44]. The M-CGH patterns for capsule biosynthesis genes (Figure 3A) show a high degree of variability for the various serovars compared to serovar 5b, except for serovar 5a. The structures of CPS from subtypes 5a and 5b have been determined [45, 46]. Both structures share a common backbone consisting of disaccharide repeating units, [→6)-α-D-Glc_pNAc(1→5)-β-KDO_p-(2→]$_n$. In addition, the CPS of serovar 5b has a lateral β-D-glucopyranosyl residue [45]. Moreover, both subtypes contain LPS 0-chain components with the same basic polysaccharide structure of a linear unbranched homopolymer of 1,6-linked β-D-galactopyranosyl residues [47]. Thus, the related structures of the capsular polysaccharides of subtypes 5a and 5b are consistant with the identical M-CGH patterns for capsule biosynthesis genes for these two serovars.

The M-CGH pattern of toxin biosynthesis genes for the reference strains of the 15 serovars is shown in Figure 3B. The pattern observed for the toxin genes is in agreement with the results of Frey and co-workers [48] who reported that the *apxIDB* genes are missing in serovar 3; *apxIAC* are missing in serovars 2, 3, 4, 6, 7, 12, 13 and 15; *apxIIAB* genes are missing in serovars 10 and 14. Consistent with previous reports [49-51], *apxIVA* was present in all serovars; as *apxIII* genes are not present in *A. pleuropneumoniae* serovar 5 strains (including L20) they were therefore not included on the AppChip2 microarray.

The genomic variability of 15 representative field isolates of *A. pleuropneumoniae* serovars 1, 7 and 15 is shown in Figure 4. For each serovar, the number of strains in which a gene is variable is plotted for each gene with the gene order corresponding to the strain L20 genome sequence. For serovars 5a, we observed only one cluster of highly variable genes which is the largest cluster (APL_0488 to APL_0525) containing many genes annotated as potential phage or prophage genes in serovar 5b.

To evaluate relationships among the 21 field isolates of serovars 1, 5, 7 and 15 and their respective reference strains, we performed hierarchical clustering to build dendrograms based upon analysis of the data excluding phage and transposase genes (Figure 5). Except for serovar 7, strains from the same serovar formed a cluster. The analysis indicated that the Ontario serovar 7 field isolates 881 and 1951 clustered separately from the Quebec and Saskatchewan serovar 7 strains. In contrast, Ontario and Saskatchewan serovar 1 field isolates were genetically very similar. As expected, the eight serovar 5a and 5b strains form a distinct cluster. These results are consistent with earlier restriction endonuclease fingerprinting analysis, which revealed limited heterogeneity amongst isolates of serovar 1 or serovar 5 whereas serovar 7 isolates showed greater variation [31].

In Canada, the most prevalent serovars are 1, 5 and 7. However, other serovars have also been isolated from sporadic outbreaks of pleuropneumonia. This is the first report describing the isolation and characterization of serovar 15 strains from field cases of porcine pleuropneumonia in North America. In previous reports, serovar 15 strains have only been isolated from pigs in Australia and Japan [15, 52]. Hierarchical clustering based on our M-CGH results showed that the three serovar 15 field strains (05-13146, 05-14657, 05-20080) are closely related to the reference strain of serovar 15 of *A. pleuropneumoniae*. These three serovar 15 field strains also had the same M-CGH pattern of toxin biosynthesis genes as reported for serovar 15 field strain isolated in Japan [52] and the reference strain (HS143). In these strains the Apx structural genes, *apxIIA*, *apxIIB* and *apxIVA*, but not *apxIA* and *apxIC*, and Apx secretion genes *apxIBD* were detected (data not shown).

The serologically non-typeable isolate, 05-15225, possessed the same M-CGH pattern of toxin biosynthesis genes as the reference strains of serovars 5a and 5b. The M-CGH pattern for capsule biosynthesis genes of the 05-15225 isolate showed little variability compared to serovar 5b. Only two genes involved in capsule biosynthesis, *cpsC* and *cpsD* had a log2 ratio value less than -1. These two genes may be deleted

or diverged in sequence in this isolate, which could explain why it was not typable by serology. Nevertheless, the dendrogram showed that 05-15225 isolate is closely related to the serovar 5 (subtypes a and b), suggesting that it can be classified in serovar 5 strains.

Other clustering methods or distances were applied to the data set in order to verify that the clusters we identified were robust (data not shown). Hierarchical clustering with Manhattan Distance metrics and K-Medians Clustering (K-MC) [53] yielded highly similar results: strains from the same serovar clustered together, with the serovar 7 strains from Ontario grouped with serovar 15 strains, although the serotype 15 13146 strain was left on its own following K-Medians Clustering. In both cases, the non-typeable 05-15225 isolate clustered with serovar 5 strains. Cluster Affinity Search Technique [54] generated the same results, however serovar 15 strains were scattered in three different clusters, and were not grouped with serovar 7 strains from Ontario.

Commercially available vaccines against *A. pleuropneumoniae*, including inactivated whole-cell bacterins and subunit vaccines, have limited efficacy and little impact on morbidity. Moreover, these vaccines confer only partial protection against the homologous serovar and generally do not confer protection against challenge with heterologous serovars [55-57]. A major focus of research for the development of new vaccines against porcine pleuropneumonia has been to identify proteins that are conserved in all 15 serovars of *A. pleuropneumoniae* and that generate cross-protection against strains of all serovars. Based on the principle that surface-exposed antigens are more susceptible to recognition by antibodies and therefore are the most suitable candidates for a vaccine, the full genome of *A. pleuropneumoniae* L20 was screened using bioinformatics predictor programs to identify open reading frames encoding putative proteins localized at the bacterial outer membrane and 45 OMPs and 48 lipoproteins were predicted [34]. Using M-CGH to identify genes that are highly conserved among the reference strains of the 15 serovars of *A. pleuropneumoniae*, as well as among field isolates, we identified 58 potential vaccine

targets (24 OMPs and 34 lipoproteins) (Table 2) that are conserved among all serovars and biovars. Among these candidates, four have been shown to be expressed *in vivo*. Using selective capture of transcribed sequences analysis (SCOTS) [58], it has been shown that *ompA* (*APL_1421*) and *APL_0829* are expressed by *A. pleuropneumoniae* in necrotic pig lung tissue. Furthermore, polyamine transport protein D (APL_0368), Omp P2 (APL_0649), OmpA (APL_1421) and outer membrane antigenic lipoprotein B (APL_1930) are required for efficient colonization of the porcine host by *A. pleuropneumoniae* as shown by signature-tagged mutagenesis (STM) experiments [59, 60].

A. pleuropneumoniae bind preferentially to cells of the lower respiratory tract, where some essential nutrients for the growth of bacteria, such as iron, are limited. *A. pleuropneumoniae* has developed several iron uptake systems including the hydroxamate siderophore receptor FhuA (APL_2016), a hemoglobin-binding receptor HgbA (APL_1047) and a transferrin receptor complex composed of two outer membrane proteins, transferrin-binding protein A (TbpA; APL_1567) and transferring-binding protein B (TbpB; APL_1568). Not only is iron essential for growth of bacteria but iron-restriction is an important signal that controls expression of many genes including some coding for virulence factors [43]. Hence, these proteins involved in iron uptake are considered as candidates for development of subunit vaccines. However, our M-CGH results showed that only one protein involved in iron uptake, TbpA, is conserved among the 15 serovars of *A. pleuropneumoniae*. This observation could explain the partial protection against infection with heterologous strains conferred by an acellular pentavalent subunit vaccine containing the TbpB of *A. pleuropneumoniae* serovar 7 [26].

The NADPH-sulfite reductase hemoprotein CysI (APL_1842) of *A. pleuropneumoniae*, a cytoplasmic protein involved in cellular metabolism, has also been shown to induce protective immunity against a homologous challenge [61]. Our CGH analysis showed that CysI is conserved among all strains tested in this study, thus this protein could represent an interesting vaccine target.

Two genes (*APL_1421* and *APL_1852)*, encoding homologs of outer membrane protein A (or OMP P5), were identified as potential vaccine targets in this study. APL_1421 and APL_1852 have 70.6% identity and showed 75.6% and 70.6% identity, respectively, to OmpA from the bovine pathogen *Mannheimia haemolytica*, which has surface exposed epitopes and is recognized by convalescent bovine sera [62, 63]. In addition to *ompA* (*APL_1421*), the outer-membrane lipoprotein LolB (APL_0777), is expressed *in vivo* by *A. pleuropneumoniae* and they have been identified and characterized as potential components of a cross-protective sub-unit vaccine against *A. pleuropneumoniae* [64]. However, Oldfield *et al.* reported that neither of these proteins was capable of eliciting protective immunity against *A. pleuropneumoniae* challenge [64]. The outer membrane protein PalA (APL_0304) was also on our list of conserved proteins. Nevertheless, it has been shown that vaccination with PalA increases the severity of *A. pleuropneumoniae* infection in vaccinated pigs [65]. Thus, the deleterious effect of PalA in vaccination and the inability of LolB and OmpA to induce protective immunity eliminate these proteins from the list of potential vaccine candidates.

Interestingly, homologs of some of the remaining conserved vaccine candidates are already under investigation as vaccine components in other bacteria (including other Pasteurellaceae). For example, the lipoprotein Plp4 from *M. haemolytica*, which had 85.3% identity with lipoprotein PlpD (APL_0460), was identified by screening antigens of *M. haemolytica* with sera from Presponse^{TM} vaccinated calves (a cell-free culture supernatant *M. haemolytica* A1 vaccine) that were protected from *M. haemolytica* A1 infections [66], thus suggesting PlpD may be a protective antigen. Another candidate, APL_0378, a glycerophosphodiester phosphodiesterase (GlpQ), showed 82% and 79.5% identity to GlpQ of *Pasteurella multocida* and protein D of *Haemophilus influenzae*, respectively. The role of GlpQ in *A. pleuropneumoniae* and *P. multocida* is unknown, however, its homolog in *H. influenzae* has been shown to mediate the acquisition of choline directly from the membranes of epithelial cells in culture and incorporate it into its own LPS [67]. *H. influenzae* protein D has been showed to elicit cross-protection against virulent heterologous strains of *H.*

influenzae in rats [68]. In contrast to *H. influenzae*, GlpQ in *P. multocida* is not surface-exposed and is unable to stimulate protective immunity, even though vaccinated animals have high antibody titers [69]. Therefore, the location of GlpQ and accessibility of GlpQ-specific antibodies should be determined in *A. pleuropneumoniae*.

OMP P4 (*ompP4*; ALP_0389) is another attractive surface exposed antigen [70] showing 65% identity to lipoprotein E (also known as OMP P4) in *H. influenzae*, which is highly conserved among both typeable and nontypeable strains [71]. In nontypeable strains of *H. influenzae* (NTHi), OMP P4 is essential for utilization of NAD and subsequent growth [72, 73]. Intranasal immunization of mice with OMP P4 in a mucosal adjuvant induces protective immune responses against NTHi infections and notably, a mucosal immune response, which reduces NTHi nasopharynx colonization [74]. These observations in *H. influenzae* suggest that lipoprotein E (APL_0389) may be an attractive candidate for a vaccine against *A. pleuropneumoniae*.

PCP (peptidoglycan-associated lipoprotein cross-reacting protein) of *H. influenzae* is under investigation since it is surface-exposed and anti-PCP serum shows bactericidal activity against several clinical isolates of type b and non-typeable *H. influenzae* [75]. Outer membrane lipoprotein SlyB (APL_0037) shows highest identity (71.4%) to this protein. However, although PCP from *P. multocida* (also surface-exposed) is recognized by convalescent chicken antiserum, it is unable to stimulate protective immunity [69].

The polyamine transport protein D, PotD (APL_0368), has extensive homology to a 38 kDa lipoprotein, Lpp38 of *M. haemolytica*. Lpp38 is surface-exposed and is recognized by sera from calves resistant to infection after natural exposure to *M. haemolytica* and by sera from calves vaccinated with M. *haemolytica* A1 outer membranes or with live bacteria [76]. Recently, in the human pathogen *Streptococcus pneumoniae*, PotD has been reported to be involved in virulence in

both an animal model of sepsis and pneumonia [77, 78]. Active immunization of mice with recombinant PotD induces a vigorous antibody response and provides a significant degree of protection against lethal pneumococcal infection [79]. These data suggest that PotD plays a role in the development of immunity to bacterial infections and may be a protective antigen.

Homologs of the outer membrane protein D15 (APL_0411) have been reported in several pathogenic bacteria including *H. influenzae*, *Haemophilus ducreyi*, *P. multocida*, *Neisseria meningitidis* and *Shigella dysenteriae* [80-83]. These studies suggest a role for these proteins in pathogenesis and immunity. Notably, it has been shown that D15 confers protection against homologous and heterologous strains of *H. influenzae* in animal models [84-86]. Similarly, Oma87, a closely related homolog in *P. multocida*, has been shown to elicit protection in animal model of infection [83]. Thus D15 is potentially an attractive vaccine target.

In this study, we have shown that M-CGH can be a useful tool to identify candidates for reverse vaccinology in order to develop subunit vaccines to *A. pleuropneumoniae*. This could assist in development of vaccines with efficacy across serovar boundaries. Future investigations will include the use of microarray transcript profiling experiments of *A. pleuropneumoniae* isolated from infected pigs to identify potential vaccine candidates that are both conserved and expressed *in vivo* during infection in pigs.

Methods

Bacterial strains

Reference strains and field isolates of *A. pleuropneumoniae* used in this study are listed in Table 1. All *A. pleuropneumoniae* strains were inoculated into Brain-Heart Infusion (BHI, Difco Laboratories, Detroit, MI) medium supplemented with NAD: either 15 μg/ml in agar or 5 μg/ml in broth. Cultures were grown at 37°C for 16-18 hours before genomic DNA isolation.

Construction of an A. pleuropneumoniae amplicon-based DNA microarray (AppChip 2)

PCR primers were designed for 1954 genes of the *A. pleuropneumoniae* L20 (serovar 5b) genome using the Primer3 program [87] controlled by an automated script as described previously [make_primers code available at http://www.ibs-isb.nrc-cnrc.gc.ca/glycobiology/group_software_e.html] [88]. Most sequences of length greater than 2000 nt were split to create two or more reporters corresponding to a single large ORF. Primer selection parameters were standardized and included similar predicted melting temperature (60 ± 2°C), uniform length (25 nt), and minimum amplicon size of 160 bp. Generation of PCR amplicons and fabrication of DNA microarrays were described previously [88]. Details on the construction of this microarray (AppChip2) are available at NCBI (GEO Accession Number GPL6658). The AppChip2 microarray comprises validated amplicons covering >92% of the ORFs longer than 160 bp in the final *A. pleuropneumoniae* L20 genome sequence (GenBank accession number CP000569).

Isolation of genomic DNA

A. pleuropneumoniae strains were harvested after growth on agar plates for 16-18 h, resuspended in H_2O, and treated with lysozyme (Roche, Laval, QC) and RNase A (Qiagen, Mississauga, ON) for 10 min at room temperature. The cell suspensions were then digested with proteinase K (MBI Fermentas, Burlington, ON) for 1 h at 37°C, and complete lysis was obtained by addition of sodium dodecyl sulfate to a final concentration of 0.1% (wt/vol). Genomic DNA, extracted from the cell lysates by two extractions with phenol-chloroform-isoamyl alcohol (25:24:1) and two extractions with chloroform, was precipitated in ethanol.

Genomic DNA labelling

Isolated genomic DNA was fragmented by nebulization. One hundred μg of DNA in H_2O and 35% glycerol (v/v) was placed in an AeroMist Nebulizer chamber (IPI Medical Products, Chicago, IL), and sheared by passing nitrogen gas through the chamber at 15 psi for 1 min. The DNA was precipitated with ethanol and suspended

in 100 µl of ddH2O. Typically, the DNA was fragmented to a range of 0.4 to 12 kb in size. Five µg of fragmented DNA were fluorescently labeled using direct chemical coupling with the Label-IT (Mirus Corp., Madison, WI) cyanine dyes Cy3 and Cy5 as recommended by the manufacturer. Probes were purified from unincorporated dyes by passing samples through Qiaquick columns (Qiagen, Mississauga, ON). Labeled DNA sample yields and dye incorporation efficiencies were assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop, Rockland, DE).

Microarray hybridizations

The hybridization profile for each strain was obtained by co-hybridizing labeled DNA from the tester strain with labeled DNA from the *A. pleuropneumoniae* serovar 5b (L20) control strain to the microarray. DNA from tester strains was labeled with Cy3 and DNA from the control strain with Cy5. Dye swaps were performed on selected strains to test for any dye-incorporation bias. Labeled samples were normalized by selecting tester/control sample pairs with similar dye incorporation efficiencies. Equivalent amounts (2 µg) of labeled tester and control samples were pooled, lyophilized, and then re-suspended in 42 µl of hybridization buffer [1 × DIGEasy hybridization solution (Roche Applied Science); 0.5 µg/µl of *Torulla* yeast tRNA (Invitrogen); 0.5 µg/µl of salmon sperm genomic DNA (Invitrogen)]. Labeled gDNA was denatured at 65°C for 5 min and applied to the microarray. Hybridizations were performed overnight at 37°C under 22 × 40-mm glass cover slips in a high-humidity chamber. Microarrays were washed 2 × 5 min at 50°C in 1 × SSC with 0.1% SDS, then 2 × 5 min at 50°C in 0.5 × SSC, and 1 × 5 min at 50°C in 0.1 × SSC. Slides were spun dry (500 × g, 5 min) and stored in lightproof containers until scanned.

Data acquisition and analysis

After hybridization with labeled gDNA, microarray slides of the 15 reference serovars were scanned using a Chipreader laser scanner (BioRad, Mississauga, ON) according to the manufacturer's recommendations. Spot quantification, signal normalization and data visualization were performed using ArrayPro Analyzer v4.5

(Media Cybernetics, Silver Spring, MD). Net signal intensities were obtained by performing local-ring background subtraction. "Tester signal" is defined as the signal intensity of the selected *A. pleuropneumoniae* reference strains labeled with appropriate fluorescent dye, while "control signal" is defined as the signal intensity of *A. pleuropneumoniae* strain 5b labeled with its appropriate fluorescent dye. The ratio of tester signal to control signal for each gene was transformed to its base 2 logarithm [89], $\log_2$ [Tester Signal/Control Signal], and is referred to as "Log2Ratio". Data from each channel were adjusted using cross-channel Loess normalization of the Log2Ratio data and low intensity and anomalous spots were flagged and removed. Data were stored and archived using the BASE BioArray Software Environment [90]. Microarray data from sets of hybridizations were exported from BASE after removal of flagged spots, Loess normalization, and averaging of data from duplicate spots on the microarray. At least three replicates of each strain were performed and the results averaged.

The microarrays for the *A. pleuropneumoniae* field strains were scanned with a Perkin-Elmer ScanArray Express scanner according to the manufacturer's recommendations. Image and data analysis were performed using TM4 suite of softwares from the J. Craig Venture Institute [91]. Raw data were generated using Spotfinder v.3.1.1. The integrated intensities of each spot, equivalent to the sum of unsaturated pixels in a spot were quantified and the integrated intensity of the local background was subtracted for each spot. The same operation was performed with the median spot intensities. Data were normalized with the MIDAS software tool using cross-channel Loess normalization. Spots with median intensities lower than 1000 were removed from the normalized data set. Intensities for duplicate spot were merged to generate the final normalized data set. The results were analyzed using the MEV software, first to check similarity of patterns of gene divergence within replicates for each serovar, then to examine data averaged across replicates of each serovar. Data were submitted to the Gene Expression Omnibus [GEO:GSE11921 and GSE14639].

To evaluate M-CGH results obtained using the different methods, we compared M-CGH data from *A. pleuropneumoniae* serovar1 versus serovar 5b hybridizations that were collected, scanned and processed using ArrayPro software or SpotFinder/MIDAS software (Figure 6). Excellent correlation between M-CGH data was obtained using the two different data acquisition and analysis methods.

In cases where more than one reporter on the chip corresponded to a single gene, the data were averaged across the reporters. Visualization and hierarchical clustering of microarray data, using Euclidean Distance metrics and Average Linkage Clustering, was performed in MEV using algorithms developed by Eisen *et al*. [92]. To examine the variation of CGH profiles between the different serovar strains, we generated sample trees as well as support trees based on bootstrapping genes with 1000 iterations. Hierarchical clustering with Manhattan Distance metrics, as well as K-Median Clustering [53] (5 clusters, 1000 iterations) and Cluster Affinity Search Technique [54] (threshold=0.55) were also performed in MEV to ensure robustness of the clusters.

List of abbreviations

AFLP : Amplified Fragment Length Polymorphism, BHI : Brain Heart Infusion, CPS : Capsular Polysaccharide, LPS : Lipopolysaccharide, M-CGH : Microarray-based Comparative Genomic Hybridization, MLEE : Multilocus Enzyme Electrophoresis NAD : Nicotinamide Adenosine Dinucleotide, OMP : Outer Membrane Protein, ORF : Open Reading Frame, PFGE : Pulsed-Field Gel Electrophoresis.

Authors' contributions

JG designed the M-CGH experiments and performed the hybridizations and data analysis with the field isolates. WAF managed the overall bioinformatics, analysed the microarray data from the 15 serovars, and contributed to the design and

construction of the microarray. VD contributed to the design of the experiments and participated in the downstream analysis. AB performed the M-CGH hybridizations of the fifteen serovars strains and assisted with the construction of the microarray. SJF assisted with the design of the microarray and the bioinformatics analyses. JIM and JWC participated in the study design and revised the manuscript. JHEN and MJ participated in the conception and supervised the design of the study and revised the manuscript. All authors read and approved the final manuscript.

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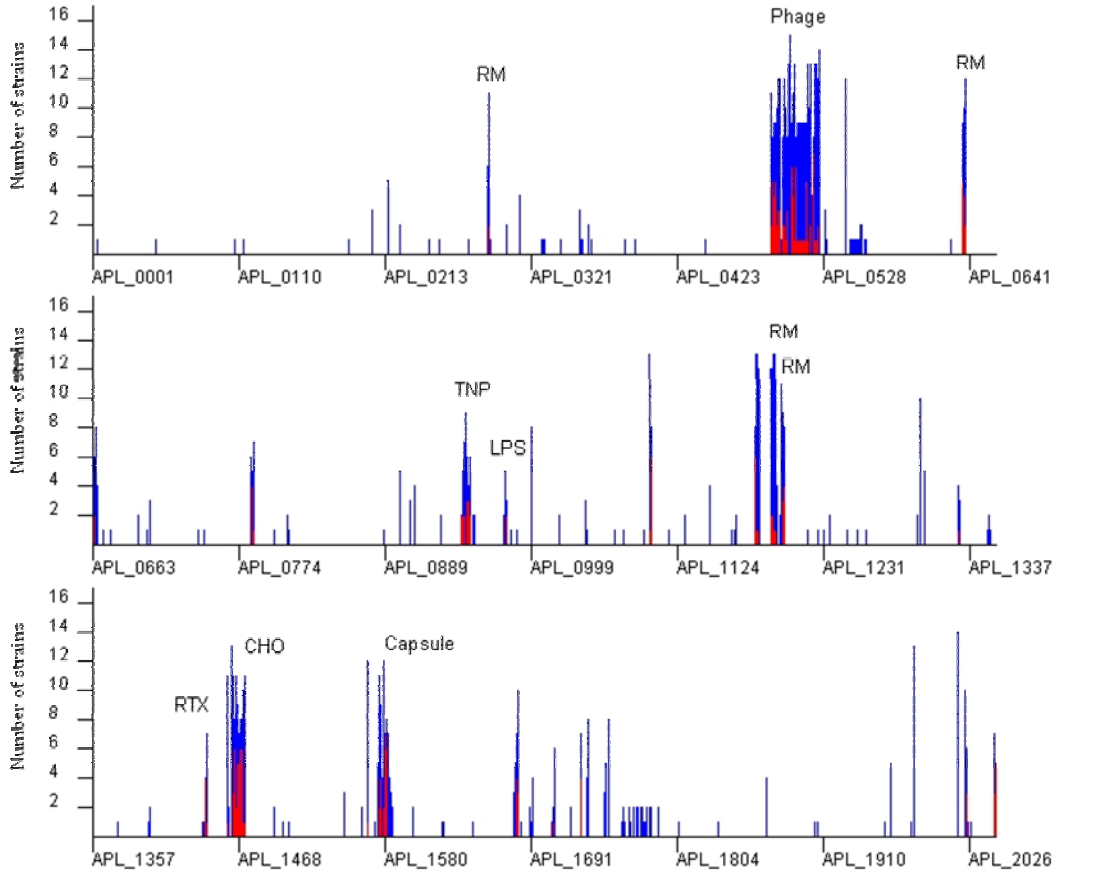

Figure 1: Number of reference strains representing the 15 serovars of *A. pleuropneumoniae* where gene is divergent or missing for each gene, ordered as in *A. pleuropneumoniae* L20 genome sequence and based on M-CGH results. (Blue: -3<Log2Ratio<-1, Red: Log2Ratio<-3)

RM, DNA restriction/modification enzymes; TNP, transposon; LPS, lipopolysaccharide biosynthesis genes; RTX, toxin genes; CHO, carbohydrate biosynthesis genes

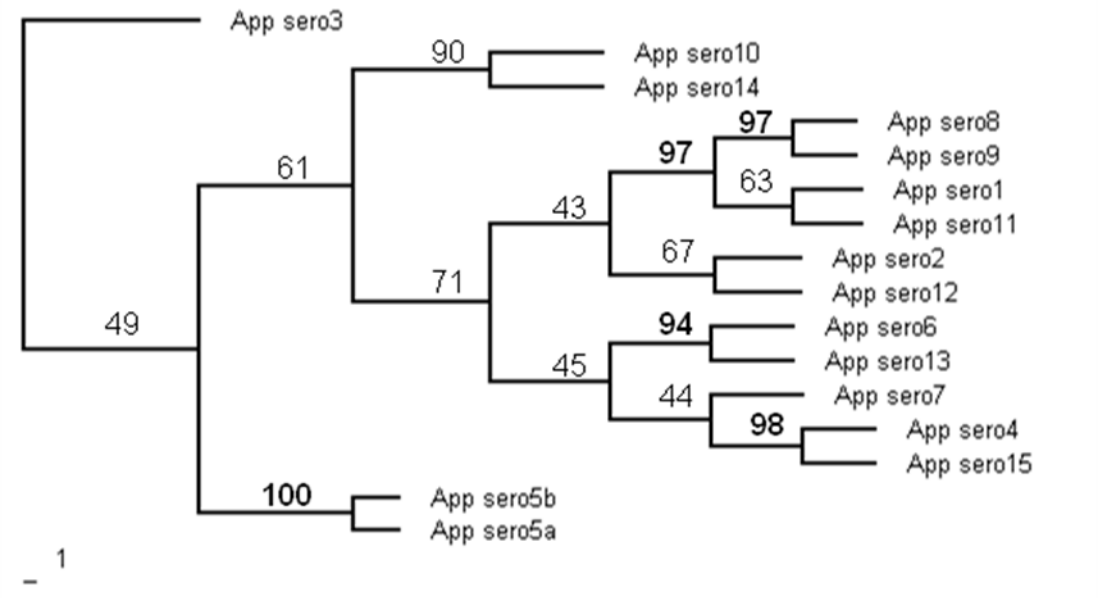

Figure 2: Hierarchical clustering of *A. pleuropneumoniae* reference strains based on M-CGH data excluding phage and transposase genes.

The dendrogram was produced using the MEV software from the J. Craig Venter Institute with Euclidean distance and average linkage clustering (n=1000 bootstrap iterations).

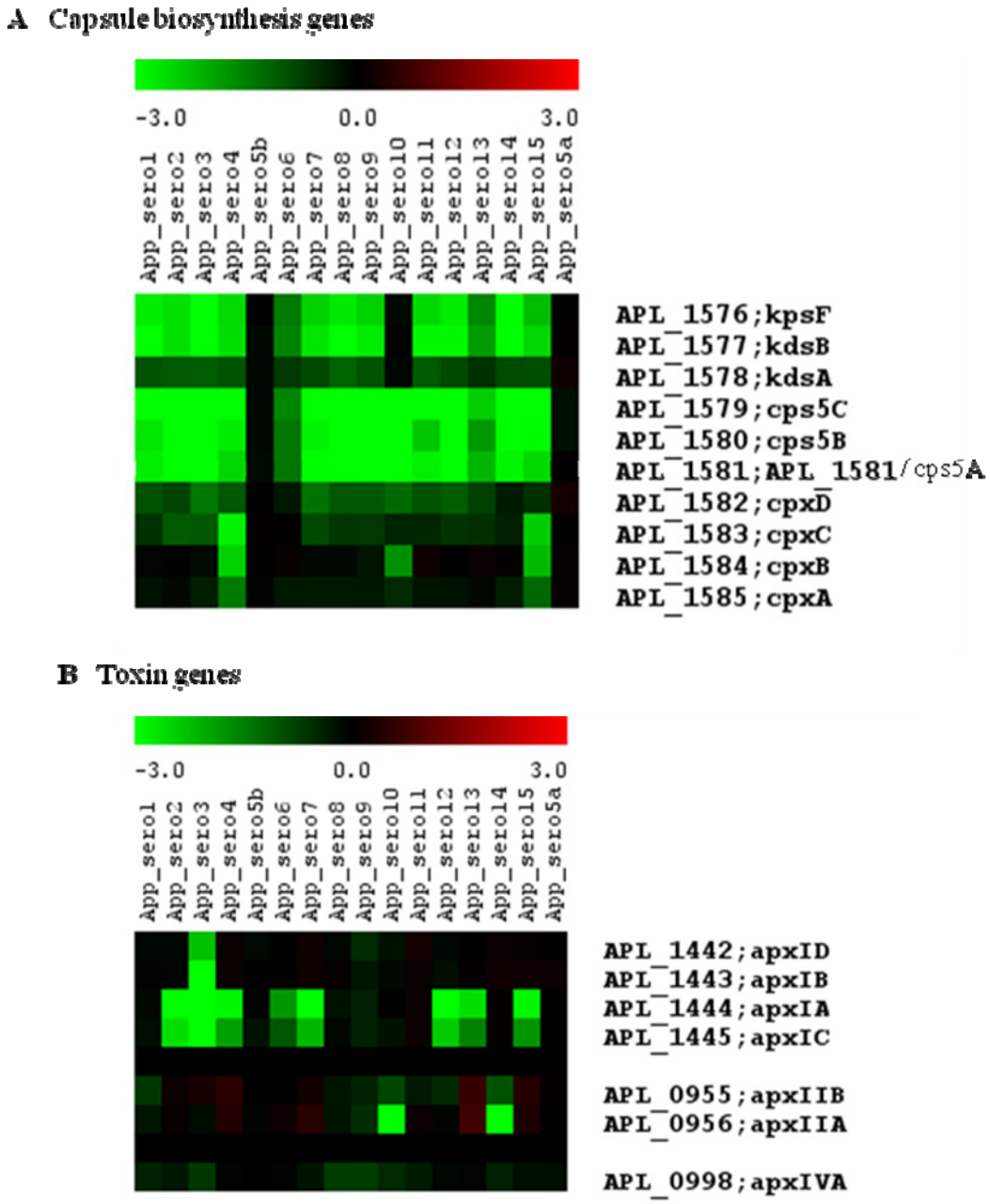

Figure 3: Variability of genes involved in toxin and capsule biosynthesis across the *A. pleuropneumoniae* reference strains representing the 15 serovars.

Green indicates that the gene is divergent in sequence or absent in the tester strain.

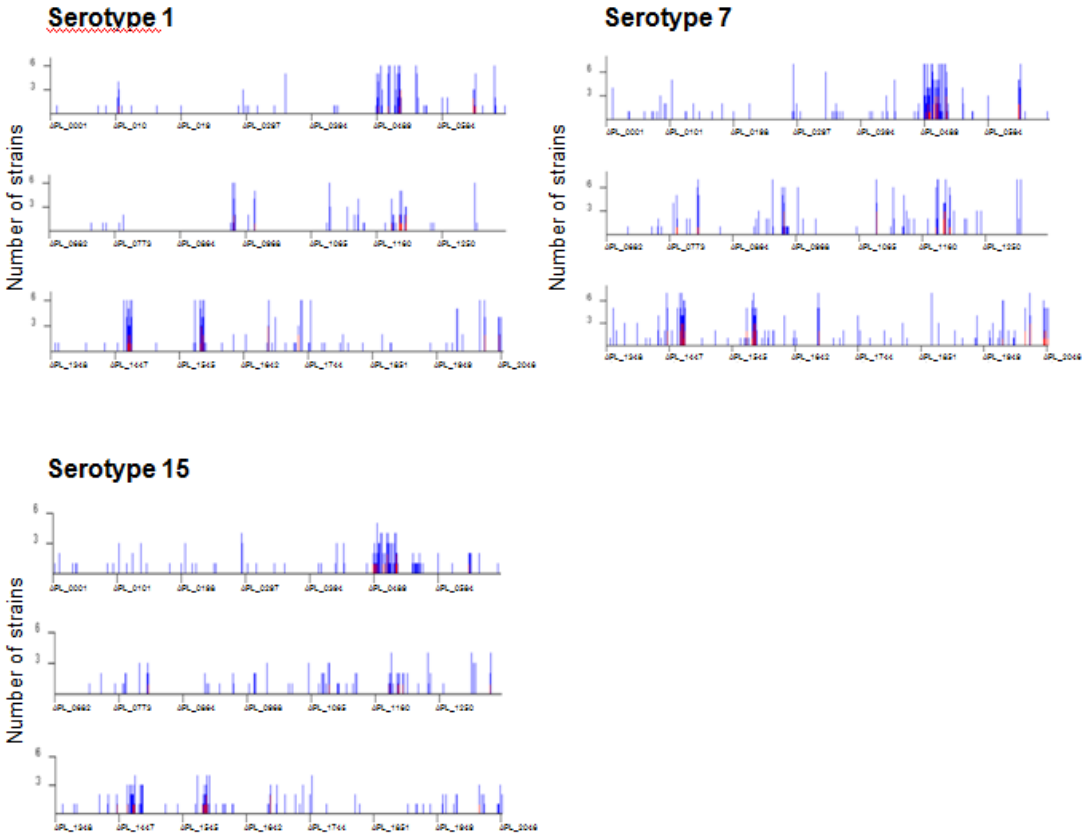

Figure 4: Number of strains from 15 fresh field isolates of *A. pleuropneumoniae* serovars 1, 7 and 15 where the sequence is divergent or absent for each gene, ordered as in *A. pleuropneumoniae* L20 genome sequence and based on M-CGH results.

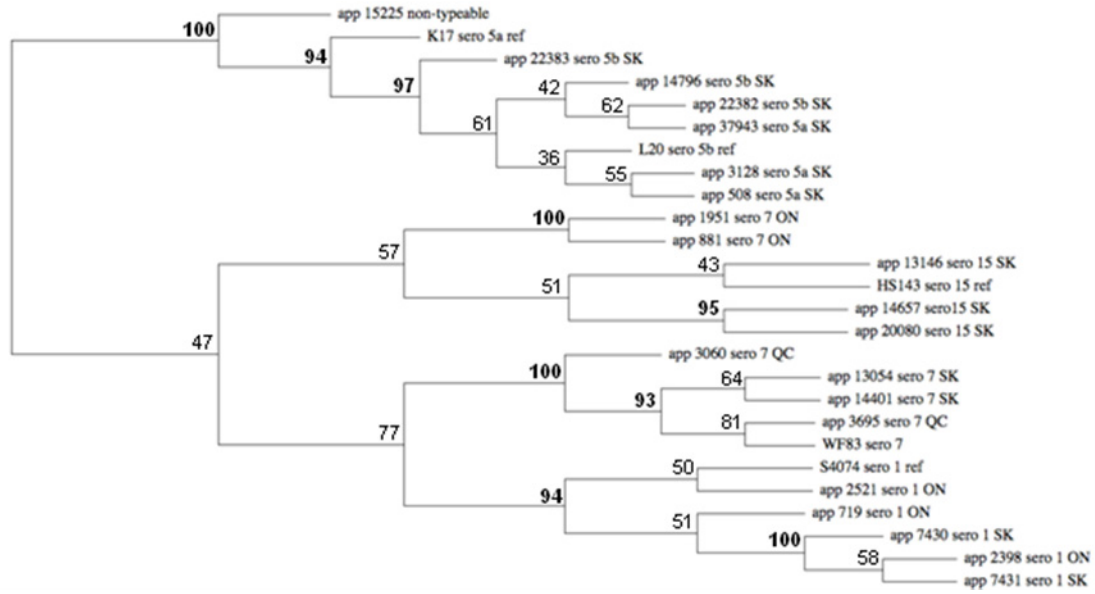

Figure 5: Hierarchical clustering of field isolates of *A. pleuropneumoniae* based on M-CGH data excluding phage and transposase genes.

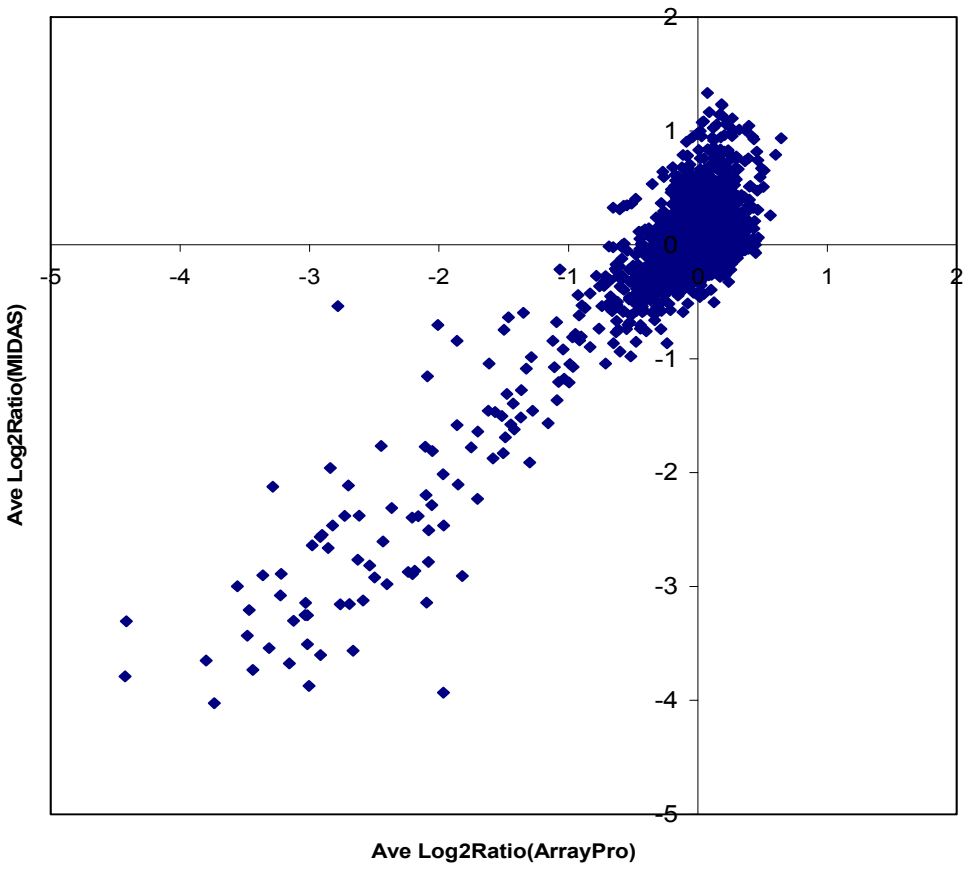

Figure 6: Comparison between M-CGH Log2Ratio data from *A. pleuropneumoniae* serovar 1 versus serovar 5b hybridizations independently collected, scanned and processed using either ArrayPro software or SpotFinder/MIDAS software. At least 2 independent hybridizations were performed for each dataset, and the processed normalized data was averaged between replicate experiments and between pairs of duplicate spots on the microarray.

Tables

Table 1: *A. pleuropneumoniae* reference strains and field isolates analyzed by M-CGH in this study.

Strains	Serovars	Source
Reference strains		All from K.R. Mittal[1]
S4074	1	
S1536	2	
S1421	3	
M62	4	
K17	5a	
L20	5b	
Femø	6	
WF83	7	
405	8	
CVJ 13261	9	
13069	10	
56153	11	
8329	12	
N273	13	
3906	14	
HS143	15	
Field strains		
05-7430, 05-7431	1	M. Ngeleka [2]
719, 2398, 2521	1	D. Slavic [3]
04-37943, 04-3128, 05-508	5a	M. Ngeleka [2]
04-14796, 03-22382, 03-22383	5b	M. Ngeleka [2]
05-3695, 06-3060	7	S. Messier [1]
04-13054, 05-14401	7	M. Ngeleka [2]
881, 1951	7	D. Slavic [3]
05-13146, 05-14657, 05-20080	15	M. Ngeleka [2]
05-15225	non-typeable	M. Ngeleka [2]

[1] Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, QC
[2] Prairie Diagnostic Services, University of Saskatchewan, Saskatoon, SK
[3] Ontario Veterinary College, University of Guelph, Guelph, ON

Table 2: *In silico* predicted OM proteins and lipoproteins from *A. pleuropneumoniae* (according to Chung et al. 2007), which are conserved amongst the 15 reference strains and the 21 field isolates tested by M-CGH in this study.

OrfID	Gene	Product
Predicted outer membrane proteins		
APL_0006	*ompP2A*	outer membrane protein P2
APL_0049	*APL_0049*	hypothetical protein
APL_0200	*hofQ*	type II secretory pathway, component HofQ
APL_0245	*APL_0245*	transferrin binding protein-like solute binding protein
APL_0257	*APL_0257*	probable outer membrane protein
APL_0276	*frpB*	iron-regulated outer membrane protein B
APL_0304	*palA*	outer membrane protein precursor PalA
APL_0410	*APL_0410*	hypothetical outer membrane protein
APL_0411	*D15*	protective surface antigen D15 precursor
APL_0460	*plpD*	lipoprotein Plp4
APL_0565	*cirA*	hypothetical ABC transporter ATP-binding protein
APL_0649	*ompP2*	Outer membrane protein P2 precursor (OMP P2)
APL_0829	*APL_0829*	hypothetical protein
APL_0840	*APL_0840*	predicted outer membrane protein
APL_0919	*irp*	iron-regulated outer membrane protein
APL_0959	*APL_0959*	hemagglutinin/hemolysin-like protein
APL_0962	*ostA*	organic solvent tolerance protein precursor
APL_1421	*ompA*	outer membrane protein P5 precursor
APL_1567	*tbpA*	transferrin-binding protein 1 Tbp1
APL_1705	*APL_1705*	FKBP-type peptidyl-prolyl cis-trans isomerase
APL_1815	*APL_1815*	hypothetical protein
APL_1852	*ompA*	Outer membrane protein P5 precursor (OMP P5)
APL_1921	*pgaA*	biofilm PGA synthesis protein PgaA precursor
APL_2002	*APL_2002*	hypothetical protein
Predicted lipoproteins		
APL_0029	*APL_0029*	ABC transporter periplasmic protein
APL_0036	*APL_0036*	hypothetical protein
APL_0037	*slyB*	outer membrane lipoprotein
APL_0116	*APL_0116*	hypothetical protein

APL_0124	*APL_0124*	hypothetical protein
APL_0156	*apbE*	thiamine biosynthesis lipoprotein ApbE precursor
APL_0227	*APL_0227*	hypothetical protein
APL_0236	*APL_0236*	putative lipoprotein
APL_0332	*hlpB*	lipoprotein HlpB
APL_0356	*APL_0356*	hypothetical protein
APL_0368	*potD2*	spermidine/putrescine-binding periplasmic protein 1 precursor
APL_0378	*glpQ*	glycerophosphoryl diester phosphodiesterase
APL_0389	*ompP4*	lipoprotein E precursor
APL_0428	*smpA*	small protein A
APL_0603	*APL_0603*	hypothetical protein
APL_0611	*APL_0611*	putative lipoprotein
APL_0642	*mltB*	membrane-bound lytic murein transglycosylase B
APL_0777	*lolB*	outer-membrane lipoprotein LolB precursor
APL_0816	*mltA*	membrane-bound lytic murein transglycosylase A precursor
APL_0873	*rlpB*	putative rare lipoprotein B
APL_0920	*APL_0920*	hypothetical protein
APL_1062	*APL_1062*	hypothetical protein
APL_1121	*APL_1121*	putative lipoprotein
APL_1152	*APL_1152*	hypothetical protein
APL_1273	*APL_1273*	putative fimbrial biogenesis and twitching motility protein PilF-like protein
APL_1297	*APL_1297*	hypothetical protein
APL_1362	*APL_1362*	hypothetical protein
APL_1404	*oapB*	opacity associated protein B
APL_1741	*mltC*	Membrane-bound lytic murein transglycosylase C precursor
APL_1875	*APL_1875*	hypothetical protein
APL_1898	*APL_1898*	hypothetical protein
APL_1913	*pepO*	neutral endopeptidase
APL_1930	*APL_1930*	Outer membrane antigenic lipoprotein B precursor
APL_1957	*APL_1957*	Lipoprotein_5 domain containing protein

Additional Data

Table 3: Mean Log2Ratio for genes that are divergent or highly divergent/absent in at least one reference strain of *A. pleuropneumoniae*.

	Log2Ratio<-1.0 (divergent)			Log2Ratio<-3.0 (highly divergent or missing)		
Gene	#Strains	Mean Log2Ratio	Serovar(s)	#Strains	Mean Log2Ratio	Serovar(s)
APL_0052	1	-1.238	3	0	0	-
APL_0106	1	-1.010	8	0	0	-
APL_0113	1	-1.395	3	0	0	-
APL_0188	1	-1.415	14	0	0	-
APL_0204	3	-1.946	3, 4, 15	0	0	-
APL_0215	4	-1.575	8, 9, 10, 11	0	0	-
APL_0246	1	-1.076	14	0	0	-
APL_0253	1	-1.128	15	0	0	-
APL_0273	1	-1.264	8	0	0	-
APL_0289	6	-1.636	2-4, 7, 14, 15	0	0	-
APL_0290	13	-2.153	1-4, 7-15	2	-3.792	3, 14
APL_0293	1	-4.161	14	1	-4.161	14
APL_0313	4	-1.122	3, 11, 13, 14	0	0	-
APL_0328	1	-1.545	10	0	0	-
APL_0329	1	-1.539	10	0	0	-
APL_0330	1	-1.122	10	0	0	-
APL_0341	1	-1.143	2	0	0	-
APL_0355	3	-1.609	8, 9, 11	0	0	-
APL_0356	1	-1.103	8	0	0	-
APL_0357	1	-1.539	8	0	0	-
APL_0363	1	-1.057	10	0	0	-
APL_0387	1	-1.055	2	0	0	-
APL_0442	1	-1.347	8	0	0	-
APL_0488	13	-2.025	1-4, 6-12, 14, 15	2	-3.084	3, 14
APL_0489	13	-2.662	1-4, 6, 8-12, 14-15	5	-3.610	2, 3, 12, 14, 15
APL_0490	13	-2.490	1-4, 7-15	4	-3.246	3, 8, 12, 14
APL_0493	14	-3.279	1-4, 5a, 6, 8-15	9	-3.915	2, 3, 5a, 8, 9, 11, 12, 14, 15
APL_0494	12	-3.532	1-3, 5a, 6, 8-12, 14, 15	10	-3.754	2, 3, 5a, 8-12, 14, 15
APL_0495	15	-3.664	1-4, 5a, 6-15	12	-4.070	2-4, 5a, 6-12, 14, 15
APL_0496	15	-3.648	1-4, 5a, 6-15	12	-3.941	2-4, 5a, 6-12, 14, 15
APL_0498	1	-1.360	5a	0	0	-
APL_0499	10	-2.785	2-4, 5a, 6, 7, 12-15	2	-3.759	3, 5a
APL_0500	14	-2.199	2-4, 5a, 6-15	2	-3.458	3, 5a
APL_0501	11	-2.184	2-4, 5a, 6, 7, 10, 12-15	1	-3.320	5a
APL_0502	11	-3.478	2-4, 5a, 6, 7, 10, 12-15	8	-4.043	2-4, 5a, 7, 12, 14, 15
APL_0503	13	-2.139	2-4, 5a, 6-9, 11-15	0	0	-
APL_0504	15	-1.921	1-4, 5a, 6-15	0	0	-
APL_0505	15	-3.040	1-4, 5a, 6-15	9	-3.567	2, 3, 5a, 7-9, 12, 14, 15
APL_0506	15	-2.585	1-4, 5a, 6-15	4	-3.539	3, 5a, 14, 15

APL_0507	14	-2.318	1-4, 5a, 6-9, 11-15	1	-3.708	5a
APL_0508	14	-3.248	1-4, 5a, 6-9, 11-15	8	-3.845	2-4, 5a, 7, 12, 14, 15
APL_0509	10	-3.799	2-4, 5a, 6, 7, 10, 12, 14, 15	9	-3.950	2-4, 5a, 7, 10, 12, 14, 15
APL_0510	10	-4.157	2-4, 5a, 6, 7, 10, 12, 14, 15	9	-4.385	2-4, 5a, 7, 10, 12, 14, 15
APL_0511	10	-2.627	2-4, 5a, 6, 7, 10, 12, 14, 15	1	-4.117	5a
APL_0512	10	-2.275	2-4, 5a, 6, 7, 10, 12, 14, 15	1	-3.974	5a
APL_0513	10	-2.564	2-4, 5a, 6, 7, 10, 12, 14, 15	1	-3.492	5a
APL_0514	10	-2.454	2-4, 5a, 6, 7, 10, 12, 14, 15	1	-3.197	5a
APL_0515	14	-3.192	1-4, 5a, 6-12, 14, 15	9	-3.717	2-4, 5a, 7, 10, 12, 14, 15
APL_0516	14	-2.036	1-4, 5a, 6-12, 14, 15	1	-3.626	5a
APL_0517	10	-1.318	1-4, 5a, 7, 10, 12, 14, 15	0	0	-
APL_0518	15	-4.323	1-4, 5a, 6-15	13	-4.668	1-4, 5a, 7-12, 14, 15
APL_0519	4	-1.158	1, 5a, 11, 12	0	0	-
APL_0520	15	-2.878	1-4, 5a, 6-15	8	-3.330	3, 4, 8, 10-12, 14, 15
APL_0522	14	-3.871	1-4, 5a, 6-12, 14, 15	13	-4.002	1-4, 5a, 7-12, 14, 15
APL_0523	14	-3.366	1-4, 5a, 6-12, 14, 15	13	-3.456	1-4, 5a, 7-12, 14, 15
APL_0524	14	-4.170	1-4, 5a, 6-12, 14, 15	12	-4.526	1-4, 5a, 8-12, 14, 15
APL_0525	14	-2.231	1-4, 5a, 6-12, 14, 15	0	0	-
APL_0532	3	-1.709	3, 10, 14	0	0	-
APL_0533	1	-1.103	3	0	0	-
APL_0546	12	-1.777	1-4, 8-15	0	0	-
APL_0549	1	-3.769	3	1	-3.769	3
APL_0550	1	-4.368	3	1	-4.368	3
APL_0551	1	-4.466	3	1	-4.466	3
APL_0552	1	-5.131	3	1	-5.131	3
APL_0553	1	-5.064	3	1	-5.064	3
APL_0554	1	-2.147	3	0	0	-
APL_0555	1	-4.039	3	1	-4.039	3
APL_0556	2	-3.760	2, 3	2	-3.760	2, 3
APL_0557	2	-3.336	2, 3	2	-3.336	2, 3
APL_0560	1	-2.696	3	0	0	-
APL_0561	1	-1.095	3	0	0	-
APL_0625	1	-1.232	10	0	0	-
APL_0633	14	-2.945	1-4, 6-15	9	-3.252	1-3, 7-10, 12, 14
APL_0635	14	-3.338	1-4, 6-15	10	-3.617	2-4, 7-10, 12, 14, 15
APL_0636	14	-3.444	1-4, 6-15	12	-3.627	1-4, 7-12, 14, 15
APL_0663	3	-1.130	8, 10, 11	0	0	-
APL_0664	8	-2.890	1, 2, 8-11, 13, 14	2	-3.396	10, 14
APL_0665	8	-2.240	1, 2, 8-11, 13, 14	0	0	-
APL_0666	4	-1.739	8, 9, 10, 14	0	0	-
APL_0670	1	-1.055	10	0	0	-
APL_0676	1	-1.199	10	0	0	-
APL_0697	2	-1.056	4, 10	0	0	-
APL_0703	1	-1.209	10	0	0	-
APL_0705	3	-1.230	8, 9, 14	0	0	-
APL_0784	6	-1.305	3, 6, 8-10, 14	0	0	-

APL_0785	9	-3.259	1, 4, 6-9, 11, 13, 14	5	-3.936	1, 8, 9, 11, 14
APL_0786	8	-2.477	1, 4, 7-9, 11, 13, 14	1	-3.350	8
APL_0803	1	-1.018	2	0	0	-
APL_0812	2	-1.842	6, 7	0	0	-
APL_0813	1	-1.755	6	0	0	-
APL_0888	1	-1.082	13	0	0	-
APL_0899	5	-1.059	3, 7, 8, 11, 14	0	0	-
APL_0906	3	-1.265	2, 10, 14	0	0	-
APL_0910	4	-1.213	8, 9, 11, 14	0	0	-
APL_0933	2	-1.999	7, 10	0	0	-
APL_0947	2	-3.202	10, 14	2	-3.202	10, 14
APL_0948	3	-2.886	4, 10, 14	2	-3.309	10, 14
APL_0949	9	-2.485	1, 4, 7-12, 14	2	-3.819	10, 14
APL_0950	9	-1.576	1, 4, 7-12, 14	0	0	-
APL_0952	9	-2.927	1, 6-12, 14	6	-3.563	1, 8-11, 14
APL_0953	7	-3.095	1, 7-11, 14	4	-3.677	8-10, 14
APL_0954	8	-2.069	1, 7-12, 14	2	-3.389	10, 14
APL_0956	2	-4.233	10, 14	2	-4.233	10, 14
APL_0957	2	-3.552	10, 14	2	-3.552	10, 14
APL_0979	2	-1.238	9, 10	0	0	-
APL_0980	5	-1.921	1, 6, 8, 9, 11	0	0	-
APL_0981	5	-2.704	1, 6, 8, 9, 11	2	-3.481	8, 9
APL_0988	1	-1.222	8	0	0	-
APL_0999	8	-1.171	2, 3, 7-11, 14	0	0	-
APL_1038	3	-1.347	2, 14, 15	0	0	-
APL_1069	1	-1.506	10	0	0	-
APL_1093	14	-2.349	1-4, 6-15	1	-3.019	8
APL_1094	14	-2.795	1-4, 6-15	6	-3.274	2, 3, 8, 10, 12, 14
APL_1107	1	-1.144	15	0	0	-
APL_1148	4	-1.144	3, 7, 8, 14	0	0	-
APL_1165	1	-1.137	10	0	0	-
APL_1167	1	-1.820	10	0	0	-
APL_1182	14	-2.825	1-4, 6-15	6	-3.485	1, 8-11, 14
APL_1183	14	-2.233	1-4, 6-15	1	-3.075	9
APL_1184	14	-2.377	1-4, 6-15	1	-3.222	10
APL_1185	12	-1.656	1-4, 7-12, 14, 15	0	0	-
APL_1193	14	-3.496	1-4, 6-15	12	-3.743	1-4, 7-12, 14, 15
APL_1194	14	-3.833	1-4, 6-15	12	-4.038	1-4, 7-12, 14, 15
APL_1195	14	-4.980	1-4, 6-15	13	-5.145	1-4, 7-15
APL_1196	14	-4.088	1-4, 6-15	13	-4.192	1-4, 7-15
APL_1197	4	-1.069	7, 8, 10, 11	0	0	-
APL_1199	2	-1.017	7, 12	0	0	-
APL_1200	11	-2.253	1-4, 7-9, 11-13, 15	0	0	-
APL_1201	12	-3.481	1-4, 6-9, 11-13, 15	9	-3.820	1-4, 7-9, 11, 12
APL_1202	12	-3.160	1-4, 6-9, 11-13, 15	8	-3.537	1-4, 8, 9, 11, 12
APL_1218	1	-1.335	2	0	0	-
APL_1227	1	-1.186	9	0	0	-
APL_1231	1	-1.084	8	0	0	-
APL_1235	2	-1.489	3, 10	0	0	-
APL_1256	1	-1.601	15	0	0	-

APL_1262	1	-1.062	9	0	0	-
APL_1299	2	-3.360	10, 14	2	-3.360	10, 14
APL_1301	10	-1.835	1-4, 7-9, 11, 13, 15	0	0	-
APL_1304	5	-1.251	3, 7, 8, 11, 14	0	0	-
APL_1329	5	-2.293	2-4, 6, 15	1	-3.081	3
APL_1330	3	-1.226	2, 3, 4	0	0	-
APL_1350	1	-1.562	8	0	0	-
APL_1351	2	-1.115	8, 11	0	0	-
APL_1352	1	-1.035	8	0	0	-
APL_1376	1	-1.066	7	0	0	-
APL_1402	2	-1.140	8, 11	0	0	-
APL_1442	1	-2.276	3	0	0	-
APL_1443	1	-4.106	3	1	-4.106	3
APL_1444	8	-3.181	2, 3, 4, 6, 7, 12, 13, 15	4	-3.805	2, 3, 7, 12
APL_1445	8	-2.083	2, 3, 4, 6, 7, 12, 13, 15	1	-3.261	3
APL_1460	12	-2.088	1, 3, 4, 7--15	1	-3.027	8
APL_1461	2	-1.305	3, 14	0	0	-
APL_1463	13	-2.102	1-4, 7-15	0	0	-
APL_1464	14	-2.810	1-4, 6-15	3	-3.503	3, 8, 11
APL_1465	14	-2.881	1-4, 6-15	8	-3.311	1-4, 8, 10, 12, 14
APL_1466	13	-2.326	1-4, 7-15	2	-3.500	8, 9
APL_1467	14	-3.189	1-4, 6-15	9	-4.011	1-4, 7-9, 11, 15
APL_1468	12	-3.202	1-4, 6-9, 11-13, 15	7	-3.622	1, 3, 4, 7, 8, 11, 15
APL_1469	14	-2.721	1-4, 6-15	8	-3.297	1-4, 7-9, 11
APL_1470	14	-2.750	1-4, 6-15	6	-3.652	1-3, 8, 9, 15
APL_1471	11	-3.734	1-4, 6-9, 12, 13, 15	10	-3.928	1-4, 7-9, 12, 13, 15
APL_1472	12	-2.388	1-4, 6-9, 11-13, 15	1	-3.420	8
APL_1506	1	-1.129	8	0	0	-
APL_1568	13	-2.020	1-4, 7-15	1	-3.240	8
APL_1573	1	-2.013	2	0	0	-
APL_1575	5	-1.992	1, 4, 12, 14, 15	0	0	-
APL_1576	13	-2.498	1-4, 6-9, 11-15	2	-3.075	3, 14
APL_1577	13	-2.695	1-4, 6-9, 11-15	4	-3.118	1, 3, 8, 14
APL_1578	4	-1.104	2, 3, 8, 11	0	0	-
APL_1579	14	-3.449	1-4, 6-15	12	-3.691	1-4, 7-12, 14, 15
APL_1580	14	-3.076	1-4, 6-15	7	-3.736	2, 3, 8-10, 12, 14
APL_1581	14	-2.792	1-4, 6-15	6	-3.152	2, 3, 8-10, 12
APL_1582	7	-1.177	3-4, 7-10, 12	0	0	-
APL_1583	4	-1.836	2-4, 15	0	0	-
APL_1584	3	-2.115	4, 10, 15	0	0	-
APL_1585	2	-1.327	4, 15	0	0	-
APL_1621	1	-1.295	15	0	0	-
APL_1622	1	-1.015	15	0	0	-
APL_1677	3	-1.147	3, 4, 8	0	0	-
APL_1678	5	-1.187	2, 7-9, 11	0	0	-
APL_1680	11	-3.123	1-4, 6-9, 11, 12, 15	7	-3.510	1-3, 7-9, 11
APL_1681	13	-3.309	1-4, 7-15	10	-3.526	1-3, 7-12, 14
APL_1683	1	-1.299	10	0	0	-
APL_1690	2	-1.234	8, 11	0	0	-
APL_1691	1	-1.027	3	0	0	-

APL_1692	4	-1.173	1, 3, 10, 14	0	0	-
APL_1706	1	-1.290	10	0	0	-
APL_1707	3	-2.257	3, 4, 10	1	-3.181	10
APL_1708	6	-1.166	1-3, 8, 9, 11	0	0	-
APL_1729	11	-3.061	1-4, 8, 9, 11-15	7	-3.333	1, 3, 8, 9, 11, 12, 14
APL_1733	4	-1.573	1, 8, 9, 11	0	0	-
APL_1734	8	-1.945	1, 3, 4, 8-12	0	0	-
APL_1745	4	-1.337	2, 8, 11, 12	0	0	-
APL_1746	5	-1.204	2, 8, 11, 12, 14	0	0	-
APL_1748	8	-1.826	1-3 8, 9, 11, 12, 15	0	0	-
APL_1869	4	-1.714	2, 7, 9, 14	0	0	-
APL_1904	1	-1.173	9	0	0	-
APL_1963	5	-1.194	3, 7, 8, 11, 14	0	0	-
APL_1980	13	-2.035	1-4, 7-15	0	0	-
APL_2016	14	-2.334	1-4, 6-15	0	0	-
APL_2023	10	-1.634	1-3, 7-11, 14, 15	0	0	-
APL_2024	9	-3.057	1-3, 7-11, 14, 15	6	-3.279	3, 7, 8, 10, 11, 14
APL_2025	1	-1.003	7	0	0	-
APL_2045	10	-3.015	1, 2, 7-9, 11-15	7	-3.266	2, 7-9, 11, 12, 14
APL_2046	10	-2.846	1, 2, 7-9, 11-15	5	-3.194	2, 8, 9, 11, 14

Annexe 3

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malT Knockout Mutation Invokes a Stringent Type Gene-expression Profile in *Actinobacillus pleuropneumoniae* in Bronchoalveolar Fluid

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Running head: *malT* role in *A. pleuropneumoniae*

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Abstract

Background

Actinobacillus pleuropneumoniae causes contagious pleuropneumonia, an economically important disease of commercially reared pigs throughout the world. To cause this disease, *A. pleuropneumoniae* must rapidly overcome porcine pulmonary innate immune defenses. Since bronchoalveolar fluid (BALF) contains many of the innate immune and other components found in the lungs, we examined the gene expression of a virulent serovar 1 strain of *A. pleuropneumoniae* after exposure to concentrated BALF for 30 min.

Results

In reverse transcription PCR differential display (RT-PCR DD) experiments, *A. pleuropneumoniae* CM5 exposed to BALF up-regulated, among other genes, a gene predicted to encode LamB, an outer-membrane transport protein of the maltose regulon. To determine the role of the *lamB* and other genes of the maltose regulon in the pathogenesis of *A. pleuropneumoniae*, knockout mutations were created in the*lamB* and *malT* genes, the latter being the positive transcriptional regulator of the maltose regulon. Relative to the *lamB* mutant and the wild type, the *malT* mutant had a significant ($P < 0.05$) decrease in growth rate and an increased sensitivity to fresh porcine serum and high concentrations (more than 0.5 M) of sodium chloride. In DNA microarray experiments, the BALF-exposed *malT* mutant exhibited a gene-expression profile resembling that of a stringent type gene-expression profile seen in bacteria facing amino acid or carbon starvation. Genes encoding proteins for protein synthesis, energy metabolism, and DNA replication were down-regulated, while genes involved in stringent response (e.g., *relA*), amino acid and nucleotide biosynthesis, biofilm formation, DNA transformation, and stress response were up-regulated.

Conclusion

These results suggest that MalT may be involved in protection against some stressors and in the transport of one or more essential nutrients in BALF. Moreover, if MalT is directly or indirectly linked to the stringent response, an important global mechanism of bacterial persistence and virulence in many bacterial pathogens, it might play a role in *A. pleuropneumoniae* pathogenesis.

Background

A. pleuropneumoniae causes contagious pleuropneumonia in pigs. The disease can occur in acute, sub-acute, or chronic form [1]. The acute form is characterized by fibrinohemorrhagic pneumonia and the sub-acute and chronic forms by pleuritis with localized necrotizing lesions. The severity and the spread of the disease depend upon the serovar and dose of the strain, and in large measure, upon the immune status of the herd [2].

A. pleuropneumoniae is well adapted to survive and replicate in the host respiratory tract. Its survival and replication requires the expression of genes encoding proteins that protect the bacterium from the host immune response and help it to acquire nutrients. Although RTX (repeats in toxin) toxins, lipopolysaccharide, capsule, and various amino acid and iron transport systems of the bacterium are essential to cause acute disease [3], it is not known how the organism survives in the face of non-cellular innate immune components that form the first line of defence in the lungs [4]. To identify *A. pleuropneumoniae* genes that are expressed in a medium that mimics, at least in part, the alveolar surface environment of the lungs, we incubated the bacterium in concentrated porcine bronchoalveolar lavage fluid (BALF). In addition to innate immune components, such as collectins, defensins, lysozyme, lactoferrin, and cathelicidin [4], BALF contains surfactant, surfactant-associated proteins, dissolved minerals, and other substances functioning in

antioxidation, lipid metabolism, and tissue repair and proliferation in the lungs [5]. Thus, genes expressed by *A. pleuropneumoniae* in porcine BALF may be important for survival and pathogenesis of the organism.

In RT-PCR DD experiments, *A. pleuropneumoniae* CM5 exposed to BALF for 30 min differentially expressed a number of genes, including seemingly a *lamB* homolog. Consistent with this finding, an earlier study had also reported that *A. pleuropneumoniae* expresses a maltose-inducible, LamB-like outer membrane protein in the host [6]. In *E. coli* and other gram-negative bacteria, *lamB* encodes an outer membrane transport protein involved in the transport and metabolism of maltose and maltodextrins. The *E. coli* maltose regulon is comprised of at least ten genes whose transcription is positively regulated by MalT in the presence of maltotriose derived from either imported maltodextrins or endogenous glycogen [7].

In addition to maltose and maltodextrin transport and metabolism, the genes of the maltose regulon have been associated, in ways less well understood, with virulence in bacteria. For example, MalF, an inner membrane maltose and maltodextrin transport protein, and MalQ, a dextrinyl transferase, have been associated with the expression of cholera toxin and toxin-co-regulated pilus in *Vibrio cholerae* [8], as has been LamB with cytopathic effect in enteropathogenic *E. coli* [9], and adhesion in enteroinvasive *E. coli* [10] and *Aeromonas veronii* [11]. Mutants of the *malE* and *malT* (transporter) genes in group A *Streptococcus* are attenuated in their ability to grow in human saliva and to metabolize α glucans and are significantly impaired in their ability to colonize the mouse oropharynx [12,13].

To elucidate the role of the predicted maltose regulon in *A. pleuropneumoniae*, *malT* and *lamB* knockout mutants were constructed and characterized phenotypically. Since MalT is a regulatory protein, the effect of its knockout on the bacterial gene expression level was also determined using DNA microarrays.

Results

Expression of maltose-regulon genes by the wild-type *A. pleuropneumoniae* CM5 in BALF

Several differentially expressed genes in *A. pleuropneumoniae* CM5 exposed to BALF for 30 min at 37°C were first presumptively identified by RT-PCR DD studies. These included genes encoding protein synthesis and hypothetical proteins (APL_068, APL_0363, and APL_0367), in addition to a cell surface protein, LamB (Figure 1). Homologs (>99% DNA identity) of the 3 hypothetical proteins are present in all the serotypes of *A. pleuropneumoniae* sequenced so far, suggesting that they might have a role in persistence or pathogenesis, but their levels of expression were not confirmed by real-time PCR or other more direct methods. The level of expression of the *lamB* gene was estimated by real-time PCR analysis to be 3.3-fold higher in BALF- than in BHI-exposed cells (Table 1). Genes of the maltose regulon that were also up-regulated (although some at very low levels) in BALF-exposed cells included *malF* and *malG* (encoding the intrinsic membrane proteins of maltose transport system), *malP* (maltodextrin phophorylase), *malQ* (amylomaltase) and *malK* (the ATP-binding cassette of the maltodextrin transporter; Table 1). For further study, we constructed *lamB* and *malT* mutants to evaluate the possible role of these genes in the survival of *A. pleuropneumoniae* CM5.

Growth curves of the *malT* and *lamB* mutants

The *malT* mutant grew slower than the wild-type organism in BHI. The growth pattern of the *lamB* mutant was, however, similar to that of the wild-type organism (Figure 2).

Effect of acarbose on the growth of the isogenic *malT* and *lamB* mutants of *A. pleuropneumoniae* CM5

To assess the effect of the *malT* knockout mutationon the functioning of the maltose regulon, the parent strain and the *malT* mutant were grown in acarbose-containing BHI in the presence or absence of maltose. Acarbose is a competitive inhibitor of maltose transport [14]. Because of the fastidious nutritional requirements of *A. pleuropneumoniae*, this experiment was performed in BHI instead of a chemically

defined medium. After 16 h of incubation in acarbose-containing BHI that was supplemented with maltose, the wild-type organism reached a significantly lower OD_{600} ($P < 0.05$) than did the *malT* mutant (Figure 3). In acarbose-containing BHI that was not supplemented with maltose, there was again, a significant difference in the growth of the two strains. The number of wild type and *malT*mutant cells was lower in acarbose-containing BHI than in the BHI containing both maltose and acarbose; however, this difference was not significant (Figure 3). The *lamB* mutant showed a trend similar to that of the *malT* mutant grown in the acarbose-containing medium, but the number of *lamB* mutant cells was lower than that of the *malT* mutant; however, this difference was not significant.

Survival of the *malT* and *lamB* mutants

Because LamB is a cell surface protein that is positively regulated by MalT, we examined the effects of serum and high concentrations of sodium chloride to better understand the role of these genes in the survival of *A. pleuropneumoniae*. The percent survival of the *malT*mutant after incubation at 37°C for 1 h in 90 and 50% porcine serum was significantly ($P < 0.05$) lower than the percent survival of the wild- type strain (Figure 4). There was no significant difference in the survival between the wild-type organism and the *lamB* mutant in either concentration of the serum. The number of cells of all the three strains (wild-type organism, *malT* and *lamB* mutants) surviving in 90% serum was higher than the number of cells surviving in 50% serum. *E. coli* DH5α did not survive in either concentration of serum.

In the maltose-supplemented BHI containing different concentrations of sodium chloride, the wild type parent, and the *malT* and *lamB*mutants showed a significant ($P < 0.05$) decrease in cell numbers after 3 h of incubation (Figure 5). The decrease in the cell number was least in the wild-type organism and greatest in the *malT* mutant. In 1 M sodium chloride, the *malT* mutant decreased in number from an initial count (prior to the addition of the salt to the medium) of 10^7 CFU/ml to a final count (3 h subsequent to the addition of the salt to the medium) of 10 CFU/ml. Even at a 2 M salt concentration, the wild-type organism decreased in

number to only 5 log CFU/ml from approximately the same initial count as that of the *malT* mutant. At salt concentrations of 1 M and above, the *lamB* mutant showed a decline in cell numbers midway between those of the numbers shown by the parent strain and the *malT* mutant. The wild-type organism, and the *malT* and *lamB* mutants were all susceptible to killing by high concentrations of sodium chloride, but this killing was greatest in the*malT* mutant (Figure 5).

Differential gene expression by the *malT* mutant in BALF

To understand the basis of the observed phenotypic differences between the *malT* mutant and the wild-type organism, gene expression profiles of the mutant and parent strains were compared using DNA microarrays. Following the incubation of the exponentially grown cultures of the mutant and wild-type organism in fresh BHI at 37°C for 30 min, no significant differences were observed in the gene expression profiles of the two strains even at low delta values. Incubation in BALF, however, resulted in a total of 223 genes being differentially expressed in the *malT* mutant at a false discovery rate (the percentage of the differentially expressed genes identified just by chance) of 1%. The differentially expressed genes included 104 up-regulated and 119 down-regulated genes and 92 of these encoded hypothetical proteins (Table 2, Additional file 1: Analyzed microarray data). In general, the genes encoding proteins involved in energy metabolism and protein biosynthesis were down-regulated (Table 3), while as those involved in amino acid and nucleotide biosynthesis, DNA transformation, and biofilm formation were up-regulated (Table 4). The *relA* gene encoding a stringent response regulatory protein was also up-regulated in the *malT* mutant. Though known as an *in vivo*-expressed RTX toxin, the *apxIVA* gene was up-regulated by the wild-type strain in BALF [15] and its expression was further increased in BALF in the *malT* mutant.

Expression of selected genes representing biological functional categories of interest was also measured by real-time PCR analysis (Table 5). A good corroboration in the context of the up- and down-regulation of the genes was found between the microarray and real-time PCR data.

Discussion

Expression of maltose-regulon genes by BALF-exposed *A. pleuropneumoniae* CM5

After exposure of *A. pleuropneumoniae* CM5 to BALF for 30 minutes, a gene that appeared to be *lamB* homologue was shown to be up-regulated by the organism in RT-PCR DD experiments (Figure 1). We selected 30 min for incubation of the organism in BALF, as the medium conditions should remain fairly constant during this time as might be seen in the animal during early infection when there is constant replenishment of alveolar fluid. As shown in real-time PCR studies, the genes encoding intrinsic membrane transport system proteins (MalF and MalG), maltodextrin phosphorylase (MalP), amylomaltase (MalQ), ATP-binding cassette of the maltodextrin transporter (MalK) of the maltose regulon were also up-regulated in BALF, although some at very low levels (Table 1). Comparison of gene expression in BALF- and BHI-incubated cells by DNA microarrays [15] showed that *malF* and *malG* were up-regulated in BALF. However, no differential expression was seen in *malT*, *malK*, *malP* or *malQ* genes. This disparate finding could be because only small quantities of these proteins are required for function, and small changes in gene expression are difficult to detect. For further study, we focused on the *lamB* and *malT*genes of the maltose regulon as LamB is a cell surface protein that lies at the host-pathogen interface and MalT is a transcriptional regulator that might control the expression of genes other than those involved in the maltose and maltodextrin transport and metabolism.

malT and *lamB* are the components of a functional maltose regulon in *A. pleuropneumoniae* CM5

All of the strains of *A. pleuropneumoniae* sequenced so far possess homologs of the maltose regulon genes *malEFG*, *malK-lamB-malM*, *malT*and *malPQ*. As demonstrated by microarray-based comparative genomic profiling, these genes are present in the reference strains of all 15 serovars of *A. pleuropneumoniae* [16]. It might be noted that maltose regulon genes are also present in two other upper

respiratory tract pathogens, *Mannheimia haemolytica* and *Haemophilus parasuis*. The arrangement of some of these genes in *A. pleuropneumoniae*, however, differs from that found in *E. coli*. As in *E. coli*, MalT appears to be a positive transcriptional regulator of *lamB* in *A. pleuropneumoniae* as demonstrated by a two-fold decrease in the expression of *lamB* in the isogenic *malT* mutant of *A. pleuropneumoniae*CM5 in BHI supplemented with maltose (Table 5). This finding is consistent with an earlier phenotypic study [6] which reported that *A. pleuropneumoniae* expresses a LamB-like outer membrane protein when maltose is added to BHI agar. Moreover, the *A. pleuropneumoniae*MalT and LamB has a high degree of amino acid similarity with MalT and LamB homologs of a number of other Gram-negative organisms. Also, MalT has a conserved DNA-binding (LuxR-like C-terminal containing helix-turn-helix) motif such as found in the *E. coli* MalT protein.

To further examine the effect of the *malT* mutation on the regulation of the maltose regulon, both the wild-type organism and the *malT*mutant were grown in the presence of acarbose. Acarbose is a pseudo-oligosaccharide similar in structure to maltotetraose and it is a competitive inhibitor of maltose transport in *E. coli*. It can inhibit maltose uptake only if maltose-transport system is first activated by maltose. Acarbose also inhibits α-amylases and α-glucosidases and is not degraded by *E. coli* [14]. In BHI supplemented with maltose, acarbose reduced the growth of the wild-type organism as well as that of the *malT* mutant (Figure 3). The reduction in the growth might have been caused either by accumulation of toxic levels of acarbose by the bacterial cells or by the inhibition of bacterial glucosidases by the accumulating acarbose, or both. The reduction was, however, significantly ($P < 0.05$) greater in the wild-type organism than in the mutant. This is perhaps due to the increased uptake of acarbose by the wild-type organism, owing to its higher activation of the maltose regulon by the intact *malT*. On the other hand, the reduction in the growth of the *malT* mutant could have been due to the non-specific entry of acarbose into the bacterial cells.

As *A. pleuropneumoniae* CM5 is not amenable to complementation it should be noted that we can not rigorously exclude the possibility that the phenotype exhibited

by the *malT* negative strain was affected by some alteration of another gene that occurred during strain construction, but this is very unlikely. That said, taken together, the above findings suggest that *A. pleuropneumoniae* has a functional maltose regulon similar to that of *E. coli*.

malT is required for optimum survival of *A. pleuropneumoniae* CM5 in serum and high concentrations of sodium chloride

In comparison with the wild-type *A. pleuropneumoniae* CM5 and *lamB* mutant, the *malT* mutant had a significantly decreased ability to survive following incubation in fresh porcine serum for 1 h; the wild-type organism, however, grew in serum to a significantly higher number (Figure 4). As resistance of *A. pleuropneumoniae* to killing by serum is predominantly due to its capsule and LPS [17,18], the decreased survival of the *malT* mutant in serum could have been due to a change in its cell surface polysaccharides or to an alteration in its general metabolism as indicated by its slower growth in BHI. Similarly, in the presence of sodium chloride concentrations of more than 0.5 M, the *malT* mutant had a significantly ($P < 0.05$) diminished ability to survive in the BHI supplemented with maltose. This result suggests that MalT-regulated genes are required for protection against the high concentrations of sodium chloride in *A. pleuropneumoniae*(Figure 5). An association has been shown to exist between the components of the maltose regulon, stress response, and hypersomolarity in *E. coli* [19], but it is not known how the maltose regulon behaves in the presence of an exogenous activator and high concentrations of the sodium chloride.

Differential gene expression of the _malT_ mutant in BALF resembles the stringent type gene-expression profile

There was no significant difference between the gene expression profile of the parent strain and the *malT* mutant after incubation of the log-phase cultures in fresh BHI for 30 min. In BALF, however, 223 genes were differentially expressed by the *malT* mutant (Table 2). The gene expression profile of the mutant resembled a metabolic downshift; genes encoding protein synthesis, energy metabolism, transport of nutrients and DNA replication were all down-regulated, while those involved in

amino acid and nucleotide biosynthesis, biofilm formation (prevalent in *A. pleuropneumoniae* field isolates [20]), DNA transformation, and the stress response were up-regulated (Tables 3 and 4). This type of gene-expression response mimics the gene-expression profile of the stringent response seen in *E. coli* and other organisms during nutrient deprivation [21-23].

Carbon starvation in *E. coli* invokes a global gene expression response, resulting in the down-regulation of the genes encoding proteins for the growth and replication of the organism and the up-regulation of the genes encoding proteins for the biosynthesis of amino acids, alternate sigma factors, biofilm components [24], as well as proteins of unknown function [25]. During amino acid starvation, the ratio of uncharged to charged tRNA increases, resulting in ribosome stalling at the A-site of the 50S ribosomal subunit. The stalling of the ribosome results in the activation of ribosome-bound RelA. RelA, a synthase and SpoT, a hydrolase with a weak synthase activity, synthesize pppGpp (guanosine 3'-diphosphate,5'-triphosphate) and ppGpp (guanosine 3', 5'-bispyrophosphate) which in turn invoke a global gene expression response including down-regulation of rRNA synthesis, such as seen in the stringent response to nutrient starvation [24].

The increased expression of *relA* and the changes in the overall gene expression profile of the *malT* mutant in BALF closely resembled the stringent-response gene-expression profile in other bacteria, including *E. coli*. Consistent with the notion of a stringent response having a role in *A. pleuropneumoniae*, all the major stringent response regulatory genes including *relA*, *spoT* and *dksA* (DnaK suppressor protein) are present in the genome of this pathogen. A *malT* knockout mutation in *A. pleuropneumoniae* could result in a stringent response because MalTis linked, directly or indirectly, to the regulation of the stringent response genes, or because it regulates the uptake of nutrient(s) in addition to maltose and maltodextrins. The latter assumption could explain the up-regulation of the *lamB* gene in BALF as a secondary response to the activation or the up-regulation of MalT for the acquisition of nutrients. The slower growth of the *malT* mutant and its increased sensitivity to the biological stressors could also be explained by changes in cell surface molecules

that result from the inability of the mutant to acquire unknown essential nutrient(s). By balancing nutrient availability with ribosome synthesis through the stringent response, bacteria can control replication, enter into a persistence mode of life, or express virulence factors, depending upon the type of bacteria [26-29].

Conclusion

Taken together, our data suggest that *A. pleuropneumoniae* CM5 has a functional maltose regulon similar to that found in *E. coli*. Although it is likely that these genes have a role in acquisition of nutrients in saliva and in the oropharynx where maltodextrins would be predicted to be found, these studies suggest that the maltose regulon could also play a significant role once the organism enters the lungs. Further, the slower growth rate and increased salt and serum sensitivity of the *malT* mutant versus *lamB* mutant suggests that MalT has a role beyond that of maltose and maltodextrin metabolism in *A. pleuropneumoniae*. This is perhaps due to the involvement of the MalT in the transport or processing of some essential nutrient(s). This assumption is further supported by the expression of the stringent type transcript profile in the *malT* mutant in BALF. MalT could also be directly or indirectly linked to the stringent response without being involved in the transport of the essential nutrient(s); however, this remains to be proven. The presence of the maltose-regulon genes in all serovars of *A. pleuropneumoniae* and in related pathogens such as *Mannheimia haemolytica* and *Haemophilus parasuis* provides further circumstantial evidence that carbohydrate metabolism mediated by the maltose regulon might play a role in the persistence, if not the pathogenesis of some respiratory tract pathogens.

Methods

Bacterial strains and media

A. pleuropneumoniae CM5 [30], and *E. coli* strains β2155 [31] and DH5α (Clontech, CA, USA) were used in this study (Tables 6 and 7). *A. pleuropneumoniae* CM5 was grown either in brain heart infusion (BHI; Becton, Dickinson and Company, Sparks, MD, USA) or Mueller-Hinton (MH; Becton, Dickinson and Company) medium, supplemented with 0.01% (wt/vol) β-nicotinamide adenine dinucleotide (NAD) as required. Transconjugation medium consisted of MH broth with 20% (wt/vol) sucrose, 10% equine serum (wt/vol), and 0.01% NAD (wt/vol). *E. coli*strains were routinely cultured in Luria-Bertani (LB) medium, but in the case of *E. coli* β2155, the medium was always supplemented with 1 mM diaminopimelic acid (DAP; Sigma-Aldrich, St. Louis, MO, USA). As required, chloramphenicol was also added at the rate of either 5.0 or 2.5 μg/ml.

Collection and concentration of bronchoalveolar lavage fluid

BALF was collected from ten high-health status pigs of approximately 15 kg in body weight. After euthanizing the pigs, the lungs of the individual animals were lavaged with 100 ml of PBS (phosphate-buffered saline), and the lung washings were collected and centrifuged to remove cell debris. The contents of the washings were then concentrated with a 5 kDa molecular weight cut off ultra-centrifugal filter device, Vivacell 70 (Vivascience Ltd., Stonehouse, GL, UK), which reduced the volume of the washings to $1/20^{th}$ that of their total initial volume. The concentrated BALF was sterilized by filtration through a 0.22 μm membrane filter (Pall Corporation, Ann Arbor, MI, USA) and kept at -80°C for long-term storage. Molecules less than 5 kDa in molecular weight were not concentrated by this method; nevertheless, the fluid still contained these substances in the concentrations found before ultrafiltration.

Reverse-transcription PCR differential display

The RT-PCR DD method described by McClelland et al. [32] was adapted to identify the differentially expressed genes of *A. pleuropneumoniae* CM5 in BALF. Briefly,

the organism was grown to an OD_{600} of 0.7 in BHI at 37°C, harvested by centrifugation, and an approximately 10^7 colony forming units (CFU) were suspended in either concentrated BALF or fresh BHI. After incubation of the cell suspensions at 37°C for 30 min, the bacteria were harvested by centrifugation and immediately subjected to RNA extraction.

RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and quantified using RNA 6000 Nano LabChip chips read in a Bioanalyzer 2100 instrument (Agilent Technologies, Santa Clara, CA, USA). The RNA was treated with Turbo RNA-free DNase (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions.

A total of 0.5 μg of RNA and 85 different combinations (Table 8) of arbitrary random primers (GenHunter Corp., Nashville, Tennessee, USA) (Table 9) were used to synthesize cDNA with Moloney Murine Leukemia Virus reverse transcriptase (M-MLV reverse transcriptase; Invitrogen). Reverse transcriptase-negative controls were run with each of the transcription reaction.

One microlitre of the reverse-transcription reaction mixture was used as a template to amplify the cDNA under relaxed PCR conditions. The same primer pairs were used in both the template cDNA synthesis and the random PCR -amplification of the template cDNA. The 20-μl PCR reaction mixtures contained 1.5 μM of each of the forward and reverse primers, 2.0 μl of 10 × PCR buffer, 200 μM of dNTP mixture, 4.0 mM MgCl2, and 2.5 U of *Taq* DNA polymerase (New England Biolabs, Pickering, ON, Canada). The PCR thermal profile included an initial random primer annealing and extension steps (denaturation 94°C for 5 min; primer annealing at 39°C for 5 min; and strand extension at 72°C for 3 min) followed by a 40-cycle PCR (denaturation 95°C for 2 min; primer annealing at 39°C for 2 min; and strand extension at 72°C for 1 min) with a final amplification step of 10 min at 72°C. PCR products of the same primer pair were run side by side on 7% polyacrylamide gels and silver stained, as described elsewhere [33], to visualize the bands representing differentially expressed genes (Figure 1). Bands representing differentially expressed

genes were scratched with a 25 gauge needle to harvest DNA. The DNA on the pointed end of the needle was dissolved in a 10 µl of PCR-grade water for 5 min. This solution of DNA served as a template for a PCR reaction in which the same protocol and the same primers were used as in the differential display PCR that generated the band. The amplified DNA was run on agarose gels and stained with ethidium bromide to visualize the bands for excision. The DNA from the excised bands was purified using QIAquick Gel Extraction Kits (Qiagen Inc., Mississauga, ON, Canada), and the purified PCR products were cloned into the pCR4-TOPO (TOPO TA Cloning Kit, Invitrogen), according to the manufacturer's instructions. The inserts were sequenced by dye terminator cycle sequencing (DNA Sequencing Facility, College of Biological Sciences, University of Guelph, Guelph, ON) and compared with the annotated genome sequences of *A. pleuropneumoniae* using Blastx available at http://blast.ncbi.nlm.nih.gov/Blast.cgi to identify the complete genes.

Construction of the *malT* knockout mutant

Based on the genome sequence of *A. pleuropneumoniae* serovar 1 strain 4074, primers were designed to amplify the entire *malT* gene (nucleotides 2118860 to 2121577). The *malT* PCR product was purified and cloned into pCR4-TOPO. The resultant plasmid was used as the template in a PCR reaction to produce a linearized plasmid with a deletion of the central 838 bp (bp 922 to bp 1760) of the *malT* gene. The amplicon was generated using Phusion *Taq* DNA polymerase (New England Biolabs), a high fidelity DNA polymerase, and the primers that annealed in back to back manner leaving a central 900 bp region of the plasmid *malT* between them. Following the gel purification of the PCR product, the *omlA-P* promoter driven chloramphenicol acetyl transferase gene (*cat*), obtained by PCR amplification of pEMOC2 [34] was blunt-end ligated with the linear plasmid. The resultant circular plasmid with the *cat* insertion in the *malT* was designated as pTopoMC. The Δ*malT*::*cat* fragment of pTopoMC was then PCR amplified with forward and reverse primers containing *Not*I and *Pst*I sites, respectively. The Δ*malT*::*cat* PCR amplicon was gel purified, digested with *Not*I and *Pst*I, and cloned into pEMOC2.

The resultant plasmid, named pEMOC2M, was electroporated into *E. coli* β2155. pEMOC2M was mobilized from *E. coli* β2155 into *A. pleuropneumoniae* CM5 using a modification of the filter mating technique described by Oswald et al. [35]. Briefly, overnight cultures of *E. coli* β2155/pEMOC2M (grown on LB agar containing 25 μg/ml chloramphenicol), and *A. pleuropneumoniae* CM5 (grown on BHI agar) were washed with 2 ml of TNM buffer (1 mM Tris-HCl, pH 7.2; 10 mM MgSO4; 100 mM NaCl). The OD_{600} of both the donor and the recipient strains was adjusted to 1 by adding TNM buffer. A 100 μl volume of the donor and 10 μl of the recipient strains were mixed by inversion, and the mixture was centrifuged to pellet the cells, which were washed and then resuspended in 1 ml of fresh TNM buffer. A 50 μl volume of the suspension was spotted onto a 0.45 μm nitrocellulose filter (Pall Corporation) placed onto the BHI agar plate containing DAP and $MgSO_4$ (10 mM). After incubation at 37°C for 6 h in an atmosphere of 5% CO_2, the filter was washed with 5 ml of BHI broth. The cells were harvested by centrifugation and re-suspended in 0.5 ml of BHI broth. After 10-fold serial dilution of the cell suspension, 50 μl of cells from each of the dilution was plated onto BHI agar plates containing chloramphenicol (5 μg/ml). After 24 h of incubation at 37°C, the individual colonies appearing on the agar plates were inoculated in 1 ml of MH broth for further incubation at 37°C for 3 h. The cell suspensions of each of the colony were plated on the MH plates containing 2.5 μg/ml chloramphenicol. These plates were incubated at 29°C for 48 h. A few colonies from each of the plates were used in colony PCR to verify the integration of the plasmid into the chromosomal *malT* geneof *A. pleuropneumoniae* CM5. The primers for the colony PCR were designed so that one primer annealed inside the integrated plasmid and the other on the nearby bacterial chromosomal DNA, thus verifying both plasmid integration and orientation.

The colonies that had undergone plasmid integration at the correct site were selected for the sucrose counter-selection. Selected individual colonies with an integrated plasmid were incubated with constant agitation in 1 ml of MH broth at 37°C until the cultures were slightly turbid. A 1 ml volume of the counter-selection medium was then added and each of the cultures was incubated for a further 5 h. A 50-μl cell

suspension from each of the ten-fold serial dilutions (10^0 to 10^7) of these cultures was then plated onto the MH agar plates containing sucrose (10%) and chloramphenicol (2.5 μg/ml). After incubation at 37°C for 48 h, colonies appearing on the plates were patched onto two BHI agar plates; one containing chloramphenicol (2.5 μg/ml) and the other, ampicillin (100 μg/ml). Chloramphenicol-resistant, ampicillin-sensitive colonies were screened for the second crossover by the PCR using the primers that annealed to the regions of the bacterial chromosome immediately flanking the *malT* gene. The predicted disruption of the *malT* gene was confirmed by Southern blotting using the wild type *malT* gene as a probe and by sequencing the PCR amplicon spanning the *cat* gene insertion. The primers and plasmids used in the construction of the *malT* mutant are given in Table 6.

Construction of the *lamB* knockout mutant

The construction of the *lamB* knockout mutant involved the same approach as described for the construction of the *malT* mutant. A central 379-bp region (bp 518 to bp 897) of the *lamB* was replaced with the *omlA-P* driven *cat* gene and the knockout mutation was confirmed by sequencing and Southern blotting. The primers and plasmids used in the construction of *lamB* mutant are given in Table 7.

Growth of the mutants

A. pleuropneumoniae CM5, and its isogenic *malT* and *lamB* mutants were grown in BHI at 37°C to monitor their growth. The OD_{600} of each of the strains was measured every hour from the lag to stationary phase of growth to construct growth curves. For doubling time calculations, culture aliquots were taken at 2, 3, and 4 h of incubation and the number of CFUs was determined by the plating of 10-fold dilutions. The data were analyzed using one way analysis of variance (ANOVA) and the means were compared using Tukey's method.

The wild-type organism and the *malT* and *lamB* mutants were also incubated in the BHI containing 0.5% (wt/vol) acarbose and 0.5% (wt/vol) maltose to assess the effect of acarbose on the growth of these strains. As the strains grew slowly in the

acarbose-containing BHI, their growth was measured after 16 h of incubation at 37°C.

Survival of the mutants in serum

Individual colonies from the overnight cultures of *A. pleuropneumoniae* CM5, the *malT* and *lamB* mutants, and *E. coli* DH5α, were incubated in 5 ml of BHI at 37°C for 2 h. A 1 ml volume of each of the cultures was centrifuged at 10,000 ×*g* for 2 min to pellet the cells before suspension in 1 ml of pre-warmed PBS. One hundred µl of a $1{:}10^5$ dilution of each culture was added to 900 µl of 100 and 55.5% fresh porcine serum (vol/vol in PBS). As a control, 100 µl of $1{:}10^5$ dilution of each culture was also added to 900 µl of heat-inactivated porcine serum (inactivated by heating at 65°C for 15 min). The number of CFU of each culture was determined after the incubation of the cultures at 37°C for 1 h. The number of the CFU surviving in fresh serum was expressed as percent survival according to the following equation:

$$\text{Percent survival} = \left(\frac{\frac{\text{CFU at 1 h of incubation in serum}}{\text{CFU at 0 h incubation}}}{\frac{\text{CFU at 1 h of incubation in heat inactivated serum}}{\text{CFU at 0 h incubation}}} \right) \times 100$$

The experiment was run in quadruplicate, and the percent-survival data were divided by 2 before being converted to arcsin values for the analysis using two-way ANOVA. Means were compared by Tukey's Method.

Survival of the mutants in sodium chloride

A. pleuropneumoniae CM5, and the *malT* and *lamB* mutants were grown to an OD600 of 0.7 in the BHI broth supplemented with 1% (wt/vol) maltose. Each of these cultures was mixed with fresh BHI containing 4 M sodium chloride in equal proportions for a final concentration of 2 M sodium chloride; cultures containing 1 and 0.5 M of the salt were prepared by the same approach. The number of CFU of each culture was calculated prior to the addition of the salt-containing BHI and 3 h subsequent to the incubation at 37°C in salt-containing medium. The experiment was

repeated four times, and the data obtained were analyzed using ANOVA. Means were compared using Tukey's Method.

Microarray experiments

The AppChip2 microarray chips used in this study, were an evolved version of the AppChip1 chip, and like its predecessor, was a part of the *A. pleuropneumoniae* 5b L20 genome sequencing project (NRC-IBS, Ottawa, Canada). For the construction of AppChip2, open-reading-frame (ORF) PCR fragments of 160-nucleotide length and above were spotted in duplicate on the microarray slides. The spots represent 2033 ORFs, covering 95% of the total ORFS, from the complete genome sequence of the organism. Spotted sheared genomic DNA from *A. pleuropneumoniae* L20 and porcine DNA were used as controls http://ibs-isb.nrc-cnrc.gc.ca/glycobiology/appchips_e.html. Further details concerning chip production are described elsewhere [36].

Based on the strain (the wild-type organism or the *malT* mutant) and the incubation medium (BHI or BALF), the microarray experiments involved three types of hybridizations: (1) Cy3-labeled cDNA from the BHI-incubated wild-type organism vs. Cy5-labeled cDNA from the BALF-incubated wild-type organism (2) Cy3-labeled cDNA from the BHI-incubated wild-type organism vs. Cy5-labeled cDNA from the BALF-incubated *malT* mutant, and (3) Cy3-labeled cDNA from the BHI-incubated wild-type organism vs. Cy5-labeled cDNA from BHI-incubated*malT* mutant. Four replications, including dye-swaps, were carried out for each type of hybridization.

cDNA was synthesized in the presence of amino-allyl-dUTP (Sigma-Aldrich, St. Louis MO, US), random octamer primers (Biocorps, Montreal, QC, Canada), SuperScript II transcriptase (Invitrogen, Carlsbad, CA, US), and the RNA (15 μg per reaction) obtained from the BALF- and BHI-incubated organisms, according to the method described by Carrillo et al. [37]. Labeling of the cDNA was carried out indirectly with one of the mono-functional NHS-ester dyes Cy3 or Cy5 (GE

Healthcare, Buckinghamshire, UK), which binds to the amino-allyl-dUTP of the cDNA. The dye labeling efficiency of cDNA was determined spectrophotometrically. The data were submitted to the Gene Expression Omnibus (GEO: GSE13006).

Microarray data analysis

Microarray image and data analysis was carried out using the TM4 Suite of software [38] for microarray analysis, (J. Craig Venter Institute, JCVI, USA) as described elsewhere [36]. Briefly, images were analyzed with Spotfinder v3.1.1. The final intensity of each spot was obtained by subtracting the background intensity from the integral spot intensity (the sum of the intensities of all the spot pixels excluding the saturated ones). The spots with intensities less than one standard deviation above their spot background intensities were eliminated from the downstream analysis, as were the ones with total intensity less than 10000. Replicate spots were analyzed subsequent to the normalization of the data using the LOWESS (locally weighted linear regression) algorithm. The genes that were thus represented by good quality spots (defined by a score assigned by the software based on the number of unsaturated pixels, shape, and signal to noise ratio of the spot) on a minimum of two replicate slides were considered for the downstream analysis using SAM (significance analysis of microarray) to identify the differentially expressed genes. The differentially expressed genes were classified depending upon their biological roles into various functional categories according to the JCVIs Comprehensive Microbial Resources (CMR) database.

Quantitative real-time PCR

The parameters of RNA capacity, optimum primer concentration, and the gene dynamic ranges were determined before carrying out the real-time PCR for the relative quantification of the target gene expression. As an endogenous control, the level of prolyl-tRNA-synthetase gene (*syp*) expression was used to normalize the target gene expression levels, since this gene exhibited the least variation in expression across various conditions in both the microarray and real-time PCR experiments. In the quantitative real-time PCR experiments, three independent

biological replicates were tested in triplicate. Calculation of the relative quantification of the target genes was done using the Comparative C_T ($\Delta\Delta C_T$) method [39]. The protocol of the PCR is given as described below:

Each 20-μl PCR reaction mixture contained 2 × Power SYBR Green PCR Master Mix (Applied Biosystems, Streetsville), 100 nM of each of forward and reverse primer, and 5 μl of template cDNA. Synthesis of the template cDNA was carried out in a 20-μl reaction mixture containing 500 ng RNA, using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), which contains random primers for the synthesis of cDNA. The real-time PCR thermal profile included the heat-activation of AmpliTaq Gold DNA Polymerase at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, and primer annealing and extension at 60°C for 1 min. The PCR reactions were carried out in 96-well plates using a StepOnePlus thermocycler (Applied Biosystems, Streetsville, ON, Canada). The primers used in the real-time PCR are given in Table 10.

List of abbreviations

BALF: bronchoalveolar lavage fluid; BHI: Brain Heart Infusion; CFU: colony forming unit(s); NaCl: sodium chloride; NAD: β-nicotinamide adenine dinucleotide; ORF: Open Reading Frame; PAG: polyacrylamide gel; PCR: polymerase chain reaction; RT-PCR DD: reverse-transcription PCR differential display; vol: volume, wt: weight.

Authors' contribution

AGL and JIM conceived and designed the experiments. AGL conducted the experiments, carried out the data analysis, and drafted the manuscript. VD carried out microarray hybridization experiments and data analysis. JHEN designed and fabricated the microarray chip, Appchip2. MJ also helped in the study design and

critically revised the manuscript. All the authors contributed to the final manuscript preparation and approved its submission for publication.

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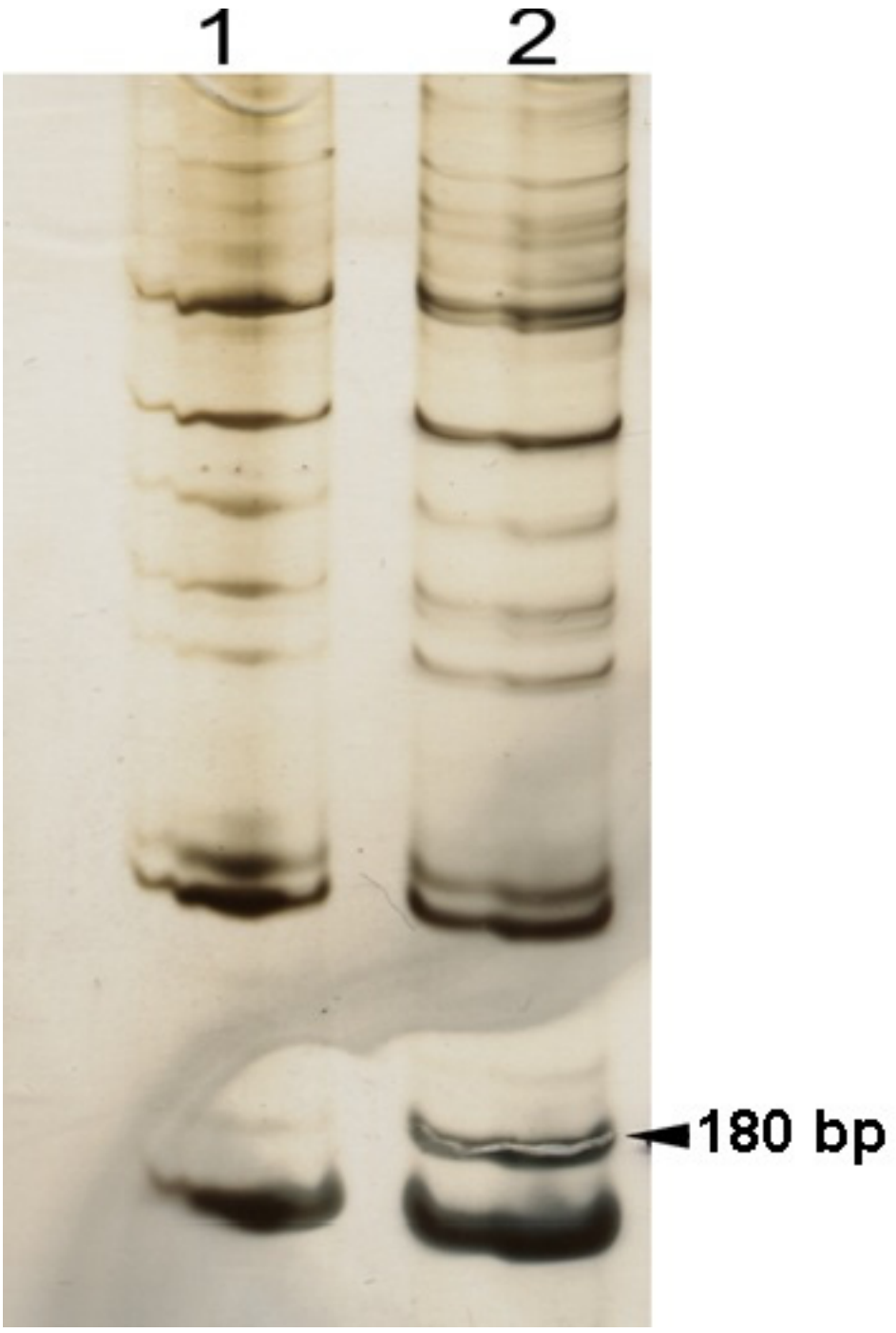

Figure 1. Silver-stained gel comparing *A. pleuropneumoniae* RT-PCR DD products in BHI broth (1) and BALF (2). The arrow points to the band representing a differentially expressed gene, which based on cloning and sequencing (see Methods), appeared to be *lamB*.

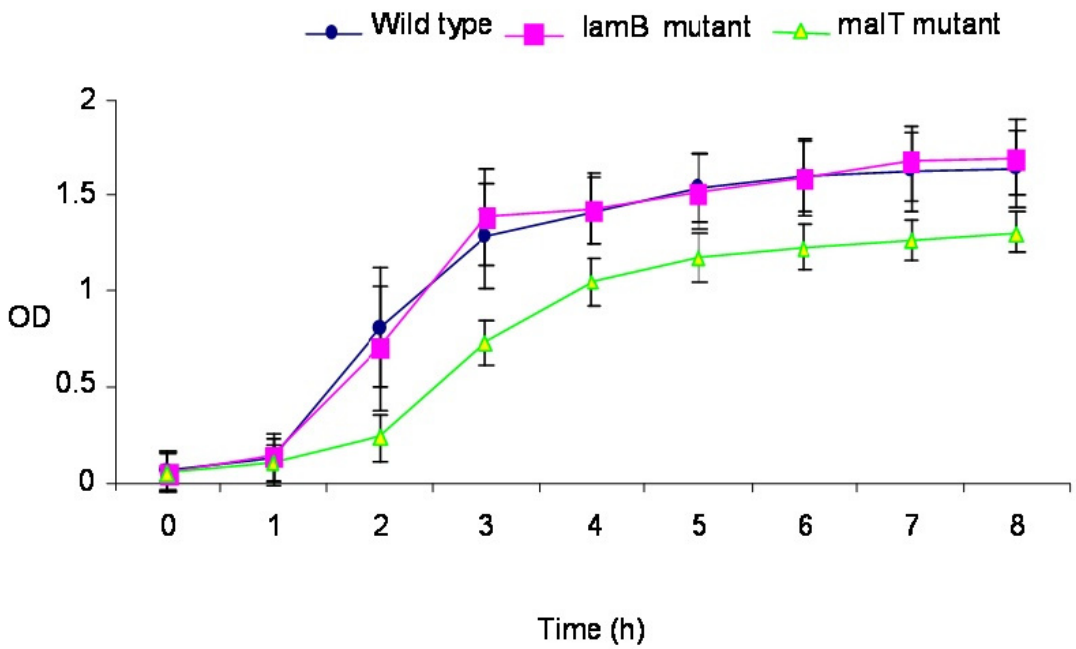

Figure 2. Growth curves of the wild type strain and *lamB* and *malT* mutants in BHI broth.

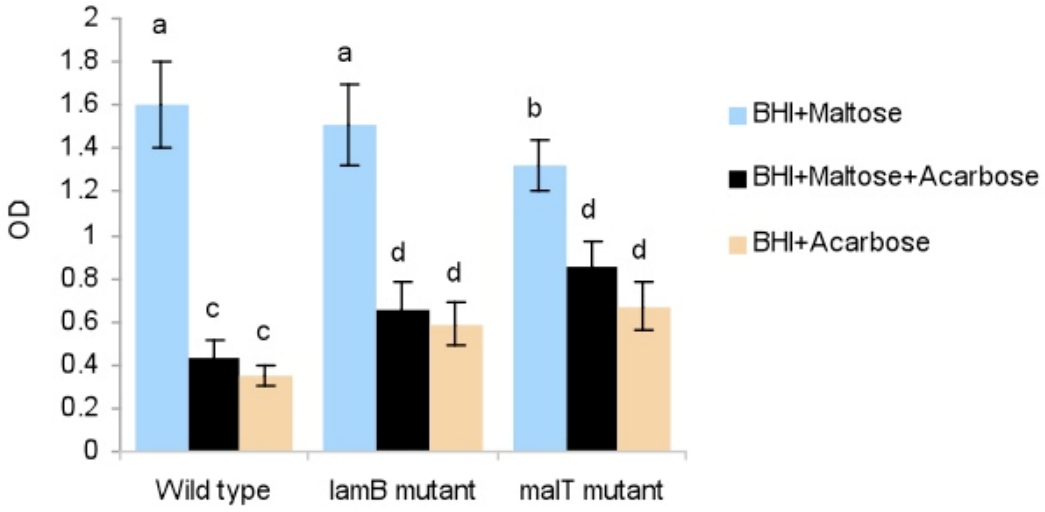

Figure 3. Overnight growth of the wild type strain and the *lamB* and *malT* mutants in acarbose or maltose. The bars with same letters on the top do not differ significantly ($P < 0.05$)

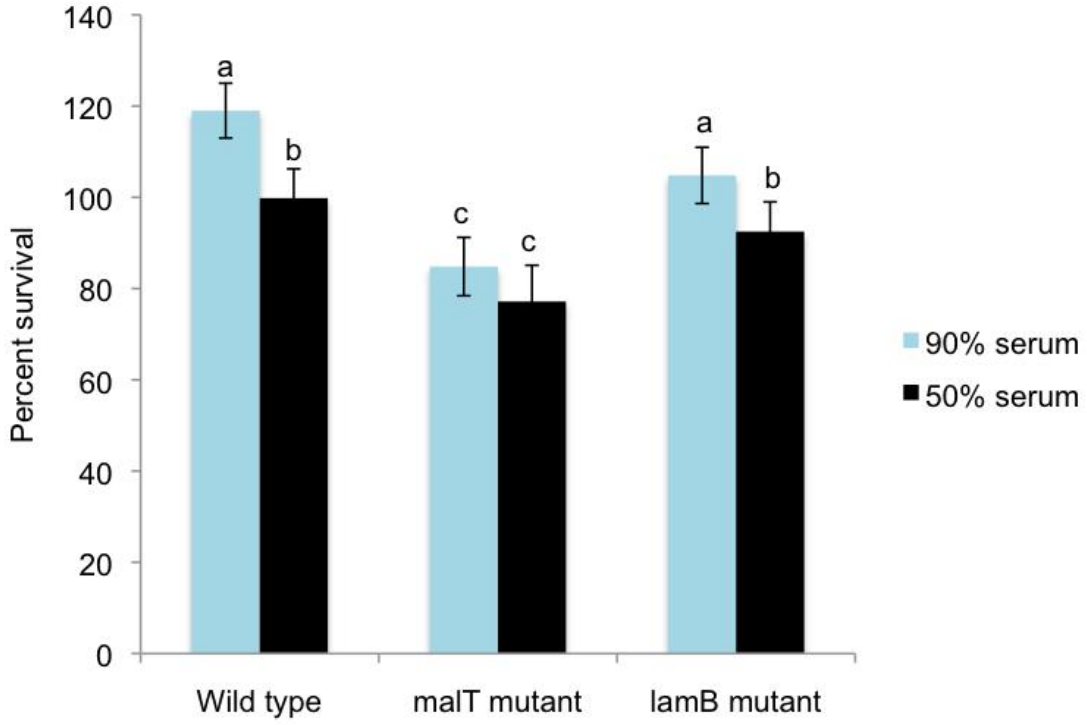

Figure 4. Percent survival of the wild type strain, and the *malT* and *lamB* mutants in porcine serum. The percent survival is the fresh-serum-surviving CFU expressed as the percent of CFU surviving in the heat inactivated serum. The strains were incubated in fresh and heat-inactivated serum for 1 h. The bars with same letters on the top do not differ significantly ($P < 0.05$)

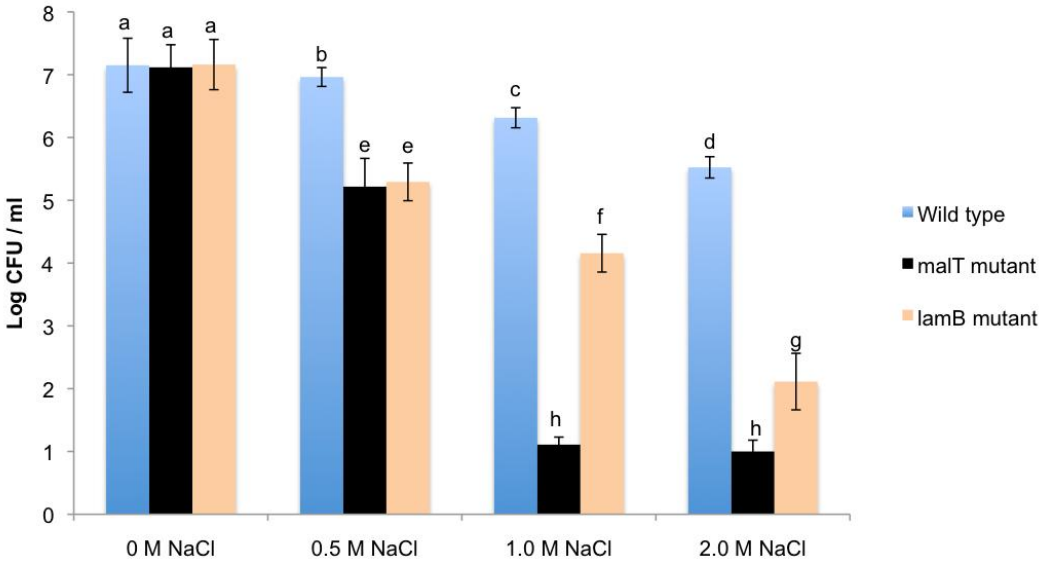

Figure 5. CFU of the wild type strain, and the *malT* and *lamB* mutants in different NaCl concentrations. The strains were incubated for 3 h in the salt-containing BHI medium. Before being exposed to NaCl, the strains were grown in maltose-containing BHI. The bars with the same letters on the top do not differ significantly ($P < 0.05$)

Tables

Table 1. Differential expression of maltose-regulon genes in BALF-exposed *A. pleuropneumoniae* CM5.

Gene	Putative function	$\Delta\Delta C_T \pm$ SD	Fold-change*
malE (T)	Periplasmic maltose binding protein	-2.82 ± 0.51	7.06 (4.95-10.05)
malE (R)		0 ± 0.84	1 (0.55-1.79)
malF (T)	Intrinsic membrane protein of maltose transport system	-2.79 ± 1.01	6.91 (3.43-13.92)
malF (R)		0 ± 0.39	1 (0.76-1.31)
malG (T)	Intrinsic membrane protein of the maltose transport system	-2.6 ± 0.40	6.06 (8-4.59)
malG (R)		0 ± 0.40	1(0.76-1.31)
malK (T)	ATP-binding protein of the maltodextrin transporter	-1.10 ± 0.39	2.14 (1.6-2.8)
malK (R)		0 ± 0.76	1(0.59-1.69)
lamB (T)	Maltoporin	-1.73 ± 0.46	3.31 (2.41-4.56)
lamB (R)		0 ± 0.35	1(0.78-1.27)
malP (T)	Maltodextrin phosphorylase	-0.85 ± 0.46	1.80(1.31-2.46)
malP (R)		0 ± 0.79	1(0.58-1.72)
malQ (T)	Amylomaltase	-0.96 ± 0.48	1.94(1.39-2.71)
malQ (R)		0 ± 0.55	1(0.68-1.46)
malT (T)	Transcriptional activator of maltose-regulon genes	-0.75 ± 0.32	1.68(1.34-2.09)
malT (R)		0 ± 0.79	1(0.58-1.72)

* Fold change is the fold increase or decrease in the level of expression of a gene in the wild type exposed to BALF (target sample, abbreviated as T) relative to the level of expression of the gene in the wild type exposed to BHI (calibrator or reference sample, abbreviated as R), as measured by real-time PCR.

Values in the parentheses represent the range in the fold change.

Table 2. Number of genes expressed differentially* within a functional category by the BALF-exposed *malT* mutant

Number of genes expressed differentially* within a functional category by the BALF-exposed *malT* mutant

Functional category	Up-regulated genes	Down-regulated genes
Protein biosynthesis	2	7
Amino acid biosynthesis	6	2
Cofactor biosynthesis	4	8
Biofilm formation	4	0
Nucleotide biosynthesis	3	0
Lipid biosynthesis	0	2
Lipid degradation	1	0
Cell envelope biosynthesis	3	10
Cellular processes	5	2
Central intermediary metabolism	0	4
DNA metabolism	3	6
Energy metabolism	7	18
Protein folding and stabilization	2	2
Regulatory proteins	7	2
Transcriptional regulators	0	5
Secretion and trafficking	4	10
Mobile and extra-chromosomal function	2	0
Unclassified and unknowns	51	41
Total	104	119

* Differential expression of a gene in the *malT* mutant is relative to the level of expression of the gene in the wild-type organism, as measured in microarray experiments.

Table 3. Protein-synthesis and energy-metabolism genes expressed differentially* by the BALF-exposed *malT* mutant

Type of the product encoded by the differentially expressed gene	Up-regulated genes	Down-regulated genes
Ribosomal proteins and their modifiers	*rpmE*	*rplQ*, *rpsQ*, *rplI*, *rpmG*
tRNA base modifiers	*queA*	*alaS*, *truD*, *trmU*,
Transcription and transcription-related factors		*deaD*, *rnc*, *rph*, *nusA*, *nusB*
Amino acid biosynthetic enzymes	*trpD*, *dapA*, *argD*, *proC*, *leuC*, *ilvH*, *tyrR*	*aroA*, *aroB*,
Periplasmic nitrate reductase (*nap* operon)		*napB*, *napG*, *napF*, *napD*, *napH*
Nitrite reductase (nrf operon)		*nrfB*, *nrfC*
Dimethyle sulfoxide reducatse (*dms* operon)		*dmsA*, *dmsB*
Hydrogenases		*hyaA*, *hybB*
Amino acid catabolism		*sdaA*, *aspA*
pyruvate formate-lyase 1-activating enzyme		*PflA*
Glycolysis and gluconeogenesis	*gpmB*, *pepC*	*FruK*
TCA cycle enzymes	*sucD*, *lpdA*	
Non-glucose hexose-monosaccharide metabolism enzymes	*mtlD*	*nagZ*
Products of central intermediary metabolism		*ureA*, *ppx*
ATP synthase		*atpC*, *atpB*
Formate dehydrogenase		*fdhE*
Products involved in fermentation	*dld*, *aldA*	
Regulatory proteins	*narP*, *sixA*, *gntR*, *cysB*, *asnC*, *gcvA*, *rseA*	*arcA*, *iclR*
Cofactors	*folA*, *folP*, *pdxY*, *thiH*	*hemB*, *chuW*, *lipA*, *ispA*, *ddc*, *dxs*, *ispE*, *iscA*

* Differential expression of a gene in the *malT* mutant is relative to the level of expression of the gene in the wild-type organism (reference sample) as measured in microarray experiments. For complete gene names and the fold changes in gene expression see Additional file 1: Analyzed microarray data.

Table 4. Nutrient-acquisition, replication and virulence genes expressed differentially* by the BALF-exposed *malT*mutant

Type of the product encoded by the differentially expressed gene	Up-regulated genes	Down-regulated genes
Biofilm-formation proteins	*pgaA, pgaC, tadF, apfB*	
Toxin	*apxIVA*	
Factors imparting resistance to antimicrobials		*ostA, ccp*
Peptidoglycan and LPS biosynthetic enzymes	*cpxD, mrdA*	*dacA, murA, mltA, mreD, fbB1, kdsB, gmhA*
Membrane proteins	*ompP1*	*ompW, oapB*
Amino acid transporters		*brnQ, sdaC*
Carbohydrate transporter	*mtlA*	*ptsB, rbsD*
Iron transport proteins	*cbiO*	*exbD2, afuB_2, frpB, yfeC, exbB2*
Protein/peptide transport proteins	*dppF*	
Other cation transporters		*ptsN*
Cell division	*fic*	
Lipid transporters	*glpF*	
Factors involved in adaptation to unusual environment	*relA*	
DNA transformation	*comEA, comF*	
DNA degradation proteins	*xseA*	
DNA replication, recombination proteins	*recG*, *rdgC*, *recJ*	*xerC*, *recR*, *priB*, *pol A*, *ligA*, *recA*
Protein-fate proteins	*htpX, prlC*	*ecfE*
Nucleotide metabolism enzymes	*purC, purD, purT*	
Phopholipid and fatty acid biosynthesis and degradation enzymes	*namA*	*accA, fabD*

* Differential expression of a gene in the *malT* mutant is relative to the level of expression of the gene in the wild-type organism (reference sample). For complete gene names and the fold changes in gene expression see Additional file 1: Analyzed microarray data.

Table 5. Verification of microarray data by real-time PCR

Gene	Putative function	$\Delta\Delta C_T \pm$ SD	Fold change by real-time PCR	Fold change by microarray[1]
dmsA(T)	Anaerobic dimethyl sulfoxide reductase chain A precursor	3.45 ± 1.41	0.091 (0.03-0.24)	0.15
dmsA(R)		0 ± 0.51	1 (0.69-1.42)	
dmsB(T)	Anaerobic dimethyl sulfoxide reductase chain B	2.54 ± 1.61	0.17 (0.05-0.52)	0.34
dmsB(R)		0 ± 0.46	1 (0.72-1.38)	
napB (T)	Nitrate reductase cytochrome c-type subunit	2.24 ± 0.41	0.21 (0.15-0.28)	0.17
napB (R)		0 ± 0.49	1 (0.71-1.40)	
napF (T)	Ferredoxin-type protein NapF	2.24 ± 0.46	0.21 (0.07-0.61)	0.09
napF (R)		0 ± 0.47	1 (0.71-1.39)	
napD (T)	Putative napD protein	2.39 ± 0.34	0.18 (0.14-0.24)	0.18
napD(R)		0 ± 0.54	1 (0.68-1.46)	
ilvH (T)	Acetolactate synthase small subunit	-2.60 ± 0.36	6.08 (4.68-7.90)	6.14
ilvH (R)		0 ± 0.45	1 (0.70-1.41)	
pgaA (T)	Biofilm PGA synthesis protein PgaA precursor	-2.04 ± 1.08	4.11 (1.94-8.70)	8.18
pgaA (R)		0 ± 0.74	1 (0.59-1.67)	
pgaC (T)	Biofilm PGA synthesis N-glycosyltransferase PgaC	-2.47 ± 0.42	5.54 (4.12-7.45)	6.23
pgaC (R)		0 ± 1.05	1(0.48-1.07)	
apxIVA(T)	RTX toxin protein	-3.01 ± 1.12	8.06 (3.69-17.61)	6.5
apxIVA(R)		0 ± 0.60	1 (0.65-1.52)	
relA (T)	GTP pyrophosphokinase	-0.95 ± 0.42	2.0 (1.44-2.56)	6.30
relA (R)		0 ± 0.59	1(0.66-1.51)	
lamB(T)[2]	Maltoporin	1.03 ± 0.39	0.49 (0.37-0.64)	na[3]
lamB (R)		0 ± 0.23	1 (0.85-1.17)	

[1]Fold change is the fold increase or decrease in the level of expression of a gene in the *malT* mutant (target sample, abbreviated as T) relative to the level of expression of the gene in the wild type (calibrator or reference sample, abbreviated as R) in BALF except for the *lamB* gene[2] whose expression was compared in BHI to examine the effect of the *malT* knockout mutation on the expression of the *lamB* gene. [3] Not applicable.

Values in the parentheses represent the range in the fold change.

Table 6. Bacterial strains, plasmids and primers used in the construction of the *malT* mutant

Bacterial strains, plasmids or primers	Characteristic or sequence	Source or Remark
E. coli DH5α	F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(rk -, mk +) *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-	Clonetech
E. coli β2155	*thr*B1004*pro thi hsdS lacZ*ΔM15 (F′lacZΔM15*lacI*^{q} *traD*36 *proA*$^{+}$ *proB*$^{+}$) Δ*dap*::*erm*(Erm^{r})*recA*::RP4-2-*tet*(Tc^{r})Mu-km(Km^{r})λpir	Reference no. 28
E. coli DH5α-pTOPOPCR-malT	DH5α harboring pCR4-TOPO containing *malT* of *A. pleuropneumiae* CM5	This work
E. coli DH5α-pTopoMC	DH5α harboring pCR4-TOPO containing Δ*malT*::*cat*	This work
E. coli DH5α-pEMOC2M	DH5α harboring pEMOC2 containing Δ*malT*::*cat*	This work
A. pleuropneumoniae CM5 3ΔmalT	MalT negative mutant of *A. pleuropneumonaie* CM5	This work
pCR4-TOPO	A linearized plasmid for cloning PCR product	Invitrogen
pEMOC2	A conjugation vector based on pBluesript SK with *mob*RP4 and Cm^{r}	Reference no. 31
pTOPOPCR-malT	pCR4-TOPO containing *malT* of *A. pleuropneumiae* CM5	This work
pTopoMC	pCR4-TOPO containing Δ*malT*::*cat*	This work
pEMOC2M	harboring pEMOC2 containing Δ*malT*::*cat*	This work
malT-L	**Atgcaagcaacattttcaaga**	The primers for the PCR amplification of the *malT* gene of *A. pleuropneumoniae CM5*
malT-R	ttagctataccccatcattctcaa	
stopupmalT-L	**Ttagttagttacgagctttttcacaccgttt**	The primers for the PCR generation of a linearized plasmid containing a deletion of 900 bp in its *malT* gene cloned in pTOPOPCR-malT.
stopupmalT-R	taactaactaatgggaatggcatcatttaga	
pnmalT-L	**Tcatctgcagatgcaagcaacattttcaaga**	The primers for the PCR amplication of the Δ*malT*::*cat* and the insertion of the *Pst*I *and Not*I sites into the PCR product.
pnmalT-R	acaatacagcggccgcttagctataccccatcattctcaa	

Table 7. Bacterial strains, plasmids and primers used in the construction of the l*amB* mutants of *A. pleuropneumoniae* CM5

Bacterial strains[1], plasmids or primers	**Characteristic or sequence**	**Source or Remark**
E. coli DH5α-pTOPOFL	DH5α harboring pCR4-TOPO containing *lamB* of *A. pleuropneumiae* CM5	This work
E. coli DH5α-TOPOΔFLcat	DH5α harboring pCR4-TOPO containing Δ*lamB*::*cat*	This work
E. coli DH5α-pEMOC2-ΔlamB	DH5α harboring pEMOC2 containing Δ*lamB*::*cat*	This work
A. pleuropneumoniae CM5 ΔlamB	LamB negative mutant of *A. pleuropneumoniae* CM5	This work
pTOPOFL	pCR4-TOPO containing *lamB* of *A. pleuropneumiae* CM5	This work
TOPOΔFLcat	pCR4-TOPO containing Δ*lamB*::*cat*	This work
pEMOC2-ΔlamB	pEMOC2 containing Δ*lamB*::*cat*	This work
CrosslamB-L CrosslamB-R	**Ggtggcgtaaaagtaggagat** tggtcattatccaccaccaa	The primers for the PCR amplification of the *lamB* gene of *A. pleuropneumoniae CM5*
stopuplamB-L stopuplamB-R	**Ttagttagttacaatattttcaacccctgcac** Taactaactaatcacgcacaaggttcaaaag	The primers for the PCR generation of a linearized plasmid containing a deletion of 400 bp in the *lamB* gene cloned in pTOPOPCR-lamB
PstcrosslamB-L NotcrosslamB-R	**Tcatctgcagggtggcgtaaaagtaggagat** acaatacagcggccgctggtcattatccaccaccaa	The primer sequences for the PCR amplication of the Δ*lamB*::*cat* and the insertion of the *PsT*I *and Not*I sites into the PCR product

Table 8. Arbitrary random primer pair combinations used in RT-PCR DD

AP17/AP18	AP17/AP19	AP17/AP20	AP17/AP21	AP17/AP21
AP17/AP21	AP17/AP22	AP17/AP23	AP17/AP24	AP17/AP24
AP17/AP24	AP18/AP18	AP18/AP19	AP18/AP19	AP18/AP20
AP18/AP20	AP18/AP21	AP18/AP21	AP18/AP22	AP18/AP22
AP18/AP23	AP18/AP23	AP18/AP24	AP19/AP18	AP19/AP20
AP19/AP21	AP19/AP22	AP19/AP23	AP19/AP23	AP19/AP24
AP20/AP18	AP20/AP21	AP20/AP22	AP20/AP23	AP20/AP24
AP21/AP24	AP21/AP18	AP21/AP22	AP21/AP23	AP22/AP18
AP22/AP23	AP22/AP24	AP23/AP18	AP23/AP24	AP24/AP18
AP41/AP18	AP41/AP42	AP41/AP43	AP41/AP44	AP41/AP45
AP41/AP46	AP41/AP47	AP41/AP48	AP42/AP18	AP42/AP43
AP42/AP44	AP42/AP45	AP42/AP46	AP42/AP46	AP42/AP47
AP43/AP18	AP43/AP44	AP43/AP45	AP43/AP46	AP43/AP47
AP43/AP48	AP43/AP48	AP44/AP18	AP44/AP45	AP44/AP46
AP44/AP47	AP44/AP48	AP45/AP18	AP45/AP46	AP45/AP46
AP45/AP47	AP45/AP48	AP46/AP18	AP46/AP47	AP46/AP48
AP47/AP18	AP47/AP48	AP47/AP48		

Table 9. Sequences of the arbitrary random primers used in RT-PCR DD

Arbitrary random primer	Sequence
AP17	AAGCTTACCAGGT
AP18	AAGCTTAGAGGCA
AP19	AAGCTTATCGCTC
AP20	AAGCTTGTTGTGC
AP21	AAGCTTTCTCTGG
AP22	AAGCTTTTGATCC
AP23	AAGCTTGGCTATG
AP24	AAGCTTCACTAGC
AP41	AAGCTTACGGGGT
AP42	AAGCTTTGCACCG
AP43	AAGCTTGAAGCGG
AP44	AAGCTTCTCCGGA
AP45	AAGCTTGGCTGAC
AP46	AAGCTTCGGTCCT
AP47	AAGCTTATGCCCG
AP48	AAGCTTGCGGTGA

Table 10. Oligonucleotide primers used in the real-time PCR

Gene	Forward primer	Reverse primer
dmsA	ATGTTGCCGGACAAGCACAAGATG	TCTCAATGGACAACGGCTACCACA
dmsB	AACAGGCATCGATTGCACCGTTAC	ACTTGGACGTGCGTGTTTATTGGC
napB	GCGCATGGCAACCTAAACATTGGT	TACAGGCTTTGCAGTAGCGGAAAC
napD	TCGGCTAAAGCAAGCTGTCTGTCA	TAGCGCAAGTGAAAGCGGACATTC
napF	ACAACCGTCTCCGCAACTTCTACA	TTGGCTACAACGGAAGAAGCATGG
ilvH	GAAAGTTTAACCGTTGCGCCGACT	ACGTTCAATATGCTCGGTAGGGCT
pgaA	GGGAACCGGTGTGAATGCAATGAA	TGTTGGAACGTTTGTGAAGACGCC
pgaC	ATCGTTGCGTTACACCAAGCGAAC	ACCGACATACTTGCCTCTTGCGAT
apxIVA	TTGGACTTCACCTGCAAACATGCC	CGGGCAAATATTCCAAAGCGCAGA
relA	TCGGACAGTTGAAGTGGGAAT	TGCAAGGCGATTACTCGGTAA
syp	AAGAAACGCCGAATGATGCACAGG	ACACCTCGATAGCACCACCTTTGT
lamB	CTGCTAAAGAGAGTTTACCGATGCCA	TGCAACATTACGGGCAGGTAAACG
malK	GCGTGTTGCAATTGGACGTACCTT	CATGGCTTCGATTTGGTCATGCGT
malM	AGCGACACCGTCAAAGACAGAACT	CCAACGTTTGGCTAAATGTGCGGA
malT	TCCTTGATGAGCTTTCGACCCACA	TAAACCGAGCACCTGCCATTCTCT
malP	ACGCTTAGCCGCCTGCTATTTAGA	CACGCATCGCCTTCTTCATGTTGT
malQ	ATGCCTATCGGCCTTTACCGTGAT	ACCGACAGAGGCATCTAGCACAAA
malE	AACCGATGAAGGACTCACAACCGT	TTTCCGCATTCGCCATAGTTGCTG
malF	TGCCGTTAATGATTGCCAGCTTCG	GCAGCCGCTAAACCAAAGTCTTGT
malG	AGTGTTACTCATGCGGACGGAAGT	GCATACGCAGCAGTGGTTGAAAGT

Supplementary data : Differentially expressed genes of the BALF-exposed *A. pleuropneumoniae malT* mutant, grouped according to biological role.

ORF No.	Gene	Predicted protein	Fold change
Amino acid biosynthesis			
APL_0699	*aroA*	3-phosphoshikimate 1-carboxyvinyltransferase	-4.4
APL_0193	*aroB*	3-dehydroquinate synthase	-2.3
APL_0899	*dapA*	dihydrodipicolinate synthase	6.72
APL_0244	*argD*	acetylornithine aminotransferase (ACOAT)	3.67
APL_0160	*proC*	pyrroline-5-carboxylate reductase	5.82
APL_0139	*leuC*	3-isopropylmalate dehydratase large subunit 2	3.46
APL_0728	*ilvH*	acetolactate synthase small subunit	6.14
APL_0797	*tyrR*	transcriptional regulatory protein TyrR	3.94
Biofilm formation			
APL_1921	*pgaA*	biofilm PGA synthesis protein PgaA precursor	8.18
APL_1923	*pgaC*	biofilm PGA synthesis N-glycosyltransferase PgaC	6.23
APL_0547	*tadF*	tight adherence protein F	6.29
APL_0879	*apfB*	fimbrial biogenesis protein	4.35
Biosynthesis of cofactors			
APL_0883	*folA*	dihydrofolate reductase	3.64
APL_1876	*folP*	Dihydropteroate synthase	3.87
APL_1988	*hemB*	delta-aminolevulinic acid dehydratase	-4.43
APL_1523	*chuW*	coproporphyrinogen III oxidase	-2.42
Biosynthesis of cofactors			
APL_1593	*lipA*	lipoyl synthase	-7.73
APL_0807	*ispA*	geranyltranstransferase	-3.27

APL_1975	*ddc*	L-2,4-diaminobutyrate decarboxylase	-3.22
APL_0207	*dxs*	1-deoxy-D-xylulose-5-phosphate synthase (DXPS)	-2.88
APL_0776	*ispE*	4-diphosphocytidyl-2-C-methyl-D-erythritolkinase	-2.38
APL_0929	*iscA*	iron-binding protein IscA	-2.14
APL_1485	*pdxY*	pyridoxamine kinase	9.78
APL_0536	*thiH*	thiazole biosynthesis protein ThiH	2.44
Cell envelope			
APL_1596	*dacA*	D-alanyl-D-alanine carboxypeptidase fraction A	-7.24
APL_1286	*murA*	UDP-N-acetylglucosamine1-carboxyvinyltransferase	-4.12
APL_0816	*mltA*	membrane-bound lytic murein transglycosylase A precursor	-3.23
APL_0945	*dacB*	penicillin-binding protein 4 precursor	-3.18
APL_0437	*mreD*	rod shape-determining protein MreD	-2.37
APL_1559	*mrdA*	penicillin-binding protein 2	4.87
APL_1472	*rfbB1*	dTDP-glucose 4,6-dehydratase	-5.13
APL_0085	*kdsB*	3-deoxy-manno-octulosonate cytidylyltransferase	-3.12
APL_1364	*gmhA*	putative phosphoheptose isomerase	-2.69
APL_1582	*cpxD*	capsule polysaccharide export protein	2.53
APL_0933	*ompP1*	putative outer membrane protein precursor	4.69

Cell envelope			
APL_1086	*ompW*	outer membrane protein W precursor	-15.25
APL_1404	*oapB*	opacity associated protein B	-3.41
Cellular processes			
APL_0405	*relA*	GTP pyrophosphokinase	6.3
APL_0962	*ostA*	organic solvent tolerance protein precursor	-2.68
APL_1406	*comEA*	DNA uptake protein	4.15
APL_2004	*comF*	Competence protein F	5.38
APL_1379	*ccp*	cytochrome c peroxidase	-8.65
APL_0860	*fic*	filamentation induced by cAMP protein Fic-like protein	2.49
APL_0998	*apxIVA*	RTX toxin protein	6.5
Central intermediary metabolism			
APL_1618	*ureA*	urease gamma subunit UreA	-4.43
APL_0708	*ppx*	exopolyphosphatase	-3.45
APL_1674	*dmsA*	anaerobic dimethyl sulfoxide reductase chain A precursor	-6.66
APL_1675	*dmsB*	anaerobic dimethyl sulfoxide reductase chain B	-2.9
DNA metabolism			
APL_0817	*xseA*	putative exodeoxyribonuclease VII large subunit	2.82
APL_1805	*xerC*	tyrosine recombinase xerC	-11.66
APL_0074	*recR*	recombination protein RecR	-4.53
APL_1170	*priB*	primosomal replication protein	-3.83

DNA metabolism			
APL_0473	*polA*	DNA polymerase I (POL I)	-3.72
APL_1302	*ligA*	DNA ligase	-2.55
APL_1143	*recA*	recombinase A	-2.52
APL_0494	*rdgC*	recombination-associated protein RdgC	3.7
APL_0459	*recJ*	single-stranded-DNA-specific exonuclease RecJ	4.39

Energy metabolism

APL_1331	*hyaA*	hydrogenase-2 small chain precursor	-14.04
APL_1486	*napB*	nitrate reductase cytochrome c-type subunit	-5.71
APL_0857	*sdaA*	L-serine dehydratase	-3.64
APL_1091	*aspA*	aspartate ammonia-lyase	-2.54
APL_0102	*nrfC*	nitrite reductase	-10.25
APL_0101	*nrfB*	cytochrome c-type protein NrfB precursor	-4.91
APL_1333	*hybB*	putative Ni/Fe-hydrogenase 2 b-type cytochrome subunit	-4.14
APL_1035	*pflA*	pyruvate formate-lyase 1-activating enzyme	-3.33
APL_1645	*atpC*	ATP synthase epsilon chain	-2.67
APL_1652	*atpB*	ATP synthase A chain	-2.62
APL_1428	*napG*	ferredoxin-type protein napG-like protein	-12.55
APL_1431	*napF*	ferredoxin-type protein NapF	-10.02
APL_1430	*napD*	putative napD protein	-5.28
APL_1427	*napH*	ferredoxin-type protein NapH-like protein	-4.2
APL_0896	*fdhE*	formate dehydrogenase accessory protein	-2.93
APL_0687	*dld*	D-lactate dehydrogenase	2.86

Energy metabolism

APL_2011	*aldA*	Putative aldehyde dehydrogenase aldA	6.32
APL_0344	*fruK*	1-phosphofructokinase	-3.09
APL_0230	*gpmB*	phosphoglycerate mutase/fructose-2, 6-bisphosphatase	5.72
APL_0339	*pepC*	phosphoenolpyruvate carboxylase	12.05
APL_0771	*lpdA*	dihydrolipoyl dehydrogenase	8.21
APL_1737	*sfsA*	sugar fermentation stimulation-like protein	-2.22
APL_1629	*mtlD*	mannitol-1-phosphate 5-dehydrogenase	2.67
APL_0451	*sucD*	succinyl-CoA ligase [ADP-forming] subunit alpha	2.85
APL_1111	*nagZ*	beta-hexosaminidase	-2.54

Lipid metabolism			
APL_1486	*accA*	acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	-8.27
APL_1993	*fabD*	malonyl CoA-acyl carrier protein transacylase (MCT)	-2.86
APL_1191	*namA*	NADPH dehydrogenase	2.41
Extrachromosomal element function			
APL_1058	APL_1058	transposase	4.2
APL_1206	APL_1206	plasmid stability-like protein	8.18
Protein fate			
APL_0412	*ecfE*	putative zinc metalloprotease	-2.05
APL_1039	*htpX*	putative protease HtpX-like protein	2.09
APL_1034	*prlC*	oligopeptidase A	3.03
APL_0433	*msrB*	methionine sulfoxide reductase B	-3.27
Protein synthesis			
APL_1785	*rplQ*	50S ribosomal protein L17	-12.97
APL_1769	*rpsQ*	30S ribosomal protein S17	-3.89
APL_1169	*rplI*	50S ribosomal protein L9	-3.48
APL_1972	*rpmG*	50S ribosomal protein L33	-3.16
APL_1821	*rpmE*	50S ribosomal protein L31	4.04
APL_0654	*alaS*	alanyl-tRNA synthetase	-3.59
APL_1926	*truD*	tRNA pseudouridine synthase D	-11.25
APL_1383	APL_1383	tRNA (guanine-N(7)-)-methyltransferase	-4.2
APL_0765	*queA*	S-adenosylmethionine: tRNAribosyltransferase-isomerase	2.72
Nucleotide metabolism			
APL_2018	*purC*	Phosphoribosylaminoimidazole-succinocarboxamidesynthase (SAICAR synthetase)	2.76
APL_1172	*purD*	phosphoribosylamine--glycine ligase	6.21
APL_1106	*purT*	putative phosphoribosylglycinamide formyltransferase 2	8.47
Regulatory proteins			

APL_0048	*arcA*	aerobic respiration control protein ArcA	-3.91
APL_0133	*cysB*	HTH-type transcriptional regulator CysB	3
Regulatory proteins			
APL_1838	*asnC*	Regulatory protein AsnC	5.22
APL_0131	*gcvA*	glycine cleavage system transcriptional activator-like protein	2.98
APL_0395	*rseA*	putative sigma-E factor negative regulatory protein	2.19
APL_0108	*iclR*	putative HTH-type transcriptional regulator	-2.39
APL_0059	*narP*	nitrate/nitrite response regulator protein	3.35
APL_0571	*gntR*	HTH-type transcriptional regulator	14.82
APL_0188	*sixA*	possible phosphohistidine phosphatase	5.22
Transcription			
APL_0575	*deaD*	cold-shock DEAD box protein A-like protein	-3.37
APL_0543	*rnc*	ribonuclease III	-5.97
APL_0055	*rph*	ribonuclease PH	-3.56
APL_0638	*nusA*	transcription elongation protein NusA	-10.95
APL_0201	*nusB*	transcription antitermination protein NusB	-5.82
Transport			
APL_1991	*brnQ*	Branched-chain amino acid transport system carrier protein braB	-7.53
APL_0856	*sdaC*	serine transporter	-6.92
APL_1319	*ptsB*	PTS system sucrose-specific EIIBC component	-5.25
APL_1669	*rbsD*	high affinity ribose transport protein RbsD	-4.43
APL_1603	*mtlA*	PTS system mannitol-specific EIICBA component	12.84
APL_0077	*exbD2*	biopolymer transport protein ExbD2	-7.52
Transport			
APL_0564	*afuB_2*	Ferric transport system permease protein	-5.82
APL_0276	*frpB*	iron-regulated outer membrane protein B	-4.78
APL_0128	*yfeC*	putative iron transport system membrane protein	-2.21

APL_0160	*cbiO*	predicted ABC transport ATP-binding protein CbiO	6.33
APL_0374	*glpF*	glycerol uptake facilitator protein	6.77
APL_0335	*ptsN*	PTS system, nitrogen regulatory IIA-like protein	-2.56
APL_0078	*exbB2*	biopolymer transport protein ExbB2	-4.83
APL_0068	*dppF*	dipeptide transport ATP-binding protein DppF	3.33
Unclassified and unknowns			
APL_1636	APL_1636	hypothetical protein	-7.21
APL_1382	APL_1382	hypothetical protein	-4.74
APL_0519	APL_0519	predicted phage minor tail protein	-4.61
APL_0104	APL_0104	autotransporter adhesin	-4.46
APL_0438	APL_0438	hypothetical protein	-3.98
APL_0196	APL_0196	hypothetical protein	-3.88
APL_0905	APL_0905	hypothetical protein	-3.67
APL_1891	APL_1891	hypothetical protein	-3.63
APL_2043	APL_2043	hypothetical protein	-3.45

Unclassified and unknowns

APL_1285	APL_1285	hypothetical protein	-3.24
APL_1231	APL_1231	hypothetical protein	-3.19
APL_0949	APL_0949	hypothetical protein	-3.12
APL_0602	APL_0602	hypothetical protein	-3.09
APL_1983	APL_1983	hypothetical protein	-3.06
APL_0305	APL_0305	hypothetical protein	-3.01
APL_0232	APL_0232	hypothetical protein	-2.74
APL_0264	APL_0264	putative ABC transporter ATP-binding protein	-2.67
APL_1272	APL_1272	predicted transcriptional accessory protein	-2.53
APL_0363	APL_0363	hypothetical protein	-2.24
APL_0355	*proP*	bicyclomycin resistance-like protein	-2.18

APL_0676	APL_0676	hypothetical protein	-2.08
APL_0668	APL_0668	hypothetical protein	-11.77
APL_1807	APL_1807	hypothetical protein	-8.69
APL_1867	APL_1867	hypothetical protein	-7.97
APL_1632	APL_1632	predicted transcriptional regulator of sugar metabolism	-7.89
APL_0293	APL_0293	putative type I site-specific restriction-modification system, R (restriction) subunit	-7.36
APL_0707	APL_0707	hypothetical protein	-7.1
APL_1660	APL_1660	mannose permease IID component	-7.01
APL_0937	APL_0937	hypothetical protein	-6.36

Unclassified and unknowns

APL_0843	APL_0843	hypothetical tRNA/rRNA methyltransferase	-6.25
APL_1573	APL_1573	2,3-diketo-L-gulonate reductase	-6.03
APL_1115	APL_1115	hypothetical protein	-5.85
APL_0637	APL_0637	hypothetical protein	-5.4
APL_0695	APL_0695	hypothetical protein	-5.4
APL_0029	APL_0029	ABC transporter periplasmic protein	-5.07
APL_0217	APL_0217	hypothetical protein	-4.11
APL_0738	APL_0738	hypothetical protein	-3.44
APL_1740	APL_1740	Probable Fe(2+)-trafficking protein	2.11
APL_1266	APL_1266	putative dehydrogenase	2.24
APL_0313	APL_0313	deoxyguanosinetriphosphate triphosphohydrolase-like protein	2.24
APL_1746	APL_1746	hypothetical protein	2.28
APL_0703	APL_0703	hypothetical ATP-dependent helicase	2.3
APL_0885	APL_0885	hypothetical protein	2.44
APL_0991	APL_0991	hypothetical protein	2.57
APL_1980	APL_1980	hypothetical protein	2.65
APL_0781	APL_0781	putative ATP-dependent helicase	2.69

APL_0858	APL_0858	hypothetical protein	2.7

Unclassified and unknowns

APL_1976	*yedF*	hypothetical protein	2.77
APL_0672	APL_0672	hypothetical protein	2.85
APL_1903	APL_1903	hypothetical protein	2.98
APL_0788	APL_0788	transcriptional regulatory protein	3.02
APL_1656	APL_1656	hypothetical protein	3.03
APL_1044	APL_1044	hypothetical protein	3.1
APL_1491	APL_1491	hypothetical protein	3.11
APL_1855	APL_1855	hypothetical protein	3.19
APL_1088	APL_1088	hypothetical protein	3.21
APL_0162	APL_0162	putative phosphatase	3.27
APL_0811	APL_0811	hypothetical protein	3.35
APL_1141	APL_1141	hypothetical protein	3.36
APL_1187	APL_1187	hypothetical protein	3.37
APL_1167	APL_1167	hypothetical protein	3.41
APL_0511	APL_0511	hypothetical protein	3.62
APL_0787	APL_0787	putative ATPase	3.71
APL_1247	APL_1247	hypothetical protein	3.73
APL_1996	*rssA*	hypothetical protein	3.78
APL_0480	APL_0480	hypothetical protein	3.83

Unclassified and unknowns

APL_1267	APL_1267	hypothetical protein	4.09
APL_0426	APL_0426	hypothetical protein	4.15
APL_1948	APL_1948	hypothetical protein	4.28
APL_0500	APL_0500	hypothetical protein	4.41
APL_1415	APL_1415	hypothetical protein	4.48
APL_0781	APL_0781	putative ATP-dependent helicase	4.83

APL_1262	APL_1262	transcriptional regulator MerR family	5.15
APL_1234	APL_1234	hypothetical protein	5.23
APL_1681	APL_1681	hypothetical protein	5.74
APL_1495	APL_1495	putative transcriptional regulator	5.75
APL_0526	APL_0526	hypothetical protein	6.16
APL_1588	APL_1588	predicted TRAP transporter solute receptor	6.4
APL_1252	APL_1252	hypothetical protein	7.34
APL_0882	APL_0882	hypothetical protein	7.37
APL_0137	APL_0137	hypothetical protein	8.01
APL_0222	APL_0222	putative lipoprotein	10.01
APL_0940	APL_0940	hypothetical protein	10.79
APL_0703	APL_0703	hypothetical ATP-dependent helicase	12.19
APL_1263	APL_1263	hypothetical protein	18.51
APL_1015	*deoC*	deoxyribose-phosphate aldolase	-3.03
APL_1037	*focA*	putative formate transporter	-2.24
APL_1654	*gidB*	methyl transferase GidB	-4.41
APL_0132	*APL_0132*	putative haloacid dehalogenase-like hydrolase	5.2
APL_0403	*engA*	GTP-binding protein EngA	3
APL_1655	*gidA*	tRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA	-2.27
APL_1977	*yedE*	hypothetical protein	3.64