Université de Montréal

RÔLE DU LIPOPOLYSACCHARIDE DANS LA PATHOGENÈSE D'*ACTINOBACILLUS PLEUROPNEUMONIAE* ET DANS SON INTERACTION AVEC LE SYSTÈME IMMUNITAIRE INNÉ

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RÉSUMÉ

Le lipopolysaccharide (LPS) est un facteur de virulence majeur d'*Actinobacillus pleuropneumoniae*, un pathogène des voies respiratoires porcines. Plusieurs mutants LPS d'*A. pleuropneumoniae* sérotype 1 affectés au niveau de l'antigène O (mutants rugueux) ou du noyau oligosaccharidique (OS) (mutants « core ») du LPS ont été précédemment générés dans notre laboratoire par insertion du transposon mini-*Tn10*. Dans ce projet, nous avons caractérisé la structure du noyau OS de 3 mutants « core » (les mutants 5.1, CG1 et CG3) par méthylation et spectrométrie de masse. Pour évaluer le rôle du LPS dans l'interaction d'*A. pleuropneumoniae* avec le système immunitaire inné, nous avons quantifié la synthèse de cytokines proinflammatoires telles que l'interleukine 6 (IL-6), le tumor necrosis factor alpha (TNF-α), l'interleukine 1 beta (IL-1β), le monocyte chemoattractant protein 1 (MCP-1) et l'interleukine 8 (IL-8) par des macrophages alvéolaires porcins (PAMs) et par 2 lignées de cellules épithéliales porcines, les NPTr (Newborn Pig Trachea) et les SJPL (St. Jude Porcine Lung). La stimulation de ces cellules par des LPS purifiés ou des bactéries tuées à la chaleur, n'a montré aucune différence entre les mutants LPS et la souche sauvage, dans leur capacité à stimuler la synthèse de cytokines proinflammatoires. Cependant, contrairement aux PAMs qui produisent toutes les cytokines testées, aucune production n'a été détectée chez les cellules de poumon (SJPL). Chez les cellules de trachée (NPTr), nous avons détecté uniquement la production d'IL-8 qui reste cependant nettement inférieure à la quantité produite par les PAMs stimulés. Ainsi, l'étude des voies intracellulaires impliquées dans l'activation des gènes de l'inflammation a permis de souligner l'absence de la sous-unité active p65 de NF-κB chez les SJPL. De plus, la stimulation des NPTr par des bactéries vivantes, en présence d'un inhibiteur de IRAK 1/4, montre que l'activation de NF-κB chez ces cellules est indépendante de la voie TLR/NF-κB, minimisant ainsi le rôle du LPS dans la stimulation des cellules épithéliales de trachée. Dans cette étude, nous avons également mis en évidence un rôle important du noyau OS dans la résistance d'*A. pleuropneumoniae* à des peptides antimicrobiens, car les 3 mutants « core » sont plus sensibles que le mutant rugueux 27.1 et la souche sauvage à ces peptides cationiques. De plus, les infections expérimentales de porc par ces mutants suggèrent que les résidus galactose (Gal I) et DD-heptose (Hep IV) du noyau OS seraient importants non

seulement pour l'adhésion mais aussi pour la virulence d'*A. pleuropneumoniae* sérotype 1. Étant donné que le mutant « core » 5.1 est très atténué et cause significativement moins de lésions pulmonaires que les autres mutants LPS d'*A. pleuropneumoniae* sérotype 1, nous avons évalué ses activités hémolytique et cytotoxique. Alors que le mutant 5.1 possède une activité hémolytique comparable à celle de la souche parentale, son activité cytotoxique pour les PAMs est significativement diminuée. Cependant, aucune diminution n'a été observée dans l'expression et la sécrétion des toxines hémolytiques et cytolytiques ApxI et ApxII chez ce mutant. Ainsi, nous en avons déduit que l'absence des résidus GalNAc-Gal II-Gal I du noyau oligosaccharidique chez le mutant 5.1, affecterait les toxines Apx au niveau de leur activité. Grâce à des tests ELISA et des expériences de résonance plasmonique de surface, nous avons démontré pour la première fois une interaction entre le LPS et les toxines ApxI et ApxII via le noyau oligosaccharidique. Nos résultats indiquent également que la région GalNAc-Gal II-Gal I du noyau externe est essentielle dans l'interaction entre le noyau oligosaccharidique et les toxines Apx. Cette étude suggère que l'interaction entre le LPS et les toxines ApxI et ApxII augmenterait l'activité cytotoxique d'*A. pleuropneumoniae* sérotype 1.

En conclusion, ce projet démontre le rôle important du noyau OS du LPS dans la pathogenèse d'*A. pleuropneumoniae* mais suggère également que d'autres constituants de la bactérie seraient impliqués dans la stimulation du système immunitaire de l'hôte.

Mots clés : *Actinobacillus pleuropneumoniae*, lipopolysaccharide, noyau oligosaccharidique, cytokines, NF-κB, peptides antimicrobiens et toxines Apx

ABSTRACT

The lipopolysaccharide (LPS) is a major virulence factor of *Actinobacillus pleuropneumoniae*, the etiological agent of porcine pleuropneumonia. We previously reported that the core oligosaccharide (OS) region of LPS is essential for optimal adhesion of the bacteria to respiratory tract cells. Rough LPS and core LPS mutants of *A. pleuropneumoniae* serotype 1 were generated by using a mini-*Tn10* transposon mutagenesis system. Here, we performed a structural analysis of the oligosaccharide region of three core LPS mutants (5.1, CG1 and CG3) that still produce the same O-antigen using methylation analyses and mass spectrometry. In order to evaluate the role of LPS in the interaction of *A. pleuropneumoniae* with the innate immune system, we performed a kinetic study of proinflammatory cytokines production such as interleukin-6 (IL-6), tumor necrosis factor (TNF-α), interleukin-1 beta (IL-1β), monocyte chemoattractant protein (MCP-1) and interleukin-8 (IL-8) by porcine alveolar macrophages (PAMs), and by two immortalized epithelial cell lines, namely the Newborn Pig Trachea (NPTr) and the St. Jude Porcine Lung (SJPL) cell lines. Upon stimulation of the cells with purified LPS or heat-killed bacteria, our results showed that the rough LPS and core LPS mutants had the same ability to stimulate the production of cytokines. However, although stimulated PAMs produced all the cytokines tested, no cytokine production was detected with the SJPL cells, and only low amounts of IL-8 was produced by the NPTr cells. Characterization of the intracellular pathways involved in the stimulation of inflammatory processes revealed that the SJPL cells lack the NF-κB p65 subunit, which is necessary for NF-κB pathway activation. Moreover, stimulation of NPTr cells pre-treated with an IRAK 1/4 inhibitor suggested that NF-κB activation occurs through a pathway independent of Toll-like receptor, minimizing the role of LPS in NF-κB-induced IL-8 production in these cells. Investigation of the involvement of LPS in the resistance of *A. pleuropneumoniae* serotype 1 to antimicrobial peptides showed that the three core LPS mutants were more susceptible to cationic peptides than both the rough LPS mutant (27.1) and the wild type parent strain. Furthermore, experimental pig infections with these mutants revealed that the galactose (Gal I) and DD-heptose (Hep IV) residues present in the outer core of *A. pleuropneumoniae* serotype 1 LPS are important for adhesion and overall virulence in the natural host. Since the core LPS

mutant 5.1 was found to be highly attenuated and caused significantly less lung lesions, we evaluated its hemolytic and cytotoxic activities. While mutant 5.1 exhibited wild type hemolytic activity, a significant decrease was observed in its cytotoxicity to PAMs. The fact that similar expression and secretion of both ApxI and II toxins was observed in this mutant suggested that the LPS core truncation could affect the toxins in their functional properties. Here we demonstrate, for the first time, a physical interaction between the core OS of LPS and the toxins ApxI and ApxII, upon ELISA and surface plasmon resonance binding assays. Our results indicate that the GalNAc-Gal II-Gal I domain of the outer core, a region missing in mutant 5.1, is critical to LPS/Apx interactions and the overall cytotoxicity of *A. pleuropneumoniae* serotype 1. In conclusion, this project demonstrates the crucial role of the LPS core OS in the pathogenesis of *A. pleuropneumoniae*, but also suggests potential involvements of other components of the bacterium in the stimulation of the host innate immune system.

Keywords: *Actinobacillus pleuropneumoniae*, lipopolysaccharide, core oligosaccharide, cytokines, NF-κB, antimicrobial peptides and Apx toxins.

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LISTE DES SIGLES ET DES ABRÉVIATIONS

AA	Alditol Acetates
ABC	ATP Binding Cassette
ACP	Acyl Carrier Protein
ADP	Adénosine DiPhosphate
Afu	Actinobacillus ferric uptake
AMP	AntiMicrobial Peptides
ADN ou DNA	Acide DésoxyriboNucléique
ANEX	ANionic EXtract
ANOVA	ANalysis Of VAriance
AP-1	Activator Protein-1
Apf	*Actinobacillus pleuropneumoniae* fimbriae
ARN ou RNA	Acide RiboNucléique
ATP	Adénosine TriPhosphate
BALF	BronchoAlveolar Lavage Fluid
BALT	BronchoAlveolar Lymphoid Tissue
BCA	BiCinchoninic Acid
BCAA	Branched-Chain Amino Acids
BHI	Brain Heart Infusion
Ca^{2+}	Calcium
CAMP	Christie, Atkin, Munch-Peterson factor
CAPE	Caffeic Acid Phenethyl Ester
CD	Circular Dichroism
CE-ESIMS	Capillary Electrophoresis-Electrospray Ionization Mass Spectrometry
CE-MS	Capillary Electrophoresis-Mass Spectrometry
CFS	Cell-Free culture Supernatant
CFU	Colony Forming Unit
CO-IP	CO-ImmunoPrécipitation

cps	capsular polysaccharide synthesis
cpx	capsular polysaccharide export
Cu	Cuivre
CyaA	Adenylate Cyclase
DIVA	Differentiating Infected from Vaccinated Animals
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	DiMethyl SulfOxide
DPBS	Dulbecco's Phosphate-Buffered Saline
EHEC	EnteroHemorragic *E. coli*
ELISA	Enzyme-Linked ImmunoSorbent Assay
EMSA	Electrophoretic Mobility Shift Assay
FBS	Foetal Bovine Serum
FDR	False Discovery Rate
Fhu	Ferric hydroxamate uptake
Fur	Ferric uptake regulator
Gal	Galactose
GalNac	N-acétyl galactosamine
GAPDH	GlycérAldehyde-3-Phosphate DésHydrogénase
GC-MS	Gas Chromatography-Mass Spectrometry
Glc	Glucose
GlcNac	N-acétyl glucosamine
gmh	glycérol-manno-heptose
Hep	Heptose
Hex	Hexose
HexNac	N-acétyl hexosamine
HgbA	Hemoglobin binding protein A
HlyA	Hemolysin Alpha
HNP	Human Neutrophil Peptide
Hsf	*Haemophilus* surface fibrils
Ig	Immunoglobulin
IL	Interleukine

IRAK-1	Interleukin-1 Receptor-Associated Kinase 1
Kb	kilobases
Kdo	2-keto-3-deoxyoctulosonic acid
IVET	*In Vivo* Expression Technology
LAL	Limulus Amebocyte Lysate
LBP	LPS Binding Protein
LC-MS	Liquid Chromatography-Mass Spectrometry
LDH	Lactate DésHydrogénase
LFA-1	Lymphocyte Function-associated Antigen-1
Lkt	Leukotoxin
LOS	LipoOligoSaccharides
LPS	LipoPolySaccharides
Lrp	Leucine-responsive regulatory protein
LT	Heat-Labile Toxin
Man	Mannose
ManNac	N-acétyl mannosamine
MEM	Minimal Essential Medium
MIC	Minimum Inhibitory Concentration
Mn	Manganèse
MOI	Multiplicity Of Infection
MRE	Mean Residual Ellipticities
MS	Mass Spectrometry
MyD88	Myeloid Differentiation factor 88
NAD	Nicotinamide Adénine Dinucléotide
NADPH	Nicotinamide Adénine Dinucléotide Phosphate
Nal	Nalidixic acid
NFκB	Nuclear Factor Kappa B
NMR	Nuclear Magnetic Resonance
NPTr	Newborn Pig Trachea
NTP	Nucleotide TriPhosphate
OD	Optical Density

ODN	CpG OligoDiNucleotide
OmlA	Outer membrane lipoprotein A
OMP	Outer Membrane Protein
OMV	Outer Membrane Vesicles
ONPG	Ortho-NitroPhenyl-β-Galactoside
ORF	Open Reading Frame
OS	OligoSaccharide
P	Phosphate
PalA	Peptidoglycan-associated lipoprotein A
PAM	Porcine Alveolar Macrophages
pBD	porcine β-Defensin
PBS-T	PBS-Tween
PCR	Polymerase Chain Reaction
PEA	PhosphorylÉthanolAmine
PGA	Poly-β-1,6-N-acétyl-D-glucosamine
PMAA	Partially Methylated Alditol Acetates
PMAP	Porcine Myeloid Antimicrobial Peptide
PMN	PolyMorphoNucléaires
PPLO	PleuroPneumonia-Like Organisms
RBC	Red Blood Cells
Rha	Rhamnose
RT	Room Temperature
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RTX	Repeat in Toxins
SCOTS	Selective Capture of Transcribed Sequences
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis
SELDI	Surface Enhanced Laser Desorption Ionisation
Sfu	*Salmonella* ferric uptake
sIgA	secretory Immunoglobulin A

SJPL	St. Jude Porcine Lung
SOD	SuperOxide Dismutase
SPR	Surface Plasmon Resonance
STM	Signature Tagged Mutagenesis
T1SS	Type 1 Secretion System
Tbp	Transferrin binding protein
TBS	Tris-Buffered Saline
TCA	TriChlorAcetic acid
TDs	Transactivation Domains
TLR	Toll-Like Receptor
TMB	3, 3`, 5, 5`-TetraMethylBenzidine
TNF	Tumor Necrosis Factor
TRAF-6	Tumor Necrosis Factor - Receptor-Associated Factor 6
UDP	Uridine DiPhosphate
Und-P	Undecaprenyl-Phosphate
Und-PP	Undecaprenyl-PyroPhosphate
ure	uréase
UTP	Uridine TriPhosphate
VTA	Vaccine-Targeting Adjuvants
WT	Wild Type
Yfu	*Yersinia* ferric uptake
Zn	Zinc

À mon père
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INTRODUCTION

La paroi bactérienne constitue l'interface entre le micro-organisme et son environnement. Elle est composée de macromolécules impliquées dans de nombreux mécanismes de virulence et notamment dans la colonisation. En effet, l'attachement des bactéries aux cellules de l'hôte est dû à la présence de molécules bactériennes, appelées adhésines. *A. pleuropneumoniae* est un pathogène des voies respiratoires porcines, responsable de la pleuropneumonie porcine (Straw, 2006). Parmi les principaux facteurs de virulence, tels que la capsule (Ward and Inzana, 1994, Ward *et al.*, 1998, Rioux *et al.*, 2000), les systèmes d'acquisition de fer (Haesebrouck *et al.*, 1997, Jacques, 2004, Bosse *et al.*, 2002) et les exotoxines Apx (Frey, 1995), le LPS est connu comme l'adhésine majeure de cette bactérie (Bélanger *et al.*, 1990). Les LPS sont des molécules présentes au niveau de la membrane externe des bactéries Gram négatives et sont généralement constitués de 3 parties : le lipide A permettant l'ancrage de la molécule dans la membrane externe, le noyau oligosaccharidique (OS) constitué du noyau interne et du noyau externe et l'antigène O composé de plusieurs unités répétées de sucres. Ainsi, il a été démontré que le noyau OS était la partie du LPS responsable des propriétés d'adhérence d'*A. pleuropneumoniae* (Rioux *et al.*, 1999, Galarneau *et al.*, 2000). De par leur localisation et leur accessibilité à la surface, les LPS interviennent non seulement dans la colonisation mais interagissent aussi avec le système immunitaire inné. En effet, la région du lipide A constituant la partie active du LPS, aussi appelé endotoxine, est impliquée dans la stimulation de l'immunité innée et contribue à l'inflammation (Akira *et al.*, 2006). D'autre part le LPS, chargé négativement, représente la cible des peptides antimicrobiens cationiques, autres composants de l'immunité innée, à la surface de la membrane (Brogden, 2005). Il a ainsi été démontré que la susceptibilité d'une bactérie à ces peptides dépendait essentiellement de la composition en sucres, en acides gras et en charges de ses LPS (Loutet *et al.*, 2006, Guo *et al.*, 1998, Raetz *et al.*, 2007). Au-delà de son interaction avec les cellules et composants de l'hôte, le LPS peut aussi contribuer à la pathogenèse de la bactérie en interagissant avec d'autres composants bactériens, tels que les protéines de la membrane externe (Ferguson *et al.*, 2000) ou encore les exotoxines. Les toxines Apx (ApxI à IV) sont des toxines hémolytiques et/ou cytolytiques sécrétées par *A. pleuropneumoniae* qui sont en grande partie responsable des lésions pulmonaires observées lors de la pleuropneumonie porcine (Choi *et al.*, 2001). Ces toxines appartiennent à la famille des toxines RTX et agissent principalement par lyse

osmotique en formant des pores dans la membrane plasmique des cellules cibles (Schmidt *et al.*, 1996, Maier *et al.*, 1996, Karakelian *et al.*, 1998). Il a été démontré que le LPS agissait en synergie avec les toxines RTX pour augmenter leur activité cytotoxique (Herlax *et al.*, 2005, Bauer and Welch, 1997). Le LPS se retrouve donc au centre de nombreux mécanismes de virulence qui pourraient permettre à *A. pleuropneumoniae* d'assurer son maintien chez l'hôte et d'exercer son pouvoir pathogène.

REVUE DE LITTÉRATURE

1. *Actinobacillus pleuropneumoniae*

1.1. Historique

Actinobacilllus pleuropneumoniae est l'agent étiologique de la pleuropneumonie porcine, une maladie responsable d'importantes pertes économiques dans l'industrie porcine (Rycroft & Garside, 2000; Straw, 2006). Cette bactérie appartient à la famille des *Pasteurellaceae* qui comprend les genres *Actinobacillus*, *Haemophilus*, *Pasteurella* et *Mannheimia*. En 1957, Pattison et coll. isolent, à partir de lésions de pleuropneumonie porcine, une bactérie exigeante en NAD (nicotinamide-adénine-dinucléotide) qui sera dénommée « *Haemophilus*-like » avant d'être inscrite sur la liste officielle des dénominations bactériennes sous le nom de *Haemophilus pleuropneumoniae* (Shope, 1964; White *et al.*, 1964). En 1978, Bertschinger et Seifert isolent de cas de pleuropneumonie une bactérie non exigeante en NAD ressemblant à *Mannheimia (Pasteurella) haemolytica* et appelée « *Pasteurella haemolytica*-like ». Ces deux bactéries présentent des analogies phénotypiques et, par hybridation ADN-ADN, Pohl *et al.* (1983) montrent que ces bactéries sont fortement apparentées à *Actinobacillus lignieresii* et plus éloignées de l'espèce type du genre *Haemophilus* (*Haemophilus influenzae*). Ils proposent donc de créer un nouveau taxon, *Actinobacillus pleuropneumoniae*, comprenant 2 biotypes : le biotype 1 pour les souches NAD-dépendantes et le biotype 2 pour les souches NAD-indépendantes qui requièrent cependant la présence de certains précurseurs du NAD (Niven & Lévesque, 1988).

1.2. Caractéristiques phénotypiques

A. pleuropneumoniae est un coccobacille Gram négatif non sporulé, non mobile, capsulé, se présentant de manière isolée, en paires ou en courtes chaînes. La bactérie est anaérobie facultative et son métabolisme est de type fermentatif. La caractérisation bactériologique de la bactérie montre qu'elle est positive pour l'oxydase, l'uréase, l'ONPG, la réduction des nitrates et des nitrites, la phosphatase alcaline ainsi que pour l'acidification du fructose, du mannose, du saccharose et du xylose. Cependant, des variants uréase-

négatifs ont exceptionnellement été isolés. Sur gélose au sang de mouton, on note une étroite zone d'hémolyse, complète pour le biotype 1 et plus faible pour le biotype 2. La taille de la zone d'hémolyse est augmentée par l'hémolysine β des staphylocoques (ou réaction de CAMP: Christie, Atkin, Munch-Peterson) (Kilian, 1976).

1.3. Biotypes et sérotypes d'*Actinobacillus pleuropneumoniae*

Comme il a été mentionné ci-dessus, les souches d'*A. pleuropneumoniae* sont réparties dans 2 biotypes. Les bactéries appartenant au biotype 1 sont NAD-dépendantes alors que celles du biotype 2 sont NAD-indépendantes. À ce jour, 13 sérotypes du biotype 1 (1 à 12 et 15) et 2 sérotypes du biotype 2 (13 et 14) ont été identifiés chez *A. pleuropneumoniae* (Blackall *et al.*, 2002; Schaller *et al.*, 2001), basés sur la structure des polysaccharides capsulaires et des lipopolysaccharides (LPS) (Perry *et al.*, 1990). Cependant, la classification des sérotypes dans les biotypes n'est pas toujours évidente car quelques sérotypes peuvent se retrouver dans les 2 biotypes (Gottschalk, 2007). Ainsi, certaines souches des sérotypes 2, 4, 7 et 9 peuvent aussi appartenir au biotype 2 et sont retrouvées uniquement en Europe (Gottschalk & Taylor, 2005). Des souches du sérotype 13 appartenant au biotype 1 ont aussi été isolées en Amérique du Nord (Tableau 1). Quelques sérotypes montrent une similarité au niveau de l'antigène O de leur LPS, ce qui expliquerait les réactions croisées observées entre les sérotypes 1, 9 et 11 (Mittal, 1990; Mittal *et al.*, 1993), les sérotypes 3, 6 et 8 (Mittal *et al.*, 1992) et les sérotypes 4 et 7 (Mittal & Bourdon, 1991). Les sérotypes 1 et 5 sont subdivisés respectivement en 1a et 1b (Jolie *et al.*, 1994) et 5a et 5b (Nielsen, 1986), selon de petites différences au niveau des antigènes capsulaires. Tous les sérotypes sont potentiellement capables de causer la maladie mais on retrouve quelques sérotypes prédominants qui sont répartis selon leur localisation géographique. Ainsi, les sérotypes 1, 5 et 7 sont prévalents en Amérique du Nord tandis que le sérotype 2 est le plus souvent retrouvé en Europe et au Japon (Dubreuil *et al.*, 2000). Le sérotype 15 récemment identifié est lui très présent en Australie (Blackall *et al.*, 2002) mais a aussi été identifié en Amérique du Nord et au Mexique (Tableau 1).

Tableau I. Sérotypes d'*A. pleuropneumoniae* et leur prévalence en Amérique du Nord. Adapté de Gottschalk, 2007.

Sérotype	Biotype	Présence en Amérique du Nord	Virulence [a]
1	I	Oui	Forte
2	I et II [b]	Oui [c]	Faible
3/6/8	I	Oui	Variable
4	I et II [b]	Oui [c]	Faible
5a	I	Oui	Forte
5b	I	Oui	Forte
7	I et II [b]	Oui [c]	Forte/Variable
9	I et II [b]	Non	-
10	I	Oui	Faible
11	I	Non	-
12	I	Oui	Variable
13	I et II [d,e]	Oui [e]	???
14	II	Non	-
15	I	Oui [e]	Variable
Non sérotypable	II	Oui [e]	Forte [f]

[a] Cette classification est basée sur les souches canadiennes et américaines et peut être significativement différente dans d'autres pays

[b] les souches des Biotypes I et II diffèrent au niveau de la virulence et de la production de toxine

[c] Uniquement les souches du Biotype I ont été isolées en Amérique du nord

[d] Uniquement les souches du Biotype II ont été isolées en Amérique du nord

[e] Observations non publiées

[f] Un seul troupeau affecté à ce jour

1.4. Transmission et pathogenèse de l'infection

A. pleuropneumoniae est une bactérie qui infecte exclusivement l'appareil respiratoire du porc et qui n'a pas été isolée chez d'autres espèces telles que l'homme, les oiseaux ou les rongeurs. La transmission de la maladie se fait par contact direct ou par transmission aérosol et survient dans la majorité des cas via l'arrivée d'un porteur asymptomatique (Straw, 2006). La transmission par voie indirecte joue un rôle important lorsque la maladie évolue selon un mode aigu ou suraigu car la bactérie est excrétée massivement dans les

sécrétions nasales qui peuvent contenir jusqu'à 10^9 UFC/ml. La progression de la maladie est influencée par plusieurs facteurs comme la virulence de la souche, la densité de l'inoculum, les conditions d'élevage (température, humidité, ventilation défectueuse et surpeuplement) et le statut immunitaire des animaux. Après une durée d'incubation variant de quelques heures à quelques jours, la maladie évolue sous 3 formes cliniques principales, soient les formes suraiguë, aiguë et chronique :

La forme suraiguë est la forme la plus grave et la mort survient généralement entre 24 et 36 heures. Des cas de mort subite sans symptôme préalable sont aussi régulièrement observés. Cette forme de pleuropneumonie est surtout retrouvée chez les porcs n'ayant aucune immunité acquise contre le micro-organisme. Elle débute par des signes généraux graves (ex : hyperthermie de l'ordre de 41 à 42°C, abattement et anorexie) et éventuellement par de la diarrhée et des vomissements de courte durée. Les animaux malades se couchent sur le sol, la peau est cyanosée dû à un choc circulatoire et en phase terminale, on note une dyspnée sévère. L'animal cherche parfois à compenser cette dyspnée en respirant par la bouche en position assise.

La forme aiguë présente une évolution moins rapide et peut mener à une forme chronique. Les animaux malades présentent une fièvre (40,5 à 41°C), un abattement, une anorexie ainsi qu'un syndrome respiratoire grave avec dyspnée et toux. L'évolution clinique est très variable d'un animal à un autre et les porcs qui survivent deviennent des porteurs asymptomatiques qui excrètent les germes restés présents au niveau des lésions pulmonaires, des amygdales et des cavités nasales.

La forme chronique se développe généralement après les signes cliniques de la phase aiguë. On note des retards de croissance, la présence ou non de fièvre et une toux sporadique. La bactérie est présente dans des lésions pulmonaires nécrotiques et peut envahir les voies respiratoires et les amygdales. Une coinfection bactérienne ou virale peut aggraver l'importance de la maladie.

Les lésions causées par la pleuropneumonie porcine sont principalement localisées à l'appareil respiratoire. Ainsi, des lésions pulmonaires bilatérales de pleurésie sérofibrineuse et de pneumonie nécrotique et fibrinohémorragique, sous forme de foyers bien délimités, sont observées au niveau des lobes cardiaques, apicaux et au moins partiellement sur les lobes diaphragmatiques. Au début de la maladie on observe des changements histopathologiques tels que la nécrose, l'hémorragie, une infiltration de neutrophiles, l'activation des macrophages et des plaquettes, une thrombose vasculaire et un oedème. De plus, la trachée et les bronches sont remplies d'un exsudat mousseux et sanguinolent (Bertram, 1985, 1986, 1988; Liggett *et al.*, 1987). Dans les formes chroniques, des nodules entourés d'une épaisse capsule de tissu conjonctif et des foyers de nécrose sont visibles notamment sur les lobes diaphragmatiques. Les lésions nécrotiques peuvent héberger des bactéries durant plusieurs mois et les animaux peuvent transmettre l'infection. Des lésions de pleurésie fibrineuse se développent et, lors de la guérison, elles sont souvent les seules lésions visibles à l'abattoir, si bien que la mise en évidence d'un pourcentage de lésions de pleurésie chronique à l'abattage permet de suspecter une infection de l'élevage par *A. pleuropneumoniae*.

1.5. Détection et traitement

Des tests sérologiques comme le test de la fixation du complément et l'ELISA sont généralement utilisés pour le titrage d'anticorps dirigés contre *A. pleuropneumoniae* chez les porcs infectés (Sidibe *et al.*, 1993). Cependant, la détection sérologique est limitée par le fait qu'elle donne une mesure indirecte de l'infection qui n'est pas forcément représentative. Cette méthode de détection est également confrontée aux problèmes de réactions croisées entre sérotypes (Perry *et al.*, 1990; Straw, 2006). La confirmation de l'infection par la culture en milieu sélectif (Jacobsen & Nielsen, 1995) de prélèvements au niveau des amygdales ou des cavités nasales est souvent nécessaire. Le sérotypage des souches isolées se fait généralement grâce à des tests de coagglutination (Mittal *et al.*, 1983a; Mittal *et al.*, 1983b; Sidibe *et al.*, 1993). Cependant, les amygdales et les cavités nasales sont des sites très colonisés par d'autres bactéries qui rendent difficiles l'isolement d'*A. pleuropneumoniae*. D'autres techniques comme l'utilisation de billes de polystyrène

superparamagnétiques (billes immunomagnétiques) couplées à des anticorps dirigés contre *A. pleuropneumoniae* (Gagné *et al.*, 1998), ont ainsi été développées pour optimiser l'isolement de la bactérie par rapport à la culture conventionnelle en milieu sélectif. Des techniques de PCR basées sur la détection de gènes codant pour des protéines d'*A. pleuropneumoniae* ont aussi été développées (Fittipaldi *et al.*, 2003; Gram & Ahrens, 1998; Savoye *et al.*, 2000; Zhou *et al.*, 2008) et ont notamment l'avantage de permettre la détection d'*A. pleuropneumoniae* dans des cultures bactériennes mixtes.

L'infection à *A. pleuropneumoniae* peut être traitée par des antibiotiques, les plus actifs étant les quinolones (danofloxacine et enrofloxacine) et les céphalosporines (ceftiofur) (Hart *et al.*, 2006; Wallgren *et al.*, 1999a, 1999b). Cependant, on observe une émergence accrue de résistance acquise à de nombreux antibiotiques chez *A. pleuropneumoniae*. Ainsi, des résistances aux sulfonamides (sulfaméthoxazole), aux tétracyclines, à la tiamuline, à la tilmicosine, aux macrolides (érythromycine) et aux β-lactames (pénicilline et ampicilline) (Matter *et al.*, 2007; Wasteson *et al.*, 1996) ont été observées. D'autre part, il a été récemment démontré que les polymères de poly-N-acetylglucosamine codés par les gènes *pga* et qui interviennent dans la formation de biofilm, augmentaient la résistance d'*A. pleuropneumoniae* aux antibiotiques (Izano *et al.*, 2007). Le traitement aux antibiotiques est également limité par le fait qu'il permet de réduire la mortalité et de limiter la perte de poids, mais ne supprime pas totalement la présence de la bactérie (Hart *et al.*, 2006).

1.6. La vaccination contre *A. pleuropneumoniae*

À ce jour, il n'existe pas de traitement totalement efficace contre l'infection à *A. pleuropneumoniae* et les techniques de prévention restent essentielles pour diminuer la prévalence de la maladie. Au-delà de la prophylaxie sanitaire qui est nécessaire pour minimiser la transmission de l'infection au sein de l'élevage (Straw, 2006), la vaccination reste la meilleure technique de prévention. Ainsi, de nombreux travaux ont été consacrés à la vaccination contre *A. pleuropneumoniae* et les récentes études offrent de nouvelles perspectives quant à la découverte d'un vaccin efficace. Ces études ont fait l'objet d'une revue récente par notre laboratoire (annexe 1).

Les premiers vaccins commercialisés dits de « première génération » sont constitués de bactéries entières tuées ou inactivées par traitement chimique ou par irradiation. Également appelés bactérines, ces premiers vaccins ont été élaborés dans le souci de présenter un maximum d'antigène de surface au système immunitaire de l'hôte tout en éliminant les problèmes de réversion observés chez les vaccins vivants. Cependant, ces bactérines n'ont pas montré une grande efficacité car elles sont confrontées à plusieurs problèmes tels que l'altération des antigènes de surface lors de l'inactivation ainsi que l'incapacité d'offrir une protection contre tous les sérotypes. En effet, l'efficacité vaccinale des bactéries tuées est limitée par le fait qu'elles ne sécrètent plus de protéines immunogènes telles que les toxines Apx (voir section 2.3) et qu'elles ne peuvent plus exprimer certains antigènes communs à tous les sérotypes qui sont exprimés uniquement *in vivo*. De ce fait, les vaccins vivants atténués et les vaccins sous-unitaires représentent les avenues de recherche les plus prometteuses dans la vaccination contre *A. pleuropneumoniae*.

Pour l'élaboration des vaccins sous-unitaires dits de « deuxième génération », l'imunogénicité et le pouvoir protecteur de plusieurs antigènes de surface d'*A. pleuropneumoniae* ont été évalués. Les études précédentes ont montré que la capsule et les LPS n'étaient pas de très bons candidats car ils n'offraient pas de protection croisée due à leur trop grande hétérogénicité entre les sérotypes. Les travaux se sont donc penchés sur les protéines ou lipoprotéines de la membrane externe qui sont relativement bien conservées entre les sérotypes. Ainsi, les lipoprotéines OmlA et PalA ainsi que des protéines de la membrane externe impliquées dans l'acquisition de fer telles que les « transferrin binding proteins » (Tbp) ont montré une protection partielle. Certaines de ces protéines ont même été incluses dans des vaccins sous-unitaires actuellement commercialisés (ex : Pleurostar™, Novartis). Au-delà des antigènes membranaires, il a été démontré que les toxines Apx sécrétées par *A. pleuropneumoniae* étaient très immunogènes et jouaient un rôle primordial dans la protection, si bien qu'elles se retrouvent, sous la forme de toxoïdes, dans la plupart des vaccins sous-unitaires commercialisés à ce jour.

Malgré les problèmes de réversion associés à l'utilisation de souches vivantes en vaccinologie, les vaccins vivants atténués représentent l'une des meilleures approches dans la recherche d'un vaccin contre *A. pleuropneumoniae*. En effet, il a été démontré que les porcs qui survivaient à l'infection étaient partiellement protégés contre les infections

croisées par d'autres sérotypes. Ceci suggère que les bactéries vivantes sont les seules à offrir une protection croisée via l'expression d'antigènes protecteurs *in vivo*. Plusieurs mutants atténués d'*A. pleuropneumoniae* dans des gènes impliqués dans le métabolisme ou encore les gènes codant pour les toxines Apx ont ainsi été développés. Cependant aucun vaccin vivant contre *A. pleuropneumoniae* n'a été commercialisé à ce jour. L'utilisation des vaccins vivants pose un autre problème qui est celui de la différentiation entre les porcs infectés et les porcs vaccinés. En effet, la plupart des vaccins vivants attenués ne sont pas affectés au niveau de leurs antigènes de surface ou de leurs facteurs immunogènes, qui sont essentiels pour une stimulation efficace du système immunitaire. Ces mutants deviennent donc sérologiquement indifférentiables des souches virulentes. Une nouvelle avancée en matière de vaccin vivant, la technologie DIVA (Differentiating Infected from Vaccinated Animals), permet de faire cette différentiation en mutant un gène codant pour un facteur immunogène exprimé chez tous les sérotypes mais pas essentiel pour la protection. Ce facteur absent chez les vaccins atténués constituerait alors un marqueur négatif permettant une discrimination sérologique entre les porcs infectés et les porcs vaccinés.

Les stratégies de vaccination telles que l'utilisation d'adjuvants et de formulations ainsi que les voies d'administration du vaccin jouent également un rôle important dans l'efficacité vaccinale.

Malgré les progrès réalisés dans le domaine des vaccins vivants atténués et sous-unitaires, l'absence sur le marché d'un vaccin efficace contre *A. pleuropneumoniae* peut s'expliquer par le fait que les connaissances sont limitées en ce qui concerne les antigènes et leur expression *in vivo*. Ainsi, des outils génétiques tels que l'IVET (*in vivo* expression technology), le SCOTS (selective capture of transcribed sequences), le « microarray » et la STM (signature tagged mutagenesis) représentent les nouvelles avenues de recherche en vaccinologie qui peuvent permettre l'identification de nouveaux antigènes exprimés *in vivo* (IVET, SCOTS, microarray) ou de gènes essentiels à la survie (STM). D'autre part le séquençage complet des génomes d'*A. pleuropneumoniae* sérotypes 3 (Xu *et al.*, 2008) et 5b (Foote *et al.*, 2008) offre de nouvelles perspectives quant à la découverte de nouveaux candidats de vaccins contre *A. pleuropneumoniae*. Ainsi, l'analyse *in silico* du génome d'*A. pleuropneumoniae* sérotype 5b a permis de prédire l'existence de 93 protéines ou lipoprotéines de la membrane externe parmi lesquelles 47 ont été identifiées par des

analyses protéomiques et dont la plupart sont des protéines nouvellement caractérisées (Chung *et al.*, 2007).

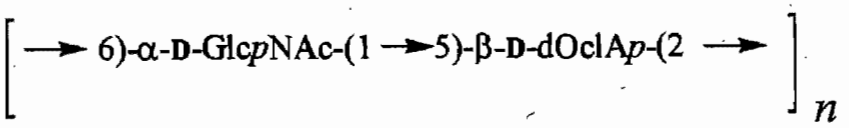
2. FACTEURS DE VIRULENCE

La pathogénicité d'*A. pleuropneumoniae*, comme chez beaucoup d'autres bactéries pathogènes, est multifactorielle. Parmi les principaux facteurs de virulence décrit à ce jour, on retrouve les polysaccharides capsulaires, les protéines et les systèmes d'acquisition de fer, les exotoxines Apx et les lipopolysaccharides (LPS). Dans cette étude nous nous sommes plus particulièrement intéressés aux LPS et aux toxines Apx.

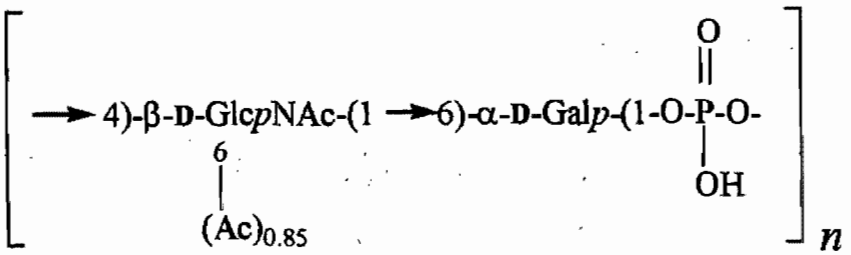
2.1. La capsule

La capsule est constituée de polymères d'unités oligosaccharidiques chargés négativement dû à la présence d'acides carboxyliques ou de phosphates (Perry *et al.*, 1990). La structure de la capsule des sérotypes 1 à 12 d'*A. pleuropneumoniae* (Altman *et al.*, 1992; Beynon *et al.*, 1991a; Beynon *et al.*, 1991b; Beynon *et al.*, 1991c; Beynon *et al.*, 1993; Dubreuil *et al.*, 2000; Perry *et al.*, 1990) et plus récemment celle des sérotypes 13 (MacLean *et al.*, 2004) et 15 (Perry *et al.*, 2005), a été élucidée. Les structures capsulaires produites par *A. pleuropneumoniae* peuvent être divisées en 3 groupes (Figure 1) : les sérotypes 5a, 5b et 10 ont une capsule constituée d'unités oligosaccharidiques répétées alors que celle des sérotypes 1, 4, 12 et 15 est constituée de polymères d'oligosaccharides joints par des liaisons phosphodiesters. Les sérotypes 2, 3, 6, 7, 8, 9, 11 et 13 possèdent une capsule constituée de polymères d'acides téichoïques liés par des liaisons phosphodiesters. Les différences retrouvées au niveau de la composition en sucre des polysaccharides capsulaires sont à l'origine de la classification des souches *d'A. pleuropneumoniae* en sérotypes (Inzana & Mathison, 1987).

SEROTYPE 5a CPS



SEROTYPE 1 CPS



SEROTYPE 2 CPS

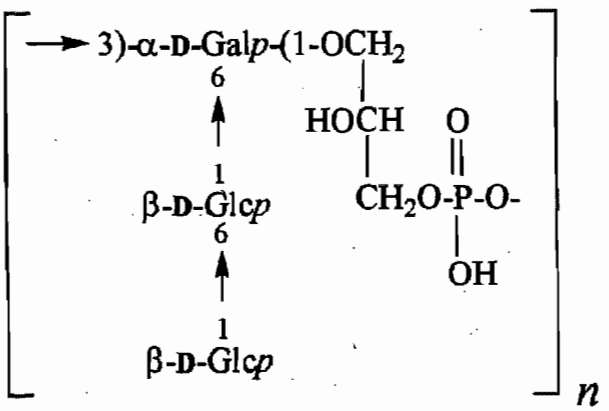


Figure 1. Représentation des 3 types de structure de polysaccharides capsulaires retrouvés chez *A. pleuropneumoniae*. La capsule du sérotype 5a, comme celle des sérotypes 5b et 10, est constituée d'unités oligosaccharidiques répétées liés par des liens glycosidiques. La capsule du sérotype 1, comme celle des sérotypes 1, 4, 12 et 15, est constituée de polymères d'oligosaccharides joints par des liaisons phosphodiesters. La capsule du sérotype 2, comme celle des sérotypes 3, 6, 7, 8, 9, 11 et 13, est constituée de polymères d'acides téichoïques liés par des liaisons phosphodiesters. Adaptée de Perry *et al.*, 1990.

En ce qui concerne la biosynthèse de la capsule, les gènes *cpxABCD* codant pour l'exportation de la capsule ont été à l'origine identifiés chez le sérotype 5a d'*A. pleuropneumoniae* (Ward & Inzana, 1997). Cette étude a ensuite permis l'identification des gènes *cps*, en amont des gènes *cpx*, qui sont impliqués dans la biosynthèse de la capsule chez *A. pleuropneumoniae* (Bandara *et al.*, 2003; Schuchert *et al.*, 2004; Ward *et al.*, 1998). Une étude récente (Jessing *et al.*, 2008) basée sur la comparaison des séquences nucléotidiques et protéiques, a montré une corrélation entre les homologies retrouvées au niveau des gènes de biosynthèse *cps* et le type de structure capsulaire produit par les sérotypes. Ainsi, des homologies de séquences sont retrouvées entre les sérotypes 2, 6, 7 et 8 et entre les sérotypes 1 et 12.

Au niveau fonctionnel, la capsule n'a aucune activité biologique intrinsèque (Fenwick *et al.*, 1986) mais elle joue un rôle important dans la pathogenèse. La capsule a des propriétés antiphagocytaires (Inzana *et al.*, 1988) et est considérée comme étant la barrière de défense principale de la bactérie contre les mécanismes humoraux de l'hôte (Inzana *et al.*, 1988; Rycroft & Cullen, 1990). Ainsi, il a été démontré que des mutants acapsulés du sérotype 5a d' *A. pleuropneumoniae* sont tués par le sérum de porc normal et sont sensibles à l'activité de la voie alterne du système du complément (Ward & Inzana, 1994; Ward *et al.*, 1998). Cependant, des travaux dans notre laboratoire ont montré qu'un mutant acapsulé d'*A. pleuropneumoniae* sérotype 1 (le mutant 33.2) est atténué au niveau de sa virulence mais est résistant au sérum de porc (Rioux *et al.*, 2000). Ceci suggère d'autres fonctions importantes de la capsule dans la pathogenèse d'*A. pleuropneumoniae*.

2.2. Les systèmes d'acquisition de fer

La plupart des bactéries ont besoin de fer pour leur croissance et ont développé des mécanismes d'acquisition de fer en raison du manque de fer libre *in vivo*. En effet, chez l'hôte le fer extracellulaire est en grande partie couplé à des glycoprotéines comme la transferrine ou la lactoferrine alors qu'au niveau intracellulaire il est stocké par des protéines contenant un noyau hème comme l'hémoglobine. Plusieurs mécanismes de captation de fer sont utilisés par *A. pleuropneumoniae* (Jacques, 2004).

A. pleuropneumoniae, comme plusieurs espèces de la famille des *Pasteurellaceae*, peut capter le fer couplé à la transferrine. Le récepteur de la transferrine chez *A. pleuropneumoniae* est constitué de 2 protéines de la membrane externe appelées « transferrin-binding proteins » (Tbp) (Gonzalez *et al.*, 1995) : TbpA (Tbp1 ou TfbB) est une protéine transmembranaire de 110 kDa et TbpB (Tbp2 ou TfbA) est une lipoprotéine de 60 kDa ancrée dans la membrane externe grâce à ses chaînes d'acides gras en N-terminal. Ces deux protéines semblent agir en synergie pour capter le fer lié à la transferrine puis la protéine TbpA permettrait le transport du fer au travers de la membrane externe suivi de sa fixation à une protéine périplasmique (Figure 2). L'énergie nécessaire au transport actif du fer est fournie par le complexe TonB-ExbB-ExbD localisé dans la membrane plasmique et TonB interagirait avec TbpA (Kenney & Cornelissen, 2002) pour permettre le passage du fer à travers la membrane externe grâce à la force proton motrice (Shultis *et al.*, 2006). Chez *A. pleuropneumoniae*, 2 systèmes TonB ont été identifiés : le système TonB1 est codé par les gènes *tonB1-exbB1-exbD1* qui font partie d'un large opéron dans lequel se retrouvent en aval les gènes *tbpBA* (Tonpitak *et al.*, 2000). Le système TonB2 est codé par les gènes *exbB2-exbD2-tonB2* qui ne sont associés à aucun gène d'acquisition de fer et sont organisés dans un ordre différent de celui du locus *tonB1* (Beddek *et al.*, 2004). Il a été démontré que les 2 systèmes étaient nécessaires pour l'acquisition du fer lié à la transferrine porcine. Cependant, seul le système TonB2 serait impliqué dans l'acquisition d'hémine, d'hémoglobine et de ferrichrome (Beddek *et al.*, 2004). Les gènes codant pour les Tbp et les 2 systèmes TonB1 et TonB2 sont surexprimés en condition de restriction en fer (Deslandes *et al.*, 2007), ce qui est certainement dû à une régulation par la protéine Fur (ferric uptake regulator) (Jacobsen *et al.*, 2005). Ces Tbp sont spécifiques pour la transferrine porcine (Ricard *et al.*, 1991), expliquant en partie la spécificité d'hôte d'*A. pleuropneumoniae*. Des études d'infection expérimentales avec des mutants d'*A. pleuropneumoniae* sérotype 7 affectés au niveau des récepteurs de la transferrine ont montré que les protéines TpbA et TpbB étaient des facteurs de virulence importants d'*A. pleuropneumoniae* car elles sont essentielles à la colonisation du tractus respiratoire du porc par la bactérie (Baltes *et al.*, 2002).

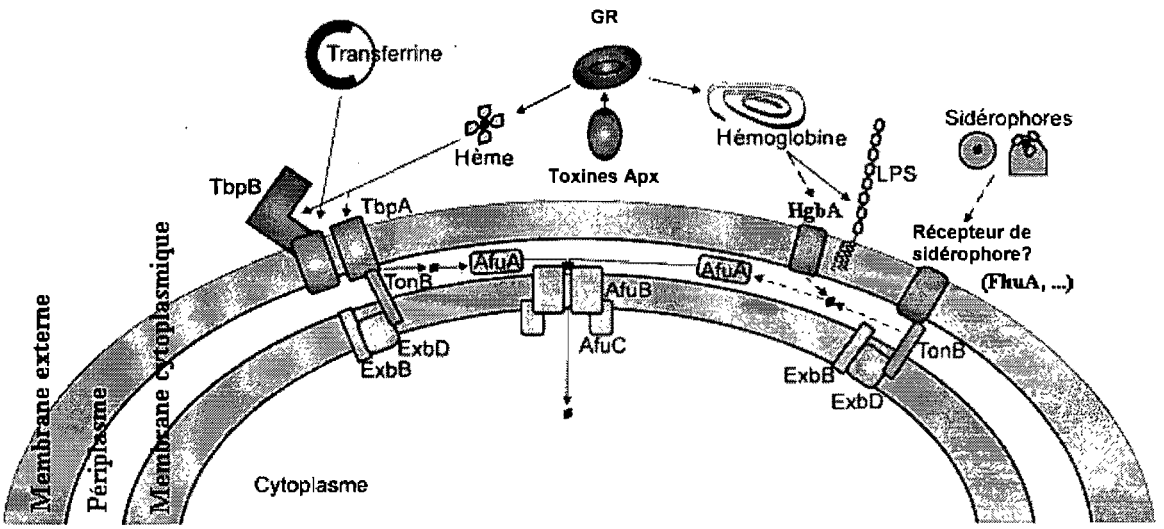


Figure 2. Représentation schématique des différents mécanismes d'acquisition du fer retrouvés chez *A. pleuropneumoniae*. GR : globules rouges. Adaptée de Bossé *et al.*, 2002.

L'acquisition du fer peut également s'effectuer à partir de l'hémoglobine (D'Silva *et al.*, 1995). Ainsi, HgbA est une protéine de la membrane externe de 105 kDa qui est impliquée dans la captation de l'hémoglobine par *A. pleuropneumoniae* (Shakarji *et al.*, 2006; Srikumar *et al.*, 2004) (Figure 2). Il a également été démontré qu'HgbA possédait des sites de fixation pour l'hème (Pawelek & Coulton, 2004) justifiant ainsi l'utilisation de l'hémine par *A. pleuropneumoniae* (Archambault *et al.*, 2003). L'analyse de la séquence en acides aminés a montré la présence d'une boîte Ton suggérant que l'énergie serait fournie par un système TonB-ExbB-ExbD (Srikumar *et al.*, 2004), notamment TonB2 (Beddek *et al.*, 2004). Le gène *hgbA* est présent chez tous les sérotypes *d'A. pleuropneumoniae* (Shakarji *et al.*, 2006; Srikumar *et al.*, 2004) et est surexprimé en condition de restriction en fer (Deslandes *et al.*, 2007). En effet, la présence d'un site de liaison pour le répresseur Fur au niveau du promoteur d'*hgbA* confirme la régulation de son expression par le fer. Des études d'infections expérimentales ont montré qu'un mutant *hgbA* du sérotype 1 était atténué (Shakarji *et al.*, 2006), suggérant ainsi un rôle important d'HgbA dans la virulence d'*A. pleuropneumoniae*. Il a aussi été démontré que le LPS d'*A. pleuropneumoniae* pouvait se

lier à l'hémoglobine grâce à son lipide A (Bélanger *et al.*, 1995). Ceci suggère que le LPS pourrait agir en synergie avec HgbA pour optimiser la captation de fer par la bactérie.

A. pleuropneumoniae peut capter le ferrichrome, un sidérophore de type hydroxamate, via un récepteur FhuA inséré dans la membrane externe (Mikael *et al.*, 2003) (Figure 2). Les sidérophores sont des molécules de faible poids moléculaire (< 1000 Da) qui ont une très forte affinité pour le fer ferrique et peuvent ainsi extraire le fer complexé aux molécules de l'hôte comme la transferrine et la lactoferrine. Certaines bactéries peuvent synthétiser des sidérophores alors que d'autres captent des sidérophores exogènes produits par d'autres bactéries ou des champignons. On distingue 2 classes de sidérophore selon le groupement chimique impliqué dans la liaison avec le fer : les hydroxamates (ex : le ferrichrome) et les catéchols (ex : entérobactine). Il a été démontré que les sérotypes 1 et 5 d'*A. pleuropneumoniae* étaient capables de produire un chélateur de fer qui ne correspond cependant ni à un catéchol, ni à un hydroxamate (Diarra *et al.*, 1996). Par contre, *A. pleuropneumoniae* peut utiliser le ferrichrome exogène via un mécanisme codé par l'opéron *fhuCDBA* (Mikael *et al.*, 2002; Shakarji *et al.*, 2006) qui contrairement à d'autres systèmes d'acquisition de fer, n'est pas régulé par le fer (Mikael *et al.*, 2003). FhuA (75 kDa) est le récepteur et le transporteur du ferrichrome à travers la membrane externe, FhuD (35.6 kDa) est une protéine chaperonne qui prend en charge le ferrichrome dans le périplasme et FhuC (28.5 kDa) et FhuB (69.4 kDa) constituent un ABC transporteur permettant l'internalisation du ferrichrome. L'énergie nécessaire au passage du Fe^{3+}-ferrichrome à travers la membrane externe d'*A. pleuropneumonaie* serait fournie par le complexe TonB2-ExbB2-ExbD2 (Beddek *et al.*, 2004) via une interaction entre FhuA et TonB2 (Pawelek *et al.*, 2006). Cependant, la captation de Fe^{3+}-ferrichrome par FhuA ne semble pas être le principal mécanisme d'acquisition de fer utilisé par *A. pleuropneumoniae* car les mutants *fhuA* n'ont pas montré d'atténuation dans un modèle d'infection expérimentale (Shakarji *et al.*, 2006).

Le fer dans le périplasme sera ensuite pris en charge par le système AfuABC (Actinobacillus ferric uptake) impliqué dans le transport des ions ferriques périplasmiques vers le cytoplasme (Figure 2). Les protéines AfuABC sont homologues à des systèmes retrouvés chez d'autres bactéries tels que SfuABC de *Serratia marcescens*, HitABC de *Haemophilia influenzae*, FbpABC de *Neisseria gonorrhoeae* et YfuABC de *Yersinia enterocolitica*. AfuA est une protéine périplasmique fixant le fer, AfuB est une perméase

insérée dans la membrane cytoplasmique et AfuC est un ABC transporteur permettant le transport actif du fer dans le cytoplasme (Chin *et al.*, 1996).

2.3. Les exotoxines Apx d'*A. pleuropneumoniae*

Les toxines Apx font partie de la famille des toxines RTX possédant des propriétés structurales et fonctionnelles communes. Ces toxines se caractérisent essentiellement par la présence d'une série de répétitions (8 à 40) d'une séquence consensus nonamérique riche en glycine (GGXGXDXUX), justifiant ainsi l'appellation RTX (repeat in toxin). À ce jour, 4 toxines Apx (ApxI à IV) ont été identifiées et jouent un rôle plus ou moins important dans la pathogenèse d'*A. pleuropneumoniae* (Frey *et al.*, 1993; Schaller *et al.*, 1999).

2.3.1. Généralités sur les toxines RTX

Les toxines RTX sont secrétées par un grand nombre de bactéries Gram négatives incluant *Escherichia coli*, des membres de la famille des *Pasteurellaceae* (*Mannheimia*, *Pasteurella* et *Actinobacillus*), *Bordetella pertussis* et *Vibrio cholerae* (Tableau 2). Ces toxines sont hémolytiques et/ou cytolytiques et agissent principalement par lyse osmotique en formant des pores dans la membrane plasmique des cellules cibles (Karakelian *et al.*, 1998; Maier *et al.*, 1996; Schmidt *et al.*, 1996). Selon leur spécificité cellulaire, les toxines RTX sont divisées en 2 catégories : les hémolysines (ex : HlyA de *E. coli* et ApxI) actives sur un large spectre de cellules et les leucotoxines (ex : LktA de *M. haemolytica* et LtxA de *A. actinomycetemcomitans*) qui sont plus spécifiques aux leucocytes (Lally *et al.*, 1999). Au-delà de la formation de pores, d'autres fonctions telles que l'apoptose (Kelk *et al.*, 2003) ou l'activation de processus inflammatoires (Grimminger *et al.*, 1991; Maheswaran *et al.*, 1993; Uhlen *et al.*, 2000) ont aussi été attribuées aux toxines RTX.

L'hémolysine alpha d'*E. coli* (HlyA) est considérée comme le prototype de la famille des toxines RTX dans la mesure où elle a fait l'objet de nombreuses études et a permis par analogie l'étude d'autres toxines RTX. La grande majorité des toxines RTX, comme HlyA, est codée par un seul opéron regroupant 4 gènes contigus *rtxCABD*. *rtxA* code pour la protoxine qui subit une maturation post-traductionnelle correspondant à une ou deux

Tableau II. Caractéristiques des toxines RTX chez différentes espèces bactériennes. Adapté de Ludwig & Goebel, 2006.

Bactéries	Toxine RTX	AH[a]	AC[a]	Taille (kDa)	Opéron
Escherichia coli	α-Hemolysin (HlyA)	+	+	110	>*hlyCABD*
	EHEC-Hemolysin (EHEC-HlyA)	+	+	107	>*hlyCABD*
Proteus vulgaris	Hemolysin (HlyA)	+	nd	110	>*hlyCABD*
Morganella morganii	Hemolysin (HlyA)	+	nd	110	>*hlyCABD*
Mannheimia haemolytica	Leukotoxin (LktA)	(+)	+	102	>*lktCABD*
Mannheimia varigena	Leukotoxin (PlLktA)	nd	+	102	>*pllktCABD*
Pasteurella aerogenes	PaxA	-	nd	107.5	>*paxCABD*
Actinobacillus pleuropneumoniae	ApxI	+	+	110	>*apxICABD*
	ApxII	+	+	102.5	>*apxIICA*
	ApxIII	-	+	113	>*apxIIICABD*
	ApxIV	+	nd	202/170	>*ORF1apxIVA*
Actinobacillus suis	ApxI	+	+	110	>*apxICABD*
	ApxII	+	+	102.5	>*apxIICA*
Actinobacillus porcitonsillarum	ApxII	+	+	102.5	>*apxIICABD*
Actinobacillus equuli	AqxA	+	+	110	>*aqxCABD*
Actinobacillus actinomycetemcomitans	Leukotoxin (LtxA)	-	+	116	>*ltxCABD*
Bordetella pertussis	AC toxin (CyaA)	+	+	177	*cyaC<>cyaABDE*
Vibrio cholerae	RtxA (VcRtxA)	-	+	484	*rtxArtxCchp<>rtxBDE*
Vibrio vulnificus	RtxA (VvRtxA)	nd	+	550	*rtxArtxCchp<>rtxBDE*
Moraxella bovis	Cytotoxin (MbxA)	+	+	99	>*mbxCABDtolC*

[a] AH : Activité hémolytique, AC : Activité cytolytique

acylations au niveau de résidus lysines. Cette acylation est médiée par le produit du gène *rtxC* (Issartel *et al.*, 1991; Stanley *et al.*, 1994). Les gènes *rtxB* et *rtxD* codent respectivement pour un ABC transporteur et sa protéine adaptatrice qui s'articulent avec une protéine de la membrane externe TolC pour constituer le système de sécrétion de type 1 (T1SS) nécessaire à l'exportation de la toxine mature à travers le périplasme (Thanabalu *et al.*, 1998; Wandersman & Delepelaire, 1990). Au niveau structural, la toxine active est généralement constituée de plusieurs domaines qui sont de N en C-terminal (Figure 3) :

- un domaine hydrophobe conservé en N-terminal qui se replie essentiellement en hélices α amphiphatiques pour constituer le domaine d'insertion dans la membrane eucaryote (Hyland *et al.*, 2001; Schindel *et al.*, 2001)
- un domaine qui constitue environ 40% de la molécule et qui semble nécessaire pour l'attachement aux cellules cibles. Ce domaine possède des résidus lysines auxquels sont liées de façon covalente une ou 2 chaînes d'acides gras (Hormozi *et al.*, 1998; Stanley *et al.*, 1994) qui joueraient un rôle dans l'attachement de la toxine aux membranes eucaryotes (El-Azami-El-Idrissi *et al.*, 2003)
- un domaine constitué de répétitions de la séquence consensus riche en glycine (Frey *et al.*, 1991; Ludwig *et al.*, 1988) qui adopte une structure stable en « β-roll » avec des brins β parallèles repliés en hélice droite et qui fixe le calcium nécessaire à l'activité de la toxine
- une séquence signal non clivée en C-terminal impliquée dans la sécrétion de la toxine par le système de sécrétion de type 1 (T1SS) (Koronakis *et al.*, 1989).

À ce jour, aucune structure 3D n'a été proposée pour les toxines RTX. Cependant, des études de modélisation informatique suggèrent que la partie N-terminale hydrophobe est composée d'hélices α amphiphatiques (Ludwig *et al.*, 1991). D'autre part, par analogie avec la protéase alcaline de *Pseudomonas aeruginosa* dont la structure 3D a été élucidée (Baumann *et al.*, 1993), on suppose que le domaine de séquences riche en glycine adopte

une structure en « β-roll » parallèle en fixant les ions Ca^{2+}. En effet, il a été démontré que le calcium jouait un rôle primordial dans l'activité de la toxine en modifiant sa structure tertiaire, favorisant ainsi son insertion dans les membranes (Bakas *et al.*, 1998; Soloaga *et al.*, 1998). D'autres rôles du calcium ont aussi été proposés, notamment dans la régulation de l'expression des gènes codant pour la toxine (Gygi *et al.*, 1992).

D'autres toxines appartenant à la famille des RTX possèdent des caractéristiques divergentes des toxines RTX conventionnelles. Ainsi, l'adenylate cyclase (CyaA) de *Bordetella pertussis* est une protéine bi-fonctionnelle de 177 kDa qui possède une activité adénylate cyclase grâce à un domaine situé à l'extrémité N-terminal de la protéine et une activité hémolytique liée au domaine RTX en C-terminal (Figure 3). CyaA possède également un nombre élevé de 40 répétitions de la séquence consensus riche en glycine. La toxine VcRtxA de *V. cholerae* est une large protéine de 484 kDa qui fonctionnellement diffère totalement des autres toxines RTX car elle joue un rôle dans la dépolymérisation de l'actine et n'est associée à aucune activité hémolytique ou cytolytique. En ce qui concerne sa structure, VcRtxA possède en C-terminal des répétitions d'une séquence consensus riche en glycines et en aspartates susceptible de fixer le calcium, qui comporte cependant neuf résidus de plus que la séquence consensus conventionnel retrouvée chez les autres toxines RTX (GGXGXDXX(V/I)XXGXXNXXX). De plus, VcRtxA possède en N-terminal une série de répétitions d'une autre séquence consensus riche en glycine de 19 acides aminés (GXAN(I/V)XT(K/H)VGDGXTVAVMX) de fonction inconnue. L'organisation des gènes codant pour CyaA et VcRtxA montre également des divergences par rapport aux autres toxines RTX, les 4 gènes étant répartis sur 2 opérons (Tableau 2).

2.3.2. Les toxines Apx

Les toxines Apx sont hémolytiques et/ou cytolytiques (Maier *et al.*, 1996) et sont différentiellement sécrétées par les 15 sérotypes d'*A. pleuropneumoniae* (Blackall *et al.*, 2002; Frey, 1995) (Tableau 3) ainsi que par d'autres espèces d'*Actinobacillus.* Parmi les 4 toxines Apx identifiées à ce jour, les toxines ApxI-III ont été les plus étudiées.

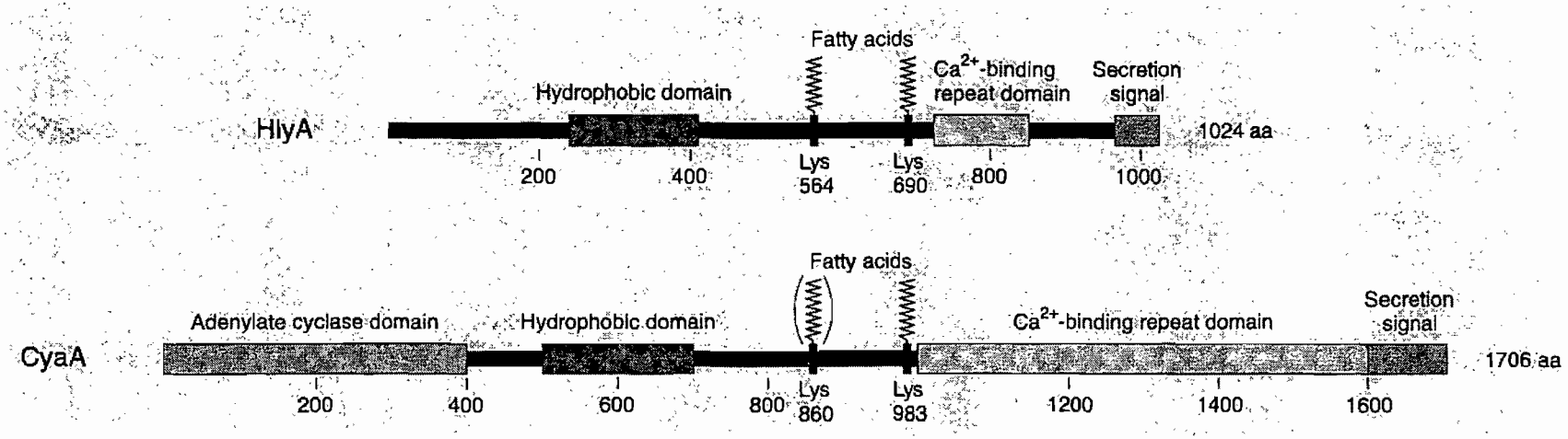


Figure 3. Structure de l'hémolysine-α (HlyA) d'*E. coli* et de l'adénylate cyclase (CyaA) de *B. pertussis*. aa : acides aminés. Adaptée de Ludwig & Goebel, 2006.

2.3.2.1. Les opérons Apx

Les toxines ApxI, ApxII et ApxIII sont respectivement codées par les opérons *apxICABD* (Frey *et al.*, 1994), *apxIICA* (Frey *et al.*, 1993; Jansen *et al.*, 1992) et *apxIIICABD* (Jansen *et al.*, 1993; Jansen *et al.*, 1994) pour lesquels les gènes sont organisés selon un schéma conventionnel semblable à l'opéron *hly* codant pour HlyA. Étant donné que l'opéron *apxII* ne possède pas les gènes de sécrétion *apxIIB* et *apxIID* qui ont probablement été perdus au cours de l'évolution, les toxines ApxI et ApxII sont toutes les deux sécrétées par le système de sécrétion d'ApxI (Frey *et al.*, 1993).

2.3.2.2. Structure

La caractérisation structurale des 3 toxines (ApxI à III) montre des caractéristiques typiques des toxines RTX incluant la région hydrophobique en N-terminal, les répétitions de la séquence consensus ainsi que la séquence signal non clivée en C-terminal. Concernant la maturation par acylation de la prétoxine en toxine active, la présence des chaînes acyles liées de façon covalente aux lysines a été démontrée uniquement pour CyaA et HlyA. La présence des gènes *apxIC*, *apxIIC* et *apxIIIC* codant pour les acyltransférases suggèrent cependant que les toxines Apx seraient également acylées.

2.3.2.3. Phénotype

La caractérisation phénotypique montre des différences entre les toxines. Ainsi, ApxI est une protéine de 105 kDa qui possède une forte activité hémolytique et est également très cytotoxique pour les cellules endothéliales, les macrophages alvéolaires et les neutrophiles (Kamp *et al.*, 1991). La toxine ApxII, avec une taille approximative de 105 kDa semblable à ApxI, a été décrite comme faiblement hémolytique et modérément cytotoxique. ApxIII est une protéine de 120 kDa qui est dépourvue d'activité hémolytique mais qui est fortement cytotoxique pour les cellules endothéliales, les macrophages alvéolaires et les neutrophiles (Rycroft *et al.*, 1991).

2.3.2.4. Homologie de séquence

La comparaison des séquences nucléotidiques de ces toxines montre une forte homologie entre ApxI, ApxIII et HlyA alors que ApxII est phylogénétiquement liée à la leucotoxine LktA de *M. haemolytica*. Au niveau des séquences en acides aminés, on retrouve également le même patron d'homologie entre les toxines. Ainsi, ApxI et ApxIII possèdent respectivement 56% et 50% d'homologie avec HlyA contre 47% pour ApxII. En revanche, ApxII est homologue à LktA à 67% contre seulement 41% pour ApxI. De plus, les toxines ApxI, ApxIII et HlyA possèdent 13 répétitions de la séquence consensus riche en glycines alors que ApxII et LktA n'en possèdent que 8. Les homologies retrouvées entre ces toxines reflètent également leur activité biologique, notamment au niveau de leur spécificité cellulaire (Lally *et al.*, 1999).

2.3.2.5. Répartition des toxines Apx chez *Actinobacillus*

ApxI est sécrétée par les sérotypes 1, 5, 9, 10 et 11 d'*A. pleuropneumoniae* (Tableau 3) ainsi que par *Actinobacillus suis* (Kamp *et al.*, 1994). Un opéron *apxICABD* avec une région promotrice tronquée a également été retrouvé chez *Actinobacillus lignieresii*. ApxII est sécrétée par tous les sérotypes d'*A. pleuropneumoniae* à l'exception des sérotypes 3 et 10 (Tableau 3), ainsi que par *A. suis*, *Actinobacillus rossii* et *Actinobacillus porcitonsillarum*. ApxIII est sécrétée par les sérotypes 2, 3, 4, 6 et 8 d'*A. pleuropneumoniae* (Tableau 3). Un opéron *apxIIICABD* a été retrouvé chez *A. rossii*, mais la toxine n'a pu être détectée dans les cultures d'*A. rossii*.

2.3.2.6. La toxine ApxIV

La toxine ApxIV est la moins caractérisée des toxines Apx et a la particularité d'être exprimée uniquement *in vivo*. En effet, elle n'a pu être détectée dans des cultures d'*A. pleuropneumoniae*, sous différentes conditions de croissance *in vitro*. En revanche, cette toxine ApxIV est potentiellement produite *in vivo* par tous les sérotypes d'*A. pleuropneumoniae* (Tableau 3) dans la mesure où les sérums provenant de porcs infectés

par tous les sérotypes réagissent avec la toxine recombinante. D'autre part, une faible activité hémolytique de la toxine ApxIV et une synergie co-hémolytique avec la sphingomyélinase (toxine bêta) de *Staphylococcus aureus* a également été mise en évidence. En ce qui concerne les gènes de biosynthèse de la toxine, aucune organisation typique en opérons n'a été retrouvée. Par contre, deux variants du gène codant pour ApxIV (*apxIV*$_{var1}$ et *apxIV*$_{var3}$) ont été retrouvés chez les sérotypes 1 et 3 d'*A. pleuropneumoniae* codant respectivement pour des protéines de 202 et 170 kDa. La séquence en acides aminés d'ApxIV$_{var1}$ révèle une protéine constituée de domaines typiquement retrouvés chez les toxines RTX avec cependant quelques divergences au niveau des séquences répétées riches en glycines. En effet, ApxIV$_{var1}$ possède un nombre élevé de 24 répétitions, justifiant ainsi la taille importante de la protéine, qui sont séparées par 4 régions correspondant à des séquences consensus de la famille des DNA Polymérases II. Cependant le rôle de cette toxine ApxIV dans la pathogenèse d'*A. pleuropneumoniae* reste à démontrer (Schaller *et al.*, 1999).

Tableau III. Génotype et expression des toxines Apx chez les sérotypes d'*A. pleuropneumoniae* (Blackall *et al.*, 2002; Frey, 1995).

Sérotypes	*apxI*		*apxII*		*apxIII*		*apxIV*	Toxines exprimées *in vitro*	Toxines exprimées *in vivo*
	CA	*BD*	*CA*	*BD*	*CA*	*BD*	*A*		
1, 5, 9 et 11	+	+	+	-	-	-	+	ApxI et ApxII	ApxI, ApxII et ApxIVA
2, 4, 6, et 8	-	+	+	-	+	+	+	ApxII et ApxIII	ApxII, ApxIII et ApxIVA
'3	-	-	+	-	+	+	+	ApxIII	ApxIII et ApxIVA
7 et 12	-	+	+	-	-	-	+	ApxII	ApxII et ApxIVA
10	+	+	-	-	-	-	+	ApxI	ApxI et ApxIVA
15	-	+	+	-	+	+	+	ApxII et ApxIII	nd

2.4. Les lipopolysaccharides (LPS)

Les LPS sont des molécules complexes de la membrane externe des bactéries Gram négatives. Le LPS, également appelé endotoxine, est composé de trois régions bien définies :

- le lipide A qui permet l'ancrage du LPS dans la membrane externe
- le noyau oligosaccharidique (OS) composé du noyau interne et externe
- la chaîne O-spécifique composé d'unités répétées de sucres

2.4.1. Lipide A

La première étape dans la synthèse du lipide A (Figure 4) correspond à une O-acylation du nucléotide UDP-GlcNAc. Cette étape correspond à l'ajout d'un résidu β-hydroxymyristate porté par un donneur ACP et est catalysée par l'acyltransférase LpxA (Anderson & Raetz, 1987). Cette acylation est suivie d'une étape de désacétylation de l'UDP-3-O-(acyl)-GlcNAc par la zinc-métalloprotéine LpxC (Jackman *et al.*, 2001). Un deuxième résidu β-hydroxymyristate est ensuite rajouté au cours d'une N-acylation catalysée par l'acyltransférase LpxD (Kelly *et al.*, 1993). La molécule UDP-2,3-diacylglucosamine obtenue est clivée par la pyrophosphatase LpxH pour donner le 2,3-diacylglucosamine-1-phosphate également appelé lipide X. La protéine LpxB catalyse ensuite la formation d'un disaccharide par la condensation du lipide X et d'une autre molécule de UDP-2,3-diacylglucosamine liés par une liaison β(1'-6) (Crowell *et al.*, 1987; Radika & Raetz, 1988). La kinase LpxK phosphoryle la molécule en position 4' du disaccharide pour donner le lipide IV_A (Ray & Raetz, 1987) auquel est ajouté 2 résidus Kdo en position 6' du disaccharide par la protéine bifonctionnelle WaaA (Clementz & Raetz, 1991). La dernière étape correspond au branchement de 2 chaînes d'acides gras secondaires (laurate et myristate) par O-acylation au niveau des hydroxyles des 2 chaînes d'hydroxymyristate du résidu glucosamine distal (Brozek & Raetz, 1990). Ces 2 acylations catalysées par la lauroyltransférase LpxL et la myristoyltransférase LpxM complète le processus de biosynthèse qui donne la molécule Kdo_2-lipide A.

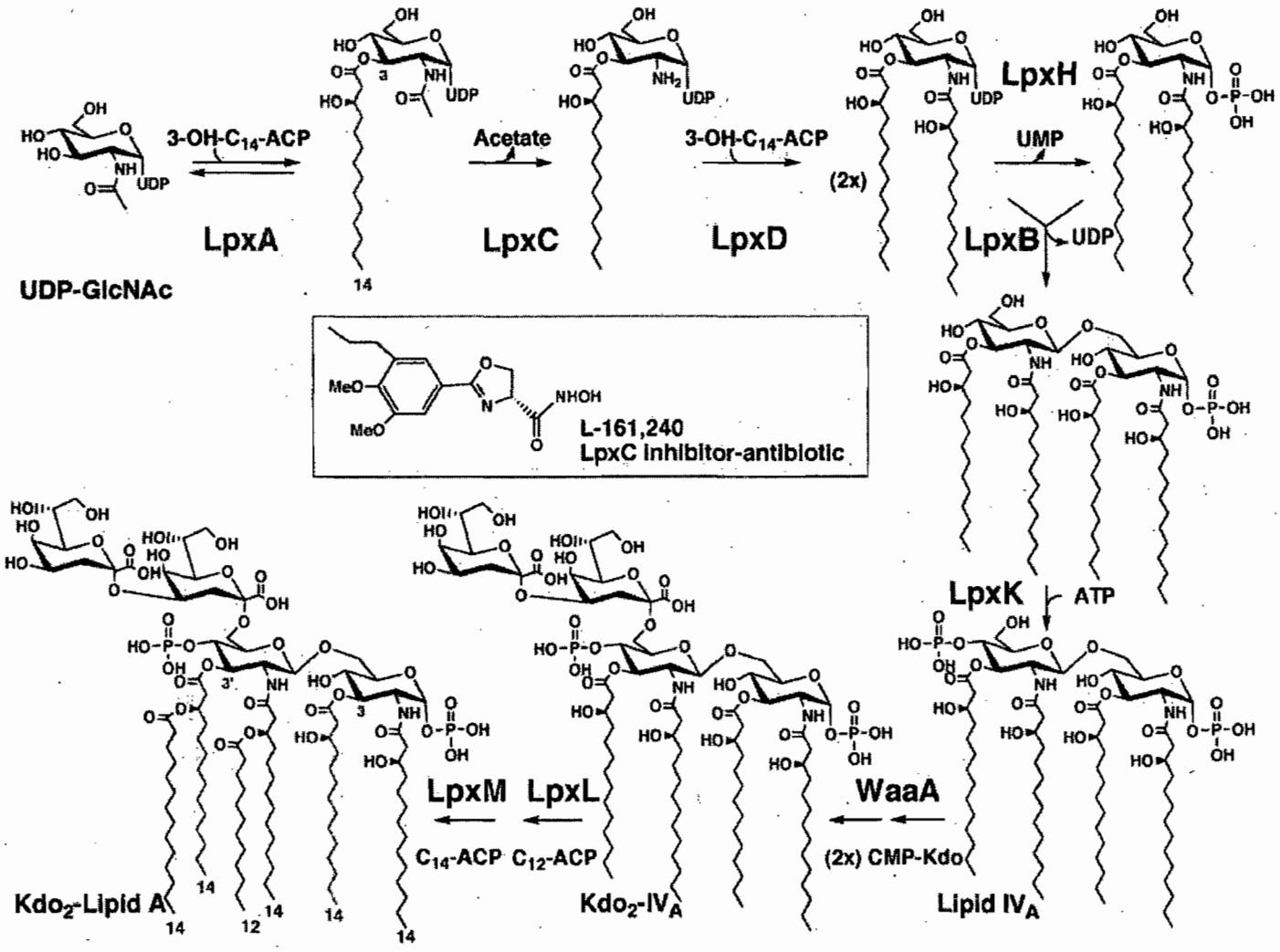


Figure 4. Structure et biosynthèse du Kdo_2-lipide A chez *E. coli* K-12. Adaptée de Raetz & Whitfield, 2002.

Le lipide A seul n'existe pas chez les cellules, le LPS minimal requis pour la viabilité bactérienne étant constitué du lipide A glycosylé et de deux Kdo (chémotype Re) (Raetz, 1996).

2.4.2. Le noyau oligosaccharidique

Le noyau OS divisé en noyau interne et externe, constitue en général la partie charnière entre le lipide A et l'antigène O des bactéries à phénotype lisse. Le noyau interne est relativement bien conservé entre les espèces et se lie au lipide A, alors que le noyau externe, plus variable, permet l'attachement de la chaîne-O spécifique. On distingue également des souches rugueuses ne possédant pas d'antigène O où le noyau externe constitue la partie proximale du LPS, ou encore d'autres bactéries produisant des lipooligosaccharides (LOS) dont le noyau est uniquement constitué du noyau interne et de quelques sucres correspondant au noyau externe (Kahler & Stephens, 1998; St Michael *et al.*, 2006).

La biosynthèse du noyau OS est sous le contrôle du locus *waa* qui est constitué de 3 parties (opéron ou gène) chez *E. coli* et *Salmonella* (Figure 5) :

- l'opéron *gmhD-* constitué des gènes *gmhD-waaFC*, est impliqué dans la biosynthèse et le transfert du L,D-heptose
- l'opéron *waaQ-*, constitué des gènes *waaQGPOTYWVL*, est nécessaire à la biosynthèse du noyau externe ainsi qu'à la modification du noyau OS. Cet opéron *waaQ* est sous le contrôle du gène *rfaH* (Schnaitman & Klena, 1993)
- le gène *waaA* codant pour une Kdo transférase bifonctionnelle (Clementz & Raetz, 1991) qui est impliquée dans l'ajout de résidus Kdo au lipide A (voir ci-dessus).

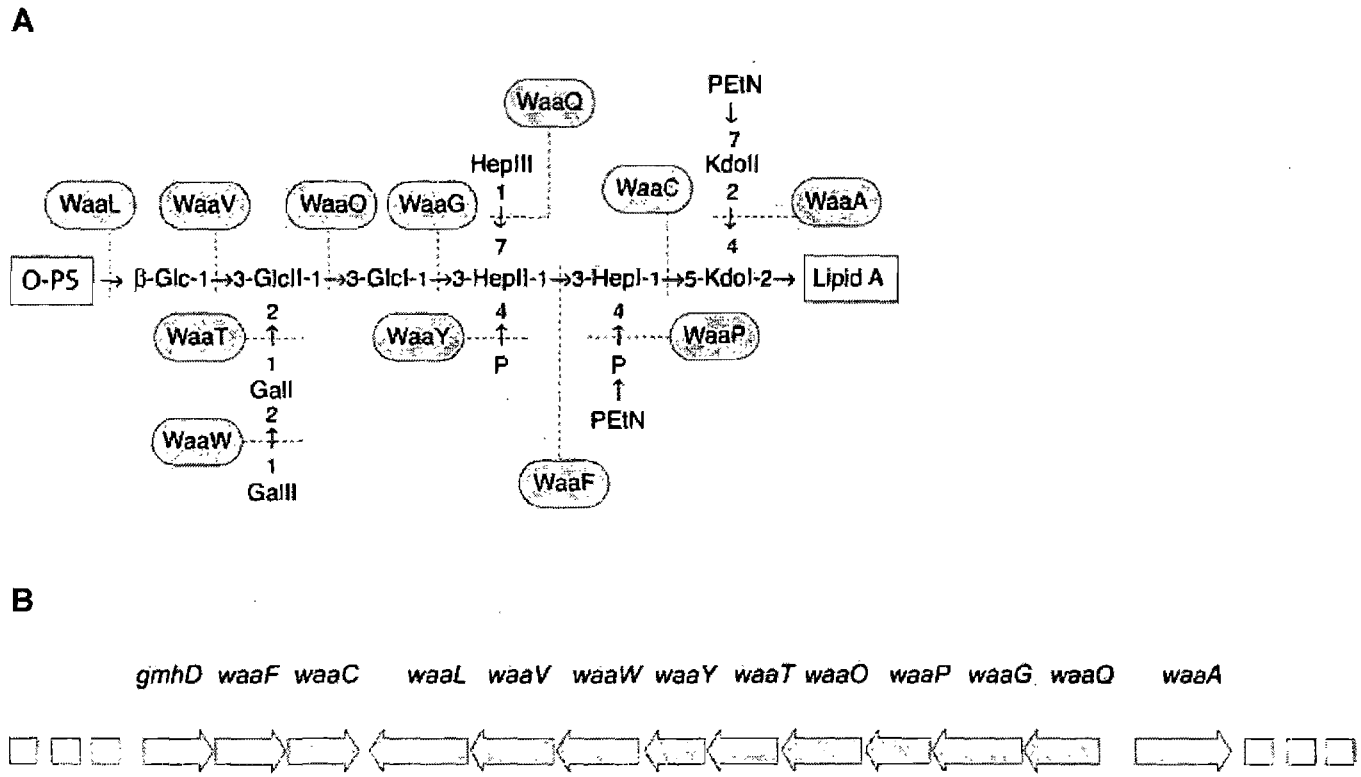


Figure 5. Structure et biosynthèse du noyau OS de type R1 d'*E. coli* (A). Organisation génétique du locus *waa* (B). Adaptée de Raetz & Whitfield, 2002.

2.4.2.1. Le noyau interne

La biosynthèse du noyau interne commence par un transfert de 2 résidus heptose Hep I et Hep II à la molécule Kdo_2-lipide A et est médiée respectivement par les 2 heptosyltransférases WaaC et WaaF qui utilisent comme substrat préférentiel l'ADP-L,D-heptose (Gronow *et al.*, 2000). La synthèse préalable d'ADP-L,D-heptose est médiée par l'activité séquentielle des protéines GmhA-HldE-GmhB-HldE qui donnent l'ADP-D-*glycéro*-D-*manno*-heptose, suivie d'une épimérisation par la protéine HldD pour donner l'ADP-L-*glycéro*-D-*manno*-heptose (Kneidinger *et al.*, 2002). Trois principales enzymes interviennent dans la modification de la région oligosaccharidique (Raetz & Whitfield, 2002) chez *E. coli* et *Salmonella* (Figure 5). Ainsi, les kinases WaaP et WaaY sont impliquées dans la phosphorylation des heptoses tandis que l'heptosyltransférase WaaQ permet l'ajout d'un résidu heptose branché à la chaîne principale. L'étude de mutants d'*E. coli* a montré que ces modifications du noyau interne nécessitaient une action séquentielle de ces 3 enzymes dans un ordre précis, soit WaaP-WaaQ-WaaY (Yethon *et al.*, 1998), faisant de WaaP l'enzyme la plus importante. En effet, il a été démontré que les mutants particulièrement affectés au niveau du gène *waaP* avaient un phénotype « deep rough » caractéristique alors que la modification apportée par la protéine ne semble pas être impliquée directement dans l'élongation du noyau OS (Figure 5). Ceci suggère donc une action concertée des enzymes de biosynthèse sous forme de complexes protéiques, qui serait essentielle à un bon assemblage du noyau OS. D'autres enzymes potentiellement impliquées dans la modification du noyau interne ont été identifiées mais leur implication fonctionnelle n'a pas encore été complètement élucidée. Ainsi, la glycosyltransférase WaaZ retrouvée chez certaines souches de *S. enterica* et de *E. coli* possède une activité α-2,4 Kdo transférase et serait potentiellement impliquée dans l'ajout du troisième résidu Kdo retrouvée dans les noyaux OS de type R2 et K-12 (Figure 6).

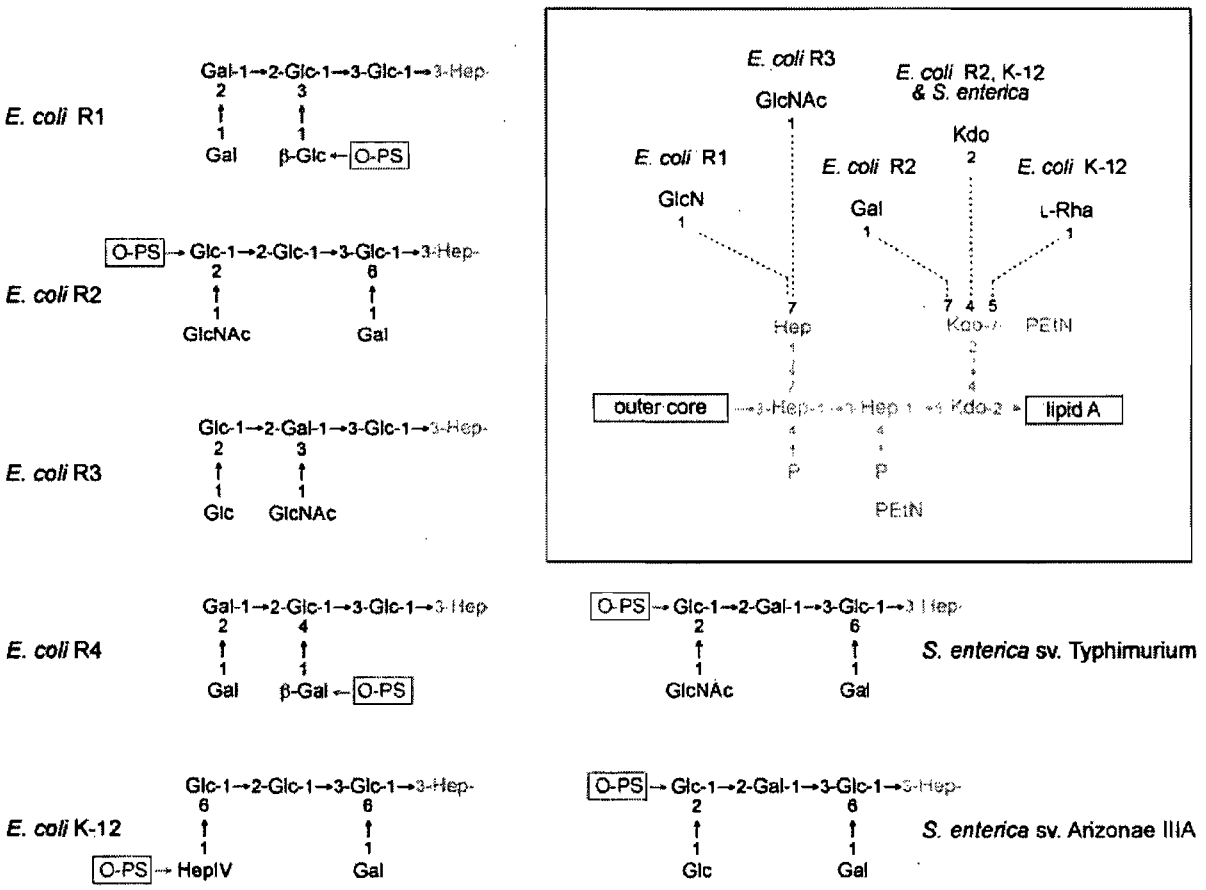


Figure 6. Structure du noyau externe des différents types de noyau OS connus chez *E. coli* et *Salmonella*. En encadré, on retrouve la partie conservée du noyau interne et les modifications spécifiques à chaque type de noyau OS. Adaptée de Raetz & Whitfield, 2002.

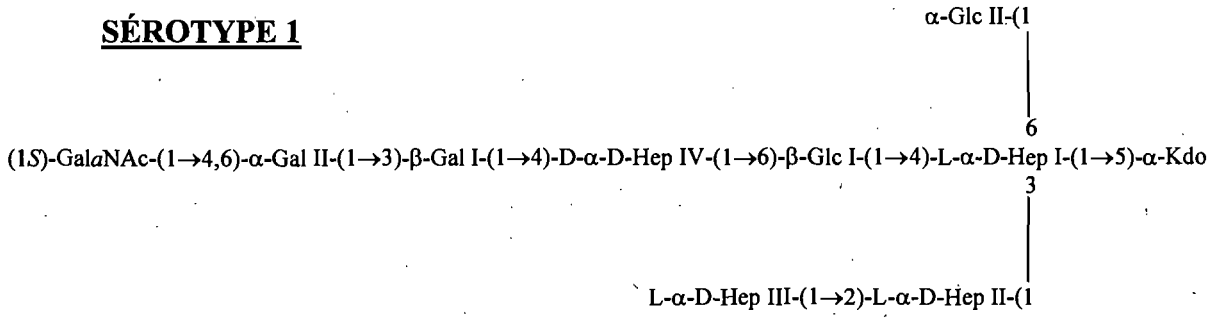
Chez *A. pleuropneumoniae* le noyau interne est très conservé entre les différents sérotypes (Michael *et al.*, 2004) (Figure 7). Il consiste en un trisaccharide de L-glycéro-D-manno-heptose lié à un résidu Kdo. L'heptose (Hep I) proximal est substitué par un résidu β-glucose en position 4 (Glc I) et un α-glucose en position 6 (Glc II). Un dernier résidu D-glycéro-D-manno-heptose (Hep IV) est ajouté en position 6 du Glc I. Une structure identique est également retrouvée chez d'autres *Pasteurellaceae* tels que *P. multocida* et *M. haemolytica* (Brisson *et al.*, 2002; St Michael *et al.*, 2005a; St Michael *et al.*, 2005b), ce qui confirme bien la faible variabilité du noyau interne. Cependant, 2 glycoformes du noyau

OS ont été récemment identifiées chez *P. multocida*, une possédant 2 Kdo et l'autre ne possédant qu'un seul Kdo phosphorylé au niveau du noyau interne (Harper *et al.*, 2007).

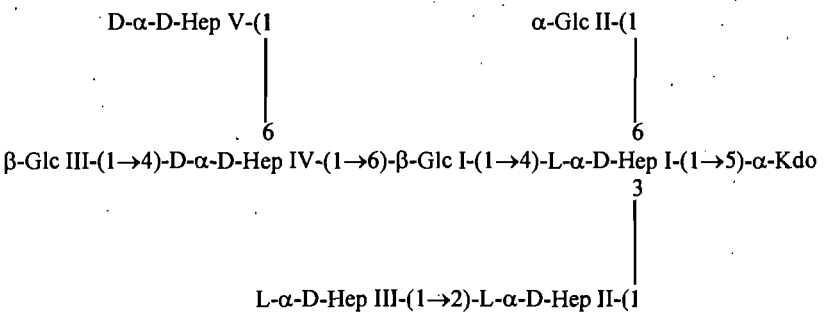
2.4.2.2. Le noyau externe

Le noyau externe est la partie la plus variable du noyau OS et on peut trouver des différences au sein d'une même espèce bactérienne. Ainsi, chez *E. coli*, on distingue 5 types de noyaux OS (R1 à R4 et K-12) dont la distinction se fait essentiellement sur la variabilité du noyau externe (Figure 6). Chez *Salmonella*, on retrouve 2 types de noyaux OS qui sont ceux des sérovars Typhimurium et Arizonae IIIA et qui présentent de fortes homologies avec ceux retrouvés chez *E. coli*. Ainsi, le core externe du serovar Thyphimurium est identique à celui du noyau de type R2 de *E. coli*. Chez *A. pleuropneumoniae*, 2 types de noyaux OS ont été identifiés selon leur mobilité électrophorétique et ils sont différentiellement répartis chez les sérotypes (Jacques *et al.*, 1996) : le noyau de type 1 qui migre plus lentement que celui du LPS Ra de *S.* Typhimurium se retrouve chez les sérotypes 1, 6, 9 et 11 alors que le noyau de type 2 migrant à la même vitesse que le noyau du LPS Ra de *S.* Typhimurium se retrouve chez les sérotypes 2, 3, 4, 5, 7, 8, 10 et 12. L'analyse de la structure du noyau OS du LPS d'*A. pleuropneumoniae* a récemment permis de montrer les divergences entre ces 2 types de noyaux (Michael *et al.*, 2004) (Figure 7) : le noyau de type 1 possède un noyau externe composé du trisaccharide (1*S*)-Gal*a*NAc-(1 → 4,6)-α-Gal-(1 → 3)-β-Gal-, branché au noyau interne en position 4 de l'Hep IV alors que le noyau de type 2 ne possède qu'un résidu D-*glycéro*-D-*manno*-heptose (Hep V) branché en position 6 de l'Hep IV, justifiant ainsi sa plus grande mobilité électrophorétique. Cependant des divergences sont également retrouvées au sein du noyau de type 2 car on constate que le sérotype 2, contrairement au sérotype 5, possède un β-glucose (Glc III) supplémentaire branché en position 4 de l'Hep IV (Figure 7).

SÉROTYPE 1



SÉROTYPE 2



SÉROTYPE 5a et 5b

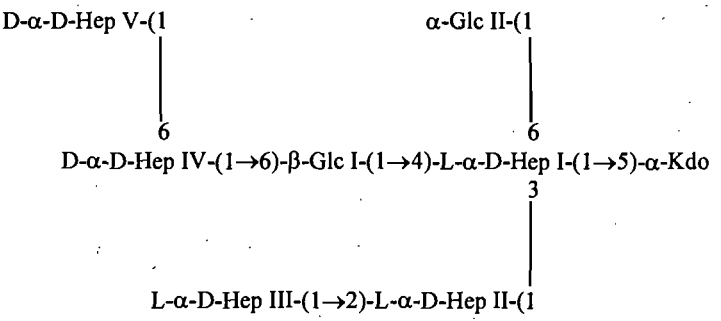


Figure 7. Structure des 2 types de noyau OS retrouvés chez *A. pleuropneumoniae* (Michael *et al.*, 2004). Le sérotype 1 possède un noyau de type 1 alors que les sérotypes 2, 5a et 5b possèdent un noyau de type 2 ayant une plus grande mobilité électrophorétique.

La biosynthèse du noyau externe est contrôlée par plusieurs gènes de l'opéron *waaQ*. Dans tous les types de noyaux identifiés chez *E. coli* et *Salmonella*, on retrouve un glucose comme premier résidu qui est ajouté par la glucosyltransférase WaaG (Figures 5 et 6). D'autres glycosyltransférases codées par les gènes *waaOTWV* vont par la suite catalyser l'élongation du noyau externe par l'ajout séquentiel de résidus glucose ou galactose essentiellement par des liaisons α. Cependant, l'élongation du noyau externe montre des divergences entre les différents types de noyaux tels que mentionnés plus haut. Ainsi, on peut observer des ajouts de sucres par des liaison β notamment dans les noyaux de type R1 et R4 (Figure 6), catalysés respectivement par la β1,3-glucosyltransferase WaaV et la β1,4-galactosyltransferase WaaX (Heinrichs *et al.*, 1998b). Les noyaux de type R2 de *E. coli* et de *Salmonella enterica* serovar Typhimurium possèdent un résidu GlcNAc terminal qui est ajouté par l'enzyme WaaK (Heinrichs *et al.*, 1998a). On observe dans le noyau de type K-12 un heptose IV terminal. Suite à l'élongation du noyau externe, une dernière étape de ligation médiée par la ligase WaaL va permettre l'attachement de la chaîne-O au noyau externe des LPS ayant un phénotype lisse (Abeyrathne *et al.*, 2005; Abeyrathne & Lam, 2007). D'autre part, il a été démontré que cette ligation nécessite non seulement la présence du résidu nécessaire à l'attachement de l'antigène O, mais également celle des résidus se trouvant à proximité du site d'attachement (Heinrichs *et al.*, 1998a; Kaniuk *et al.*, 2004). Même si la plupart des études ont démontré le rôle du noyau externe dans l'attachement de la chaîne-O, le noyau interne peut également servir de site d'attachement à l'antigène O. Ainsi, un site d'attachement d'un résidu galactose de l'antigène O a été identifié au niveau du noyau interne en position 7 de l'Hep III du noyau OS d'*A. pleuropneumoniae* sérotype 5a (Michael *et al.*, 2004)

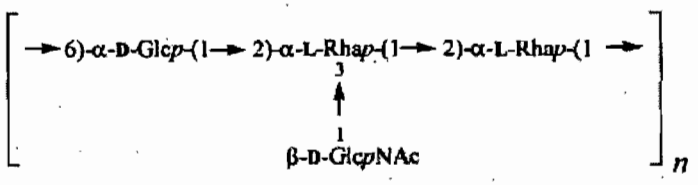
2.4.3. L'antigène O

La chaîne O-spécifique est la partie la plus variable du LPS et les différences observées au niveau de la structure des polysaccharides O peuvent être à l'origine d'une classification des souches en sérotypes chez une même espèce. Ainsi, *E. coli* produit environ 170 sérotypes O et on dénombre environ 46 sérogroupes chez *S. enterica*.

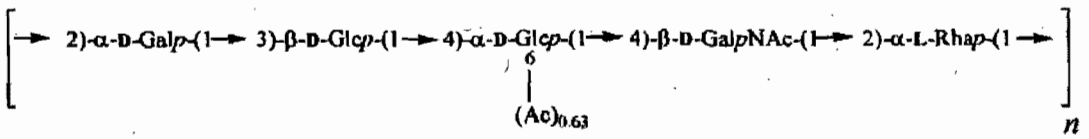
Chez *A. pleuropneumoniae* l'antigène O participe également avec les polysaccharides capsulaires à l'identification des différents sérotypes (voir section 1.3). L'antigène O consiste en un polymère d'unités répétées comprenant 1 à 8 résidus glycosyl (Jacques, 1996). La présence et le nombre d'unités répétées déterminent le profil du LPS sur gel d'électrophorèse (Byrd & Kadis, 1989). Une souche bactérienne présentant de longues chaînes est dite lisse alors qu'une bactérie sans antigène O est qualifiée de rugueuse. Certaines souches ne présentant pas toutes les longueurs de chaînes d'une souche lisse sont dites semi-rugueuses. Ainsi, chez *A. pleuropneumoniae*, les sérotypes 2, 4 et 7 ont un LPS lisse, les sérotypes 1 et 5 ont un LPS semi-rugueux et les sérotypes 3 et 6 ont un LPS rugueux. Certains LPS, appelés lipooligosaccharides (LOS), tels que ceux retrouvés chez *Neisseria*, ne possèdent pas d'antigène O et ne possèdent que quelques sucres constituant le noyau OS (Mandrell & Apicella, 1993).

La structure du LPS d'*A. pleuropneumoniae* a été précédemment identifiée pour les sérotypes 1 à 12 (Dubreuil *et al.*, 2000; Perry *et al.*, 1990) (Figure 8). L'antigène O du sérotype 1 est un polymère branché d'unités répétées d'un tétrasaccharide composé de α-L-rhamnopyranosyl, de α-D-glycopyranosyl et de 2-acétamido-2-désoxy-β-D-glucose (2:1:1). Plus récemment, l'antigène O du sérotype 13 a été identifié comme un polymère branché d'unités répétées d'un tétrasaccharide composé de résidus de l-rhamnose, de 2-acétamido-2-désoxy-D-galactose, et de D-galactose (1:1:2) (MacLean *et al.*, 2004). L'antigène O du sérotype 14 est un polymère branché d'unités répétées d'un disaccharide composé d'une chaîne de β-D-galactofuranose substitué en position 2 par un résidu α-D-galactopyranose (Perry & MacLean, 2004). La structure de l'antigène O du sérotype 15 est semblable à celle des sérotypes 3 et 8 et consiste en un polymère linéaire d'unités répétées d'un pentasaccharide constitué de 2 résidus D-glucose et de 3 résidus D-galactose (Perry *et al.*, 2005) (Figure 8).

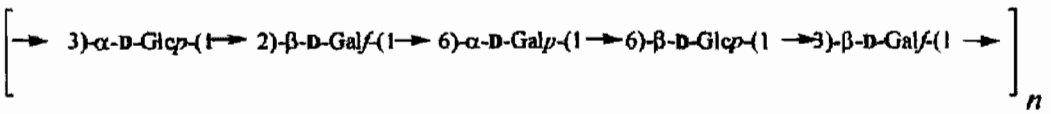
SEROTYPE 1 LPS O-CHAIN



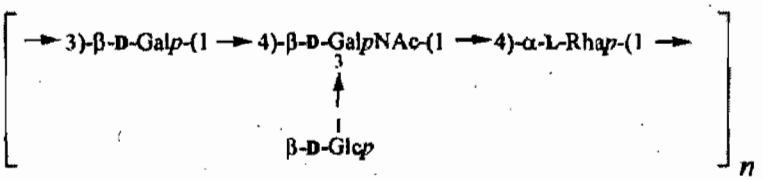
SEROTYPE 2 LPS O-CHAIN



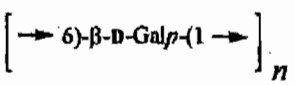
SEROTYPE 3 LPS O-CHAIN



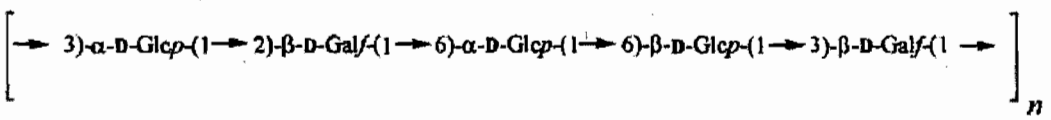
SEROTYPE 4 LPS O-CHAIN



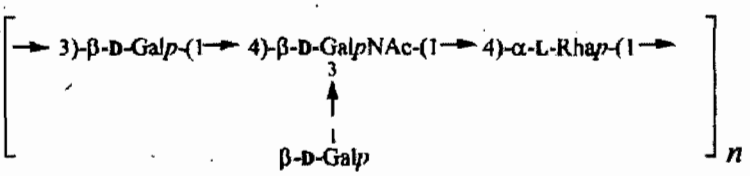
SEROTYPE 5 LPS O-CHAIN



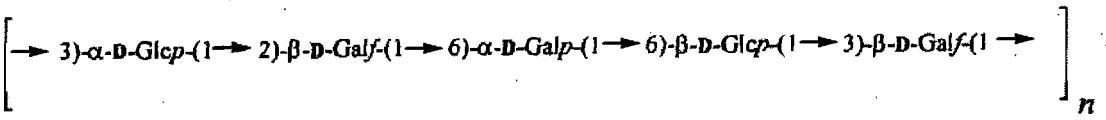
SEROTYPE 6 LPS O-CHAIN



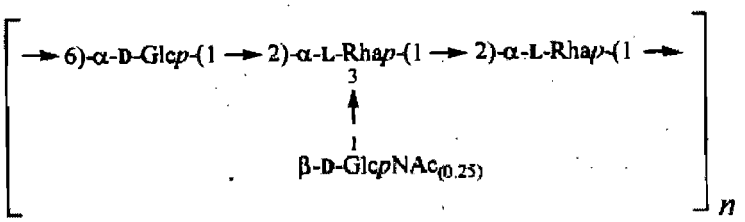
SEROTYPE 7 LPS O-CHAIN



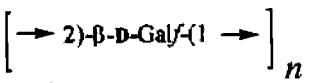
SEROTYPE 8 LPS O-CHAIN



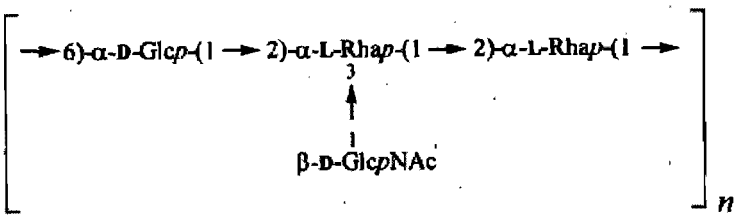
SEROTYPE 9 LPS O-CHAIN



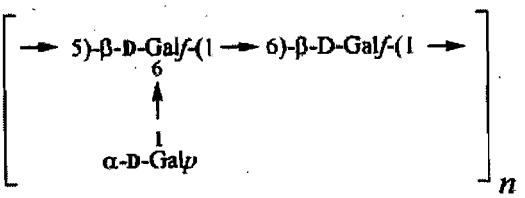
SEROTYPE 10 LPS O-CHAIN



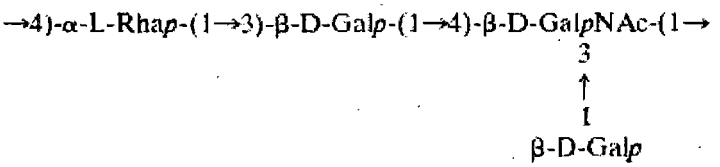
SEROTYPE 11 LPS O-CHAIN



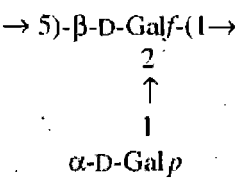
SEROTYPE 12 LPS O-CHAIN



SEROTYPE 13 LPS O-CHAIN



SEROTYPE 14 LPS O-CHAIN



SEROTYPE 15 LPS O-CHAIN

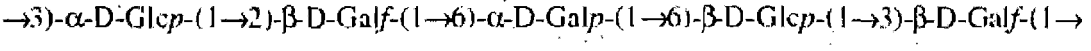


Figure 8. Structure de l'antigène O chez les sérotypes d'*A. pleuropneumoniae*. Adaptée de MacLean *et al.*, 2004; Perry *et al.*, 1990; Perry & MacLean, 2004; Perry *et al.*, 2005.

La grande majorité des enzymes impliquées dans la biosynthèse et l'assemblage de l'antigène O est codée au niveau du chromosome par les loci *wb* (rfb)* qui présentent un large polymorphisme reflétant la diversité de structure des polysaccharides O. Ces gènes *wb** codent essentiellement pour des glycosyltransférases et des enzymes impliquées dans le processus d'exportation du LPS (Reeves, 1994). Chez *A. pleuropneumoniae* sérotype 1, une région de 18 cadres ouverts de lecture potentiellement impliquée dans la biosynthèse de l'antigène O a été identifiée (Labrie *et al.*, 2002).

L'antigène O est synthétisé indépendamment du lipide A et du noyau OS sur un transporteur lipidique, l'undécaprényl phosphate (und-P), associé à la membrane interne (Whitfield, 1995). Les polysaccharides O sont ainsi synthétisés dans le cytoplasme par des glycosyltransférases à partir de nucléotides et sont par la suite transférés du transporteur lipidique und-P au noyau-lipide A, qui est ancré dans la partie périplasmique de la membrane externe. Ceci suggère donc l'existence d'un système qui permettrait le passage des polysaccharides O dans le périplasme. Trois voies de biosynthèse de l'antigène O basées sur leur mécanisme d'exportation sont ainsi proposées :

- La voie Wzy dépendante
- La voie ABC transporteur dépendante
- La voie synthase dépendante

Ces voies diffèrent les unes des autres par leur mécanisme d'exportation et de polymérisation mais possèdent en commun l'initiation de la biosynthèse de l'antigène O dans le cytoplasme et le processus final de ligation au noyau-lipide A dans le périplasme.
Le processus d'initiation de la biosynthèse de l'antigène O est relativement bien conservé entre les espèces. L'initiation commence par le transfert d'un sucre phosphorylé au transporteur lipidique und-P. Les enzymes d'initiation les mieux caractérisées sont la galactose-1-phosphate (Gal-1-P) transférase WbaP de *S. enterica* (Wang *et al.*, 1996) et la N-acétylglucosamine-1-phosphate (GlcNAc-1-P) transférase WecA de *E. coli* (Meier-Dieter *et al.*, 1992). Ces enzymes utilisent comme substrats l'UDP-Gal ou l'UDP-GlcNAc pour catalyser la formation d'und-PP-Gal pour WbaP et d'und-PP-GlcNAc pour WecA. Par ailleurs, ces enzymes possèdent un spectre d'activité assez large car elles interviennent

également dans la synthèse de la capsule ou d'exopolysaccharides, et ne sont pas forcément très spécifiques. En effet, il a été démontré que la GlcNAc-1-P transférase WecA pouvait également catalyser la formation de und-PP-GalNAc chez certaines souches de *E. coli* (Amor & Whitfield, 1997).

2.4.4. Assemblage et transport des LPS

La biosynthèse des LPS lisses peut être divisée en 2 étapes indépendantes, qui sont la synthèse du noyau-lipide A et la synthèse de l'antigène O. La première étape de la synthèse du noyau-lipide A correspond à la formation de la molécule de Kdo_2-lipide A qui se retrouve ancrée dans la partie cytoplasmique de la membrane interne par l'intermédiaire des chaînes d'acyles. Le noyau OS est ensuite rajouté au Kdo_2-lipide A par des glycosylations séquentielles au niveau du cytoplasme. Ce noyau lipide-A va ensuite être pris en charge par l'ABC transporteur MsbA (Doerrler *et al.*, 2001; Polissi & Georgopoulos, 1996; Zhou *et al.*, 1998) qui agit comme une flipase pour permettre le passage du noyau-lipide A du coté périplasmique où aura lieu la ligation avec l'antigène O médiée par la ligase WaaL. L'assemblage de l'antigène O et son passage dans l'espace périplasmique peuvent se dérouler selon 3 voies :

2.4.4.1. La voie Wzy dépendante

Cette voie représente la voie classique de biosynthèse de l'antigène O chez *S. enterica* sérogroupes B et E1 (Raetz, 1996; Reeves *et al.*, 1996). Dans ce modèle, l'élongation se fait par l'ajout d'unités oligosaccharidiques nouvellement synthétisées à l'extrémité réductrice de la chaîne en croissance (Figure 9A). Ainsi, suite à l'initiation par WbaP qui permet l'ajout du premier galactose, 2 autres glycosylations cytoplasmiques catalysées par les transférases WbaU et WbaN vont conduire à la formation du trisaccharide Man-Rha-Gal qui constituera une unité oligosaccharidique (Raetz, 1996). Dans le cas des souches de *S. enterica* sérogroupe B, une glycosylation supplémentaire catalysée par WbaV va permettre le branchement d'un didésoxyhexose (abéquose) sur le résidu galactose. L'unité oligosaccharidique attachée au transporteur lipidique und-PP va ensuite être transloquée

dans le périplasme par la flippase Wzx (Liu *et al.*, 1996) et la polymérisation va s'opérer par le transfert d'un polymère constitué de plusieurs répétitions d'unité oligosaccharidique lié à un autre und-PP, à l'unité oligosaccharidique nouvellement synthétisée. Cette polymérisation est catalysée par l'antigène O polymérase Wzy (Daniels *et al.*, 1998) et la longueur de la chaîne-O est sous le contrôle d'une protéine régulatrice Wzz appartenant à la sous-famille des PCP-1 qui sont des copolymérases de polysaccharide (Whitfield & Larue, 2008). En effet, il a été proposé que Wzz interagirait avec la polymérase Wzy et la flippase Wzx, pour favoriser une extension maximale de la chaine-O dans le périplasme avant la ligation au noyau-lipide A (Batchelor *et al.*, 1991).

Chez *A. pleuropneumoniae*, le séquençage du génome de la souche JL03 de sérotype 3 a permis de détecter la présence des gènes *wzz*, *wzy* et *wzx* (Xu *et al.*, 2008). Ceci suggère que l'antigène O des sérotypes 3, 8 et 15, qui sont identiques (Perry *et al.*, 2005), serait synthétisé par la voie Wzy dépendante.

2.4.4.2. La voie ABC transporteur dépendante

Cette voie est le plus souvent destinée aux chaînes-O ayant une structure linéaire non branchée et se caractérise par l'ajout séquentiel de sucres à l'extrémité non-réductrice de la chaîne en croissance. L'élongation totale du polymère se déroule exclusivement dans le cytoplasme par des glycosylases et ne nécessite aucune polymérase (Figure 9B). Tous les polysaccharides O synthétisés par cette voie, dont les polymères de polymannose de *E. coli* O8, O9, O9a (Kido *et al.*, 1995; Kido *et al.*, 1998; Rick *et al.*, 1994) et de *Klebsiella pneumoniae* O3 et O5, sont initiés par la GlcNAc-1-P transférase WecA. Ainsi, l'und-PP-GlcNAc sert d'amorce à l'extension de la chaîne et le résidu GlcNAc qui sera transféré au noyau-lipide A lors de la ligation n'est présent qu'une seule fois au début de la chaîne, contrairement à la voie Wzy dépendante où le GlcNAc serait ajouté à chaque unité oligosaccharidique. L'étape suivante correspond à l'ajout d'un ou de plusieurs sucres servant d'adaptateur entre l'und-PP-GlcNAc et les unités répétées. Ainsi, chez *E. coli* O9, l'adaptateur correspond à un résidu mannose ajouté par WbdC (Kido *et al.*, 1995) alors que chez plusieurs sérotypes de *K. pneumoniae*, la galactosyltransférase bifonctionnelle WbbO (Guan *et al.*, 2001) permet le transfert de 2 galactoses à l'amorce und-PP-GlcNAc. La

phase d'élongation est ensuite catalysée par des glycosyltransférases mono- ou bifonctionelles qui vont permettre l'ajout d'un ou de plusieurs sucres liés entre eux de façon particulière. En effet, une unité oligosaccharidique peut se définir selon la nature des liaisons entre les sucres, notamment lorsque le polymère est formé d'un seul type de sucre comme c'est le cas pour les polymères de polymannose. Suite à la polymérisation, la chaîne-O liée à l'und-PP est transloquée à travers la membrane cytoplasmique par un système ABC transporteur constitué de l'ATPase Wzt et du pore transmembranaire Wzm (Cuthbertson *et al.*, 2005; Cuthbertson *et al.*, 2007). Dans cette voie, la longueur des chaînes n'est pas contrôlée par la copolymérase Wzz et les mécanismes de terminaison de l'élongation sont encore mal connus.

Chez *A. pleuropneumoniae* sérotype 1, une région de 18 cadres ouverts de lecture (ORFs) impliquée dans la biosynthèse des polysaccharides O a été identifiée et les protéines codées par les ORFs 10 et 11 présentent des homologies de séquences avec des ABC transporteurs (Labrie *et al.*, 2002). D'autre part, la comparaison des régions impliquées dans la biosynthèse de l'antigène O entre les sérotypes 1, 3 et 5 a révélé la présence du gène *wzm* chez les sérotypes 1 et 5b (Xu *et al.*, 2008) suggérant que la synthèse de l'antigène O se ferait par la voie ABC transporteur dépendante pour ces 2 sérotypes .

2.4.4.3. La voie synthase dépendante

Cette voie est très peu caractérisée et le seul exemple connu de polysaccharides O synthase dépendantes, est l'homopolymère de N-acétylmannosamine qui constitue l'antigène O:54 de *S. enterica* sérovar Borreze (Keenleyside *et al.*, 1994; Keenleyside & Whitfield, 1996). Les 2 premières étapes de biosynthèse sont similaires à la voie ABC transporteur dépendante notamment avec une initiation médiée par WecA (Keenleyside *et al.*, 1994) et l'ajout d'un adaptateur ManNAc à l'und-PP-GlcNAc catalysé par la transférase WbbE (Keenleyside *et al.*, 2001). Par la suite, la synthase WbbF va catalyser l'extension de la chaîne polysaccharide tout en exportant simultanément le polymère à travers la membrane cytoplasmique (Keenleyside & Whitfield, 1996). Cependant, les mécanismes exacts d'exportation à travers la membrane interne et de terminaison de l'élongation ne sont pas encore connus.

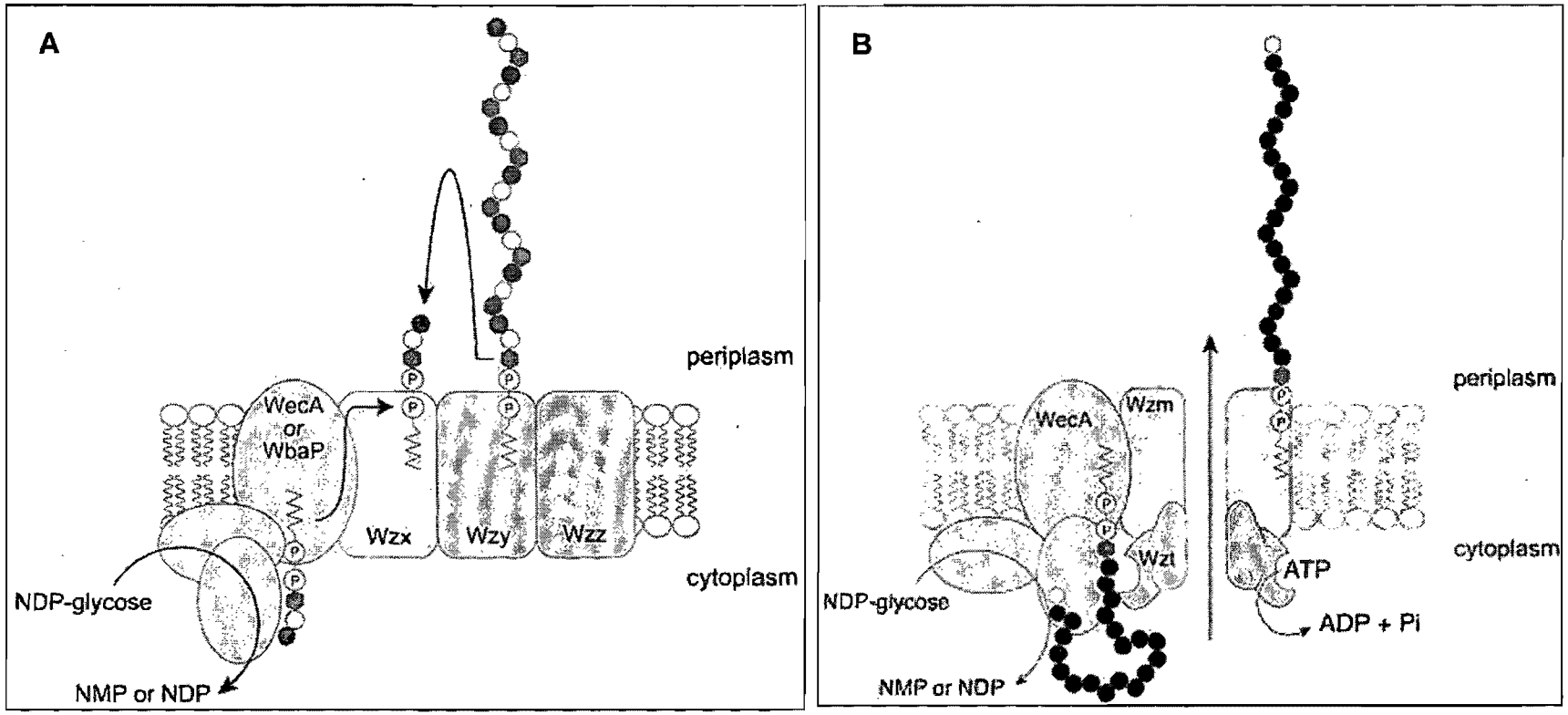


Figure 9. Les 2 grandes voies de biosynthèse de l'antigène O. La voie Wzy dépendante (A) et la voie ABC transporteur dépendante (B). Adaptée de Raetz & Whitfield, 2002.

2.4.5. Exportation des LPS vers la membrane externe

Le mécanisme par lequel les LPS passent à travers le périplasme pour s'insérer dans la membrane externe est encore inconnu. Cependant, l'existence d'un modèle d'assemblage et de translocation des polysaccharides capsulaires à travers le périplasme (Bliss & Silver, 1996; Whitfield & Roberts, 1999) suggère un modèle semblable pour les LPS. De plus, le caractère hydrophobe du lipide A rendrait le passage du LPS seul à travers le milieu aqueux du périplasme thermodynamiquement défavorable, ce qui conforte encore l'idée d'une machinerie de translocation. Ainsi, de récentes études ont montré un rôle important du complexe protéique Imp/RlpB (Wu *et al.*, 2006) ainsi que l'implication des protéines LptA et LptB (Sperandeo *et al.*, 2007) dans le transport des LPS de la membrane interne à la membrane externe.

2.5. Autres facteurs de virulence

Des fimbriae de type IV constitués de sous-unités protéiques appelées ApfA, ont été identifiés à la surface d'*A. pleuropneumoniae* (Zhang *et al.*, 2000b). La biosynthèse du fimbriae de type IV est codée par l'opéron *apfABCD* qui présente de fortes homologies avec les gènes retrouvés chez *Haemophilus ducreyi*. Le gène *apfA* code pour un précurseur de 16 kDa possédant une séquence signal de 13 acides aminés qui est ensuite clivée pour donner la protéine mature ApfA de 14.5 kDa. Le clivage de la séquence signal se ferait par une séquence signal peptidase codée par le gène *apfD*. Les protéines ApfB, ApfC et ApfD semblent être respectivement les équivalents des protéines PilB, PilC et PilD de *Pseudomonas aeruginosa* (Stevenson *et al.*, 2003). Il a été démontré que ces fimbriae de type IV d'*A. pleuropneumoniae* étaient exprimés *in vivo* et lors de l'adhésion aux cellules épithéliales, mais pas dans des conditions normales de croissance *in vitro* (Boekema *et al.*, 2004).

A. pleuropneumoniae est capable de sécréter des protéases dans le milieu qui peuvent également être relachées dans des vésicules de membrane externe (Negrete-Abascal *et al.*, 2000). Une métalloprotéase zinc-dépendante de 101 kDa a été identifiée chez *A.*

pleuropneumoniae (Garcia Gonzalez *et al.*, 2004). Cette protéase peut s'oligomériser ou être clivée en des protéines de plusieurs tailles conservant leur activité protéolytique (Negrete-Abascal *et al.*, 1994; Negrete-Abascal *et al.*, 1998). Il a été démontré que cette protéase pouvait hydrolyser les immunoglobulines (IgA et IgG) et l'hémoglobine porcine et qu'elle était présente chez tous les sérotypes (Negrete-Abascal *et al.*, 1998).

Deux formes de superoxyde dismutase (SOD) codées par les gènes *sodA* et so*dC* ont été identifiées chez *A. pleuropneumoniae* (Langford *et al.*, 1996). SodA est une SOD manganèse dépendante (Mn SOD) qui n'est pas exprimée en condition anaérobie. SodC est une SOD cuivre, zinc-dépendante (Cu,Zn SOD) exprimée de façon constitutive. La caractérisation de SodC a montré la présence d'une séquence signal en N-terminal de la protéine qui permettrait son exportation dans le périplasme où elle serait localisée. Le rôle exact de SodC dans la virulence d'*A. pleuropneumoniae* n'est pas connu mais elle permettrait de pronlonger la survie de la bactérie dans les phagosomes en éliminant les ions superoxyde produits par les phagocytes (Kroll *et al.*, 1995; Langford *et al.*, 1996) (Forest *et al.*, 2000). Cependant, un mutant *sodC* d'*A. pleuropneumoniae* n'a montré aucune atténuation de virulence dans un modèle d'infection expérimentale (Sheehan *et al.*, 2000).

L'uréase chez *A. pleuropneumoniae* est codée par une région d'ADN de 6.3 kb comprenant les gènes de structure *ureABC* et les gènes accessoires *ureEFGD* en aval (Bosse & MacInnes, 1997). L'importance de l'uréase dans la pathogenèse *d'A. pleuropneumoniae* est controversée. En effet, les premières études ont démontré qu'*A. pleuropneumoniae* ne requérait pas l'activité uréase pour le développement de la pleuropneumonie aiguë chez le porc (Tascon Cabrero *et al.*, 1997). Cependant, d'autres travaux plus récents ont montré que 2 mutants uréase-négatifs affectés au niveau du gène *cbiK*, impliqué dans le transport du cofacteur nickel ou au niveau du gène accessoire *ureG*, étaient atténués (Bosse & MacInnes, 2000). L'utilisation d'un mutant *ureC* dans un modèle d'infection expérimentale a montré un rôle de l'uréase dans la persistance de la bactérie au niveau des poumons, en affectant la réponse immunitaire (Baltes *et al.*, 2001). D'autre part, l'implication du gène *ureC* dans la conception d'un vaccin atténué DIVA suggère

également un rôle de l'uréase dans la pathogenèse d'*A. pleuropneumoniae* (Maas *et al.*, 2006; Tonpitak *et al.*, 2002)

2.6. Les mutants LPS d'*A. pleuropneumoniae* sérotype 1

Plusieurs mutants LPS d'*A. pleuropneumoniae* sérotype 1 ont été générés dans notre laboratoire par mutagenèse par transposition à l'aide du transposon mini-Tn*10*. Suite au criblage d'une banque de mutants en présence de novobiocine dans le but d'obtenir des mutants LPS, 7 mutants rugueux (1.1, 15.1, 51.1, 44.1, 27.1, 24.1 et 36.1) et 4 mutants du noyau ou « mutants core » (5.1, CG1, CG3, CG5) on été isolés (Galarneau *et al.*, 2000; Rioux *et al.*, 1999). Les mutants rugueux ont été identifiés par l'absence de réactivité avec un anticorps monoclonal dirigé contre l'antigène O alors que les mutants core, qui possèdent encore leur antigène O, ont été identifiés selon leur mobilité électrophorétique. L'analyse du site d'insertion du mini-Tn*10* a permis l'identification des gènes affectés dans chaque mutant.

Les gènes mutés chez les mutants rugueux se retrouvent dans une région de 18 cadres ouverts de lecture (ou ORFs) (Labrie *et al.*, 2002) qui est homologue à un cluster de gènes chez *A. actinomycetemcomitans* Y4 sérotype b, impliqué dans la biosynthèse des polysaccharides O. Ainsi, les mutants 1.1 et 15.1 sont mutés au niveau de l'ORF12 qui code pour une protéine présentant des homologies avec des glycosyltransférases. Le mutant 51.1 et le mutant 44.1 sont mutés respectivement dans l'ORF16 et l'ORF17 qui codent pour des protéines homologues aux rhamnosyltransférases. Les mutants 27.1, 24.1 et 36.1 sont mutés dans l'ORF18 qui code pour une protéine qui serait impliquée dans l'initiation de la synthèse du polysaccharide.

Le mutant core 5.1 est muté dans le gène *galU* qui code pour l'UTP-α-D-glucose-1-phosphate uridylyltransférase, une enzyme impliquée dans la synthèse de l'UDP-glucose. Cette enzyme présente respectivement 87% et 83% d'homologies avec celles retrouvées chez *H. ducreyi* et *H. influenzae*. Le gène muté chez le mutant CG 1 code pour une protéine ayant respectivement 25% et 29% d'homologie avec la galactosyltransférase WlaC et la N-acétylgalactosamine transférase WlaE de *Campylobacter jejuni*. Les mutants CG3 et CG5 sont tous les deux mutés dans le gène *lbgB* codant pour une D-glycéro-D-manno-heptosyl

transférase. La caractérisation phénotypique de ces mutants core suggère des niveaux de troncation différents du noyau OS selon la migration électrophorétique. Ainsi, le mutant CG1 serait le moins tronqué, suivi du mutant 5.1 et des mutants CG3 et CG5.

L'étude des propriétés biologiques de ces mutants a montré que tous les mutants LPS étaient sensibles au sérum de porc suggérant un rôle général du LPS dans la résistance au sérum. L'étude des mutants rugueux a démontré que la perte de l'antigène O n'avait pas d'effet sur l'adhérence de la bactérie aux cellules de l'appareil respiratoire du porc, et sur la virulence de la bactérie dans un modèle d'infection expérimentale chez le porc. Par contre, le mutant core 5.1 présente une adhérence diminuée et une virulence atténuée (Rioux *et al.*, 1999). De plus, les mutants core CG3 et CG5 sont également moins adhérents (Galarneau *et al.*, 2000). Cependant, il a été démontré que le mutant CG1, qui semble être le moins tronqué des mutants core, n'était pas affecté au niveau de ses capacités d'adhésion. L'ensemble de ces résultats suggère que le noyau OS du LPS, et non l'antigène O, jouerait un rôle important dans la pathogenèse d'*A. pleuropneumoniae* et que le niveau de troncation du noyau OS serait critique pour les propriétés de virulence du LPS.

3. RÔLE DU LPS DANS LA PATHOGENÈSE

3.1. LPS et adhérence bactérienne

Le processus d'attachement de la bactérie à la cellule hôte est considéré comme l'une des premières étapes de la colonisation et de l'infection. Plusieurs travaux ont suggéré un rôle du LPS dans l'adhérence bactérienne (Genevaux *et al.*, 1999; Jacques, 1996) et les LPS semblent aussi jouer un rôle dans la colonisation du tractus intestinal (Martindale *et al.*, 2000). Par contre, son rôle comme adhésine chez *E. coli* reste controversé car l'utilisation d'anticorps dirigés contre les antigènes O des LPS O111 et O157 diminue l'adhérence des EHEC à des cellules en culture alors que la préincubation de cellules avec des LPS n'inhibent pas l'adhésion (Paton *et al.*, 1998). Cependant, chez *A. pleuropneumoniae*, le LPS représente une adhésine majeure permettant la colonisation du tractus respiratoire du porc par la bactérie. En effet, il a été démontré que des LPS purifiés pouvaient bloquer l'adhérence d'*A. pleuropneumoniae* à des sections de trachée de porc (Bélanger *et al.*,

1990; Paradis *et al.*, 1994). D'autres études ont souligné l'importance du LPS et non de la capsule dans l'adhérence d'*A. pleuropneumoniae*. Ainsi, il a été proposé que la capsule masquerait les sites d'adhésion au niveau des LPS car les souches ayant une capsule épaisse étaient moins adhérentes (Bélanger *et al.*, 1992; Bélanger *et al.*, 1994). De plus, il a été démontré que le mutant acapsulé 33.2 d'*A. pleuropneumoniae* sérotype 1 était plus adhérent à des sections de trachée de porc que la souche sauvage (Rioux *et al.*, 2000). La propriété d'adhérence du LPS d'*A. pleuropneumoniae* avait tout d'abord été associée à la partie polysaccharidique et non au lipide A (Paradis *et al.*, 1994). Par la suite, l'étude de mutants LPS *d'A. pleuropneumoniae* sérotype 1 a permis de restreindre la propriété d'adhérence au noyau OS du LPS (Galarneau *et al.*, 2000; Rioux *et al.*, 1999). Étant donné le rôle du LPS comme adhésine majeure d'*A. pleuropneumoniae*, l'attachement de la bactérie lors de la colonisation suppose la présence de récepteurs spécifiques à la surface des cellules respiratoires porcines. Ainsi, il a été démontré que le LPS se fixait spécifiquement au phosphatidylétanolamine de la membrane des cellules eucaryotes (Jeannotte *et al.*, 2003).

3.2. LPS et système immunitaire inné

3.2.1. Rôle du LPS dans l'inflammation

Le but ultime de l'immunité innée est la reconnaissance du pathogène par des cellules inflammatoires telles que les macrophages conduisant à la phagocytose et à l'induction de médiateurs de l'inflammation permettant de détruire le micro-organisme (Bals & Hiemstra, 2004). Cependant, lors d'une infection importante, la surproduction de composés inflammatoires entraîne une destruction massive des tissus et des vaisseaux pouvant conduire au choc septique (Russell, 2006). Le LPS contribue ainsi à la pathogenèse d'*A. pleuropneumoniae* en stimulant l'inflammation grâce aux propriétés d'endotoxine du lipide A. En effet, le lipide A du LPS est reconnu par le TLR-4 (toll-like receptor 4) à la surface membranaire des cellules inflammatoires telles que les macrophages (Miller *et al.*, 2005), induisant ainsi une cascade de transduction de signaux conduisant à l'inflammation (Akira *et al.*, 2006) (Figure 10). Au niveau pulmonaire, l'immunité innée est médiée par des leucocytes comme les neutrophiles et les macrophages alvéolaires mais également par les

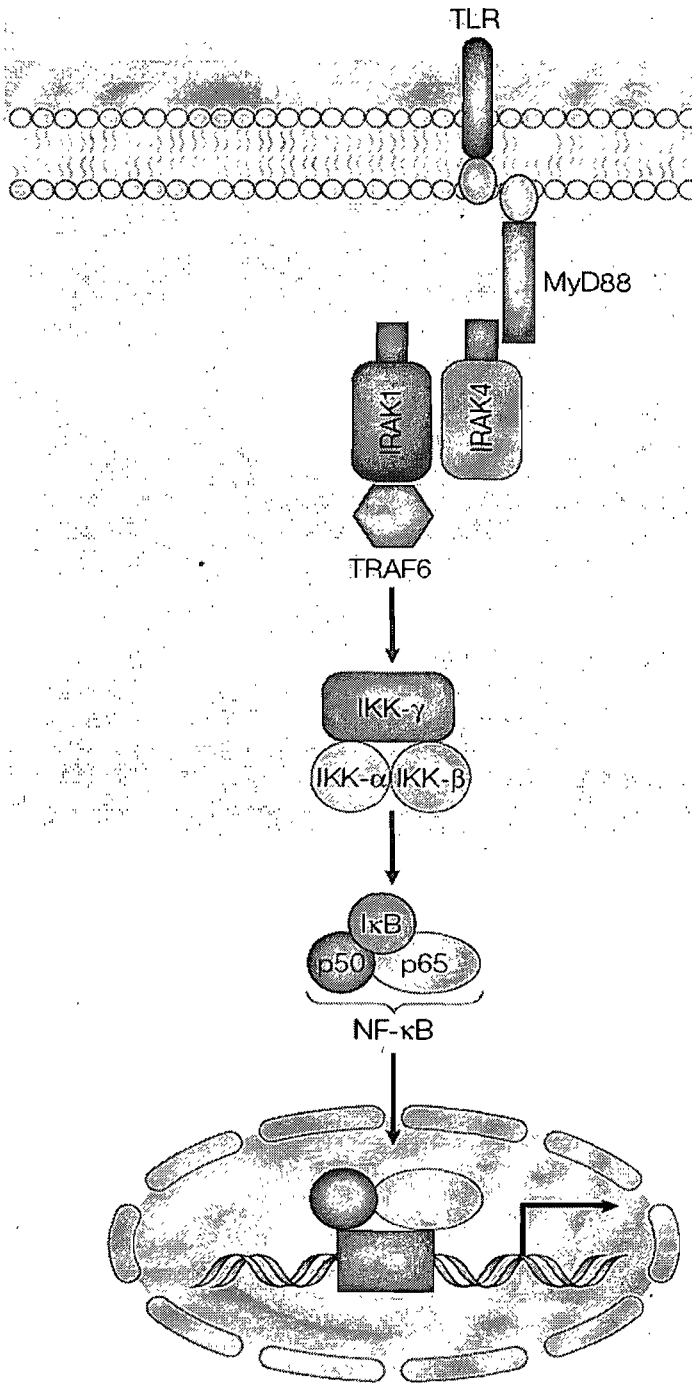


Figure 10. Voie de signalisation des « Toll-like receptors » (TLRs). La stimulation du domaine extracellulaire du TLR entraîne une cascade de transduction de signaux impliquant les protéines MyD88, IRAK4, IRAK1, TRAF6 et toute une série d'autres intermédiaires, menant à la phosphorylation du complexe IκB kinase (IKK). Le complexe phosphoryle à son tour IκB qui permet la translocation de l'hétérodimère actif p50/p65 de NF-κB dans le noyau. Adaptée de Miller *et al.*, 2005.

cellules épithéliales des voies respiratoires et des alvéoles pulmonaires (Zaas & Schwartz, 2005). Ainsi, il a été démontré que la stimulation de macrophages alvéolaires porcins par des LPS d'*A. pleuropneumoniae* entraînait la production de IL-1, TNF-α et IL-8 (Huang *et al.*, 1999) et que la présence de cytokines inflammatoires lors d'une infection par *A. pleuropneumoniae* était associée à l'intensité des lésions pulmonaires (Choi *et al.*, 1999). D'autre part, ces cytokines inflammatoires ont aussi été détectées dans des lavages bronchoalvéolaires de poumons provenant de porcs infectés par *A. pleuropneumoniae* (Baarsch *et al.*, 1995).

L'activation du TLR-4 par le LPS est complexe et requiert au moins 3 autres protéines, le CD14, le LBP (LPS binding protein) et le MD-2. La reconnaissance du LPS par TLR-4 dépend de MD-2 alors que la sensibilité du récepteur TLR-4/MD-2 pour le LPS est augmentée par le CD14 et la LBP (Shimazu *et al.*, 1999; Underhill & Ozinsky, 2002). La complexité de l'interaction LPS/TLR-4 est encore accentuée par le polymorphisme de la région extracellulaire de TLR-4 impliquée dans la reconnaissance ainsi que par la diversité de structure du lipide A chez les espèces bactériennes. En effet, le TLR-4 chez l'humain est plus spécifique que celui de la souris et par conséquent réagit moins aux différents types de lipide A (Miller *et al.*, 2005). Ceci a donc une implication directe sur la résistance ou la sensibilité de l'organisme à certaines infections. Il a été démontré que la longueur et le nombre de chaînes d'acyles du lipide A déterminait la conformation du LPS et par conséquent modifiait la spécificité de reconnaissance du LPS par le TLR-4 (Schromm *et al.*, 1998; Schromm *et al.*, 2000). Ainsi, le lipide A hexa-acylé constitué d'acides gras à 12 ou 14 atomes de carbone adopte une structure conique qui a un pouvoir stimulateur maximal chez l'humain (Schromm *et al.*, 2000). Ce lipide A hexa-acylé se retrouve chez la plupart des bactéries dont *A. pleuropneumoniae*. À l'inverse, des LPS possédant un lipide A constitué uniquement de 4 à 5 chaînes d'acides gras avec souvent 16 à 18 atomes de carbones, comme ceux retrouvés chez *Helicobacter pylori, Legionella pneumophila, Yersinia pestis* ou encore *Francisella*, stimulent moins les TLR-4 humains et peuvent même agir comme antagonistes (Golenbock *et al.*, 1991; Moran *et al.*, 1997; Smith *et al.*, 2003). Cette diversité retrouvée au niveau de la structure du lipide A peut s'avérer intéressante du point de vue de la bactérie. En effet, le LPS hexa-acylé peut contribuer à la pathogenèse de la bactérie en stimulant une réponse inflammatoire qui entraîne par la

même occasion la destruction des tissus de l'hôte, comme c'est le cas pour les lésions pulmonaires observées lors d'infections par *A. pleuropneumoniae*. À l'inverse, un LPS possédant un lipide A tétra- ou penta-acylé peut permettre à la bactérie d'échapper au système immunitaire de l'hôte pour pouvoir exercer son pouvoir pathogène. Certaines bactéries peuvent même réguler et moduler la structure de leur lipide A selon l'environnement dans lequel elles se trouvent. Ainsi *Y. pestis* produit un LPS très acylé à 21°C qui est résistant aux peptides antimicrobiens présents chez les insectes alors qu'à 37°C, elle produit un LPS tétra-acylé qui n'est pas reconnu par le TLR-4 humain (Miller *et al.*, 2005). Suite à la reconnaissance du LPS par le domaine extracellulaire de TLR-4, le domaine intracellulaire TIR (Toll/interleukin-1 (IL-1) receptor) interagit avec la protéine adaptatrice MyD88 (myeloid differentiation factor 88) (Figure 10). La stimulation de MyD88 permet le recrutement et l'activation des protéines IRAK-1 (interleukin-1 receptor-associated kinase 1) et TRAF-6 (TNF-receptor-associated factor 6) menant ensuite à deux voies de signalisation distinctes. Une voie mène à l'activation des facteurs de transcription AP-1 alors que l'autre voie mène à l'activation du facteur de transcription NF-κB (Medzhitov, 2001; Takeda & Akira, 2005). Ces facteurs de transcription vont ensuite activer les gènes codant pour les médiateurs de l'inflammation tels que les cytokines inflammatoires ou encore les peptides antimicrobiens (Diamond *et al.*, 1996; Diamond & Bevins, 1998).

NF-κB est constitué de l'association en homo- ou hétérodimères de 5 protéines Rel : p65 (RelA), c-Rel, p50, p52 et RelB (Ghosh *et al.*, 1998). On distingue une voie classique et une voie alternative d'activation de NF-κB. L'hétérodimère p50/p65, qui est le plus abondant et le plus actif des complexes (Baeuerle & Henkel, 1994), est impliqué dans la voie classique alors que la voie alternative est elle représentée par le complexe p52/RelB (Figure 11). Parmi les 5 sous unités, p65, RelB et c-Rel possèdent un domaine de transactivation en C-terminal qui est essentiel à l'activation des gènes. À l'inverse, les protéines p50 et p52 ne possèdent pas de domaine de transactivation et ne peuvent donc pas agir seules comme facteur de transcription (Lernbecher *et al.*, 1993). De plus, p50 et p52 appartiennent à la famille des IκB qui sont des inhibiteurs de la voie NF-κB. Ainsi, il a été démontré que les homo- ou hétérodimères de p50 et p52 réprimaient la transcription *in vivo* de gènes contrôlés par NF-κB (Lernbecher *et al.*, 1993).

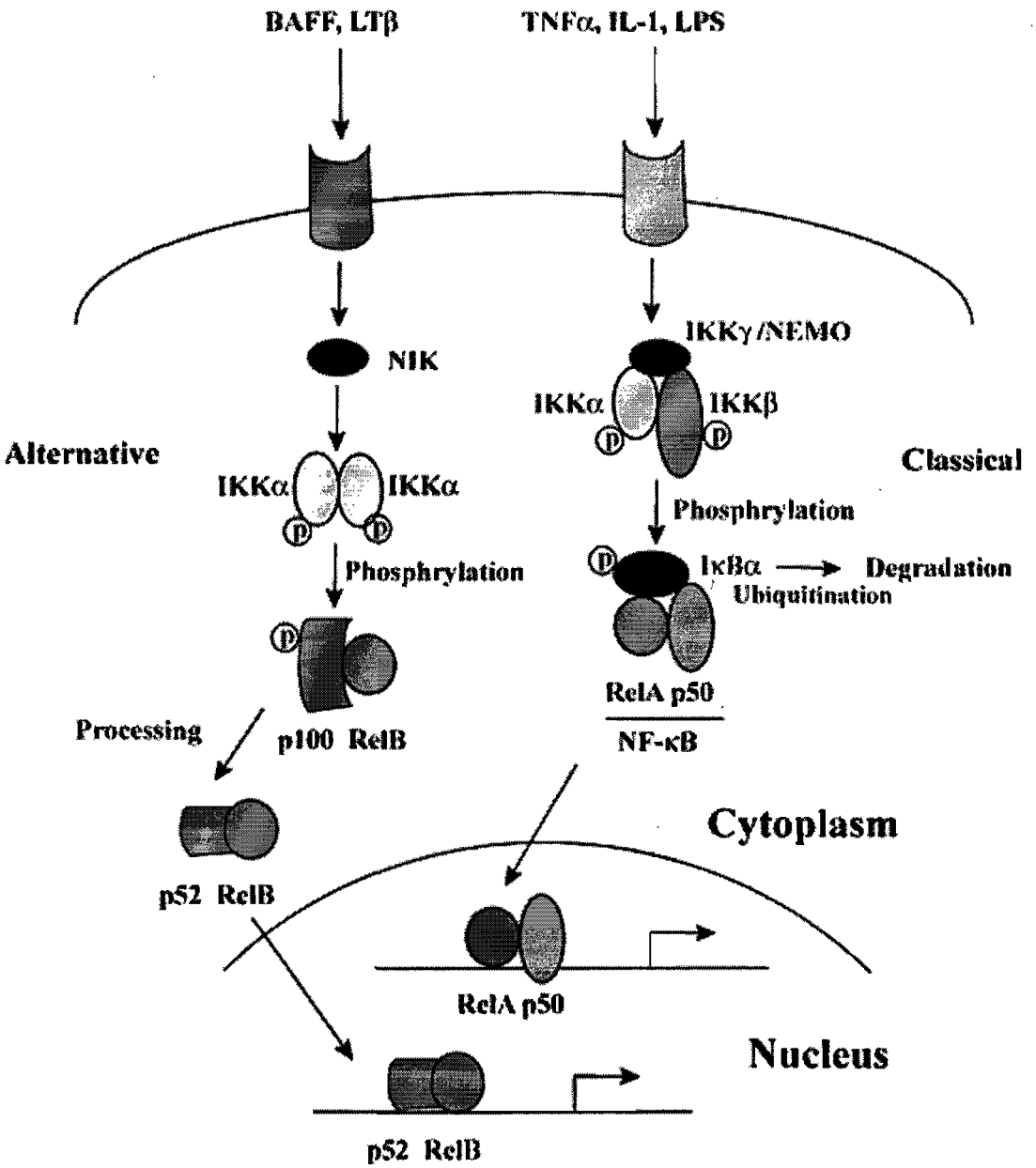


Figure 11. Voies classique et alternative de signalisation de NF-κB. La voie classique, représentée par la translocation du complexe p50/RelA(p65), peut être activée par le LPS, le TNF-α et l'IL-1β. La voie alternative, correspondant à la translocation de l'hétérodimère p52/RelB, peut être activée par la lymphotoxine (LT) β ou le BAFF (B cell activating factor belonging to the TNF family). NIK : « NF-κB inducing kinase ». Adaptée de Maeda & Omata, 2008.

3.2.2. LPS et peptides antimicrobiens

Les peptides antimicrobiens (AMPs), également appelés antibiotiques peptidiques, sont de petites molécules (12 à 100 acides aminés) produites par un grand nombre d'espèces (vertébrés, invertébrés, plantes et bactéries) et qui sont actifs contre les bactéries, les virus et les champignons. Ces peptides sont généralement amphiphatiques et possèdent donc des régions hydrophiles leur permettant d'être solubles en milieu aqueux et des régions hydrophobes leur permettant d'interagir avec les lipides membranaires. La plupart des AMPs sont cationiques et s'attachent aux bactéries grâce à des interactions électrostatiques avec les charges négatives de la membrane bactérienne, notamment les LPS et les acides téichoïques (Brogden, 2005; Zhang *et al.*, 2000a). Cependant, il existe aussi des peptides anioniques qui sont surtout retrouvés au niveau du tractus respiratoire, notamment dans le surfactant, les lavages bronchoalvéolaires et les cellules épithéliales des voies respiratoires. Ces peptides anioniques ont une petite taille qui peut varier entre 630 et 3400 Da, possèdent des régions riches en aspartate ou en glutamate et ont généralement besoin du zinc comme cofacteur pour leur activité bactéricide (Brogden *et al.*, 1996; Brogden *et al.*, 1998; Brogden *et al.*, 1999). Il a aussi été démontré que certains fragments de protéines, comme la lactoferricine correspondant à la partie cationique N-terminale de la lactoferrine, possédaient des propriétés antimicrobiennes (Arnold *et al.*, 1982; Ganz, 2004). À la différence des antibiotiques qui sont synthétisés enzymatiquement, les AMPs sont directement codés par des gènes. On distingue 3 grandes classes de peptides antimicrobiens basées sur leur composition en acides aminés et leur structure:

- les peptides linéaires sans cystéines qui adoptent une structure en hélice α (ex : cécropine et magainine)
- les peptides avec des cystéines formant des ponts disulfures et possédant une structure en feuillets β (ex : défensine et protégrine)
- les peptides riches en certains acides aminés comme la proline, l'arginine, la phénylalanine ou le tryptophane (ex : PR-39 et indolicidine). Ces peptides ne possèdent pas de cystéines et sont linéaires.

Les mécanismes d'action des AMPs sont très divers et dépendent principalement de leur taille, leur charge, leur structure et leur caractère hydrophobe ou amphiphatique (Brogden, 2005). Les AMPs sont tout d'abord attirés par les charges négatives présentes à la surface bactérienne. Ainsi, chez les bactéries Gram positives on retrouve les acides téichoïques alors qu'au niveau de la membrane externe des bactéries Gram négatives on retrouve les LPS. Les peptides anioniques, malgré leur charge, sont également attirés par les membranes bactériennes car ils sont souvent complexés au zinc (Zn^{2+}) (Brogden *et al.*, 1996). Suite à l'attraction, les AMPs vont s'attacher et s'accumuler parallèlement à la surface de la bactérie. À ce jour on connaît 3 principaux modèles utilisés par les AMPs pour augmenter la perméabilité membranaire :

Lorsque le ratio peptide/lipide augmente, les peptides attachés de façon parallèle à la double couche lipidique vont se réorienter perpendiculairement à la surface bactérienne pour s'insérer dans la membrane et former des pores grâce à des hélices α transmembranaires. Les hélices sont orientées de façon à ce que les acides aminés hydrophobes interagissent avec les lipides membranaires et que les régions hydrophiles forment le lumen du pore. Ce mécanisme appelé « barrel-stave model » (Figure 12A) est utilisé notamment par l'alaméthicine (Bechinger, 1999; Yang *et al.*, 2001).

Dans le modèle « carpet-like » (Figure 12B), les peptides s'accumulent en restant parallèles à la membrane pour former une structure en carpette recouvrant ainsi la surface. La membrane est ensuite désintégrée par la formation de micelles ou encore la formation de pores « toroïdaux » lorsque la concentration en peptides est très élevée. Ce modèle est utilisé par des AMPs comme la dermaseptine S (Pouny *et al.*, 1992), la cécropine (Gazit *et al.*, 1995; Shai, 1995), la mélittine (Naito *et al.*, 2000), la caérine 1.1 (Wong *et al.*, 1997) et l'ovispirine (Yamaguchi *et al.*, 2001).

Le modèle « toroidal-pore » (Figure 12C), consiste en la formation de pores transmembranaires qui sont cependant différents de ceux retrouvés dans le modèle « barrel-stave ». En effet, les têtes polaires des phospholipides de la double couche lipidique s'associent aux régions hydrophiles des hélices transmembranaires pour former le lumen du pore alors que dans le modèle « barrel-stave », la lumière du pore n'est formée que des régions hydrophiles des hélices. Ce modèle « toroidal-pore » est utilisé notamment par la magainine-2 (Yang *et al.*, 1998), la protégrine-1 (Yamaguchi *et al.*, 2002), la mélittine (Lee

et al., 2004; Yang *et al.*, 2001), LL-37 (Henzler Wildman *et al.*, 2003) et la MSI-78 (Hallock *et al.*, 2003).

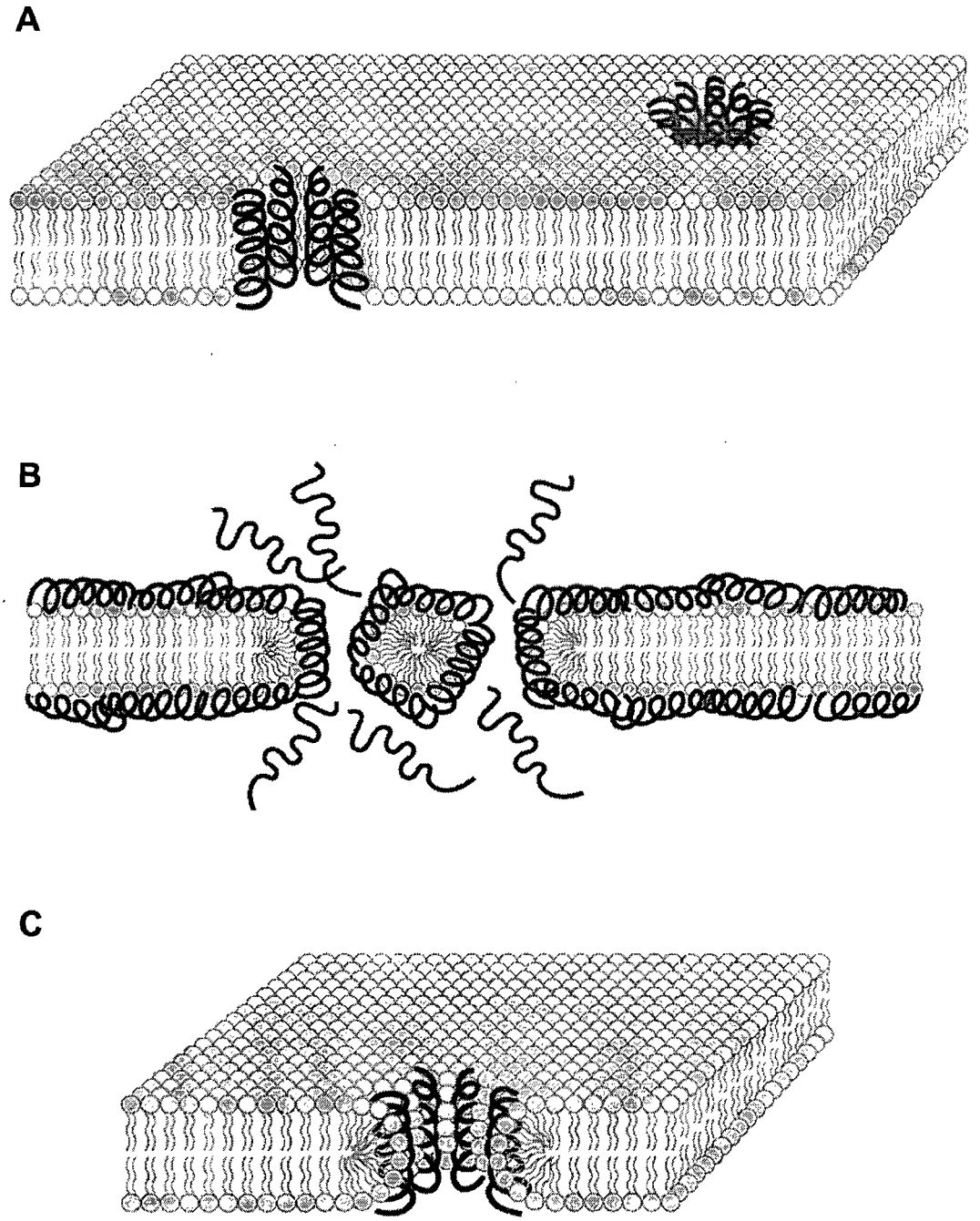


Figure 12. Représentation des trois principaux modèles utilisés par les AMPs pour augmenter la perméabilité membranaire. Le modèle « barrel-stave » (A), le modèle « carpet-like » (B) et le modèle « toroidal-pore ». Adaptée de Brogden, 2005.

Au-delà de l'augmentation de la perméabilité membranaire, qui représente le mécanisme classique par lequel les AMPs tuent les bactéries, d'autres fonctions leur sont attribuées. En effet, certains peptides comme la buforine II (Park *et al.*,, 2000) peuvent traverser la membrane cytoplasmique sans la perméabiliser pour agir sur des cibles intracellulaires et inhiber les mécanismes bactériens tels que la division cellulaire, la synthèse de la paroi, la synthèse protéique ou encore des activités enzymatiques. De même, l'indolicidine inhibe la synthèse d'ADN et d'ARN chez *E. coli* et est aussi impliquée dans le blocage de la division cellulaire (Subbalakshmi & Sitaram, 1998). Le peptide PR-39 bloque la synthèse protéique et induit la dégradation de certaines protéines impliquées dans la réplication de l'ADN (Boman *et al.*, 1993). Les défensines HNP-1 et HNP-2 (human neutrophil peptide) réduisent la synthèse d'ADN, D'ARN et de protéines et inhibent la synthèse de la β-galactosidase périplasmique (Lehrer *et al.*, 1989). La mersacidine inhibe la synthèse du peptidoglycane en interférant avec la réaction de transglycosylation (Brotz *et al.*, 1998).

Chez le porc, à l'exception de la cécropine P1 (Sipos *et al.*, 1992) et de la NK lysine (Andersson *et al.*, 1995), tous les AMPs identifiés appartiennent à la famille des défensines et des cathélicidines (Brogden *et al.*, 2003; Selsted & Ouellette, 2005; Zhang *et al.*, 2000a). Les défensines sont des peptides cationiques de 3 à 5 kDa qui se caractérisent par la présence de 6 ou 8 résidus cystéines formant des ponts dissulfures intramoléculaires. Selon la position des cystéines et les liaisons formant les ponts, on distingue les défensines α, β et θ. Au niveau structural, les défensines sont généralement constituées de 2 à 3 feuillets β anti-parallèles avec ou sans hélice α (Hoffmann *et al.*, 1999). À ce jour, aucune α- ou θ-défensine n'a été identifié chez le porc. On retrouve cependant 2 β-défensines, pBD-1 et pBD-2 qui ont été respectivement identifiées dans les cellules épithéliales de langue (Shi *et al.*, 1999) et les cellules épithéliales de l'intestin (Veldhuizen *et al.*, 2008). D'autre part, des études bioinformatiques ont suggéré l'existence d'autres β-défensines porcines (Sang *et al.*, 2006).

Les cathélicidines se caractérisent par une proséquence conservée en N-terminal qui présente de fortes homologies avec la cathéline (Ritonja *et al.*, 1989) et un domaine C-terminal très variable qui constitue le peptide mature. Les cathélicidines sont synthétisées et stockées dans les granules des cellules polymorphonucléaires (PMNs) sous forme de

propeptides qui seront ensuite maturées par une élastase endogène lors de l'activation et de la dégranulation des PMNs. Les cathélicidines porcines incluent le peptide PR-39, les protégrines 1-5, les prophénines-1,2 et les PMAP-23,36,37 (Brogden *et al.*, 2003).

En ce qui concerne le lien entre le LPS et les AMPs dans la pathogenèse bactérienne, il existe plusieurs mécanismes de résistance aux AMPs basés sur la modification de la surface bactérienne. Certaines bactéries Gram négatives peuvent modifier leur LPS pour empêcher l'attachement ou l'insertion des AMPs dans la membrane. En effet, la neutralisation des charges négatives présentes sur les phosphates du lipide A permet de diminuer les interactions électrostatiques nécessaires à l'attachement des peptides cationiques et ainsi augmenter la résistance de la bactérie aux AMPs. *E. coli* et *Salmonella* modifient leur lipide A en ajoutant des résidus chargés positivement au niveau des phosphates tels que l'aminoarabinose ou la phosphoéthanolamine, pour réduire la charge négative globale du LPS (Raetz *et al.*, 2007; Trent, 2004). D'autres bactéries comme *Rhizobium leguminosarum* possèdent des phosphatases qui enlèvent les phosphates au niveau du disaccharide du lipide A (Karbarz *et al.*, 2003). Ainsi, la diminution des phosphates au niveau du LPS pourrait être un mécanisme de résistance aux peptides cationiques. Pour empêcher l'insertion des AMPs dans la membrane, certaines bactéries réduisent également la fluidité membranaire en augmentant les interactions hydrophobes entre les LPS par l'ajout de chaînes d'acyles supplémentaires au lipide A. Ainsi, *S. typhimurium* produit un LPS hepta-acylé grâce à l'ajout d'un palmitate par la palmitoyltransférase PagP, lui permettant ainsi d'être plus résistante aux AMPs (Guo *et al.*, 1998). La plupart des modifications du lipide A sont sous le contrôle du régulateur global PhoP/PhoQ (Ernst *et al.*, 1999; Raetz *et al.*, 2007; Trent, 2004). Malgré l'importance de la structure et de la charge du lipide A dans la résistance aux AMPs, d'autres études ont également souligné un rôle important du noyau OS du LPS. Il a été démontré qu'un mutant *phoP* de *Y. pestis*, n'exprimant plus l'isoforme du LOS qui possède un galactose terminal au niveau du noyau, était plus sensible à la cécropine, au mastoparan et à la polymyxine B (Hitchen *et al.*, 2002). Les mutants deep rough *rfaH*, *waaG* et *waaP* de *S. enterica* sont plus sensibles à la polymyxine B (Nagy *et al.*, 2006; Yethon *et al.*, 2000). Les mutants core *galU* de *V. cholerae* (Nesper et al., 2001) et *lpsB* de *S. meliloti* (Campbell *et al.*, 2002) sont également plus sensibles aux AMPs. Chez *Burkholderia cenocepacia*, l'étude de mutants affectés au niveau des gènes *hldA* and *hldD*

impliqués dans la biosynthèse du noyau OS, a montré l'importance du noyau interne dans la résistance à la polymyxine B, la mélittine, et la défensine HNP-1 (Loutet *et al.*, 2006). L'ensemble de ces travaux suggère donc un rôle important du LPS et en particulier du lipide A et du noyau OS, dans la pathogenèse bactérienne.

3.3. Synergie entre le LPS et les toxines RTX

Comme il a été mentionné ci-dessus, le LPS est un facteur de virulence important impliqué dans de nombreux mécanismes de virulence chez *A. pleuropneumoniae*. Au-delà de son rôle direct dans la pathogenèse en tant qu'adhésine ou endotoxine et même dans la résistance aux peptides antimicrobiens, le LPS peut également interagir avec d'autres facteurs de virulence. Ainsi, chez *E. coli*, une corrélation a été retrouvée entre l'expression de la protéine OmpA et la capacité de cette protéine à interagir avec le LPS (Beher *et al.*, 1980). La synthèse et l'assemblage des porines OmpC et OmpF au niveau de la membrane externe est affectée chez une souche possédant un LPS avec un core oligosaccharidique tronqué (Ried *et al.*, 1990). Une interaction entre le LPS et le récepteur du ferrichrome FhuA a également été mise en évidence chez *E. coli* (Ferguson *et al.*, 2000).

En ce qui concerne les toxines RTX, de nombreux travaux ont démontré une synergie entre le LPS et ces toxines chez plusieurs bactéries. La perfusion de poumons de lapins avec du LPS accroît l'effet de l'hémolysine HlyA d'*E. coli* dans l'augmentation de la perméabilité vasculaire et la formation d'œdème pulmonaire (Schutte *et al.*, 1997). Il a été démontré que la toxine Lkt de *M. haemolytica* et le LPS agissent de concert pour activer l'expression de l'intégrine LFA-1 par les PMNs, contribuant ainsi à l'inflammation pulmonaire lors de la pasteurellose bovine (Leite *et al.*, 2003). L'activation du système immunitaire par l'adénylate cyclase CyaA de *Bordetella pertussis* (Boyd *et al.*, 2005; Ross *et al.*, 2004) et la leukotoxine Lkt de *M. hemolytica* (Lafleur *et al.*, 2001) est également augmentée par la présence de LPS. Le rôle complémentaire que joue le LPS dans le pouvoir pathogène des toxines RTX permet donc de suggérer l'existence d'une association physique entre ces 2 facteurs de virulence d'un point de vue physiologique. En effet, les tests de dosage du LPS (LAL, Limulus amebocyte lysate) ont montré que le LPS était souvent retrouvé comme contaminant dans les préparations de toxines purifiées, ce qui

conforte l'idée d'une éventuelle association entre le LPS et les toxines RTX (Czuprynski & Welch, 1995; Stevens & Czuprynski, 1995). D'autre part, cette association jouerait un rôle direct, par des mécanismes qui ne sont pas complètement élucidés, sur l'activité cytolytique et/ou hémolytique de la toxine. Ainsi, la formation de complexes entre le LPS et la toxine Lkt permettrait de stabiliser et d'augmenter l'activité cytolytique de la toxine (Li & Clinkenbeard, 1999; Zecchinon *et al.*, 2005). Concernant la spécificité de l'interaction LPS/RTX et les régions du LPS impliquées dans cette interaction, les études restent controversées. En effet, on pourrait facilement concevoir des interactions entre le lipide A du LPS et les régions hydrophobes de la toxine, notamment la partie N-terminale et les chaînes acyles (voir section 2.3). Ainsi, il a été proposé que le LPS et l'hémolysine HlyA interagissent via des interactions hydrophobes pour augmenter la stabilité et l'activité de la toxine (Herlax *et al.*, 2005). Par ailleurs, l'expression de la toxine recombinante rHlyA chez des souches lisses et rugueuses de *Salmonella typhimurium* et *Klebsiella pneumoniae* a montré que la présence de l'antigène O était fortement corrélée à l'efficacité de la toxine (Camprubi *et al.*, 1990).

De nombreux travaux chez *E. coli* ont également souligné l'importance du noyau OS dans la synergie entre le LPS et les toxines RTX grâce à l'utilisation de mutants. Ces mutants sont ainsi affectés différemment selon le gène muté et les résidus tronqués par la mutation. Un mutant *rfaC* possédant un LPS sans noyau externe et avec un noyau interne tronqué constitué uniquement de Kdo (Schnaitman & Klena, 1993) possède une toxine HlyA qui est affectée au niveau de son expression, sa sécrétion et son activité hémolytique (Bauer & Welch, 1997). Un autre mutant du noyau interne *rfaP* qui lui possède encore son noyau externe (Schnaitman & Klena, 1993) est affecté uniquement au niveau de son activité hémolytique extracellulaire (Bauer & Welch, 1997; Stanley *et al.*, 1993). D'autres études ont montré que le noyau externe serait également impliqué dans la modulation de l'activité hémolytique. Ainsi, le mutant du noyau externe *rfaJ* montre une légère réduction de son activité hémolytique (Bauer & Welch, 1997; Schnaitman & Klena, 1993). Un mutant *galU* ne possédant plus de noyau externe (Sanderson & Stocker, 1981) est affecté au niveau de la sécrétion de HlyA (Wandersman & Letoffe, 1993) alors qu'un autre mutant du noyau externe *rfaH* est affecté au niveau de l'expression, de la sécrétion et de l'activité de la toxine (Bailey *et al.*, 1992; Leeds & Welch, 1996; Wandersman & Letoffe, 1993).

L'étude de ces mutants a permis de montrer l'importance du noyau OS dans la synthèse et l'activité de l'hémolysine HlyA. Un modèle d'interaction physique entre le LPS et HlyA impliquant le noyau interne a même été proposé (Bauer & Welch, 1997). Cependant, aucune étude à ce jour n'a démontré une interaction directe entre le noyau OS du LPS et une toxine RTX, qui par conséquent modulerait l'activité de la toxine.

En ce qui concerne la contribution du LPS à l'activité de la toxine, différents mécanismes ont été proposés. Il a été démontré que le LPS augmente la stabilité de l'hémolysine HlyA en la protégeant contre la dénaturation thermique ou chimique (Herlax *et al.*, 2005). Une autre étude propose que les charges négatives présentes sur le LPS serviraient de réservoir de calcium nécessaire à l'activité de la toxine (Ostolaza & Goni, 1995). Un autre rôle possible du LPS serait d'empêcher l'autoagrégation des toxines. En effet, il a été démontré que les toxines RTX ont tendance à former des agrégats inactifs et que la dispersion de ces agrégats par des agents chaotropiques permettait de restaurer l'activité (Ostolaza *et al.*, 1991; Ostolaza *et al.*, 1997; Soloaga *et al.*, 1998; Waurzyniak *et al.*, 1994). Ainsi, la présence des résidus polaires chargés négativement au niveau du LPS augmenterait les répulsions entre les molécules de LPS ce qui réduirait ainsi l'agrégation des toxines dans le cas d'une interaction LPS/RTX (Herlax *et al.*, 2005). Il a été démontré que les mutants core *rfaP* et *rfaC* qui sont moins hémolytiques, expriment une hémolysine (HlyA) qui à tendance à s'agréger (Bauer & Welch, 1997; Stanley *et al.*, 1993). Ceci suggère que le noyau OS serait éventuellement impliqué dans une interaction entre le LPS et HlyA pour empêcher l'autoagrégation de la toxine et la maintenir sous une forme active.

Les toxines Apx sont des facteurs de virulence importants d'*A. pleuropneumoniae* dans la mesure où elles sont en grande partie responsables de la destruction des tissus et des lésions pulmonaires observées lors de la pleuropneumonie porcine (Choi *et al.*, 2001). Cette synergie qui a été proposée entre le LPS et les toxines RTX chez d'autres bactéries suggère que le LPS d'*A. pleuropneumoniae*, en particulier le noyau OS, serait également impliqué dans une interaction LPS/Apx qui modulerait l'expression, la sécrétion ou l'activité des toxines Apx.

4. APPROCHE ET DÉMARCHE SCIENTIFIQUE

4.1. Analyses structurale et biologique des mutants LPS d'*A. pleuropneumoniae* sérotype 1.

Plusieurs mutants LPS d'*A. pleuropneumoniae* sérotype 1 ont été générés par mutagenèse par transposition (voir section 2.6). La caractérisation de ces mutants a permis de suggérer que le noyau OS, contrairement à l'antigène O, jouerait un rôle important dans l'adhérence et la virulence de la bactérie. Ainsi, le mutant « core » 5.1, contrairement au mutant rugueux 27.1, est affecté au niveau de son adhérence et de sa virulence. Cependant, des variations phénotypiques ont été observées chez les mutants « core ». L'analyse électrophorétique a montré des différences de migration de la région noyau-lipide A de ces mutants « core », suggérant des niveaux de troncation différents du noyau OS. D'autre part, l'étude de l'adhérence a montré que le mutant CG1 qui semble être le moins tronqué n'est pas affecté alors que les mutants 5.1, CG3 et CG5 sont moins adhérents. Ces résultats suggèrent que l'absence de certains sucres au niveau du noyau OS affecterait les mécanismes de virulence de la bactérie. Nous voulons déterminer les modifications au niveau de la structure du noyau OS chez les différents mutants « core » et étudier leurs implications dans la pathogenèse et la virulence d'*A. pleuropneumoniae*. D'autre part, le LPS est impliqué dans plusieurs mécanismes de virulence des bactéries en interagissant avec le système immunitaire inné de l'hôte (voir sections 3.2.1 et 3.2.2). Nous voulons donc évaluer le rôle du LPS dans l'interaction d'*A. pleuropneumoniae* avec l'immunité innée :

- Nous allons déterminer la structure du noyau OS des mutants « core » 5.1, CG1 et CG3.
- Nous allons étudier la virulence des mutants core CG1 et CG3 par des infections expérimentales et la comparer à celle du mutant 5.1.
- Nous allons étudier la sensibilité des mutants LPS d'*A. pleuropneumoniae* sérotype 1 à des peptides antimicrobiens.

- Nous allons aussi évaluer le rôle du LPS dans la capacité d'*A. pleuropneumoniae* à stimuler la synthèse de cytokines proinflammatoires par des macrophages alvéolaires porcins et par deux lignées de cellules épithéliales porcines (SJPL et NPTr).

4.2. Étude du rôle du noyau oligosaccharidique dans la synergie entre le LPS et les toxines ApxI et ApxII.

Les toxines Apx sont des facteurs de virulence importants d'*A. pleuropneumoniae* qui sont principalement responsables des lésions pulmonaires. Nos précédentes études d'infection expérimentale ont montré que le mutant « core » 5.1 est très atténué et cause significativement moins de lésions pulmonaires chez le porc. En se basant sur les précédentes études qui suggèrent l'importance du noyau OS dans la synergie entre le LPS et les toxines RTX (section 3.3), nous avons voulu étudier l'effet de la troncation du noyau OS chez le mutant 5.1 sur l'activité hémolytique et cytotoxique d'*A. pleuropneumoniae* sérotype 1 et sur des interactions éventuelles entre le LPS et les toxines ApxI et ApxII :

- Nous allons étudier l'activité hémolytique et cytotoxique du mutant 5.1
- Nous allons évaluer l'expression des gènes codant pour les toxines ApxI et ApxII et leur système de sécrétion ainsi qu'évaluer la quantité intracellulaire et extracellulaire des toxines ApxI et ApxII
- Nous allons enfin étudier la possibilité d'une éventuelle interaction entre le LPS et les toxines ApxI et ApxII.

MÉTHODOLOGIE ET RÉSULTATS

Article 1

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Truncation of the LPS outer core affects susceptibility to antimicrobial peptides and virulence of *Actinobacillus pleuropneumoniae* serotype 1

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Running title: Core oligosaccharide structure and properties of *A. pleuropneumoniae* LPS mutants

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ABSTRACT

We previously reported that the core oligosaccharide region of the lipopolysaccharide (LPS) is essential for optimal adhesion of *Actinobacillus pleuropneumoniae*, an important swine pathogen, to respiratory tract cells. Rough LPS and core LPS mutants of *A. pleuropneumoniae* serotype 1 were generated by using a mini-*Tn10* transposon mutagenesis system. Here, we performed a structural analysis of the oligosaccharide region of three core LPS mutants that still produce the same O-antigen using methylation analyses and mass spectrometry. We also performed a kinetic study of proinflammatory cytokines production such as IL-6, TNF-α, IL-1β, MCP-1 and IL-8 by LPS-stimulated porcine alveolar macrophages which showed that purified LPS of the parent strain, the rough LPS and core LPS mutants, had the same ability to stimulate the production of cytokines. Interestingly, an *in vitro* susceptibility test of these LPS mutants to antimicrobial peptides showed that the three core LPS mutants were more susceptible to cationic peptides than both the rough LPS mutant and the wild type parent strain. Furthermore, experimental pig infections with these mutants revealed that the galactose (Gal I) and DD-heptose (Hep IV) residues present in the outer core of *A. pleuropneumoniae* serotype 1 LPS are important for adhesion and overall virulence in the natural host, while deletion of the terminal GalNAc-Gal II disaccharide had no effect. Our data suggest that an intact core-lipid A region is required for optimal protection of *A. pleuropneumoniae* against cationic peptides and that deletion of specific residues in the outer LPS core results in the attenuation of the virulence of *A. pleuropneumoniae* serotype 1.

INTRODUCTION

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, an infection characterized by hemorrhagic, fibrinous, and necrotic lung lesions. This highly contagious disease is responsible for substantial economic losses in the swine industry (1). The disease may be acute, subacute or chronic and is normally transmitted by chronically infected pigs. However, it has been reported that pigs may be subclinically infected without presenting clinical signs (2,3) and these pigs are thought to be the main cause of *A.*

pleuropneumoniae dissemination (1). Fifteen serotypes of *A. pleuropneumoniae* based on capsular antigens have been identified and the most predominant in Quebec are serotypes 1, 5, and 7 (4). Among the many virulence factors of *A. pleuropneumoniae*, namely the RTX toxins (5), the capsule and the outer membrane proteins (6-8), the lipopolysaccharides (LPS) are known to be important, due to their involvement in the adhesion to host cells (9,10), and are also known to play a role in the stimulation of the host immune system. LPS are complex molecules composed of three well-defined regions: (i) lipid A, anchored in the outer membrane; (ii) the core oligosaccharide containing 2-keto-3-deoxyoctulosonic acid (Kdo) and heptose residues; and (iii) the O-antigen which is a polysaccharide consisting of repeating units. The structure of *A. pleuropneumoniae* serotype 1 O-antigen has been described as branched tetrasaccharide repeating units composed of two α-L-rhamnopyranosyl, one α-D-glycopyranosyl and one 2-acetamido-2-deoxy-β-D-glucose residues (11). More recently, the structures of the core oligosaccharide of LPS from *A. pleuropneumoniae* serotypes 1, 2, 5a and 5b were elucidated using NMR spectroscopy and mass spectrometry (12), which revealed a conserved inner-core structure consisting of a trisaccharide of L-*glycero*-D-*manno*-heptose residues linked to a Kdo residue and substituted at different positions as shown previously (12). The study also allowed the identification of a novel open-chain GalNAc residue in the outer core of serotype 1 (Fig. 1A).

We have previously generated 4 core LPS and 7 rough LPS mutants of *A. pleuropneumoniae* serotype 1 by using a mini-*Tn10* mutagenesis system and the gene affected by the transposon was identified in each of the mutants (13-15). Characterization of the rough LPS mutants allowed us to identify a cluster of 13 genes involved in O-polysaccharide biosynthesis in *A. pleuropneumoniae* serotype 1 (15). The genes affected in the core LPS mutants were *galU* in mutant 5.1 (13), which encodes an UTP-α-D-glucose-1-phosphate uridylyltransferase, a gene involved in outer core elongation with galactose in mutant CG1 (14) and a gene coding for a D-*glycero*-D-*manno*-heptosyltransferase in mutants CG3 and CG5 (14). All these core LPS mutants still express an O-chain although their core oligosaccharide is apparently truncated, based on gel mobility (14). Characterization of the surface properties of these LPS mutants demonstrated that O-antigen deletions in rough mutants have no effect on the adhesion of the bacteria to frozen

tracheal sections of pigs, while an intact core oligosaccharide region seems to be required for optimal adherence (7,13). Interestingly, *in vivo* experiments in pigs showed that the core LPS mutant 5.1 that still produce an O-antigen was less virulent while no difference was observed between the rough mutant 27.1 lacking O-antigen and the parent strain (13-15). These observations suggest that the LPS core oligosaccharide could play a major role in colonization and pathogenesis of *A. pleuropneumoniae* in pigs.

Another important feature of LPS molecules is their interaction with the host immune system. The innate immunity is the first barrier for invading organisms, and inflammation is known to be of special importance in this first line of defence (16). Porcine pleuropneumonia is characterized by an intensive inflammation with infiltration of phagocytic cells such as neutrophils and alveolar macrophages, which are the main cells found in affected lungs (17). Previous *in vivo* studies have shown that *A. pleuropneumoniae* infection is associated with the production of a large amount of inflammatory mediators (18,19) and proinflammatory cytokines such as IL-1, TNF-α, IL-8 and IL-6 (20,21) which could be responsible, at least in part, for the lung injuries and tissue destruction observed in porcine pleuropneumonia (20,22). LPS are known as mediators of inflammation (16) as they interact with immune cells and activate the production of inflammatory cytokines (23,24) by these cells. Thus, by interacting with the immune system and promoting inflammation, LPS could be an important factor in the development of lesions observed in porcine pleuropneumonia. We sought to examine if a defined mutation within the LPS could result in a decrease in LPS-induced cytokine production and consequently cause an attenuation of the virulence of *A. pleuropneumoniae*. One other important component of the innate immune system is antimicrobial peptides, that can either be released by epithelial cells or by phagocytic cells (25). Most of these peptides are small cationic peptides that have been isolated from various biological sources (26); they act mainly by forming pores in bacterial membranes (27). They are classified into three major classes on the basis of their amino acids composition and their three-dimensional structure: linear α-helical peptides without cysteines (cecropin from pigs, magainin from frogs); peptides with a predominantly β-sheet structure with cysteines linked by disulfide bridges (defensin, protegrin from pigs); peptides with high contents of particular amino acids (PR-39 from pigs) (28). As a result of their positive charges, these peptides have a high affinity for

negatively charged-LPS that form the external leaflet of the outer membrane of Gram-negative bacteria. Thus, a modification within these LPS molecules could result in an increase of susceptibility to these peptides.

Considering the potential role played by the LPS core oligosaccharide in *A. pleuropneumoniae* pathogenesis, the purpose of the present study was to determine the core oligosaccharide structure of three different core LPS mutants of *A. pleuropneumoniae* serotype 1 (5.1, CG1 and CG3) by using capillary electrophoresis-electrospray ionization mass spectrometry (CE-ESIMS) in combination with methylation analysis and compare those structures to that of the wild type parent strain. To consider the effect of these mutations on the interaction of *A. pleuropneumoniae* with immune system components, a kinetic study of proinflammatory cytokines production was performed on porcine alveolar macrophages stimulated with either the LPS mutants or the wild type parent strain. The susceptibility of the core LPS mutants to antimicrobial peptides was also evaluated by using an *in vitro* assay to determine the minimum inhibitory concentration (MIC) of various cationic peptides. Finally, experimental pig infections were performed with these core LPS mutants.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacterial strains used in this study are shown in Table 1. *A. pleuropneumoniae* S4074 Nal^r was grown on BHI (Difco Laboratories, Detroit, Mich.) agar plates supplemented with 15 μg NAD per ml, and 30 μg nalidixic acid (Nal) per ml. Transpositional mutants (Table 1) were grown on BHI agar plates supplemented with 15 μg/ml of NAD, 30 μg/ml of nalidixic acid and 75 μg/ml of kanamycin. *E. coli* K88ac was grown on LB (Difco Laboratories) agar plates. Liquid cultures of *A. pleuropneumoniae* strains (wild type and mutants) were done in BHI broth supplemented with 5 μg/ml of NAD, while the *E. coli* strain was grown in LB broth.

Isolation of LPS

The LPS was isolated as described previously (12), giving 110 mg for mutant 5.1, 44 mg for mutant CG1, and 35 mg for mutant CG3. 36 mg of mutant 5.1 LPS, 30 mg of mutant CG1 LPS, and 35 mg (all but 0.2 mg used) of mutant CG3 LPS were each purified down a column of Bio-Gel P-2 and eluted with water as described previously (12).

Deacylation of LPS

0.2 mg of LPS of each mutant was O-deacylated as described previously (12) and examined by capillary electrophoresis mass spectrometry (CE-MS).

Isolation of the core oligosaccharide

The sugar containing fractions of each mutant LPS following column chromatography were pooled and treated with 3.5 ml of 2% acetic acid at 100°C for 2 h, giving core oligosaccharide (19.3 mg for mutant 5.1, 15.6 mg for mutant CG1, and 21.3 mg for mutant CG3). The samples were purified on a Bio-Gel P-2 column as described previously (12). Core fractions (fractions 19-24) eluting off the column slightly after the O-chain (fractions 15-17) were combined and lyophilized.

Microanalysis of cells

0.2 mg of cells of each mutant was suspended in 200 µl of water and 2 µl of 1 mg/ml proteinase K was added. The samples were left at 37°C for 5 h, then heated to 75°C for 10 min (to destroy the enzyme) and lyophilized. The samples were resuspended in 200 µl of 20 mM ammonium acetate buffer pH 7.5 and 1 µl of DNase and 2 µl of RNase (both 10 mg/ml) was added and left at 37°C for 6 h and lyophilized. The samples were then deacylated as described above for purified LPS and sent for CE-MS.

Analytical methods and Mass spectrometry

The structural analysis was performed as described previously (12).

Isolation of porcine alveolar macrophages (PAMs)

PAMs were obtained from two healthy pigs that originated from herds known to be serologically free for all serotypes of *A. pleuropneumoniae*, by means of lung lavage as described previously (29). Briefly, the pigs were pre-anesthetized through an intramuscular injection of 22 mg/kg ketamine and 4 mg/kg stresnil and euthanized intravenously with 60 mg/kg of pentobarbital. Three bronchoalveolar lavages were performed for each pig with a total of 300 ml of sterile pyrogen-free PBS per lavage. The fluid lavage was centrifuged at 800 x g for 10 min and the cell pellets were washed and resuspended in DMEM (Gibco 12430-054, Burlington, ON, Canada) supplemented with 40% fetal bovine serum (FBS; Gibco). The cells were cultured in 24-well tissue culture plates (Becton, Dickinson) and unadherent cells were removed by washing the plates 3 times with PBS. The cell concentration was then ajusted to 2 x 10^7/ml in a medium containing 80% DMEM and 20% DMSO and cryopreserved in liquid nitrogen until used.

Stimulation of cytokine production

Before the experiment, PAMs were quickly thawed in water bath at 37°C, washed and resuspended in complete DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml gentamicin, 0.1 mM MEM non-essential amino acids (all purchased from Gibco) and 1 mM sodium pyruvate (Sigma, Oakville, ON, Canada). Cell count was re-evaluated on the basis of trypan blue dye exclusion and the cell concentration was adjusted to 1 x 10^6/ml. PAMs were then dispensed in 24-well tissue culture plates and incubated overnight at 37°C in 5% CO_2. For the kinetic study the PAMs were classified into three groups, unstimulated cells as a negative control, cells stimulated with 1 µg/ml of purified LPS or 10^9 CFU/ml of heat-killed bacteria and incubated at 37°C in 5% CO_2. Purification of LPS was performed according to the Darveau-Hancock procedure (30,31) and the products were separated and analyzed by SDS-PAGE and immunoblotting as previously described (13). Purified LPS of the wild type parent strain and all the LPS mutants (Table 1) were used in this study. As a positive control of the stimulation, purified LPS of *E. coli* 0127:B8 (Sigma) was also used. Heat-killed bacteria from all the *A. pleuropneumoniae* strains shown in Table 1 were also used in this study and prepared as described previously (32) by incubating the organisms at 60°C

for 45 min. Stimulated or unstimulated (control) samples were collected at different time intervals (0.5, 1, 2, 4, 6, 12, 24 and 48 h). At each sampling, cell-free supernatants were collected, aliquoted and stored at -20°C for ELISA cytokine quantification. Cells were then treated for total RNA extraction for RT-PCR analysis of cytokine gene expression. As a control of the LPS-stimulated cytokines production, a 24 h incubation time of PAMs with purified LPS was carried out as described above, in the presence of 10 μg/ml polymyxin B (Sigma), an LPS inhibitor. A cytotoxicity test based on lactate dehydrogenase dosage in the supernatants showed that neither the purified LPS nor the heat-killed bacteria have a cytotoxic effect on the PAMs during the incubation times (data not shown). This test was performed using a CytoTox 96 LDH kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Enzyme-linked immunosorbent assays (ELISA) for cytokines

IL-1β was quantified using the immunoassay kit Swine IL-1β (Biosource International, Camarillo, CA), as specified by the manufacturer. TNF-α, IL-6 and IL-8 were measured by sandwich ELISA, using the following pair-matched monoclonal antibodies from R&D Systems (Minneapolis, MN), according to the manufacturer's recommendations. TNF-α: monoclonal anti-porcine TNF-α and biotinylated anti-porcine TNF-α; IL-6: polyclonal anti-porcine IL-6 and biotinylated anti-porcine IL-6; IL-8: monoclonal anti-human IL-8 and biotinylated anti-porcine IL-8. Twofold dilutions of recombinant porcine TNF-α and IL-6 (78 to 5000 pg/ml, R&D Systems) and porcine IL-8 (18 to 600 pg/ml, R&D Systems) were included as standard curves in each ELISA plate (Nunc, VWR, Ville Mont Royal, QC, Canada). Sample dilutions giving optical density readings in the linear portion of the appropriate standard curve were used to quantify the levels of each cytokine in the samples. Standard and sample dilutions were added in duplicate wells to each ELISA plate, and all analysis were performed at least four times for each individual stimulation assay. Plates were read in a Power Wave X 340 (Biotek Instruments Inc, Winooski, VT, USA) microplate reader.

Total RNA extraction

Macrophages were resuspended and lysed by repeated pipetting in 1 ml Trizol reagent (Invitrogen, Burlington, ON, Canada). Total RNA was extracted as specified by the manufacturer. The final RNA pellet was resuspended in 25 µl of DEPC-treated water, and RNA concentration and purity was measured using an Ultrospec 2100 pro UV/visible spectrophotometer (Biochrom Ltd, Cambridge, England). A PCR reaction using primers for a constitutive gene, β2-microglobulin (Table 2), was performed with the RNA samples as described below, to detect any DNA contamination. RNA was stored at -80°C for future use.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) for porcine cytokines

Reverse transcription of RNA into cDNA was performed in a 40 µl total volume containing: 1 µg of sample RNA, 400 ng random primers (Roche Diagnostics, Laval, QC, Canada), 0.5 mM dNTP mix (Amersham Biosciences, Baie d'Urfé, QC, Canada), 1X first-strand buffer, 10 mM DTT, 80 U RNAguard Rnase inhibitor (Amersham), 400 U Superscript II RnaseH reverse transcriptase (Invitrogen). Briefly template RNA, dNTPs and random primers were incubated at 65°C for 5 min followed by a step at 4°C. All components except the reverse transcriptase were then added and the sample was incubated at 25°C for 10 min and 42°C for 2 min. Superscript reverse transcriptase was finally added and the sample was incubated for 50 min at 42°C. A final heating step at 70°C for 15 min was also performed for enzyme inactivation. The newly synthesized cDNA samples were stored at -20°C until used for PCR reactions.

The oligonucleotide primers used for the detection of cDNA specific to porcine TNF-α, IL-1β, IL-6, MCP-1, IL-8 and primers for control housekeeping genes β2-microglobulin (β2-M) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are represented in Table 2. It is worth noting that the cytokine fragment and the appropriate constitutive gene fragment (Table 2) were amplified in the same reaction to minimize the risk of variations. The PCR mixtures for amplification of cDNA were performed in a 25 µl total volume containing: 0.4 mM dNTP mix (Amersham), 1X PCR buffer, 0.16 mM of forward and reverse primers for the cytokine and the housekeeping gene, 1 µl of cDNA template and 2.5 U Taq polymerase

(Amersham). The PCR reaction was performed in a Biometra *Tpersonal* thermocycler as follows: Initial denaturation for 3 min at 94°C followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C and elongation for 30 s at 72°C. The reaction was completed with a final elongation step at 72°C for 7 min. A negative PCR control (all components except cDNA) was included in all PCR reactions. 10 µl of the PCR products were separated by electrophoresis in 2% agarose gel with ethidium bromide, visualized in a MultiImage Light Cabinet apparatus and band intensity was determined by use of an Alpha imager 2000 software (Alpha Innotech Corporation). Correct size was verified by comparison with a 100-bp ladder (Roche Diagnostics). The relative band intensity was used to determine the cytokine/housekeeping gene ratio expressed as arbitrary units.

In vitro susceptibility test

One or two colonies of the strains to be tested were grown in 5 ml broth with shaking at 150 rpm for 4 h to an OD_{600nm} of 0.7. The cultures were then diluted in fresh media to 5 x 10^5 CFU/ml and used as the inoculum for the susceptibility test. Polymyxin B, protamine, cecropin P1, melittin, mastoparan (all from Sigma) and protegrin-1 (provided by R.I. Lehrer, UCLA School of Medicine) were assayed at final concentrations of 0.8 to 100 µg/ml for melittin, mastoparan and protegrin; 0.08 to 10 µg/ml for cecropin P1; 0.01 to 2 µg/ml for polymyxin B and 8 to 1000 µg/ml for protamine. Magainin-1, indolicidin and bacitracin, all purchased from Sigma, were also used in this study (Table 3). Serial dilutions of the peptides were prepared in sterile water and 10 µl of the dilutions were dispensed in duplicate in sterile 96-well polystyrene microtiter plates (Corning Incorporated, NY, USA); 90 µl of the bacterial inoculum was then added to each peptide-containing well and the mixture was incubated at 37°C. The MIC was determined as the lowest concentration of the compound that did not allow visible growth after 18-24 h of incubation. Growth was evaluated visually and also measured at 620 nm with a Dynatech MR5000 microplate reader (Dynatech Laboratories Inc, Chantilly, VA, USA). As controls, wells without peptides or without bacteria were carried out with each experiment. A *E. coli* K88ac strain was used as a control of the peptides' antimicrobial activity.

Experimental pig infection

Fourteen specific-pathogen-free 9-week old piglets (AFSSA Ploufragan, France) were used and separated into 2 groups of five pigs and 1 group of four pigs housed separately in a pathogen-free environment. The pigs were infected at 12 weeks of age; one group received the parent strain S4074 Nal^r, one group received the LPS core mutant CG1 and the last group received the LPS core mutant CG3. Pigs were challenged once intranasally with a total of 1 ml (0.5 ml per nostril) of a 6 h culture containing approximately 1 x 10^6 CFU. Animals were monitored daily for fever, cough, dyspnea and anorexia; blood samples were also collected weekly for serum analysis using an immunoenzymatic ELISA assay (Swinecheck App 1, 9, 11, Biovet, St-Hyacinthe, QC, Canada) for the detection of antibodies against LPS of *A. pleuropneumoniae* serotype 1. The performances of the animals (Daily Average Weight Gain) during the experiment were also evaluated. Pigs were euthanized 15 days after infection and a necropsy was carried out with every pig for macroscopic and microscopic examination of lesions within the lungs. For histological preparation, lung samples were fixed in 10% buffered formalin, embedded in paraffin and 6 µm thick sections were cut and stained with haematoxylin, phloxin and saffron. Lungs, tonsils and nasal cavities were cultured for reisolation of challenge bacteria using trypticase-soy (Difco) agar enriched with 1 mg/ml of crystal violet, 1 mg/ml of lincomycin, 128 mg/ml of bacitracin, 1 mg/ml of NAD, 5% (v/v) sheep blood, and 5% (w/v) yeast extract. Isolates were inoculated on selective media described above to confirm that the mutant strains or the parent strain used for challenge were indeed isolated. PCR detection of the bacteria based on the amplification of the *omlA* gene coding for an outer membrane protein of *A. pleuropneumoniae*, was also performed with lung samples (33).

RESULTS

Structural analysis of the core oligosaccharide of the 3 A. pleuropneumoniae core LPS mutants

Silver-stained SDS gels of purified LPS of the wild type parent strain and the LPS mutants (Fig. 2) confirmed the differences previously observed in the migration of low-molecular-mass bands corresponding to the core-lipid A region (13,14): the rough LPS mutant 27.1

(Lane 3) had the same mobility than the parent strain (Lane 1), the core LPS mutant 5.1 showed an additional band of lower molecular-mass (Lane 4) and the core oligosaccharide region of mutants CG1 (Lane 5) and CG3 (Lane 6) migrated faster than that of the wild type parent strain, with a faster migration for mutant CG3. The parent strain and the acapsular mutant 33.2 (Lane 2) showed similar migration profiles as they elaborate the same LPS (34). Western blot analysis using the monoclonal antibody 5.1 G8 F10 directed against the O-antigen of *A. pleuropneumoniae* serotype 1 (Fig. 3) showed clearly that purified LPS from the three core LPS mutants 5.1, CG1 and CG3 still have the high molecular mass O-chains (Lanes 4, 5 and 6). It is also interesting to note that comparable amount of these O-chains are observed in purified LPS from the parent strain (Lane 1). The rough LPS mutant 27.1 showed no reactivity with the monoclonal antibody (Lane 3) as it lacks the O-antigen. As mentioned above, the acapsular mutant 33.2 and the parent strain generate the same LPS. As a result, purified LPS from these two strains both contain the O-antigen (Lanes 1 and 2).

As recently reported (12), MS of the O-deacylated LPS sample of *A. pleuropneumoniae* serotype 1 gave three peaks at m/z = 2794 (lipid A, 1Kdo, 4Hep, 4Hex and HexNAc), m/z = 2874 (+PO4), and m/z = 2996 (+PEA +PO4) while the core oligosaccharide of the parent strain MS gave a mass of 1840 that corresponds to Kdo, 4Hep, 4Hex, HexNAc (Table 4 and Fig. 1A). The sugar analysis of the alditol acetates sample (AA) revealed the presence of Glc, Gal, DD-Hep, and LD-Hep in the ratio 2:1.5:1:2:0. In order to determine the linkage pattern of the molecule, partially methylated alditol acetates (PMAA) analysis by Gas Chromatography-Mass Spectrometry (GC-MS) was carried out; the sugars obtained were terminal Glc, 6-Glc, 3-Gal, terminal LD-Hep, 4/6-Gal, 4-DD-Hep, 2-LD-Hep and 3/4/6-Hep in the ratio of 1:1.5:1:0.5:1:1:1:1 and the proposed structure is shown in Fig 1A. Further sophisticated NMR studies had also been used to identify the nature of the HexNAc which was found to be a novel open-chain GalNAc (12).

In the present study, the structural analysis of the three core LPS mutants was investigated using CE-ESIMS in combination with methylation analysis and compared to the parent strain. The MS of the deacylated LPS sample of the mutant CG1 gave two peaks at m/z = 2508 (lipid A, 1Kdo, 1PO4, 4Hep, and 3Hex) and m/z = 2632 (+PEA). The major peak in the core was m/z = 1474 (1Kdo, 4Hep, and 3Hex), but there were minor amounts of

m/z = 1312 (-1Hex) and 1120 (-Hex and Hep) (Table 4). The AA sample contained 1Glc, 1.2Gal, 0.5DD-Hep, 0.5LD-Hep and the PMAA analysis revealed t-Glc, 6-Hex, t-Hep, 4-Hep, 2-Hep, and 3/4/6-Hep in the ratio of 1:1.5:0.1:0.5:0.5:1. Compared to the parent structure, this mutation has effected the addition of the terminal GalNAc-Gal II disaccharide (Fig. 1B) which is in agreement with the insertion of the mini-*Tn10* in *wlaC*, a gene involved in outer core elongation with galactose (14).

MS of the deacylated LPS of the mutant 5.1 gave *m/z* = 2346 as the most intense peak and this corresponds to a structure of lipid A, 1Kdo, 1PO4, 4Hep, 2Hex. There were also peaks at 2470 (+PEA), 2508 (+Hex), 2632(+Hex and PEA), 2672 (+2Hex), 2714 (+Hex and HexNAc, small peak), 2874 (+2Hex and HexNAc), and 2996 (+2Hex, HexNAc, and PEA) (Table 4). The core MS gave *m/z* = 1150 (1Kdo, 4Hep, 1Hex), 1312 (+Hex), 1474 (+2Hex) and 1678 (+2Hex and HexNAc) (Table 4). The AA analysis revealed 2Glc: 2.1Gal: 1DD-Hep: 2LD-Hep and the PMAA showed the presence of t-Glc, 6-Hex, 3-Hex, t-Hep, 4/6-Hex, 4-Hep, 2-Hep, and 3/4/6-Hep in a ratio of 1:1:0.25:0.1:0.25:0.25:0.5:0.5. The most prominent and truncated glycoform of this mutant contains one less Hex residue than mutant CG1, and compared to the parental structure lacks both GalNAc-Gal II and Gal I (Fig. 1C). This major truncated glycoform is in agreement with the mutation of *galU* involved in synthesis of UDP-glucose (13) and with the faster migrating low-molecular-mass band observed in SDS-PAGE profile of core LPS mutant 5.1 (Fig. 2; Lane 4). However the structural data (Table 4) also showed that this mutant produces small amounts of a full sized core-lipid A that migrated with the same mobility as the parent strain (Fig. 2; Lane 4), as glycoforms consistent with extension beyond this Gal I residue are still observed.

MS of the deacylated LPS sample of the mutant CG3 gave several peaks all based on the *m/z* = 2154 (lipid A, 1Kdo, 1PO4, 3Hep, and 2Hex). The others were *m/z* = 2278 (+PEA), 2316 (+Hex), and 2402 (+2PEA). The core only contained *m/z* = 1120 (1Kdo, 3Hep, and 2Hex) (Table 4). The AA sample had 1Glc: 0.5Gal: 0.1DD-Hep: 1LD-Hep and the PMAA contained t-Glc, 6-Hex, t-Hep, 2-Hep, and 3/4/6-Hep in the ratio of 2:0.1:0.25:1:1. In this mutant we see the loss of one Hep compared to the most truncated and common glycoform of the 5.1 mutant and this is reflected in the AA analysis by the loss of most of the DD-Hep. These data suggest that the tri-LD-heptosyl inner core is intact

while the DD-Hep from the extension of Hep I has been lost (Fig. 1D). Again, this is in agreement with the insertion of the mini-*Tn10* in *lbgB*, a gene coding for a heptosyltransferase (14).

It is also worth noting that the O-chain is still attached in all 3 core LPS mutants as evidenced by Western blots with O-chain specific antibodies (Fig. 3), sugar analysis of non-fractionated LPS and elution profile of the core hydrolysate during column chromatography (data not shown), suggesting that the point of attachment of the O-chain has not been compromised by the different mutations.

Kinetic of cytokines production

The data obtained from the kinetic study (Fig. 4 and 5) showed that incubation of PAMs with purified LPS resulted in an increase in proinflammatory cytokines production when compared to the unstimulated condition. The level of cytokines produced was measured by ELISA for TNF-α, IL-6, IL-1β and IL-8 (Fig. 4). We can observe that the amount of cytokines produced tends to increase with time except for IL-6. It is worth noting that the level of IL-6 measured in the stimulated conditions is comparable to the unstimulated condition (Fig. 4A). This suggests that there was no LPS-induced IL-6 production or that the amount produced was too low to be detected by the ELISA test used. The amount of IL-1β also increased with time but the highest level is reached at 24 h of incubation since a decrease is observed at 48 h incubation time (Fig. 4D). It is also interesting to see that the level of IL-8 was particularly high (Fig. 4C) when compared to the other cytokines. However no significant difference was noticed between the parent strain and the mutants since purified LPS from the rough LPS mutant, the core LPS mutants, and the wild type parent strain seem to stimulate equally the production of cytokines by PAMs (Fig. 4). These results also show that the LPS of *E. coli* and the LPS of *A. pleuropneumoniae* serotype 1 exhibited comparable stimulatory activities. Moreover, stimulation of alveolar macrophages with heat-killed bacteria of the wild type parent strain and the LPS mutants gave similar results (data not shown).

RT-PCR data showed an early expression of all the cytokines tested since the level of mRNA reached its peak value at 2 h poststimulation and declined shortly after (Fig. 5). A second peak of lower intensity (IL-6, TNF-α, MCP-1 and IL-8) or greater intensity (IL-1β)

is also observed at 12 h poststimulation which is probably due to an autoactivation process caused by the increase of cytokines in the media. It is interesting to mention that this early production of mRNA is in good correlation with the ELISA test since the amount of cytokines produced tends to be higher in the late phase of the stimulation (Fig. 4). Here again, the kinetics of gene expression for each cytokine was identical in all LPS-stimulated conditions (Fig. 5) and these observations confirm the data obtained from ELISA assays. However, the quantification of mRNA showed clearly that there is an LPS-induced IL-6 expression (Fig. 5A) when compared to the unstimulated condition which was undetectable in ELISA. In Figure 5F we can estimate the intensity of the bands which illustrates the difference between stimulated and unstimulated conditions while no obvious variations were found among the LPS-stimulated conditions. Once more, the experiment with heat-killed bacteria gave similar RT-PCR results (data not shown). To further evaluate the importance of LPS in the activation of cytokines production, a stimulation experiment was carried out by incubating the PAMs for 24 h with purified LPS in the presence of polymyxin B, an LPS inhibitor. The results indicate that the addition of polymyxin B to LPS-stimulated PAMs produced a significant decrease in IL-6, TNF-α, MCP-1 and IL-1β gene expression since the level of mRNA for each of these cytokines tends to drop down to the unstimulated condition values (data not shown). These data confirm that the production of cytokines by PAMs was indeed induced by LPS. Moreover, the decrease of mRNA appeared to be similar in all the LPS-stimulated conditions. This suggests that polymyxin B had the same inhibition effect on purified LPS from the parent strain and the LPS mutants, but also on the LPS of *E. coli*.

Overall, our data indicate that the LPS of all the mutants tested exhibited the same capacity to stimulate cytokines production as the LPS of the parent strain.

Susceptibility to antimicrobial peptides

The susceptibility test was carried out with the three LPS core mutants (5.1, CG1 and CG3) and two additional mutants of *A. pleuropneumoniae* serotype 1 previously characterized by our group were also added to the study: an acapsular mutant 33.2 (34) and a rough LPS mutant 27.1 lacking O-antigen (13,15). The MIC values obtained (Table 5) showed that the acapsular mutant (33.2), the rough LPS mutant (27.1), and the wild type parent strain

exhibited similar MICs for all the cationic peptides shown in Table 5, suggesting that the absence of O-antigen of the LPS or the absence of the capsule have no effect on the susceptibility of *A. pleuropneumoniae* serotype 1 to antimicrobial peptides. However, the data also indicate that the three core LPS mutants (5.1, CG1, and CG3) were more susceptible than the parent strain to almost all the peptides tested. Interestingly, this suggests a role of the outer core in the resistance of *A. pleuropneumoniae* serotype 1 to antimicrobial peptides. It is worth noting that the largest difference of susceptibility between the core LPS mutants and the wild type strain was observed with protegrin-1 (Table 5), a peptide present in porcine neutrophils.

The experiment was also carried out with a few other peptides that are cited in Table 3. Magainin-1 and indolicidin were tested with the parent strain at 100 μg/ml, but no susceptibility was observed at this concentration. However, it is important to mention that the *E. coli* control strain was susceptible to these two peptides, respectively at 50 μg/ml and 25 μg/ml, which confirms that the resistance of the *A. pleuropneumoniae* strain was not due to an alteration of the peptides' activity. Bacitracin was also used and showed a MIC of 250 μg/ml for both the parent strain and the mutants. This is probably due to the site of action of bacitracin which affects mostly the cell wall synthesis. Thus a modification of LPS should not affect the activity of bacitracin.

Experimental pig infection

The virulence of the core LPS mutants CG1 and CG3 and the parent strain 4074 Nal^r was evaluated in pigs. After challenge with the three strains, the mortality recorded within the 14 days was high in the mutant CG1-infected group (100%) and the parent strain-infected group (75%) as compared to the mutant CG3-infected group in which only one pig died (20%) (Table 6). Three of the five pigs infected with mutant CG1 died in less than 2 days after infection, while only one pig infected with mutant CG3 died and only on day 4 post-infection. As observed in Table 6, the body temperature was higher than normal for all the infected pigs; however, for almost all the pigs that survived, the fever tended to decrease after the fourth day post-infection, except for the parent strain-infected pigs which exhibited a high temperature until 8 days after infection. Dyspnea, anorexia and in a few cases, cough, were observed in all infected pigs. Moreover, the performances analysis of

the animals was also carried out and showed that pigs infected with the parent strain and the mutant CG1 were more affected than pigs infected with the mutant CG3, in term of daily average weight gain (Table 6).

Upon macroscopic examination, typical fibrinohemorrhagic lesions compatible with *A. pleuropneumoniae* infection (35) were observed in all infected pigs (Table 6) and the severity of the lesions observed was approximately equal between the three groups. However, the lungs' weight was also evaluated (Table 6) and the average weight was found to be lower for pigs infected with the core LPS mutant CG3; this could be due to a less intensive inflammation which would result in a decrease of oedema and accumulation of fibrin and blood.

Microscopic examination of histological samples of the lungs revealed changes compatible with porcine pleuropneumonia. Fibrinohemorrhagic pleuresia and pneumonia was associated with leucocytes infiltration and pus formation. The interlobular septum was thickened with oedema fluid and inflammatory exudates. Central necrotic zones within the pulmonary tissue were often surrounded by a high density of inflammatory cells and a certain amount of thrombosed and necrotic lymphatic vessels were also found. It should be noted that the pigs infected with the mutant CG3 showed characteristic lesions of the chronic form of *A. pleuropneumoniae* infection while typical acute phase lesions were observed in mutant CG1 infected-pigs that died earlier. *A. pleuropneumoniae* was detected in the lungs of all infected pigs either by PCR or culture (Table 6). Bacterial culture from nasal cavities and tonsils showed that bacteria were not isolated in a large number of pigs infected with the mutant CG3 (Table 6), and interestingly, these pigs survived until the end of the experiment suggesting that the attenuation of the virulence could be due to an insufficient colonization of the host airways by this mutant strain.

Finally, antibodies against *A. pleuropneumoniae* serotype 1 were detected one week after infection, in the serum of all pigs that survived, and the amount of antibodies tended to increase in the second week (data not shown). It is worth noting that the growth curves in liquid culture for the parent strain and the 2 LPS core mutants were identical (data not shown), suggesting that the difference observed in the virulence of these strains was not a matter of growth. The results of this experimental infection study showed clearly that the virulence of mutant CG3 is attenuated when compared to the wild type parent strain or the

mutant CG1. In combination with the structural analysis of the core oligosaccharide, the data suggest an important role of the outer core residues Gal I and Hep IV, which are missing in the mutant CG3 but present in mutant CG1 (Fig. 1D and B), in the pathogenesis of *A. pleuropneumoniae*.

DISCUSSION

The structural analysis of the core oligosaccharide of the three LPS core mutants has brought additional information on the different LPS biosynthesis genes that were inactivated by the transposon insertion. The analysis of the core of mutant CG1 revealed the loss of the terminal GalNAc-Gal II disaccharide when compared to the parent strain. The core of mutant 5.1 is one hexose more truncated than that of CG1 due to the loss of a galactose residue (Gal I) which also prevents the addition of the terminal disaccharide. Mutant CG3 was found to have the most truncated LPS core as it lacks a DD-Hep (Hep IV) which is essential for the further addition of the galactose (Gal I and Gal II) and GalNAc residues. These structural analyses correlate with the SDS-PAGE profiles, since the LPS of mutant CG3 has the fastest migration of the core-lipid A region (Fig. 2). The genes affected by the mutations had previously been identified as genes involved in LPS biosynthesis. In the mutant CG1, the gene affected encodes a protein with 25% of homology with the WlaC protein of *Campylobacter jejuni*, which is a galactosyltransferase (36). A good correlation was also observed between the sugar deletions within the core structure and the genes affected in the mutants 5.1 and CG3. *galU* (mutant 5.1) is the structural gene for UTP-α-D-glucose-1-phosphate uridylyltransferase, an enzyme involved in the synthesis of UDP-glucose (37) which is also found in *Haemophilus ducreyi* and *Haemophilus influenzae* (87% and 83% identity respectively) while *lbgB* (mutant CG3) encodes a D-glycero-D-manno-heptosyltransferase (38).

The results of the experimental infections reveal that the core LPS mutant CG3 seems to be less virulent than the parent strain with only 20% of mortality. Moreover, previous studies by our group have shown that the virulence of the core LPS mutant 5.1 was also attenuated, with no mortality recorded, less lung lesions and also less fever (13). These data are of interest since the two most truncated outer core mutants (5.1 and CG3; Fig. 1C and

D) are both less virulent than the parent strain; this suggests that the Gal I-Hep IV- region of the core plays an important role in the virulence of *A. pleuropneumoniae* serotype 1. Furthermore, previous *in vitro* adhesion experiments using frozen tracheal sections of pigs have demonstrated that the mutants 5.1 and CG3 were both less adherent than the parent strain (13,14). Thus, it is tempting to speculate that a defect in colonization of the respiratory tract is responsible, at least in part, for the attenuation of virulence.

Previous studies have shown the important role of LPS in stimulating the production of proinflammatory cytokines which were found to be highly produced during *A. pleuropneumoniae* infection (20,21). It is reasonable to believe that LPS mutants could be altered in their capacity to stimulate cytokines production and this could have an effect on the pathogenesis of *A. pleuropneumoniae*. The kinetic study of cytokines production has shown that the LPS mutants have the same ability than the wild type parent strain to stimulate the synthesis of cytokines by alveolar macrophages. These findings indicate that mutations within the O-antigen or the core oligosaccharide of LPS have no effect on the ability of *A. pleuropneumoniae* to stimulate cytokines production by immune cells. This is to be expected considering that the endotoxic portion of LPS is the lipid A (39). Since rough LPS and core LPS mutants of *A. pleuropneumoniae* serotype 1 have an identical lipid A they are not affected in their stimulatory activities. Therefore, truncation of the outer core has no effect on lipid A activity. We can assume that the attenuation of the virulence of both LPS core mutants 5.1 and CG3 is not due to a decreased ability to stimulate production of proinflammatory cytokines. Interestingly, the measurement of cytokine production showed a high level of IL-8 in response to LPS stimulation. This elevated amount of IL-8 can be correlated with the massive infiltration of neutrophils in the lungs during *A. pleuropneumoniae* infection (17) since IL-8 is the major chemoattractant of these cells (23,40).

LPS molecules are the major targets of cationic peptides which, most commonly, form disrupting channels in the outer membrane of Gram-negative bacteria and gain access to the plasma membrane (41). Thus, the stability of the outer membrane depends mainly on the good arrangement of the LPS, which is known to involve ionic interactions with bivalent cations (26). Previous studies have shown the important role of Mg^{2+} in tightening the LPS packing within the membrane but also in its capacity to interfere with the binding of the

peptides to the negative charges of the LPS (42). This suggests that a mutation in the LPS could alter the integrity of bacterial surface, due to a modification in the ionic interactions, rendering the bacteria more susceptible to antimicrobial peptides. It has also been demonstrated that the core oligosaccharide rather than the O-antigen, is an important feature in the resistance of *Vibrio cholerae* and *Sinorhizobium meliloti* to such peptides (43,44) and our findings with *A. pleuropneumoniae* serotype 1 LPS mutants confirm these trends. Our results also showed that the acapsular mutant 33.2 exhibited the same susceptibility than the parent strain to all the peptides tested. This suggests that the capsule is not of major importance in the resistance of *A. pleuropneumoniae* to antimicrobial peptides while previous studies on *Klebsiella pneumoniae* had shown its protecting role (45). Interestingly, the core mutants do not always share the same pattern of MIC, depending on the peptides. Since these core mutants are affected in different biosynthesis genes (13,14), we can assume that the lack of specific sugar residues in the outer core is a determining point in the resistance/susceptibility of *A. pleuropneumoniae* to antimicrobial peptides, as previously described with the regulation of lipooligosaccharide (LOS) structure in *Yersinia pestis* (46). Finally, the *in vitro* test showed that antimicrobial peptides of different species (Table 3) are active against *A. pleuropneumoniae*. The results also revealed that the susceptibility of *A. pleuropneumonie* to these peptides is not dependant on their structure since α-helical peptides (cecropin P1, melittin and mastoparan) or peptides with a β-sheet structure (protegrin-1) both inhibited the growth of *A. pleuropneumoniae* serotype 1.

We have characterized the structure of the core oligosaccharide of three core LPS mutants of *A. pleuropneumoniae* serotype 1, previously generated using transposon mutagenesis. The present results confirm that the genes identified are indeed involved in the biosynthesis of outer core LPS of *A. pleuropneumoniae* serotype 1. *In vitro* assay using antimicrobial peptides showed the importance of the LPS outer core in the resistance of the bacteria to innate defence components of the host, while structural analyses in combination with *in vivo* experiments in the natural host allowed us to determine that some residues of the outer core are particularly important in the pathogenesis of *A. pleuropneumoniae*. To the best of our knowledge, this is the first demonstration of the importance of LPS outer core residues, in the virulence of a bacterial pathogen for its natural host.

FOOTNOTES

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Table 1. Bacterial strains used in the present study.

Strains	Relevant traits	Source or reference
Actinobacillus pleuropneumoniae [a]		
S4074 Nal^r	Serotype 1 (Nal^r), parent strain	(13)
27.1 [b]	LPS O-antigen mutant	(13)
5.1 [b]	LPS core oligosaccharide mutant	(13)
CG1 [b]	LPS core oligosaccharide mutant	(14)
CG3 [b]	LPS core oligosaccharide mutant	(14)
33.2 [b]	Acapsular mutant	(34)
E. coli K88ac	K12 with fimbriae F4ac	Our collection

[a] The nalidixic acid resistant (Nal^r) strain was obtained from the reference strain *A. pleuropneumoniae* S4074 serotype 1 (K.R. Mittal, Faculté de médecine vétérinaire, Université de Montréal)

[b] All the mutants derived from *A. pleuropneumoniae* serotype 1 S4074 Nal^r

Table 2. Nucleotide sequences of the primer sets used to amplify porcine cytokine genes, the size of the PCR products are represented.

Gene	Oligonucleotide sequence		PCR product (bp)
TNF-α	For	5′-CAC TGA GAG CAT GAT CCG AG -3′	470
	Rev	5′-GGC TGA TGG TGT GAG TGA GG -3′	
IL-1β	For	5′-TCA GGC AGA TGG TGT TCT GTC -3′	430
	Rev	5′-GGT CTA TAT CCT CCA GCT GC -3′	
IL-6	For	5′-GGA ACG CCT GGA AGA AGA TG -3′	470
	Rev	5′-ATC CAC TCG TTC TGT GAC TG -3′	
MCP-1	For	5′-ATT AAT TCT CCA GTC ACC TG -3′	420
	Rev	5′-AAC ACC AGT AGT CAT GGA GG -3′	
IL-8	For	5′-TGC AGC TTC ATG GAC CAG -3′	350
	Rev	5′-TGT TGC TTC TCA GTT CTC TTC -3′	
GAPDH	For	5′-CAC TGG TGT CTT CAC GAC-3′	295
	Rev	5′-GCC ATC CAC AGT CTT CTG -3′	
B2M	For	5′-CTG CTC TCA CTG TCT GG -3′	295
	Rev	5′-ATC GAG AGT CAC GTG CT -3′	

Table 3. Overview of antimicrobial peptides used in this study.

Peptide	Sequence	Structure	Origin
Magainin-1	GIGKFLHSAGKFGKAFVGZIMKS	α-Helix	frog
Melittin	GIGAILKVLATGLPTLISWIKNKRKQ		honey bee
Mastoparan	VDWKKIGQHILSVL		*Polistes jadwigae*
Cecropin P1	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR		pig
Protegrin-1	RGGRLCYCRRRFCVCVGR	β sheet	pig
Indolicidin	ILPWKWPWWPWRR	linear	cow

Table 4. Negative ion CE-ESIMS data[a] and proposed compositions of *O*-deacylated LPS and core oligosaccharides from *A. pleuropneumoniae* serotype 1 parent strain and mutants CG1, 5.1 and CG3.

Strain	Observed Ions (*m/z*)		Molecular Mass (Da)		Relative	Proposed Composition
	$(M-2H)^{2-}$	$(M-3H)^{3-}$	Observed	Calculated	Intensity[b]	
Parent	1396	930	2794	2792.65	0.4	HexNAc, 4Hex, 4Hep, Kdo, Lipid A-OH
O-deac	1436	957	2874	2872.63	1.0	HexNAc, 4Hex, 4Hep, Kdo, Lipid A-OH, *P*
	1497	998	2996	2995.68	0.75	HexNAc, 4Hex, 4Hep, Kdo, Lipid A-OH, *P*, *P*Etn
CG1	1253	835	2508	2507.29	0.55	3Hex, 4Hep, Kdo, Lipid A-OH, *P*
O-deac	1315	876	2632	2630.34	1	3Hex, 4Hep, Kdo, Lipid A-OH, *P*, *P*Etn
5.1	1172	781	2346	2345.14	1	2Hex, 4Hep, Kdo, Lipid A-OH, *P*
O-deac	1234	822	2470	2468.19	0.5	2Hex, 4Hep, Kdo, Lipid A-OH, *P*, *P*Etn
	1253	835	2508	2507.29	0.9	3Hex, 4Hep, Kdo, Lipid A-OH, *P*
	1315	876	2632	2630.34	0.75	3Hex, 4Hep, Kdo, Lipid A-OH, *P*, *P*Etn
	1335	899	2672	2669.44	0.85	4Hex, 4Hep, Kdo, Lipid A-OH, *P*
	1355	903	2714	2710.48	0.3	HexNAc, 3Hex, 4Hep, Kdo, Lipid A-OH, *P*
	1436	957	2874	2872.63	0.85	HexNAc, 4Hex, 4Hep, Kdo, Lipid A-OH, *P*
	1497	998	2996	2995.68	0.6	HexNAc, 4Hex, 4Hep, Kdo, Lipid A-OH, *P*, *P*Etn
CG3	1076	717	2154	2152.97	0.8	2Hex, 3Hep, Kdo, Lipid A-OH, *P*
O-deac	1138	758	2278	2276.02	1.0	2Hex, 3Hep, Kdo, Lipid A-OH, *P*, *P*Etn
	1157	-	2316	2315.12	tr	3Hex, 3Hep, Kdo, Lipid A-OH, *P*
	1200	-	2402	2399.07	0.5	2Hex, 3Hep, Kdo, Lipid A-OH, *P*, 2*P*Etn
Parent	919	-	1840	1840.65	1	HexNAc, 4Hex, 4Hep, Kdo
Core OS	958	-	1919	1820.63	0.05	HexNAc, 4Hex, 4Hep, Kdo, *P*
CG1	560	-	1120	1120.99	tr	2Hex, 3Hep, Kdo

Strain	Observed Ions (*m/z*)		Molecular Mass (Da)		Relative	Proposed Composition
	$(M-2H)^{2-}$	$(M-3H)^{3-}$	Observed	Calculated	Intensity[b]	
Core OS	656	-	1312	1313.16	0.25	2Hex, 4Hep, Kdo
	737	-	1474	1475.31	1	3Hex, 4Hep, Kdo
5.1	573	-	1150	1150.51	0.7	Hex, 4Hep, Kdo
Core OS	655	-	1312	1312.66	1	2Hex, 4Hep, Kdo
	737	-	1474	1475.31	0.5	3Hex, 4Hep, Kdo
	838	-	1678	1678.50	0.1	HexNAc, 3Hex, 4Hep, Kdo
CG3 Core OS	560	-	1120	1120.99	1	2Hex, 3Hep, Kdo

Average mass units were used for calculation of molecular weight based on proposed composition as follows: Hex, 162.15; HexNAc, 203.19; Hep, 192.17; Kdo, 220.18; *P*Etn, 123.05; *P*, 79.98. The average molecular weight of *O*-deacylated lipid A (Lipid A-OH) is 952.00.

[a] Data acquired by CE-ESIMS on a crystal Model 310 CE instrument interfaced to an API 3000 triple quadrupole mass spectrometer (Perkin-Elmer / Sciex) fitted with a bare fused silica capillary column and using 30 mM morpholine acetate (pH 9.0) containing 5% methanol as the separation buffer.

[b] Measured relative to the largest peak in the triply charged region.

Table 5. Minimum inhibitory concentration of various cationic peptides tested with *Actinobacillus pleuropneumoniae* serotype 1 parent strain, acapsular mutant 33.2, rough LPS mutant 27.1 and core LPS mutants 5.1, CG1 and CG3.

	MIC (μg/ml)					
Strain	Polymyxin B	Protamine	Mastoparan	Melittin	Cecropin P1	Protegrin-1
Parent	0.5 - 1	250	100	50	5	50
33.2 (CPS$^-$)	0.5	250	50 - 100	50	5	50
27.1 (rough LPS)	0.5	250	50	25 - 50	5	50
5.1 (*galU*$^-$)	0.125	62.5	25	12.5	2.5	6.25
CG1 (*wlaC*$^-$)	0.25	125	50	12.5 - 25	2.5	12.5
CG3 (*lbgB*$^-$)	0.25	125	50	12.5	2.5	6.25

Table 6. Results of the experimental infections in pigs.

Strain	Number of pigs (*n*)	Mortality (%) [a]	Fever [b]		Average daily gain [c]	Pigs with lung lesions (%) [d]	Average lungs weight [e]	Bacterial isolation (%) [f]		
			% of pigs	Duration (days)				Lungs	Nasal cavities	Tonsils
Parent	4	75	100	8	- 225	100	902.4	100	100	100
Mutant CG1	5	100	100	4	- 514	100	1079.0	100	80	100
Mutant CG3	5	20	100	4	- 87	100	822.2	100	40	40

[a] Represents the percentage of dead and sacrificed pigs that had reached a severe stage of the disease within 15 days.

[b] $T > 40°C$.

[c] Daily average gain 8 days after infection (in g)

[d] Macroscopic and microscopic examination.

[e] Compared to ~ 500 g for non-infected control pigs of the same age.

[f] Represents the percentage of pigs in which *A. pleuropneumoniae* was isolated from lungs, tonsils and nasal cavities.

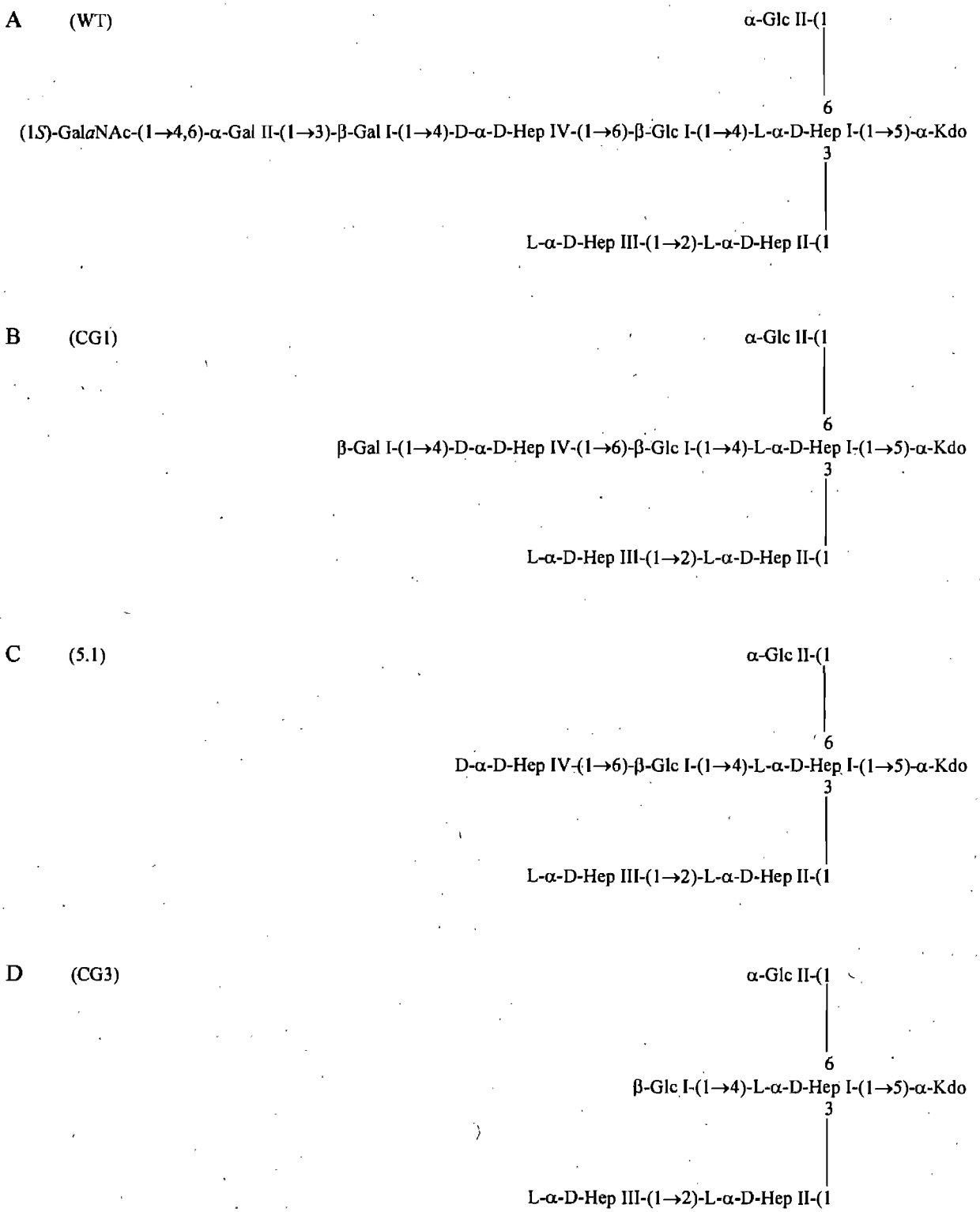

Fig 1. Structural representation of the core oligosaccharide from *A. pleuropneumoniae* serotype 1 parent strain (A), the core LPS mutant CG1 (B), the core LPS mutant 5.1 (C) and the core LPS mutant CG3 (D).

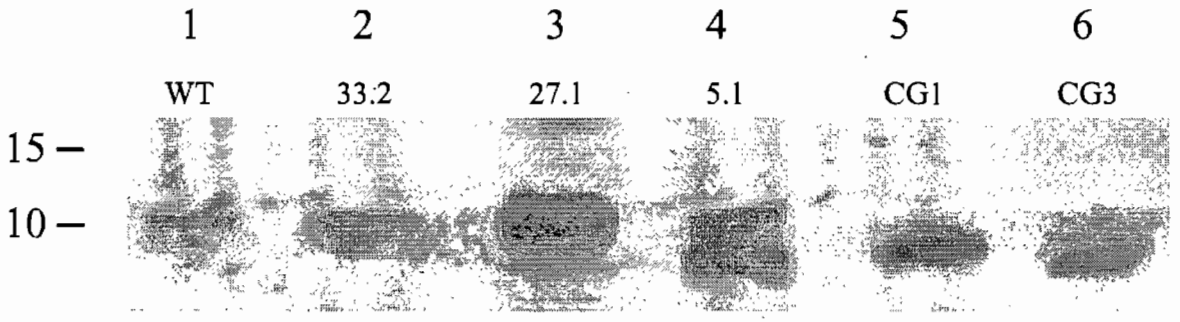

Fig 2. Silver-stained SDS-PAGE profile of purified LPS from *A. pleuropneumoniae* parent strain 4074 Nal^r and LPS mutants. Lane 1, parent strain; lane 2, acapsular mutant 33.2; lane 3, rough LPS mutant 27.1; lane 4, core LPS mutant 5.1; lane 5, core LPS mutant CG1; lane 6, core LPS mutant CG3. This gel shows the difference of migration of the core-lipid A between the wild type parent strain and the LPS mutants. Molecular mass markers (in Kilodaltons) are indicated on the left.

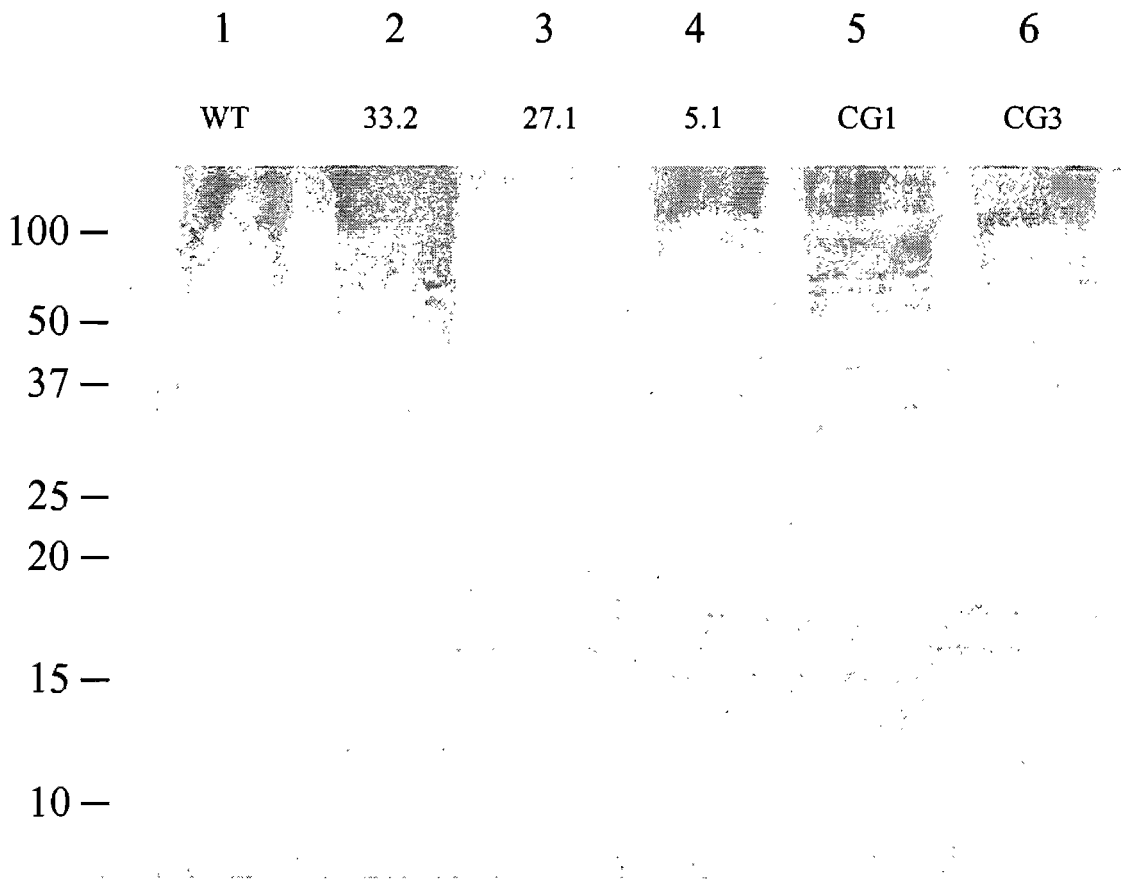

Fig 3. Immunoblot of purified LPS from *A. pleuropneumoniae* parent strain 4074 Nal^r, acapsular mutant and LPS mutants. The immunoblot was probed with monoclonal antibody 5.1 G8 F10 against *A. pleuropneumoniae* serotype 1 O-antigen. Lane 1, parent strain; lane 2, acapsular mutant 33.2; lane 3, rough LPS mutant 27.1; lane 4, core LPS mutant 5.1; lane 5, core LPS mutant CG1; lane 6, core LPS mutant CG3. Molecular mass markers (in Kilodaltons) are indicated on the left.

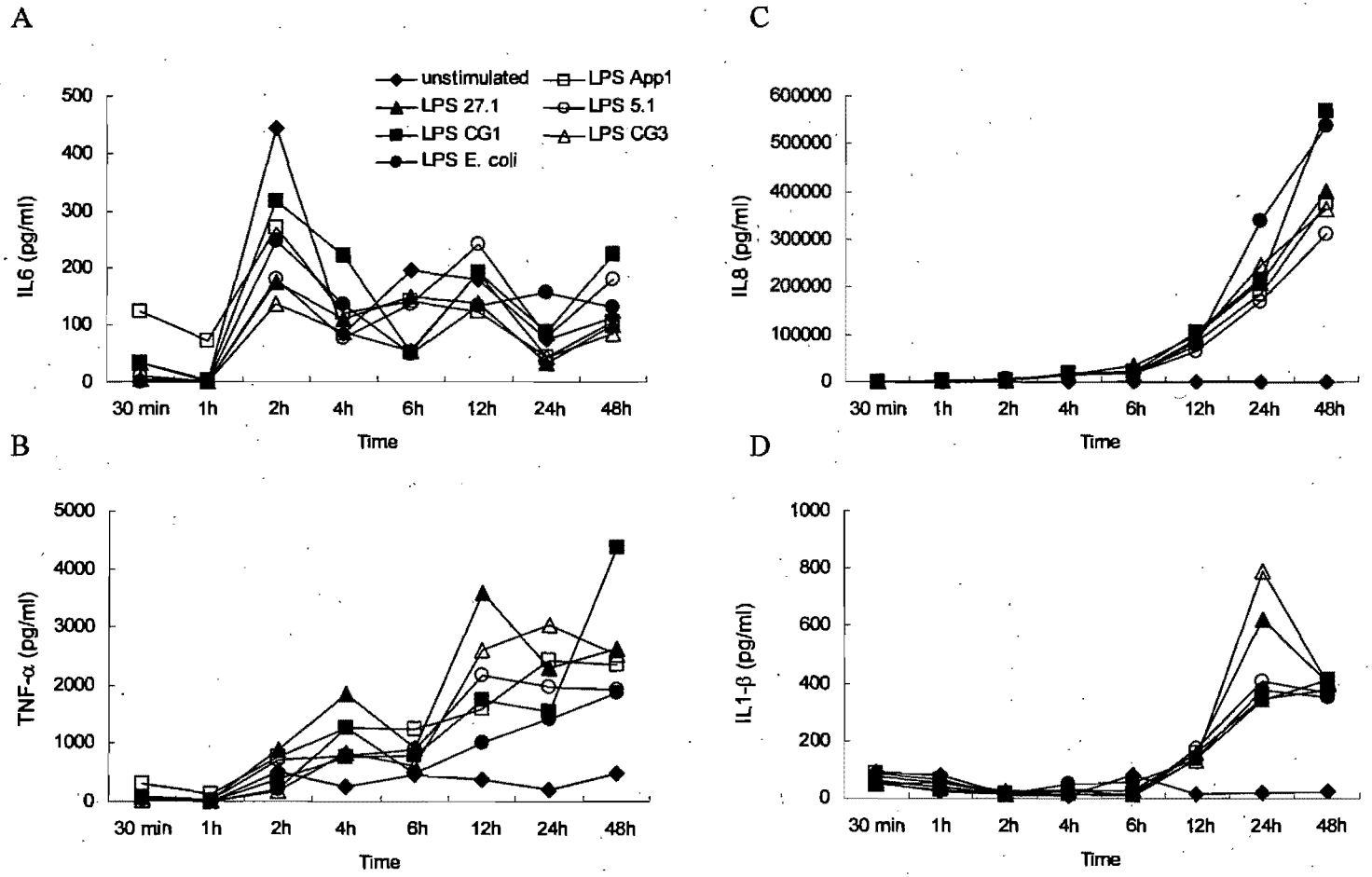

Fig 4. Time course of production of IL-6 (A), TNF-α (B), IL-8 (C) and IL-1β (D) by unstimulated and stimulated PAMs with 1 μg/ml of purified LPS from the wild type parent strain *A. pleuropneumoniae* serotype 1, the LPS mutants, and *E.coli* (used as a positive control). Culture supernatant fluids were collected at different time intervals and assayed for cytokine production by ELISA. Data are expressed in pg/mL.

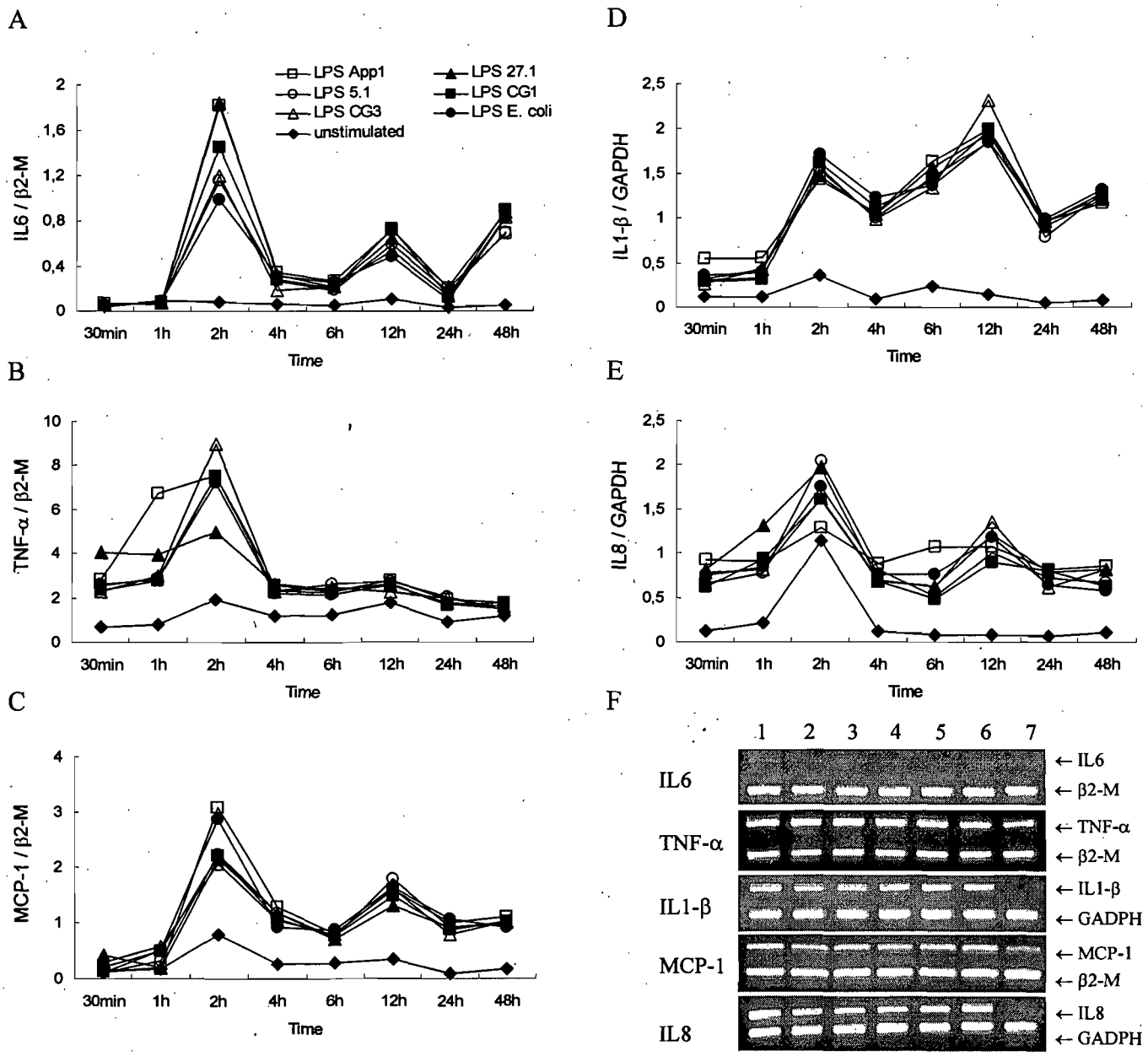

Fig 5. Kinetics of IL-6 (A), TNF-α (B), MCP-1 (C), IL-1β (D), IL-8 (E) mRNA expression. Cytokine gene expression levels were determined by RT-PCR in unstimulated and LPS-stimulated PAMs at different times of incubation. Data are represented as a ratio of cytokine/β2-M for IL-6, TNF-α, and MCP-1 and a ratio of cytokine/GAPDH for IL-1β and IL-8. The gels in (F) represent the bands intensities of the PCR products migration of RT-PCR analysis for each cytokine after 6 h of incubation. PAMs were stimulated with purified LPS of the parent strain (Lane 1), the rough LPS mutant 27.1 (Lane 2), the core LPS mutant 5.1 (Lane 3), the core LPS mutant CG1 (Lane 4), the core LPS mutant CG3 (Lane 5), *E. coli* (Lane 6) or unstimulated (Lane 7).

Article 2

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

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

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¶Participation aux expériences de stimulation de la synthèse de cytokines proinflammatoires et à l'écriture du manuscript.

ABSTRACT

Host-pathogen interactions are of great importance in understanding the pathogenesis of infectious microorganisms. We developed in vitro models to study 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 adhesion 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 *A. suis*, *H. parasuis* and *Pasteurella multocida*. Our results demonstrate that interactions of *A. pleuropneumoniae* with host epithelial cells seem 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 coccobacillus are presently known based on surface polysaccharides (66). The virulence of this pathogen is accomplished by the help of many factors including exotoxins, endotoxin,

capsule polysaccharides, adhesins, outer membrane proteins such as transferrin-binding proteins and other iron-acquisition systems.

The four pore-forming exotoxins of *A. pleuropneumoniae*, called Apx, are cytolytic and/or hemolytic (24, 60). 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 (23).

As demonstrated by Jacques *et al.*, lipopolysaccharides (LPS) are the major molecule responsible for adhesion, principally the core oligosaccharide region (8, 39, 53, 56). However other putative adhesins have also been described. For instance, type IV fimbriae expressed under specific conditions in different serotypes (75), a 60-kDa collagen-binding protein (17), as well as a 55-kDa OMP (71), 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 (20). 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 lead to the loss of its invasive capacity. *Haemophilus parasuis* has also been shown to be invasive to porcine brain microvascular endothelial cells (51, 72). 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 (28, 44). 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. In fact, *A. pleuropneumoniae* stimulates the production of cytokines such as IL-1β, IL-8 and TNF-α which are detected in alveolar fluid and tissue lesions (66). Likewise, a study by Ramjeet *et al.* 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 bacterial cells (56). IL-8, a neutrophil chemoattractant, is of particular interest as neutrophil accumulation at the infection site is a characteristic symptom of porcine pleuropneumonia (66).

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 (16). 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. Other in vivo studies based on SCOTS, IVET or STM technology (3, 4, 26, 37, 63) 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) (18) and the St. Jude Porcine Lung (SJPL) (62) 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 (18, 62).

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 infection models using these cell lines and investigated host-pathogen interactions including adherence, invasion, and bacterial transcriptomic profile, as well as cell death, cytokine production and nuclear factor expression 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. The *A. pleuroneumoniae* serotype 1 reference strain S4074 was used in all tests. 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 *Haemophilus 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, Bresceia, Italy) (18) 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) (62) was grown at 37°C in 5% CO_2 in Dulbeco's modified Eagle's medium (DMEM) (Gibco), supplemented with 10% FBS, 1% sodium pyruvate and 1.5% MEM non-essential amino acids solution (Gibco).

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). Briefly, after each incubation time, supernatants were collected to evaluate the LDH released from epithelial cells. Supernatant of non-infected cells was used as a negative control, while total lysis of cells by a treatment with 2% triton was used as the 100% cytotoxicity positive control. The samples were read in a Power Wave X340 (Biotek Instruments Inc, Winooski, VT) microplate reader at a wavelength of 490 nm. The results were 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). A bacterial suspension was added to a confluent monolayer of cells grown in flasks at an 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 culture medium was collected to ensure that non-adherent apoptotic cells would not be lost, and adherent cells were scrapped off the flasks and added to the rest of the medium. The cells were tested following manufacturer's specification. Briefly, for the cell death detection ELISA, the cells were pelleted and resuspended in PBS to a concentration of 1×10^5, washed and incubated in the provided incubation buffer. The

lysed cells were then centrifuged at 20 000g for 10 min and the supernatant was used for the ELISA. Plates were read at 405 nm in a Power Wave X340 (Biotek Instruments Inc) microplate reader. For the caspase-3 Westerns, cells were pelleted and resuspended in lysis solution (40 mM Tris-HCl (pH 6.8), 275 mM NaCl, 20% glycerol, and 2% IGEPAL). Following a 30 min incubation on ice, the cells were centrifuged at 13000 rpm for 20 min. The supernatant was heated 5 min at 95°C with loading buffer. The samples were loaded on a 12% (w/v) polyacrylamide gel and migrated at 100 V. The proteins were transferred to a nitrocellulose membrane (BIO-RAD) using a Trans-blot SD semi-dry transfer apparatus (BIO-RAD). The membrane was blocked 1 h at room temperature in 2% skim milk and then incubated overnight at 4°C with 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 wells of 4-well LabTekII chamber microscopy slides (Nunc, Naperville, IL) and incubated overnight. One ml of a 2.5 $\times$ 10^6 CFU/ml suspension of *A. pleuropneumoniae* S4074 was added to the wells, and the slides were then incubated 2 h at 37°C, 5% CO_2. Four washes with Dulbecco's phosphate-buffered saline (DPBS) (Gibco) were performed to remove non-adherent bacteria. The cells were then fixed 10 min in methanol and stained 30 min with Giemsa (Sigma, St. Louis, MS). Four washes with DPBS were performed to remove the excess stain 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 $\times$ 10^5 epithelial cells were seeded into wells of 24-well tissue culture plates (Sarstedt, Numbrecht, Germany) and incubated overnight. 500 µl of an overnight culture of the bacteria to be studied was inoculated in a fresh 5 ml broth of the adequate medium and allowed to grow until an OD_{600nm} of 0.6 was reached. The bacteria were resuspended in the appropriate cell culture medium to a concentration of 2.5 $\times$ 10^6 CFU/ml. One ml of this suspension was added to each well containing the epithelial cells (MOI of 10:1) and the

plates were incubated from 1 to 3 h. Non-adherent bacteria were removed by rinsing the wells four times with DPBS. Cell with associated bacteria were released from the wells by adding 100 µl of 1X trypsin-EDTA (Gibco) and resuspended in 900 µl DPBS buffer. Serial dilutions of the samples were performed and plated on agar plates to determine the number of bacteria that adhered to the epithelial cells.

Statistical analysis. Statistical analyzes were performed using a general linear model; an extension of the ANOVA models. $P<0.005$ was considered statistically significative.

Invasion assay. 2.5×10^5 epithelial cells were seeded into wells of 24-well tissue culture plates and incubated overnight. One ml of a 2.5×10^7 CFU/ml bacterial suspension was added to each well containing the epithelial cells (MOI of 100:1) and in wells containing no epithelial cells as negative controls. Plates were incubated for 1 to 3 h. Non-adherent bacteria were removed by rinsing the wells four times with DPBS buffer. To remove any extracellular bacteria, 1 ml of culture medium 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 the wells twice with DPBS buffer. Cells were then lysed with 100 µl of sterile dH_2O. The water solution containing the lysed cells as well as any invading bacteria was then plated on agar plates and incubated overnight.

Protein profiling of SJPL and NPTr cells in contact with *A. pleuropneumoniae*. Two 175 cm^2 tissue culture flasks were seeded with a confluent monolayer of cells. 500 µl of an overnight culture of *A. pleuropneumoniae* S4074 was used to inoculate a fresh 5 ml broth which was incubated until an OD_{600nm} of 0.6 was reached. 25 ml of DMEM culture medium with or without 1×10^7 CFU/ml of bacteria was added to the flasks. Both flasks were then incubated 3 h at 37°C in 5% CO_2. Following incubation, the flasks were washed 3 times with DPBS, and 500 µl of a lysis solution (20 mM MOPS, 0.5% triton and protease inhibitors) was added. Using cell scrappers, the cells were removed from the flasks and transfered to microcentrifuge tubes on ice. Sonication treatments of ~180 joules were 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 min in a Sorvall

RC M100 ultracentrifuge. The supernatant was preserved and analyzed for protein concentration using the Bradford assay (Bio-Rad, Hercules, CA). 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 phospho-sites and kinases (Kinex bioinformatics inc., Vancouver, BC). The samples with and without incubation with bacteria were differentially labelled and hybridized side-by-side on the same microarray. Qualitative and semi-quantitative analyzes 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 with *A. pleuropneumoniae* at a MOI of 1:10 for 30 min, 1 and 3 h. Uninfected cells were used as control. Cell stimulation was terminated by the addition of cold PBS. Nuclear proteins were extracted as follows. Sedimented cells were resuspended in 400 µl cold buffer A (10 mM HEPES, pH 7·9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride). After a 15 min incubation on ice, 25 µl of NP-40 (10%) was added to each sample which were vortexed for 30 sec and then centrifuged 1 min at 13 000 rpm. Supernatants were discarded and cell pellets resuspended in 50 µl cold buffer C (20 mM HEPES-KOH, pH 7.9, 4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulphonyl fluoride) and incubated on ice 15 min. Cell debris were removed by centrifugation at 11 000 g for 15 min at 4°C. The supernatant was stored at −70°C until further use. Protein concentrations were determined by Bradford assay (Bio-Rad). 6 µg of nuclear proteins were incubated 20 min at room temperature in 1 µl binding buffer (100nm Hepes (pH 7.9), 40% v/v glycerol, 10% w/v Ficoll, 250 mM KCl, 10 mM DTT, 5 mM EDTA, 250 mM NaCl, and 10 mg/ml BSA) and 200 ng/µl poly (dI-dC), 0.02% bromophenol blue with 1 µl of the label 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) 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 Biotech) at room temperature 1 h, and complexes resolved on standard non-denaturing 5% (w/v) polyacrilamide gel. For the IRAK inhibition assays, the cells were pre-incubated for 1 h with 50 μM IRAK 1/4 inhibitor, 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.* (56). 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. ELISAs for the detection of pro-inflammatory cytokines were performed using the same technique and antibodies as described by Ramjeet *et al.*(56). 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 (56). As a control, NF-κB inhibitions were performed where cells were pre-incubated for 1 h at 37°C in 5% CO_2 with 25 μg/ml caffeic acid phenethyl ester (CAPE) 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 3 h with 100 μl of an OD_{600} of 0.6 culture of *A. pleuropneumoniae* (MOI of 10:1). Planktonic bacteria were harvested in the culture supernatant while

adherent bacteria were harvested with the epithelial cells 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 -80°C 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 Ambion's Turbo DNase to ensure that contaminant DNA was eliminated from the samples.

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. pleuoropneumoniae* 5b L20 genome (16, 22). 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 the tested and control conditions, namely planctonic 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. Microarray analysis was carried out using the TM4 Suite of softwares (TIGR) and the SAM algorithm, using a false discovery rate (FDR) value of 0% (59). 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 (55).

RESULTS

Effect of bacterial infection on viability of epithelial cells. Tissues death is known to occur during porcine pleuropneumonia. This phenomenon involves either necrosis or apoptosis. 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 (30 min, 1 hr, 2 hrs, 3 hrs and 4 hrs) with *A. pleuropneumoniae* strain S4074 representing serotype 1. LDH cytotoxicity assays to detect necrosis were first performed (Figure 1). Important cytotoxicity was observed after an incubation of 4 hours (up to 80% at an MOI of 10:1) or with an MOI of 100:1 (up to 40% at 3 hrs) (data not shown). An MOI of 10:1 and incubation times not surpassing 3 hrs were chosen for subsequent tests as a result of low cytotoxicity in these conditions. 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 hours of bacterial infection at an MOI of 10:1 (Figures 2 and 3).

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 4). The increase of adherence over time is well demonstrated in the adherence assay, with an increase of about 1 log every hour (Figure 5).

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 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. 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 (data not shown). Amongst the protein differentially expressed, IKKα, IKKβ and 3 different MEKK proteins were detected and directed our research towards the NF-κB pathway, and ultimately to production of cytokines by the epithelial cells.

NF-κB and AP-1 induction and cytokine production. An EMSA detecting the induction of NF-κB was performed on both cell lines following incubation with *A. pleuropneumoniae* S4074 as this signalling pathway is involved in inflammatory response. 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 apparition of bands of higher density in the upper part of the gel, corresponding to a band shift (Figure 6A). 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 6B). To further investigate the bacterial-based induction of the NF-κB transcription factor in downstream pathways we evaluated the cytokine production by both cell lines in stimulated conditions. Incubations for up to 48 hrs 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 hours (Figure 7) in comparison to 800 pg/ml with purified LPS (data not shown). 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 (33). The level of NF-κB induction in comparison to non inhibited cells consequently demonstrated indirectly if the activation of Toll receptors by the bacteria is responsible for this induction. Our results indicate that for

the SJPL cells, NF-κB induction occurs through the Toll receptor pathway but for the NPTr cells, NF-κB induction is through a different pathway (data not shown). An EMSA was also performed to detect the induction of AP-1 following an incubation with heat-killed bacteria, but revealed that although AP-1 was identified in the nuclear proteins of both cell lines, no induction was detected in comparison to the uninfected cells (data not shown).

***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 hrs. 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 (Table 6).

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 : subunits of the formate dehydrogenase (*fdnGHI*) and nitrate reductase (*nrfABC*), which are essential for anaerobic respiration on nitrate (42, 74), and subunits of the fumarate reductase (*frdACD*), which allows fumarate to serve as a terminal electron acceptor (13), 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 dehydrogenase, alcohol dehydrogenase I (ap2149) and malate:quinine oxidoreductase (ap1574), are also involved in anaerobic respiration, the latter being controlled by the ArcA-ArcB two component system (70). Genes *aspA* and *dmsA* were also up-regulated. The "Transport and Binding Proteins" class was the second most affected with 12 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 protein involved in the ABC-transport of molybdate

(30). Gene *ap0495*, coding for a putative autotransporter adhesin, showed a 1.9 fold induction.

Down-regulated genes during planctonic 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 (16) were also down-regulated. ORFs ap2142 – ap2145, which code for a putative second hemoblogin receptor system and are likely transcriptionally linked, were repressed, as well as ORFs ap0796-ap0798-ap0801, 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 *NMB1668*. Once again, genes involved in anaerobic respiration were shown to be up-regulated. The *nrdG* gene codes for an enzyme which activates the NrdD reductase under anaerobic conditions and is essential for anaerobic growth in *E. coli (21, 27)*. This system plays an essential role in anaerobic synthesis of desoxyribonucleotides. Other enzymes coding for hydrogenases (*hybAB*) 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*, (14), 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 8). 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 (data not shown) (72).

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 to screen 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, IKKβ and MEKKs. NF-κB consist

of homo- or heterodimer composed of the five mammalian Rel proteins, p65, c-Rel, p50, p52 and RelB (29), 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 (48). 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* (48). 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 or p65 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 and p65 in the NPTr cells and SJPL cells respectively. 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. Indeed, the NF-κB binding site sequence within the promoter region of the human IL-8 (5'-GTGGAATTTCC-3') is closer to the p65 binding consensus (5'-GGGRNTTTCC-3') than that of p50 (5'-GGGGATYCCC-3') (47). 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 (46). Different pathways can activate NF-κB, the most frequent in bacterial infection being through the classical pathway through Toll-like receptors activation by the LPS of the bacterium (52). 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 homodimer are activated instead of the IKKβ in the classical pathway leading to NF-κB2/p100 phosphorylation. This is likely as this modification creates the production of p52 (52), 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, including LTβR, BAFF-R, CD40 and CD30 (52). 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, 36), 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 controlled 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 (25) 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 (64). The induction of anaerobic metabolism genes might only reflect the diversity of substrates from which *A. pleuropneumoniae* can generate its energy. Overall, *A. pleuropneumoniae* growth was increased during planktonic life over SJPL as compared to growth in cell culture medium.

Furthermore, a gene with possible involvement in virulence was also identified. Gene *ap0495* 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 (67), and the putative *Mannheimia haemolytica* Hsf protein (49). In *Yersinia enterocolitica*, YadA mediates adhesion to various types of host cells, and is crucial for pathogenicity (32). In *Haemophilus influenzae* serotype b, Hsf (*Haemophilus* surface fibrils) is considered as the major nonpilus adhesion (65), and was found to be associated with adherence to human epithelial cells (6, 31). 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 (15) 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 (16). 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 of enzymes and ferritin-bound ferric iron.

Some genes with possible involvement in virulence also came up as down-regulated during planktonic growth. Capsular polysaccharides are thought to play an important role in the virulence of *A. pleuropneumoniae* (12), where it could contribute to the survival of the bacteria inside the macrophages, and thereby avoid clearance. However, it has been shown on many occasions that acapsular mutants show higher level of adherence to various cell types (9-11, 38, 57). 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. Repression of 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 (16) and might therefore simply follow the same trend as other iron-acquisition 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. Despite years of research, no formal protein adhesin has been confirmed up to now in *A. pleuropneumoniae* (12). According to our list of up-regulated gene, the production of such proteins does seem to be induced by adhesion to SJPL cells. Genes *tadB* and *rcpA* are part of a large operon which, in *A. actinomycetemcomitans*, is composed of 14 genes (43) and mediates non-specific adhesion to solid surfaces, whether they are biological surfaces or not (19), and were shown to be essential for the development of the disease in a rat model (61). The genetic

organization of the *A. pleuropneumoniae tad* locus is identical to that of *A. actinomycetemcomitans* (69). Although it is suspected that the *tad* genes might be translated 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 arises after continued passage on rich medium (58) as mutations often appear in the promoter region of gene *flp-1* (69). 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 it 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 (35). Biofilms have been known to be involved in abiotic surface and intercellular adhesion, protection against host defence mechanism and antimicrobial components (73) as well as virulence (45). To this day, the only components that have been clearly shown to be involved in *A. pleuropneumoniae* adhesion to biological lung surfaces are LPS (1, 9, 41, 53, 54).

Surprisingly, 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 (*bisC*, *ap0762*, *nrfB*, *frdD*). Genes putatively regulated by HlyX have been shown to be induced by bronchoalveolar lavage fluid from infected pigs (36), 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,* as weakly hemolytic mutant exhibit significant loss of virulence for mice and pigs (68). These toxins are thought to be responsible for most of the pathological consequences of the infection (12). 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 (40), and that the iron response regulator, Fur, seems to have variable effects depending on calcium concentration in the culture medium (34). 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 (16). 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. Close contact between the bacteria and their target cells might offset the necessity to transcribe RTX toxins in order to acquire certain essential nutrients that, such as iron, can only be found inside host cells.

Adherence is seen in both models for all *A. pleuropneumonaie* strains and serotypes tested but at different levels. It is interesting to note that field strains adhere more to the cell lines than the reference strain of the same serotype. Differences were also noticed between the two cell lines for a given strain, indicating preferences for certain strains to a given cell line. No invasion is noticed for *A. pleuropneumoniae*, even though close relatives, such as *A. actinomycetemcomitans* and *H. parasuis*, are known to be invasive (20, 50, 72).

Taken together, these results demonstrate the numerous events that occur during an *A. pleuropneumoniae* infection in both the host and the microbe. When *A. pleuropneumoniae* is in the presence of the host epithelial cells, many virulence genes are up-regulated including genes coding for the putative adhesins Hsf and Pga, while capsular polysaccharide associated genes are down regulated possibly exposing adhesins usually hidden by a thick capsule. Incubation with *A. pleuropneumoniae* then leads, 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.

Overall these results showed the efficacy of the model 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 including some coding for putative adhesins. 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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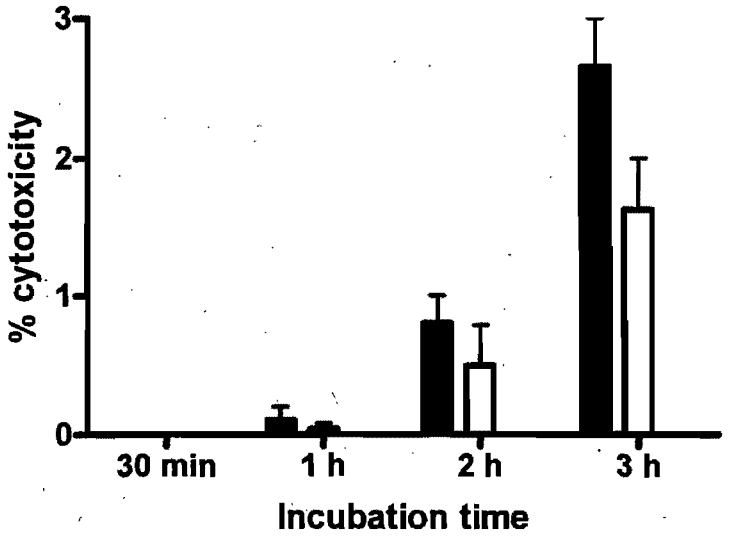

Figure 1. SJPL (filled bars) and NPTr (empty bars) cells were assessed for cytotoxicity following an infection with *A. pleuropneumoniae* strain S4074 at an MOI of 10:1.

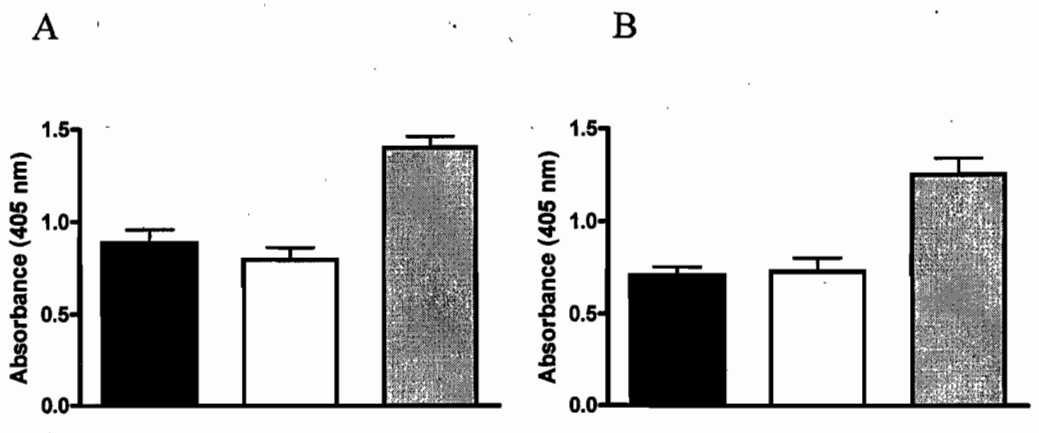

Figure 2. SJPL (A) and NPTr (B) cells were assessed for the formation of apoptotic oligonucleosomes following 3 h of incubation with *A. pleuropneumoniae* S4074 at an MOI of 10:1 (black). Untreated cells (white) and cells treated with camptothecin (grey), were also assessed as controls.

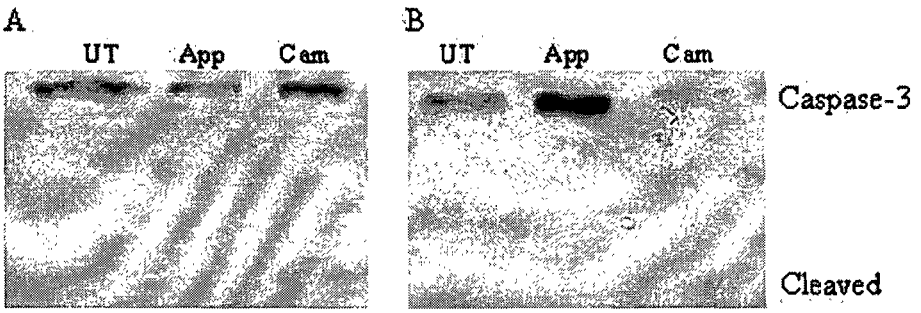

Figure 3. Representative Western blot image illustrating caspase-3 cleavage upon treatment of SJPL (A) and NPTr (B) cells with camptothencin (Cam) but not following an infection with *A. pleuropneumoniae* S4074 (App) compared to control untreated cells (UT).

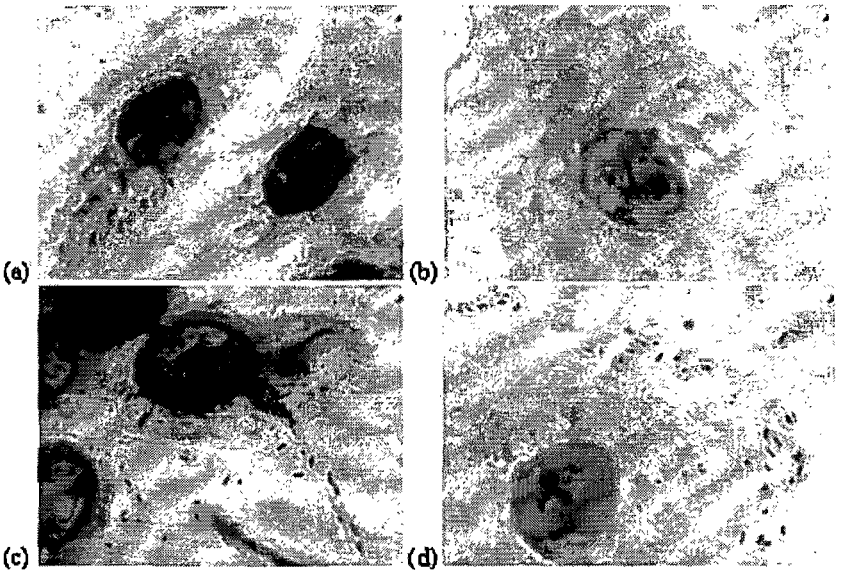

Figure 4. NPTr (a and c) and SJPL (b and d) cells stained with Giemsa in the presence (c and d) or absence (a and b) of *A. pleuropneumoniae* S4074 seen through a Leica DMR microscope at a magnification of 1000X.

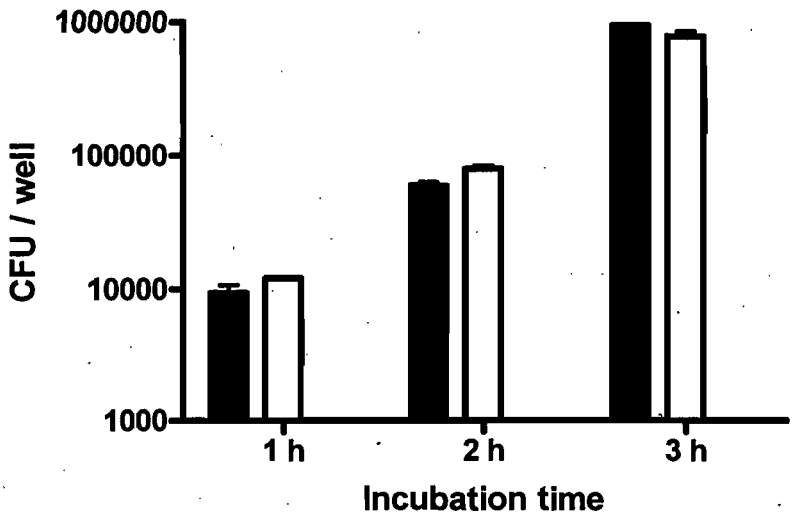

Figure 5. Adherence of *A. pleuropneumoniae* S4074 to SJPL (filled bars) and NPTr (empty bars) cells from 1 to 3 h.

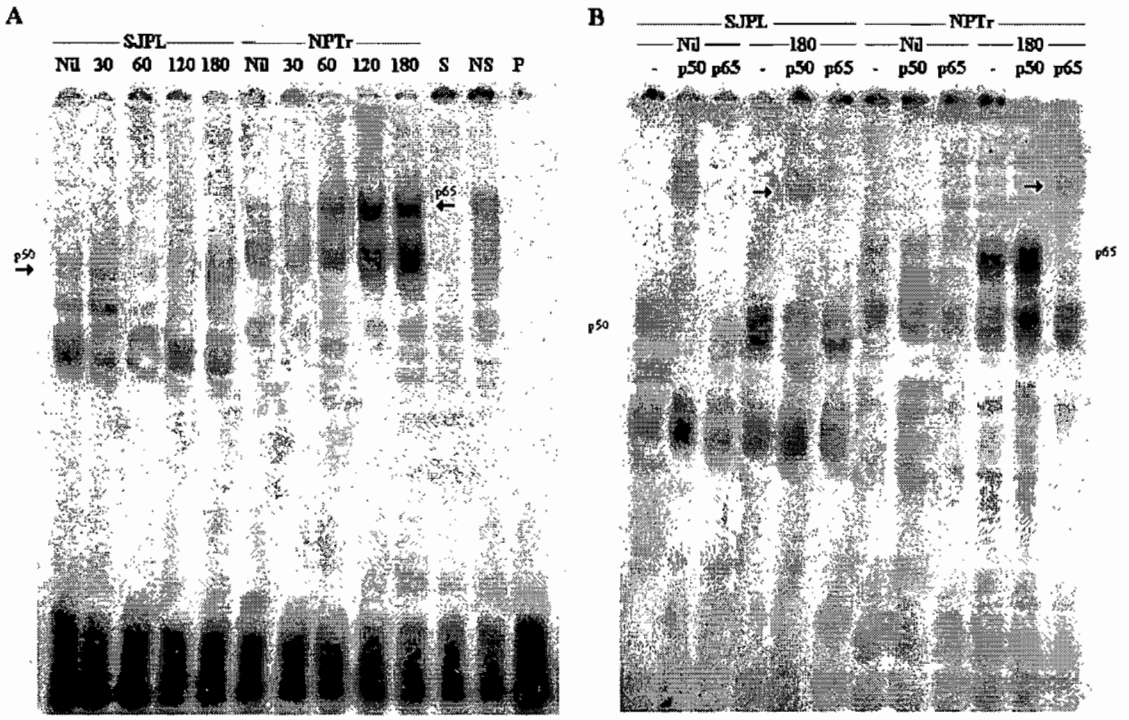

Figure 6. EMSA (A) and supershift assay (B) performed on nuclear proteins of SJPL and NPTr cells following an incubation with *A. pleuropneumoniae* S4074 or not treated for control (Nil). For the supershift assay (B), proteins where incubated with p50 antibodies, p65 antibodies, or no antibodies (-). Arrows demonstrate the subunits p50 and p65 (A) and the subunit band shifts (B).

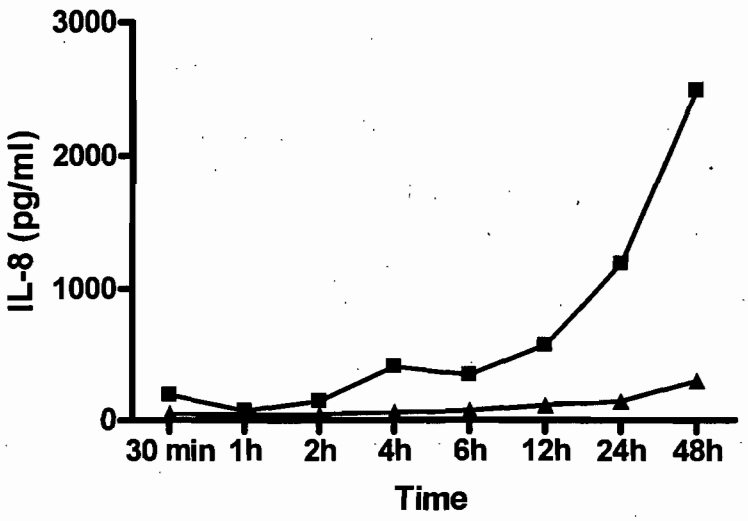

Figure 7. Production of IL-8 by NPTr cells following an induction with heat-killed *A. pleuropneumoniae* S4074 (■) and when not stimulated (▲).

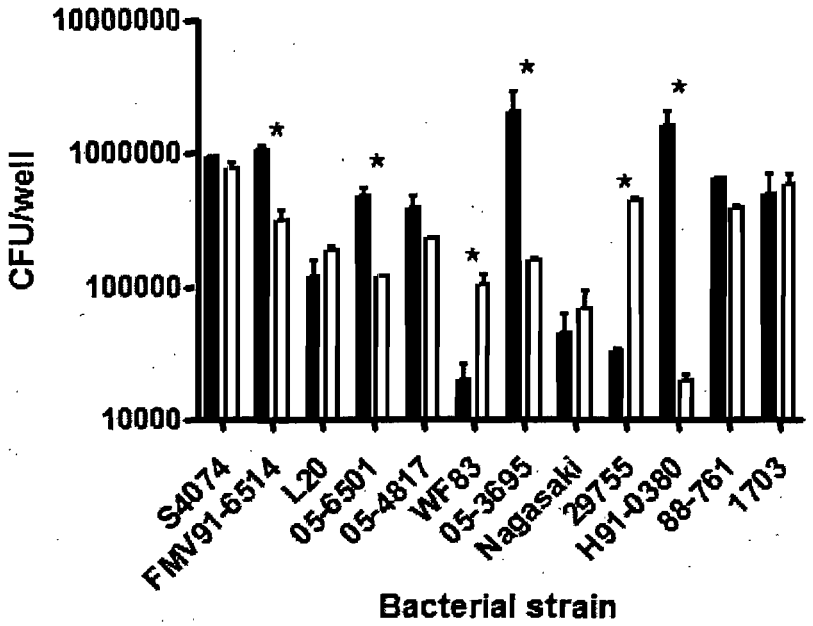

Figure 8. Adherence of twelve *Pasteurellaceae* to the SJPL (filled bars) and NPTr (empty bars) cell line after 3 h of incubation. The strains include *A. pleuropneumoniae* serotype 1 S4074 and FMV91-6514, *A. pleuropneumoniae* serotype 5b L20 and 05-6501, *A. pleuropneumoniae* serotype 5a 05-4817, *A. 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.

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)	K.R. Mittal[a]
A. pleuropneumoniae 05-4817	5a	K.R. Mittal[a]
A. pleuropneumoniae 05-6501	5b	K.R. Mittal[a]
A. pleuropneumoniae 05-3695	7	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			
ap2022	*udp*	COG2820: Uridine phosphorylase, probable outer membrane protein, possible efflux protein	3.937
ap2015	*ap2015*	COG2717: Predicted membrane protein, conserved hypothetical protein	3.388
ap1595	*ap1595*	Unassigned protein	3.214
ap0164	*ap0164*	COG1611: Predicted Rossmann fold nucleotide-binding protein	2.726
ap2168	*yedF*	COG0425: Predicted redox protein, regulator of disulfide bond formation	2.669
ap0100	*ap0100*	DUF1260 domain containing protein	2.020
ap1532	*ap1532*	Unassigned protein	1.946
ap0519	*ap0519*	DUF74 domain containing protein	1.943
ap2106	*ap2106*	predicted enzyme related to aldose 1-epimerase	1.939
ap1270	*ap1270*	DUF526 domain containing protein	1.933
ap0495	*ap0495*	COG5295: Autotransporter adhesin	1.918
ap1829	*cof*	COG0561: Predicted hydrolases of the HAD superfamily	1.842
ap1345	*ap1345*	DUF479 domain containing protein, hypothetical protein	1.811
ap1780	*ap1780*	DUF533 domain containing protein	1.724
ap2066	*ap2066*	Dala_Dala_ligas multi-domain protein	1.703
ap1395	*ap1395*	Transposase_mut domain containing protein	1.555
Biosynthesis of cofactors			
ap1793	*ap1793*	COG0310: ABC-type Co2+ transport system, permease component	2.419
ap1044	*iscS*	COG1104: Cysteine sulfinate desulfinase/cysteine desulfurase and related enzymes, iron-sulfur cluster assembly	2.374
ap1043	*iscU*	COG0822: NifU homolog involved in Fe-S cluster formation	2.011
ap1723	*hemL*	COG0001: Glutamate-1-semialdehyde aminotransferase	1.619
Energy metabolism			
ap2014	*ap2014*	COG2041: Sulfite oxidase and related enzymes	13.073
ap0996	*fdnG*	COG0243: Anaerobic dehydrogenases, typically selenocysteine-containing	12.558
ap0999	*fdnI*	COG2864: Cytochrome b subunit of formate dehydrogenase	10.946
ap0998	*fdnH*	COG0437: Fe-S-cluster-containing hydrogenase components 1	6.0988
ap0761	*ap0761*	COG0277: FAD/FMN-containing dehydrogenases	5.442
ap0108	*nrfA*	COG3303: Formate-dependent nitrite reductase, periplasmic cytochrome c552 subunit	5.039
ap0121	*putA*	COG4230: Delta 1-pyrroline-5-carboxylate dehydrogenase	4.634
ap0109	*nrfB*	nitrate reductase, cytochrome-C type protein	4.295
ap1222	*aspA*	aspartate ammonia-lyase	4.254
ap1692	*frdC*	COG3029: Fumarate reductase subunit C	3.713
ap1269	*pgi*	COG0166: Glucose-6-phosphate isomerase	3.136
ap2149	*ap2149*	COG1064: Zn-dependent alcohol dehydrogenases	2.992
ap1574	*ap1574*	COG0579: Predicted dehydrogenase	2.802
ap1536	*mauG*	COG1858: Cytochrome c peroxidase	2.796
ap1611	*fbp*	COG0158: Fructose-1,6-bisphosphatase	2.528

Locus Tag	Gene	Description	Fold Change
		COG1053: Succinate dehydrogenase/fumarate reductase,	
ap1694	*frdA*	flavoprotein subunit	2.420
ap0206	*pykA*	COG0469: Pyruvate kinase	2.322
		COG2084: 3-hydroxyisobutyrate dehydrogenase and related	
ap1337	*ap1337*	beta-hydroxyacid dehydrogenases	2.270
ap0110	*nrfC*	COG0437: Fe-S-cluster-containing hydrogenase components 1	2.250
ap1691	*frdD*	COG3080: Fumarate reductase subunit D	2.178
ap0541	*maeA*	malate oxidoreductase (NAD)	2.074
ap1848	*dmsA*	dimethyl sulfoxide reductase	1.934
ap0538	*ap0538*	COG0778: Nitroreductase	1.912
ap0091	*trxB*	COG0492: Thioredoxin reductase	1.486
Transport and binding proteins: cations and iron			
ap1418	*copA*	COG2217: Cation transport ATPase	1.518
Transport and binding proteins : others			
ap0123	*putP*	COG0591: Na+/proline symporter	6.218
		COG0607: Rhodanese-related sulfurtransferase, putative	
ap1437	*argH*	periplasmic protein	2.894
ap1310	*pnuC*	COG3201: Nicotinamide mononucleotide transporter	2.448
ap0416	*glpT*	GlpT	2.331
ap1406	*recX*	COG0471: Di- and tricarboxylate transporters	2.237
		COG1263: Phosphotransferase system IIC components,	
ap1473	*ptsB*	glucose/maltose/N-acetylglucosamine-specific	2.206
ap0500	*lctP*	COG1620: L-lactate permease	2.204
		COG0725: ABC-type molybdate transport system, periplasmic	
ap0285	*modA*	component	2.049
		COG1122: ABC-type cobalt transport system, ATPase	
ap1791	*cbiO*	component	1.902
ap1795	*cbiK*	putative periplasmic binding protein CbiK	1.823
ap2088	*yrhG*	COG2116: Formate/nitrite family of transporters	1.718
		COG2386: ABC-type transport system involved in cytochrome	
ap1529	*ccmB*	c biogenesis, permease component	1.596
Regulatory functions			
ap0124	*yiaJ*	COG1414: Transcriptional regulator	2.832
ap0436	*mclA*	COG3073: Negative regulator of sigma E activity	2.260
ap0921	*glpR*	COG1349: Transcriptional regulators of sugar metabolism	2.175
ap1798	*bioB*	COG0471: Di- and tricarboxylate transporters	1.876
		Beta-galactosidase (Lactase) gb|AAB17954.1| beta-	
ap1118	*lacZ*	galactosidase	1.654
Transcription			
ap0435	*rpoD*	RNA polymerase sigma-70 factor	2.135
ap0626	*rhlB*	COG0513: Superfamily II DNA and RNA helicases	1.911
Purines, pyrimidines, nucleosides, and nucleotides			
ap0716	*cpdB*	UshA protein	2.087
Protein fate			
ap0828	*degS*	protease DegS	1.768
ap0975	*pepE*	COG3340: Peptidase E	1.686

Locus Tag	Gene	Description	Fold Change
Protein synthesis			
ap0539	*rimK*	COG0189: Glutathione synthase/Ribosomal protein S6 modification enzyme (glutaminyl transferase)	3.093
ap0165	*ap0165*	COG0042: tRNA-dihydrouridine synthase	2.456
Cellular processes			
ap0004	*sodC*	superoxide dismutase, Cu/Zn	3.003
ap0274	*sodA*	COG0605: Superoxide dismutase	2.356
ap1392	*ap1392*	COG1966: Carbon starvation protein, predicted membrane protein	2.098
ap1563	*oapA*	COG3061: Cell envelope opacity-associated protein A	1.793
Cell envelope			
ap1658	*ftpA*	COG0783: DNA-binding ferritin-like protein (oxidative damage protectant), fine tangled pili major subunit (24 kDa surface protein)	6.378
ap0132	*thdF*	Unassigned protein	2.523
Fatty acids and phospholipids metabolism			
ap0439	*lcfA*	COG0318: Acyl-CoA synthetases (AMP-forming)/AMP-acid ligases II	1.746
Mobile and extrachromosomal element functions			
ap1665	*ap1665*	COG2801: Transposase and inactivated derivatives	2.0782984
ap1110	*ap1110*	COG2801: Transposase and inactivated derivatives	1.8143865
ap1105	*ap1105*	COG2801: Transposase and inactivated derivatives	1.6804825
DNA metabolism			
ap1277	*recA*	COG0468: RecA/RadA recombinase	1.491
Central intermediary metabolism			
ap0125	*ap0125*	COG0212: 5-formyltetrahydrofolate cyclo-ligase	2.607
ap0414	*glpK*	COG0554: 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			
ap1544	*suhB*	COG1399: Predicted metal-binding, possibly nucleic acid-binding protein	-2.431
ap0056	*gptA*	COG1217: Predicted membrane GTPase involved in stress response	-2.386
ap0209	*ap0209*	COG2901: Factor for inversion stimulation Fis, transcriptional activator	-2.209
ap0192	*lapB*	COG0402: Cytosine deaminase and related metal-dependent hydrolases	-2.056
ap2047	*sdaA*	COG3774: Mannosyltransferase OCH1 and related enzymes	-2.027
ap2146	*ap2146*	Unassigned protein, hypothetical protein	-2.000
Energy metabolism			
ap2032	*lldD*	COG1304: L-lactate dehydrogenase (FMN-dependent) and related alpha-hydroxy acid dehydrogenases	-3.284
ap0659	*guaA*	GuaA protein	-2.629
ap1363	*fldA*	flavodoxin	-2.276
Transport and binding proteins: cations and iron			
ap1176	*hgbA*	hemoglobin-binding protein A precursor	-10.713
ap1175	*hgbA*	hemoglobin-binding protein A precursor	-7.980
ap1740	*tonB1*	Periplasmic energy transducing protein TonB1, links inner and outer membranes	-5.319
ap2142	*PM0741*	COG1629: Outer membrane receptor proteins, mostly Fe transport	-3.517
ap0083	*exbD2*	biopolymer transport protein ExbD2	-3.499
ap2143	*PM0741*	COG1629: Outer membrane receptor proteins, mostly Fe transport	-2.874
ap1177	*hugZ*	heme utilization protein	-2.3871
ap0295	*yfeA*	iron (chelated) ABC transporter, periplasmic-binding protein	-2.339
ap0294	*yfeB*	putative chelated iron transport system ATP-binding protein	-2.251
ap0796	*pilQ*	COG4607: ABC-type enterochelin transport system, periplasmic component	-2.178
ap2144	*thrB*	COG1629: Outer membrane receptor proteins, mostly Fe transport	-2.163
ap0798	*fbpB*	COG4606: ABC-type enterochelin transport system, permease component	-1.993
ap2145	*thrB*	COG1629: Outer membrane receptor proteins, mostly Fe transport	-1.891
ap0801	*thrB*	COG4604: ABC-type enterochelin transport system, ATPase component	-1.760
ap0144	*yfeD*	putative iron transport system membrane protein	-1.718
Transport and binding proteins : others			
ap0330	*tolQ*	COG0811: Biopolymer transport proteins	-2.584
ap0741	*ap0741*	COG0672: High-affinity Fe2+/Pb2+ permease	-2.406

Locus Tag	Gene	Description	Fold Change
ap1549	*ap1549*	COG3715: Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	-2.135
ap1550	*ptnD*	COG3716: Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IID	-2.043
ap1755	*cpxB*	capsule polysaccharide export inner-membrane protein	-1.863
ap1049	*folK*	Unassigned protein	-1.829
ap1756	*cpxA*	capsule polysaccharide export ATP-binding protein	-1.572
ap1754	*cpxC*	capsule polysaccharide export inner-membrane protein	-1.549
Transcription			
ap0708	*nusA*	COG0195: Transcription elongation factor	-3.997
ap0644	*pnp*	COG1185: Polyribonucleotide nucleotidyltransferase (polynucleotide phosphorylase)	-2.375
ap0844	*rnb*	COG4776: Exoribonuclease II	-1.665
ap0608	*rnc*	COG0571: dsRNA-specific ribonuclease	-1.539
Purines, pyrimidines, nucleosides, and nucleotides			
ap0167	*nrdA*	COG0209: Ribonucleotide reductase, alpha subunit	-2.556
ap0661	*guaB*	COG0516: IMP dehydrogenase/GMP reductase	-2.474
ap0166	*nrdB*	COG0208: Ribonucleotide reductase, beta subunit	-2.438
ap0278	*gpt*	COG0503: Adenine/guanine phosphoribosyltransferases and related PRPP-binding proteins	-2.316
ap0731	*purK*	COG0026: Phosphoribosylaminoimidazole carboxylase (NCAIR synthetase)	-2.091
ap1309	*purD*	COG0151: Phosphoribosylamine-glycine ligase	-1.883
ap2213	*purC*	COG0152: Phosphoribosylaminoimidazolesuccinocarboxamide (SAICAR) synthase	-1.717
ap0934	*udk*	COG0572: Uridine kinase	-1.396
Protein fate			
ap1671	*tig*	COG0544: FKBP-type peptidyl-prolyl cis-trans isomerase (trigger factor)	-3.849
ap0402	*Ssa1*	autotransporter serine protease	-2.279
ap1604	*clyIB*	COG2274: ABC-type bacteriocin/lantibiotic exporters, contain an N-terminal double-glycine peptidase domain	-1.753
Protein synthesis			
ap1895	*rplK*	50S ribosomal protein L11	-5.061
ap0709	*infB*	COG0532: Translation initiation factor 2 (IF-2; GTPase)	-4.243
ap1953	*rplF*	COG0097: Ribosomal protein L6P/L9E	-3.954
ap1897	*rplJ*	COG0244: Ribosomal protein L10	-3.833
ap1727	*rpsT*	30S ribosomal protein S20	-3.720
ap1944	*rplV*	50S ribosomal protein L22	-3.039
ap0633	*rpsB*	COG0052: Ribosomal protein S2	-2.842
ap1558	*rpsG*	COG0049: Ribosomal protein S7	-2.714
ap0542	*rplY*	COG1825: Ribosomal protein L25 (general stress protein Ctc)	-2.364
ap1942	*rplW*	COG0089: Ribosomal protein L23	-2.330
ap1943	*rplB*	COG0090: Ribosomal protein L2	-2.265
ap1941	*rplD*	COG0088: Ribosomal protein L4	-2.254
ap0245	*infC*	COG0290: Translation initiation factor 3 (IF-3)	-2.192
ap1952	*rpsH*	COG0096: Ribosomal protein S8	-2.100
ap1954	*rplR*	COG0256: Ribosomal protein L18	-2.020
ap0441	*ksgA*	COG0030: Dimethyladenosine transferase (rRNA methylation)	-1.950

Locus Tag	Gene	Description	Fold Change
ap1559	*rpsL*	30S ribosomal protein S12	-1.914
ap2164	*rpmG*	COG0267: Ribosomal protein L33	-1.902
ap0043	*ap0043*	COG0536: Predicted GTPase	-1.721
ap1372	*infA*	COG0361: Translation initiation factor 1 (IF-1)	-1.716
ap0751	*efp*	COG0231: Translation elongation factor P (EF-P)/translation initiation factor 5A (eIF-5A)	-1.708
ap1960	*rpsM*	COG0099: Ribosomal protein S13	-1.705
ap1540	*ap1540*	COG0220: Predicted S-adenosylmethionine-dependent methyltransferase	-1.688
ap0711	*truB*	COG0130: Pseudouridine synthase	-1.660
ap1639	*tyrS*	COG0162: Tyrosyl-tRNA synthetase	-1.646
ap1970	*rplS*	COG0335: Ribosomal protein L19	-1.521
Cellular processes			
ap0740	*phpA*	COG2837: Predicted iron-dependent peroxidase	-2.630
Cell envelope			
ap1046	*proQ*	COG2067: Long-chain fatty acid transport protein	-8.199
ap0721	*galU*	COG1210: UDP-glucose pyrophosphorylase	-2.114
ap2182	*murA*	COG3671: Predicted membrane protein	-1.951
ap1200	*hexC*	COG2252: Permeases	-1.817
Fatty acids and phospholipids metabolism			
ap2048	*accB*	COG0511: Biotin carboxyl carrier protein	-2.570
ap2049	*accC*	COG0439: Biotin carboxylase	-2.444
ap2075	*fabA*	COG0764: 3-hydroxymyristoyl/3-hydroxydecanoyl-(acyl carrier protein) dehydratases	-2.426
ap1542	*plsX*	COG0416: Fatty acid/phospholipid biosynthesis enzyme	-1.922
Amino acid biosynthesis			
ap0213	*aroK*	COG0703: Shikimate kinase	-2.401
ap1663	*thrC*	COG0498: Threonine synthase	-2.083
DNA metabolism			
ap0080	*recR*	COG0353: Recombinational DNA repair protein (RecF pathway)	-1.634
Central intermediary metabolism			
ap1672	*yibN*	COG0607: Rhodanese-related sulfurtransferase	-2.860
ap0194	*dksA*	COG1734: DnaK suppressor protein	-1.845
ap0383	*glgA*	COG0297: 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			
ap0635	*ap0635*	COG2194: Predicted membrane-associated, metal-dependent hydrolase	3.083
ap1622	*ap1622*	Hypothetical protein	2.882
ap1865	*ap1865*	COG3477: Predicted periplasmic/secreted protein	2.609
ap1634	*ap1634*	COG2148: Sugar transferases involved in lipopolysaccharide synthesis	2.450
ap1537	*ap1537*	Uncharacterized conserved protein	2.310
ap2196	*ap2196*	hypothetical protein APL_2002	2.301
ap0836	*ap0836*	MscS family small conductance mechanosensitive ion channel	2.181
ap1745	*ap1745*	hypothetical protein APL_1575	1.940
ap0463	*ap0463*	UDP-glucose--lipooligosaccharide glucosyltransferase	1.929
ap2038	*pqiA*	Uncharacterized paraquat-inducible protein A	1.746
ap0238	*ap0238*	Hypothetical protein	1.640
Biosynthesis of cofactors			
ap1331	*hemC*	Hydroxymethylbilane synthase	4.524
ap0864	*ispE*	COG1947: 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate synthase	2.913
Energy metabolism			
ap2032	*lldD*	COG1304: L-lactate dehydrogenase (FMN-dependent) and related alpha-hydroxy acid dehydrogenases	6.7560
ap2001	*rpe*	D-ribulose-phosphate-3 epimerase	6.687
ap1486	*hyaA*	COG1740: Ni,Fe-hydrogenase I small subunit	3.366
ap1860	*fucK*	COG1070: Sugar (pentulose and hexulose) kinases	3.195
ap1864	*fucO*	COG1454: Alcohol dehydrogenase, class IV	2.887
ap1859	*fucI*	COG2407: L-fucose isomerase and related proteins	2.812
ap1488	*hybB*	hydrogenase 2 cytochrome b type component	2.796
ap1140	*kdgK*	2-keto-3-deoxygluconokinase	2.334
ap1000	*fdhE*	formate dehydrogenase accessory protein FdhE	2.007
Transport and binding proteins: cations and iron			
ap1974	*fecE*	ATP-binding component of citrate-dependent iron(III) transport protein	6.113
ap2145	*NMB1668*	COG1629: Outer membrane receptor proteins, mostly Fe transport	3.271
ap2174	*corA*	COG0598: Mg2+ and Co2+ transporters	3.001
ap0083	*exbD2*	biopolymer transport protein ExbD2	2.346
Transport and binding proteins : others			
ap0072	*dppC*	COG1173: ABC-type dipeptide/oligopeptide/nickel transport systems, permease components	12.073
ap0974	*ap0974*	COG3069: C4-dicarboxylate transporter	6.308
ap1889	*ap1889*	COG1297: Predicted membrane protein	4.639
ap0210	*ap0210*	COG0733: Na+-dependent transporters of the SNF family	4.638

Locus Tag	Gene	Description	Fold Change
ap0339	*ap0339*	COG0488: ATPase components of ABC transporters with duplicated ATPase domains	2.331
ap2031	*cysA*	COG1118: ABC-type sulfate/molybdate transport systems, ATPase component	1.938
ap1400	*sapF*	COG4167: ABC-type antimicrobial peptide transport system, ATPase component	1.858
ap1234	*ap1234*	COG0795: Predicted permeases	1.844
ap0283	*modC*	COG4148: ABC-type molybdate transport system, ATPase component	1.799
ap0649	*ap0649*	COG2814: Arabinose efflux permease	1.773
Regulatory functions			
ap1230	*ap1230*	COG0602: Organic radical activating enzymes	7.175
ap2154	*hflX*	GTP-binding protein hflX	2.005
Transcription			
ap0195	*pcnB*	COG0617: tRNA nucleotidyltransferase/poly(A) polymerase	2.566
Purines, pyrimidines, nucleosides, and nucleotides			
ap0863	*prsA*	COG0462: Phosphoribosylpyrophosphate synthetase	8.463
ap0106	*ilvG*	Acetolactate synthase isozyme II large subunit	6.897
ap0470	*purF*	COG0034: Glutamine phosphoribosylpyrophosphate amidotransferase	1.812
Protein fate			
ap1921	*ffh*	COG0541: Signal recognition particle GTPase	6.861
ap2091	*dnaJ*	Chaperone protein dnaJ	3.383
ap1485	*hypF*	COG0068: Hydrogenase maturation factor	2.080
ap1256	*ap1256*	COG0520: Selenocysteine lyase	2.068
Protein synthesis			
ap0037	*ap0037*	GTP-dependent nucleic acid-binding protein EngD	17.924
ap0642	*deaD*	COG0513: Superfamily II DNA and RNA helicases	6.254
ap0542	*rplY*	COG1825: Ribosomal protein L25 (general stress protein Ctc)	3.429
ap1480	*murZ*	COG0621: 2-methylthioadenine synthetase	2.357
ap1243	*rumB*	23S rRNA (uracil-5-)-methyltransferase RumB	2.145
ap0953	*infC*	COG0620: Methionine synthase II (cobalamin-independent)	2.078
Cellular processes			
ap2109	*pgaB*	Biofilm PGA synthesis lipoprotein PgaB precursor	7.257
ap0013	*ftsL*	COG3116: Cell division protein	3.349
ap2110	*pgaC*	Biofilm PGA synthesis N-glycosyltransferase PgaC	2.454
Cell envelope			
ap0617	*tadB*	Flp pilus assembly protein tight adherence protein TadB	2.453
ap2024	*murI*	COG0796: Glutamate racemase	2.340
ap0018	*murD*	COG0771: UDP-N-acetylmuramoylalanine-D-glutamate ligase	2.113
ap1769	*ap1769*	COG0772: Bacterial cell division membrane protein	2.096
ap1722	*wecA*	COG0472: UDP-N-acetylmuramyl pentapeptide phosphotransferase/UDP-N-acetylglucosamine-1-phosphate transferase	1.577
ap0621	*rcpA*	rough colony protein A	1.559

Locus Tag	Gene	Description	Fold Change
Fatty acids and phospholipids metabolism			
ap2048	*accB*	COG0511: Biotin carboxyl carrier protein	8.665
ap2049	*accC*	COG0439: Biotin carboxylase	4.016
ap0991	*ap0991*	COG0183: Acetyl-CoA acetyltransferase, 3-ketoacyl-CoA thiolase	3.675
Amino acids biosynthesis			
ap0352	*metC*	COG0626: Cystathionine beta-lyases/cystathionine gamma-synthases	9.278
ap0106	*ilvG*	Acetolactate synthase isozyme II large subunit	6.897
ap1613	*serA*	COG0111: Phosphoglycerate dehydrogenase and related dehydrogenases	3.896
ap0523	*trpB*	COG0133: Tryptophan synthase beta chain	3.274
ap0157	*leuC*	COG0065: 3-isopropylmalate dehydratase large subunit	3.253
ap2141	*proA*	COG0014: Gamma-glutamyl phosphate reductase	2.846
ap1282	*trpG*	putative anthranilate synthase component II	2.723
ap0272	*thrB*	COG0083: Homoserine kinase	2.459
DNA metabolism			
ap1336	*ap1336*	COG0610: Type I site-specific restriction-modification system, R (restriction) subunit and related helicases	3.251
ap1334	*ap1334*	COG0286: Type I restriction-modification system methyltransferase subunit	2.816
ap1281	*rmuC*	DNA recombination protein rmuC homolog	2.462
ap0979	*holA*	COG1466: DNA polymerase III, delta subunit	2.022
ap0313	*hsdM2*	COG0286: Type I restriction-modification system methyltransferase subunit	2.005
Central intermediary metabolism			
ap2240	*sseA*	COG2897: Rhodanese-related sulfurtransferase	1.942
ap2026	*cysJ*	COG0369: Sulfite reductase, alpha subunit (flavoprotein)	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			
ap2067	*ap2067*	Esterase domain containing protein	-6.273
ap1436	*ap1436*	-	-3.076
ap0052	*ap0052*	Unassigned protein	-2.982
ap1087	*ap1087*	YCII domain containing protein	-2.980
ap1231	*ap1231*	Unassigned protein	-2.928
ap0782	*ap0782*	Unassigned protein	-2.293
ap1522	*ap1522*	COG2823: Predicted periplasmic or secreted lipoprotein	-2.174
ap0126	*ap0126*	DUF710 domain containing protein	-2.144
ap1553	*ap1553*	Unassigned protein	-2.125
ap0843	*ap0843*	Unassigned protein	-2.098
ap0993	*ap0993*	-	-1.757
Energy metabolism			
ap0484	*gapA*	glyceraldehyde-3-phosphate dehydrogenase	-4.981
ap0996	*bisC*	COG0243: Anaerobic dehydrogenases, typically selenocysteine-containing	-4.853
ap0859	*lpdA*	COG1249: Pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamide dehydrogenase (E3) component, and related enzymes	-4.765
ap1536	*mauG*	COG1858: Cytochrome c peroxidase	-4.447
ap1103	*tktA*	COG0021: Transketolase	-3.790
ap0998	*ap0998*	COG0437: Fe-S-cluster-containing hydrogenase components 1	-3.627
ap1402	*pgk*	COG0126: 3-phosphoglycerate kinase	-3.625
ap1611	*fbp*	COG0158: Fructose-1,6-bisphosphatase	-3.315
ap0200	*gloA*	COG0346: Lactoylglutathione lyase and related lyases	-3.046
ap2112	*tpiA*	COG0149: Triosephosphate isomerase	-3.023
ap1222	*aspA*	aspartate ammonia-lyase	-2.722
ap0762	*ap0762*	COG0243: Anaerobic dehydrogenases, typically selenocysteine-containing	-2.687
ap1132	*adh2*	COG1012: NAD-dependent aldehyde dehydrogenases	-2.642
ap0860	*aceF*	COG0508: Pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamide acyltransferase (E2) component, and related enzymes	-2.620
ap1269	*pgi*	COG0166: Glucose-6-phosphate isomerase	-2.595
ap0541	*maeA*	malate oxidoreductase (NAD)	-2.416
ap1691	*frdD*	COG3080: Fumarate reductase subunit D	-2.267
ap0109	*nrfB*	nitrate reductase, cytochrome-C type protein	-2.231
ap1401	*fba*	COG0191: Fructose/tagatose bisphosphate aldolase	-2.124
Transport and binding proteins : others			
ap0429	glpR	COG2503: Predicted secreted acid phosphatase	-2.509
ap1791	cbiO	COG1122: ABC-type cobalt transport system, ATPase component	-2.108
ap0500	lctP	COG1620: L-lactate permease	-2.068
ap0803	asnA	COG0306: Phosphate/sulphate permeases	-1.816
ap0882	Cj0850c	COG0477: Permeases of the major facilitator superfamily	-1.702

Locus Tag	Gene	Description	Fold Change
Regulatory functions			
ap0726	*hlyX*	COG0664: cAMP-binding proteins - catabolite gene activator and regulatory subunit of cAMP-dependent protein kinases	-2.727
ap0699	*cpxR*	COG0745: Response regulators consisting of a CheY-like receiver domain and a winged-helix DNA-binding domain	-2.367
Purines, pyrimidines, nucleosides, and nucleotides			
ap0856	*ap0856*	5'-nucleotidase / UDP-sugar diphosphatase	-3.470
ap0716	*cpdB*	UshA protein	-2.778
ap1135	*deoD*	COG0813: Purine-nucleoside phosphorylase	-2.247
Protein fate			
ap1289	*ap1289*	COG0612: Predicted Zn-dependent peptidases	-2.401
ap1617	*slyD*	FkbP-type peptidyl-prolyl cis-trans isomerase	-2.313
Protein synthesis			
ap0825	rpsA	COG0539: Ribosomal protein S1	-1.608
Cellular processes			
ap1606	*apxIC*	ApxI toxin maturation protein C	-4.117
ap1069	*apxIIA*	RTX domain containing protein	-4.052
ap0004	*sodC*	superoxide dismutase	-3.193
Cell envelope			
ap1658	*ap1658*	COG0783: DNA-binding ferritin-like protein (oxidative damage protectant)	-4.223
ap0722	*cpsG*	COG1109: Phosphomannomutase	-3.375
ap1598	*ap1598*	Unassigned protein	-2.751
ap0132	*ap0132*	Unassigned protein	-2.566
Central intermediary metabolism			
ap0715	*ackA*	COG0282: Acetate kinase	-3.190
ap2085	*ppa*	COG0221: Inorganic pyrophosphatase	-2.207

Table 6. Indirect comparison between *A. pleuropneumoniae* transcript profiling during adherence to SJPL cells and *A. pleuropneumoniae* transcript profiling during planktonic growth over SJPL cells (149 genes).

Locus Tag	Gene	Description	Fold Change
ap0037	*ap0037*	COG0012: Predicted GTPase, probable translation factor	42.512
ap1176	*ap1176*	TonB-dependent receptor, putative	33.534
ap2032	*lldD*	COG1304: L-lactate dehydrogenase (FMN-dependent) and related alpha-hydroxy acid dehydrogenases	25.876
ap2048	*accB*	COG0511: Biotin carboxyl carrier protein	21.417
ap1046	*ap1046*	COG2067: Long-chain fatty acid transport protein	19.307
ap0072	*dppC*	COG1173: ABC-type dipeptide/oligopeptide/nickel transport systems, permease components	15.857
ap1175	*ap1175*	TonB-dependent receptor, putative	14.422
ap0352	*metC*	COG0626: Cystathionine beta-lyases/cystathionine gamma-synthases	13.237
ap0642	*ap0642*	COG0513: Superfamily II DNA and RNA helicases	12.421
ap2049	*accC*	COG0439: Biotin carboxylase	10.091
ap2109	*cDA1*	COG0726: Predicted xylanase/chitin deacetylase	9.511
ap0210	*ap0210*	COG0733: Na+-dependent transporters of the SNF family	8.985
ap1921	*ffh*	COG0541: Signal recognition particle GTPase	8.555
ap0542	*rplY*	COG1825: Ribosomal protein L25 (general stress protein Ctc)	8.428
ap1230	*ap1230*	COG0602: Organic radical activating enzymes	7.955
ap1740	*ap1740*	COG0810: Periplasmic protein TonB, links inner and outer membranes	7.678
ap0974	*ap0974*	COG3069: C4-dicarboxylate transporter	7.158
ap1558	*rpsG*	COG0049: Ribosomal protein S7	6.978
ap0083	*exbD*	biopolymer transport protein	6.410
ap0106	*ilvG*	COG0028: Thiamine pyrophosphate-requiring enzymes	6.259
ap2001	*rpe*	D-ribulose-phosphate-3 epimerase	6.130
ap2145	*ap2145*	COG1629: Outer membrane receptor proteins, mostly Fe transport	5.987
ap0739	*MW0319*	NULL	5.379
ap1727	*rpsT*	30S ribosomal protein S20	4.914
ap2142	*ap2142*	COG1629: Outer membrane receptor proteins, mostly Fe transport	4.879
ap1613	*serA*	COG0111: Phosphoglycerate dehydrogenase and related dehydrogenases	4.664
ap0958	*sdaA*	COG1760: L-serine deaminase	4.428
ap0740	*ap0740*	COG2837: Predicted iron-dependent peroxidase	3.868
ap0082	*tonB*	COG0810: Periplasmic protein TonB, links inner and outer membranes	3.834
ap1672	*ap1672*	COG0607: Rhodanese-related sulfurtransferase	3.825
ap0294	*yfeB*	COG1121: ABC-type Mn/Zn transport systems, ATPase component	3.810
ap0043	*ap0043*	COG0536: Predicted GTPase	3.717
ap0295	*yfeA*	COG0803: ABC-type metal ion transport system, periplasmic component/surface adhesin	3.702
ap0272	*thrB*	COG0083: Homoserine kinase	3.677
ap2091	*dnaJ*	COG0484: DnaJ-class molecular chaperone with C-terminal Zn finger domain	3.665
ap2141	*proA*	COG0014: Gamma-glutamyl phosphate reductase	3.345

Locus Tag	Gene	Description	Fold Change
ap0524	*trpA*	COG0159: Tryptophan synthase alpha chain	3.343
ap1177	*hugZ*	COG0748: Putative heme iron utilization protein	3.266
ap1537	*ap1537*		3.256
ap0797	*fetB2*	NULL	3.064
ap0167	*nrdA_1*	COG0209: Ribonucleotide reductase, alpha subunit	3.022
ap2082	*ap2082*	COG0730: Predicted permeases	2.932
ap1200	*ap1200*	COG2252: Permeases	2.719
ap1140	*rbsK*	COG0524: Sugar kinases, ribokinase family	2.616
ap0211	*Dam*	COG0338: Site-specific DNA methylase	2.590
ap2005	*menB*	COG0447: Dihydroxynaphthoic acid synthase	2.468
ap1565	*Psd*	COG0688: Phosphatidylserine decarboxylase	2.434
ap0313	*hsdM2*	COG0286: Type I restriction-modification system methyltransferase subunit	2.414
ap0178	*ap0178*	COG0477: Permeases of the major facilitator superfamily	2.308
ap1689	*lysA*	COG0019: Diaminopimelate decarboxylase	2.070
ap1234	*ap1234*	COG0795: Predicted permeases	2.006
ap1969	*trmD*	COG0336: tRNA-(guanine-N1)-methyltransferase	1.844
ap0150	*cysB*	COG0583: Transcriptional regulator	1.792
ap1732	*ap1732*	COG0716: Flavodoxins	-1.735
ap1125	*nhaP*	COG0025: NhaP-type Na+/H+ and K+/H+ antiporters	-1.772
ap0871	*uvrA*	COG0178: Excinuclease ATPase subunit	-1.803
ap0782	*ap0782*	Unassigned protein	-2.176
ap0882	*ap0882*	COG0477: Permeases of the major facilitator superfamily	-2.206
ap0536	*ap0536*	Unassigned protein	-2.277
ap0884	*mmcQ*	MmcQ protein	-2.307
ap0303	*ap0303*	COG0084: Mg-dependent DNase	-2.312
ap1435	*ap1435*	COG0488: ATPase components of ABC transporters with duplicated ATPase domains	-2.392
ap0993	*ap0993*		-2.416
ap0843	*ap0843*	Unassigned protein	-2.501
ap1553	*ap1553*	Unassigned protein	-2.513
ap1395	*ap1395*	Transposase_mut domain containing protein	-2.530
ap1723	*hemL*	COG0001: Glutamate-1-semialdehyde aminotransferase	-2.583
ap1605	*ap1605*	COG2931: RTX toxins and related Ca2+-binding proteins	-2.594
ap0726	*ap0726*	COG0664: cAMP-binding proteins - catabolite gene activator and regulatory subunit of cAMP-dependent protein kinases	-2.615
ap1665	*ap1665*	COG2801: Transposase and inactivated derivatives	-2.622
ap0257	*bolA*	putative transcriptional regulator, BolA	-2.759
ap1829	*Cof*	COG0561: Predicted hydrolases of the HAD superfamily	-2.762
ap0168	*ap0168*	transformation locus protein OrfG homolog	-2.768
ap1510	*ap1510*	COG1881: Phospholipid-binding protein	-2.849
ap1036	*Fdx2*	COG0633: Ferredoxin	-3.013
ap0126	*ap0126*	DUF710 domain containing protein	-3.085
ap0165	*ap0165*	COG0042: tRNA-dihydrouridine synthase	-3.095
ap1043	*iscU*	COG0822: NifU homolog involved in Fe-S cluster formation	-3.214
ap2194	*ap2194*	COG0697: Permeases of the drug/metabolite transporter (DMT) superfamily	-3.228
ap1345	*ap1345*	DUF479 domain containing protein	-3.241
ap1129	*hemX*	putative uroporphyrinogen III C-methyltransferase	-3.261
ap1694	*frdA*	COG1053: Succinate dehydrogenase/fumarate reductase, flavoprotein subunit	-3.280
ap1653	*ap1653*	COG2077: Peroxiredoxin	-3.286

Locus Tag	Gene	Description	Fold Change
ap0439	*lcfA*	COG0318: Acyl-CoA synthetases (AMP-forming)/AMP-acid ligases II	-3.336
ap1617	*slyD*	FkbP-type peptidyl-prolyl cis-trans isomerase	-3.348
ap1586	*napB*	COG3043: Nitrate reductase cytochrome c-type subunit	-3.420
ap1522	*ap1522*	COG2823: Predicted periplasmic or secreted lipoprotein	-3.435
ap1103	*tktA*	COG0021: Transketolase	-3.520
ap0715	*ackA*	COG0282: Acetate kinase	-3.525
ap1511	*ap1511*	COG1881: Phospholipid-binding protein	-3.533
ap0538	*ap0538*	COG0778: Nitroreductase	-3.594
ap1402	*pgk*	COG0126: 3-phosphoglycerate kinase	-3.610
ap1539	*ap1539*	DUF469 domain containing protein	-3.719
ap1473	*ptsB*	COG1263: Phosphotransferase system IIC components, glucose/maltose/N-acetylglucosamine-specific	-3.744
ap1231	*ap1231*	Unassigned protein	-3.784
ap1793	*ap1793*	COG0310: ABC-type Co2+ transport system, permease component	-3.883
ap0495	*ap0495*	COG5295: Autotransporter adhesin	-3.898
ap1406	*ap1406*	COG0471: Di- and tricarboxylate transporters	-3.936
ap0803	*ap0803*	COG0306: Phosphate/sulphate permeases	-3.986
ap1563	*oapA*	COG3061: Cell envelope opacity-associated protein A	-3.989
ap0762	*ap0762*	COG0243: Anaerobic dehydrogenases, typically selenocysteine-containing	-4.060
ap1791	*cbiO*	COG1122: ABC-type cobalt transport system, ATPase component	-4.064
ap1133	*groEL*	COG0459: Chaperonin GroEL (HSP60 family)	-4.137
ap0519	*ap0519*	DUF74 domain containing protein	-4.176
ap0500	*lldP*	COG1620: L-lactate permease	-4.187
ap0539	*rimK*	COG0189: Glutathione synthase/Ribosomal protein S6 modification enzyme (glutaminyl transferase)	-4.192
ap0416	*glpT*	GlpT	-4.257
ap1270	*ap1270*	DUF526 domain containing protein	-4.302
ap0124	*ap0124*	COG1414: Transcriptional regulator	-4.329
ap0164	*ap0164*	COG1611: Predicted Rossmann fold nucleotide-binding protein	-4.379
ap1691	*frdD*	COG3080: Fumarate reductase subunit D	-4.627
ap2168	*yedF*	COG0425: Predicted redox protein, regulator of disulfide bond formation	-4.665
ap0541	*maeA*	malate oxidoreductase (NAD)	-4.743
ap0499	*ap0499*	COG0247: Fe-S oxidoreductase	-4.891
ap0856	*ap0856*	5'-nucleotidase / UDP-sugar diphosphatase	-4.920
ap1310	*pnuC*	COG3201: Nicotinamide mononucleotide transporter	-5.061
ap0410	*Lnt*	COG0815: Apolipoprotein N-acyltransferase	-5.074
ap0206	*pykA*	COG0469: Pyruvate kinase	-5.134
ap0716	*cpdB*	UshA protein	-5.264
ap0052	*ap0052*	Unassigned protein	-5.304
ap1244	*eno*	COG0148: Enolase	-5.533
ap1044	*iscS*	COG1104: Cysteine sulfinate desulfinase/cysteine desulfurase and related enzymes	-5.848
ap1595	*ap1595*	Unassigned protein	-6.035
ap0859	*lpdA*	COG1249: Pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamide dehydrogenase (E3) component, and related enzymes	-6.253

Locus Tag	Gene	Description	Fold Change
ap0802	*ap0802*	COG1392: Phosphate transport regulator (distant homolog of PhoU)	-6.300
ap0860	*aceF*	COG0508: Pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamide acyltransferase (E2) component, and related enzymes	-6.453
ap0125	*ap0125*	COG0212: 5-formyltetrahydrofolate cyclo-ligase	-6.505
ap0132	*ap0132*	Unassigned protein	-6.726
ap0274	*sodA*	COG0605: Superoxide dismutase	-6.761
ap1069	*ap1069*	RTX domain containing protein	-6.818
ap0200	*gloA*	COG0346: Lactoylglutathione lyase and related lyases	-7.031
ap2022	*udp*	COG2820: Uridine phosphorylase	-7.087
ap0121	*putA*	COG4230: Delta 1-pyrroline-5-carboxylate dehydrogenase	-7.148
ap1087	*ap1087*	YCII domain containing protein	-7.192
ap1269	*Pgi*	COG0166: Glucose-6-phosphate isomerase	-7.204
ap1611	*Fbp*	COG0158: Fructose-1,6-bisphosphatase	-8.280
ap1692	*frdC*	COG3029: Fumarate reductase subunit C	-8.376
ap0004	*sodC*	superoxide dismutase	-8.629
ap1216	*clpB*	COG0542: ATPases with chaperone activity, ATP-binding subunit	-9.154
ap0108	*nrfA*	COG3303: Formate-dependent nitrite reductase, periplasmic cytochrome c552 subunit	-9.800
ap0109	*nrfB*	nitrate reductase, cytochrome-C type protein	-9.809
ap2067	*ap2067*	Esterase domain containing protein	-15.462
ap1437	*ap1437*	COG0607: Rhodanese-related sulfurtransferase	-15.518
ap1536	*mauG*	COG1858: Cytochrome c peroxidase	-16.073
ap1658	*ap1658*	COG0783: DNA-binding ferritin-like protein (oxidative damage protectant)	-19.604
ap0999	*fdnI*	COG2864: Cytochrome b subunit of formate dehydrogenase	-20.914
ap2014	*ap2014*	COG2041: Sulfite oxidase and related enzymes	-21.875
ap0998	*ap0998*	COG0437: Fe-S-cluster-containing hydrogenase components 1	-25.335
ap0996	*bisC*	COG0243: Anaerobic dehydrogenases, typically selenocysteine-containing	-39.375

Article 3

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Mutation in the LPS outer core biosynthesis gene, *galU*, affects LPS interaction with the RTX toxins ApxI and ApxII and cytolytic activity of *Actinobacillus pleuropneumoniae* serotype 1

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Running title: *A. pleuropneumoniae* LPS/Apx interaction

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SUMMARY

Lipopolysaccharides and Apx toxins are major virulence factors of *Actinobacillus pleuropneumoniae*, a pathogen of the respiratory tract of pigs. Here, we evaluated the effect of LPS core truncation in haemolytic and cytotoxic activities of this microorganism. We previously generated a highly attenuated *galU* mutant of *A. pleuropneumoniae* serotype 1 that has an LPS molecule lacking the GalNAc-Gal II-Gal I outer core residues. Our results demonstrate that this mutant exhibits wild type haemolytic activity but is significantly less cytotoxic to porcine alveolar macrophages. However, no differences were found in gene expression and secretion of the haemolytic and cytotoxic toxins ApxI and ApxII, both secreted by *A. pleuropneumoniae* serotype 1. This suggests that the outer core truncation mediated by the *galU* mutation affects the toxins in their cytotoxic activities. Using both ELISA and SPR binding assays, we demonstrate a novel interaction between LPS and the ApxI and ApxII toxins via the core oligosaccharide. Our results indicate that the GalNAc-Gal II-Gal I trisaccharide of the outer core is fundamental to mediating LPS/Apx interactions. The present study suggests that a lack of binding between LPS and ApxI/II affects the cytotoxicity and virulence of *A. pleuropneumoniae*.

INTRODUCTION

Actinobacillus pleuropneumoniae is the etiological agent of porcine pleuropneumonia, a highly contagious respiratory disease responsible for major economic losses in the swine industry (Straw, 2006). The disease is characterized by hemorrhagic, fibrinous, and necrotic lung lesions and the clinical features range from acute to chronic. To date, fifteen serotypes of *A. pleuropneumoniae* have been described based on capsular antigens (Dubreuil *et al.*, 2000; Blackall *et al.*, 2002); all serotypes are capable of causing disease, although differences in virulence have been observed (Frey, 1995; Jacobsen *et al.*, 1996). The virulence of the bacteria is mediated by the coordinated action of several virulence factors, namely the capsule (Ward and Inzana, 1994; Ward *et al.*, 1998; Rioux *et al.*, 2000), outer membrane proteins (OMPs) involved in iron uptake (Haesebrouck *et al.*, 1997; Bosse *et al.*,

2002; Jacques, 2004), Apx toxins (Frey, 1995) and lipopolysaccharides (LPS) (Jacques, 1996; Jacques and Paradis, 1998).

Apx toxins are Ca^{2+}-dependent haemolytic and/or cytotoxic toxins, members of the pore-forming RTX (repeats in toxin) toxin family (Maier *et al.*, 1996). To date, four different Apx toxins (ApxI to IV) have been described and each of the fifteen serotypes of *A. pleuropneumoniae* secretes various combinations of the Apx toxins (Schaller *et al.*, 1999; Blackall *et al.*, 2002). The 105 kDa toxins ApxI and ApxII, which are both secreted by *A. pleuropneumoniae* serotype 1, are encoded on polycistronic operons *apxICABD* (Frey *et al.*, 1994) and *apxIICA* (Jansen *et al.*, 1992; Frey *et al.*, 1993), respectively. In these operons, the A gene encodes the protoxin, while C encodes an acyltransferase involved in the post-translational activation of the protoxin in the cytoplasm (Issartel *et al.*, 1991; Stanley *et al.*, 1994). The B and D genes encode an ATPase and a protein adaptator respectively, which interact with the outer membrane protein TolC to constitute the specific type I secretion system that actively secrete the Apx toxins (Thanabalu *et al.*, 1998). The secretion of both ApxI and ApxII occurs via the same secretion system encoded by the genes *apxIB* and *apxID* of the *apxI* operon (Frey *et al.*, 1993). Apx toxins exhibit typical features of RTX toxins including: (i) the N-terminal hydrophobic region containing hydrophobic and amphipathic α-helices involved in pore formation (Ludwig *et al.*, 1991); (ii) the glycine-rich Ca^{2+}-binding nonapeptide repeats, which adopt a parallel β-roll structure (Baumann *et al.*, 1993); and (iii) the C-terminal uncleaved secretion signal. While ApxI is strongly haemolytic and cytotoxic, ApxII is weakly haemolytic and moderately cytotoxic (Kamp *et al.*, 1991). Studies indicate that Apx toxins have a major contribution in lung lesions observed during porcine pleuropneumonia (Choi *et al.*, 2001) and that the production of ApxI and ApxII are commonly associated with the most virulent strains such as *A. pleuropneumoniae* serotype 1 (Frey, 1995).

LPS are complex molecules composed of three well-defined regions: (i) lipid A, anchored in the outer membrane; (ii) the core oligosaccharide (OS) containing 2-keto-3-deoxyoctulosonic acid (Kdo) and heptose residues; and (iii) the O-antigen which is a polysaccharide consisting of repeating units. We have recently elucidated the core OS structures of LPS from *A. pleuropneumoniae* serotypes 1, 2, 5a and 5b using NMR and Mass Spectrometry (Michael *et al.*, 2004), revealing a conserved inner-core structure

consisting of a trisaccharide of L-*glycero*-D-*manno*-heptose residues linked to a Kdo residue and substituted at different positions (Michael *et al.*, 2004). The outer core of serotype 1 differs from the other serotypes as it is composed of a trisaccharide containing an open-chain GalNAc residue (Fig. 1).

Beyond their direct role in virulence, LPS molecules are known to interact with several bacterial proteins such as OMPs (Ferguson *et al.*, 2000) and exotoxins. There are previous reports of synergism between LPS and RTX toxins. For example, the *E. coli* haemolysin (HlyA), which is the prototype RTX toxin, and LPS together enhance pulmonary damage in perfused rabbit lungs (Schutte *et al.*, 1997). Cooperation of LPS with other RTX toxins in immunological activities was also found in the case of *Mannheimia haemolytica* leukotoxin (Lkt) (Lafleur *et al.*, 2001; Leite *et al.*, 2003) and *Bordetella pertussis* adenylate cyclase toxin (CyaA) (Ross *et al.*, 2004; Boyd *et al.*, 2005). Lkt cytolytic activity is also thought to be stabilized and enhanced by the formation of LPS/Lkt complexes (Li and Clinkenbeard, 1999; Zecchinon *et al.*, 2005). In the case of a specific binding of LPS to RTX toxins many studies have suggested the involvement of different regions of LPS in the activity of the toxins. It was proposed that hydrophobic interactions between HlyA and LPS, which might involve the lipid A and the N-terminal hydrophobic domain of the toxin, increase the stability and activity of the toxin (Herlax *et al.*, 2005). The presence of the O-antigen was shown to strongly correlate with the activity of HlyA as rough strains of *Salmonella typhimurium* and *Klebsiella pneumoniae* expressing recombinant HlyA were less haemolytic (Camprubi *et al.*, 1990). The core OS region of LPS might also be involved in the efficacy of RTX toxins as deep rough mutations, such as those in *rfaH*, *rfaJ*, *rfaP* and *galU* genes, affected the secretion or activity of HlyA (Wandersman and Letoffe, 1993; Stanley *et al.*, 1993; Leeds and Welch, 1996; Bauer and Welch, 1997).

We previously generated a core LPS mutant 5.1 of *A. pleuropneumoniae* serotype 1 by insertion of a mini-*Tn10* transposon in the gene *galU*, encoding an UTP-α-D-glucose-1-phosphate uridylyltransferase (Rioux *et al.*, 1999). This mutant was found to be affected in its resistance to antimicrobial peptides, its adhesion capabilities and overall virulence (Rioux *et al.*, 1999; Ramjeet *et al.*, 2005). Considering the important role of Apx toxins in the pathogenesis of *A. pleuropneumoniae* and based on the suggested role of LPS core OS in the activity of RTX toxins, we evaluated the expression, secretion and activity of Apx

toxins of this highly attenuated core LPS mutant. In the present study, we demonstrate physical interactions between LPS and both ApxI and II toxins via the core OS. The GalNAc-Gal II-Gal I region of core OS, that is missing in mutant 5.1 (Ramjeet *et al.*, 2005) (Fig. 1), is identified as a critical domain for LPS/Apx interactions, which might play a fundamental role in the cytotoxicity and pathogenesis of *A. pleuropneumoniae* serotype 1.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

The bacterial strains used in this study are shown in Table 1. *A. pleuropneumoniae* S4074 Nal^r was grown on BHI (Difco Laboratories, Detroit, MI) agar plates supplemented with 15 μg NAD per ml, and 30 μg nalidixic acid (Nal) per ml. The transpositional mutant (Table 1) was grown on BHI agar plates supplemented with 15 μg/ml of NAD, 30 μg/ml of nalidixic acid and 75 μg/ml of kanamycin. Liquid cultures of all *A. pleuropneumoniae* strains used in this study (Table 1) were done in BHI broth supplemented with 5 μg/ml of NAD.

Isolation of LPS and core OS

LPS and core OS were isolated as described previously (Michael *et al.*, 2004; Ramjeet *et al.*, 2005). LPS were purified down a column of Bio-Gel P-2 and core OS were purified following acetic acid treatment of LPS. Samples of LPS and core OS from the wild type and the mutant were lyophilized then resuspended in pyrogen free water at a concentration of 1 mg/ml for later use. The absence of protein contamination in the samples was confirmed by NMR spectroscopy and silver-stained SDS-PAGE (data not shown).

Haemolysis assay

Fresh blood samples were collected from sheep and horse in Alsever's anticoagulant and used within 3 days for making red blood cells (RBC) suspension. Cell-free culture supernatants (CFS) from each strain were harvested at different growth phases (OD_{600nm} ranging from 0.1 to 1.1) and serial two-fold dilutions of the samples were tested for haemolytic activity. Briefly, 500 μl of the diluted toxin-containing culture supernatants were mixed to an equal volume of 1% RBC resuspended in TS buffer (10 mM Tris

hydrochloride, 0.9% NaCl pH 7.5) supplemented with 20 mM $CaCl_2$. The mixture was incubated for 2 h at 37°C and placed overnight at 4°C to allow cell sedimentation. Haemoglobin-containing supernatants were then harvested for optical reading at 540 nm. A haemolysis negative control with toxin-free BHI was carried out in each experiment and subtracted to the OD values of samples. One haemolytic unit is defined as the amount of material which lyses 50% of RBC in 1 ml of a 0.5% suspension under the assay conditions described above. Haemolytic activities are expressed in haemolytic units/ml (HU/ml).

Cytolysis assay

The cytolytic activity of exponential phase (OD_{600nm} of 0.7) toxin-containing culture supernatants from each strain was evaluated on porcine alveolar macrophages (PAMs) obtained as described previously (Ramjeet *et al.*, 2005). Before the experiment, PAMs were quickly thawed in water bath at 37°C, washed and resuspended in complete DMEM (Gibco 12430-054, Burlington, ON, Canada) supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml gentamicin, 0.1 mM MEM non-essential amino acids (all purchased from Gibco) and 1 mM sodium pyruvate (Sigma, Oakville, ON, Canada). Cell count was re-evaluated on the basis of trypan blue dye exclusion and the cell concentration was adjusted to 1 x 10^6/ml. PAMs were then dispensed in 24-well tissue culture plates and incubated overnight at 37°C in 5% CO_2. Briefly, 1 ml of CFS diluted tenfold in TS buffer was added to 1 x 10^6 adherent PAMs. $CaCl_2$ was added to a final concentration of 10 mM and the plates were incubated for 4 h at 37°C in 5% CO_2. A cytotoxicity test based on lactate dehydrogenase (LDH) dosage was performed with the cell-free supernatants using a CytoTox 96 LDH kit (Promega, Madison, WI) according to the manufacturer's protocol. Incubation of PAMs with TS buffer without CFS was used as a negative control, while total lysis of cells by a treatment with 2% Triton X-100 was used as the 100% cytotoxicity positive control. A kinetic analysis of PAMs cytolysis by recombinant Apx toxins was performed as followed: rApxI or rApxII were diluted at final concentrations of 14 µg/ml and 2 µg/ml respectively in 500 µl total volume of TS buffer supplemented with 10 mM $CaCl_2$. The toxins were incubated with or without 2 µg of purified LPS from the wild type strain or the mutant 5.1 for 1 h at 4°C. The preincubated samples were then added to 1 x 10^6 adherent PAMs and the cytotoxicity was monitored as

described above at several time points (1, 2, 3 and 4 h). As a control, the cytotoxic effect of the LPS molecules on PAMs was also investigated by incubating the cells with purified LPS alone and the residual activity was subtracted to the values.

Real-time quantitative RT-PCR

Expression analysis of the genes coding for ApxI and ApxII toxins was performed using real-time quantitative RT-PCR. Briefly, RNA samples were extracted from each strain grown to an OD_{600nm} of 0.3 and subjected to a DNase treatment with TURBO DNase (Ambion, Austin, TX) to avoid DNA contamination. The oligonucleotide primers used for the detection of *apxIC*, *apxIA*, *apxIB*, *apxID*, *apxIIC* and *apxIIA* are represented in Table 2. RT-PCR reactions were performed in a 16-place Cepheid Smart Cycler® System, using the QuantiTect® SYBR® Green RT-PCR Kit (Qiagen Inc., Chatsworth, CA). For absolute quantification, PCR fragments corresponding to each gene tested were cloned in the pGEM-T Easy vector (Promega) and used as standards.

Anti-Apx antibodies

Rabbit polyclonal antibody against the toxin ApxI used in this study was a generous gift from Dr. J. Frey (Institute of Veterinary Bacteriology, University of Bern, Bern, Switzerland). Monospecific rabbit polyclonal antibody against the ApxII toxin was obtained by immunizing a rabbit 2 times subcutaneously with 50 µg of recombinant ApxII at 2-week intervals. Both immunizations contained Freund incomplete adjuvant. Antibody-containing serum collected after bleeding of the animal was tested for reactivity in a Dot blot analysis using recombinant ApxII. Immunoblot of TCA-concentrated CFS from *A. pleuropneumoniae* serotype 10 secreting only ApxI and serotype 12 secreting only ApxII, probed with either the anti-ApxI or the anti-ApxII, confirmed that there was no cross-reactivity between the antibodies (data not shown).

Expression and secretion of Apx toxins

Each bacterial strain was grown to exponential phase to an OD_{600nm} of 0.7. To evaluate the intracellular production of Apx toxins, total cell lysate extracts were prepared by resuspending bacterial pellets in equal volumes of Laemmli buffer. To investigate the

extracellular expression of Apx toxins, 250-fold concentrated CFS were prepared following protein precipitation with 10% TCA (trichloroacetic acid). Total protein concentration of all the TCA-concentrated samples were determined by bicinchoninic acid (BCA) protein assay kit (Pierce) and adjusted to 1 mg/ml. All samples were separated by SDS-PAGE using a 7.5% polyacrylamide separating gel and transferred to a nitrocellulose membrane for immunoblotting. Immunoblots were probed with rabbit polyclonal antibodies directed against the toxins ApxI or ApxII.

Co-immunoprecipitation

Bacterial cell-free culture supernatants (CFS) of the wild type parent strain ant the mutant 5.1 (OD_{600nm} of 0.7) were incubated with or without (negative control) monoclonal antibody 5.1 G8 F10 directed against *A. pleuropneumoniae* serotype 1 LPS O-antigen (Rioux *et al.*, 1997) for 1 h at 4°C under slow rotation. Protein A-linked agarose beads (EZview Red Protein A Affinity Gel, Sigma) blocked for 1 h with 2% BSA were then added and the mixture was incubated for an additional hour. After centrifugation, the pellet containing LPS-co-immunoprecipitated proteins was washed 3 times with PBS, resuspended in Laemmli buffer and analyzed by SDS-PAGE. An immunoblot using rabbit polyclonal antibodies was performed to detect the presence of the toxins ApxI and ApxII. The pulled-down samples were also analyzed by silver-stained SDS-PAGE for LPS detection.

Cloning and expression of apxICA and apxIICA

The structural genes *apxICA* and *apxIICA* were amplified from genomic DNA of *A. pleuropneumoniae* serotype 1 (Table 1) by PCR with *Pfx50* high fidelity DNA polymerase (Invitrogen, Burlington, ON, Canada) in a Biometra *Tpersonal* thermocycler according to manufacturer's instructions. Oligonucleotide primers ApxICA-For (5'-CACCATGAGTAAAAAAATTAATGGATTTG-3') and ApxICA-Rev (5'-AGCTGCTTGTGCTAAAGAATAACTC-3)' for *apxICA*, and ApxIICA-For (5'-CACCATGATGCTAAAAAATGATTTTAACG-3') and ApxIICA-Rev (5'-AGCGGCTCTAGCTAATTGAATATTATTCG-3') for *apxIICA*, were used for amplification. PCR fragments were purified with a QIAquick Gel Extraction Kit (Qiagen)

and cloned into the expression vector pET101/D-TOPO (Invitrogen), which allowed the addition of a six-His tag at the C terminal end of the structural proteins ApxIA and ApxIIA. The plasmids pET101/D-TOPO-ApxICA and pET101/D-TOPO-ApxIICA obtained were sequenced using T7 primers and introduced into *E. coli* BL21 (DE3) (Invitrogen) for overexpression. Each of the recombinants was grown in an LB medium containing 50 μg/ml ampicillin at 37°C. When the absorbance at 600 nm reached 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture medium to a final concentration of 100 μM. After growth for 4 h at 37°C in the presence of IPTG, bacterial cultures were centrifuged at 13000 x g for 15 min at 4°C and cell pellets were frozen at –20°C until inclusion bodies preparation.

Purification of inclusion bodies and renaturation of rApxI and rApxII

Frozen bacterial pellet from 1 liter of induced culture was dissolved in 20 ml of lysis buffer (20 mM Tris-HCl pH 7.5, 1% Triton X-100, 10 mM EDTA) supplemented with lysosyme at a final concentration of 100 μg/ml. The mixture was incubated in ice-bath for 30 min and sonicated in 4 rounds of 1 min with pulse 2 s and interval 3 s. The lysate was then centrifuged at 25000 x g for 10 min at 4°C and the supernatant was discarded. The pellet was washed twice with lysis buffer and then resuspended in 20 ml of 50 mM Tris pH 8, 100 mM NaCl. After centrifugation at 25000 x g for 10 min at 4°C , the pellet was dissolved in 2 ml solubilization buffer (6 M guanidium hydrochloride GuHCl, 0.1 M Tris pH 8.6) for 1 h at 4°C under slow rotation. The supernatant containing denaturated recombinant ApxI or ApxII was collected after centrifugation at 25000 x g for 20 min at 4°C. Renaturation of the toxins was performed by diluting the denaturated proteins 100-fold in 30 ml refolding buffer (880 mM L-arginine, 55 mM Tris, 21 mM NaCl, 0.88 mM KCl; pH 8.2) under slow stirring overnight at 4°C. After 5 min centrifugation at 3700 x g to pellet precipitated non-renaturated proteins, the supernatant was concentrated in an Amicon Ultra-15 centrifugal filter device with a 50 kDa cut off (Millipore) and buffer exchanged in 0.02 M sodium phosphate buffer (pH 7.4) using PD-10 Desalting columns (Amersham Biosciences). The purity of the recombinant proteins was confirmed by SDS-PAGE and staining with Coomassie blue. A Western blot analysis using an anti-HisG horseradish peroxidase-coupled antibody (Invitrogen) was also used to confirm the presence of both rApxI and

rApxII (data not shown). To test the renaturation of the purified toxins, far-UV circular dichroism (CD) was performed to look at the secondary structure of the proteins and the haemolytic activity of the toxins was evaluated as described above.

Far-UV CD spectroscopy

Far-UV circular dichroism (CD) spectra from purified rApxI and rApxII were recorded in phosphate buffer, pH 7.4, in a 0.1-cm-path-length cuvette between 190 and 260 nm, using a spectropolarimeter (Jasco Spectroscopic Co. Ltd.; model J-810). For each spectrum, three accumulations were averaged and the contribution of buffer to the measured ellipticity was subtracted. Thermal denaturation spectra were obtained at 10°C intervals between 30°C and 90°C using the temperature control unit. The CD spectra of the recombinant toxins exposed to different detergents (0.1% Triton X-100, 0.1% Tween-20, 0.1% DDM, 0.1% Empigen, 0.1% octyl glucoside; all purchased from Calbiochem) were obtained similarly. Ellipticities were converted to mean residual ellipticities (MRE). CD data were analyzed on Dicroweb (http://www.cryst.bbk.ac.uk/cdweb/html/home.html) by using the CDSSTR algorithm.

ELISA binding assay

To qualitatively monitor the binding specificities of purified LPS to Apx toxins, Nunc Maxisorp ELISA plates (Nunc, VWR, Ville Mont Royal, QC, Canada) were coated overnight at 4°C with 10 μg of purified LPS per well derived from the wild-type and the mutant. LPS (1 mg/ml) was diluted in 0.05 M carbonate buffer containing 0.1% $MgCl_2$ (pH 9.8). Plates were washed three times with PBS containing 0.05% (v/v) Tween-20 (PBS-T) and non-specific binding sites were blocked for 1 h at room temperature (RT) with 1% casein-PBS. After three washing steps in PBS-T, plates were incubated for 1 h at RT with 100 μl of either ApxI or ApxII at concentrations of 15 μg/ml and 5 μg/ml respectively. Rabbit polyclonal anti-ApxI or anti-ApxII antibodies were added at dilution 1:1000 following washing with PBS-T and plates were incubated for 1 h at 37°C. Primary antibody was detected after 1 h of incubation at 37°C with a commercial goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Mississauga, ON). To ensure that equal amounts of LPS were coated in each well, LPS-coated wells were incubated with the primary monoclonal antibody 5.1 G8 F10

directed against *A. pleuropneumoniae* serotype 1 LPS O-antigen followed by a detection with a commercial goat anti-mouse IgG + IgM (H + L) conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories). A 3,3',5,5'-tetramethylbenzidine (TMB) solution (Sigma) was used for the revelation of immune complexes and plates were read at 450 nm in a Power Wave X 340 (Biotek Instruments Inc, Winooski, VT) microplate reader. Analyses were performed in duplicate at least five times. To evaluate the binding capacity of LPS core OS to Apx toxins, a competition binding assay was performed. Purified core OS from the wild type (core OS WT) was added as a competitor at increasing concentrations (0, 2, 10, 20 and 100 μg/ml) in toxin preparations prior to incubation with wild type LPS. The inhibitory effect of the core OS WT and the core OS 5.1 on the LPS binding to the toxins was also compared by using the purified core OS at 100 μg/ml. The competition experiments were repeated at least three times. A negative control without coated LPS was added in each experiment and subtracted to binding values.

Surface Plasmon Resonance (SPR) binding assay

Binding interactions between WT (1840 Da) or mutant 5.1 (1312 Da) core OS and ApxI/II (105 kDa) were also examined in real-time using BIACORE 3000 instrumentation (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Experiments were performed on research-grade CM5 sensor chips at 25°C using filtered (0.2 μm) and degassed HBS-EP (10 mM Hepes pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) Tween-20). Protein-grade detergents (10% (v/v) Tween-20, 10% (v/v) DDM) were from Calbiochem; all other chemicals were reagent grade quality. Prior to all experiments, protein concentrations were determined using the BCA assay and purity (>95%) was assessed by SDS-PAGE (12.5% polyacrylamide) under reducing (5% (v/v) 2-mercaptoethanol) conditions, followed by silver staining (data not shown). Far-UV CD spectroscopy and haemolysis analysis confirmed that the toxins were renaturated and functional. Complementary CD analysis also demonstrated that selected SPR assay conditions (ie. HBS-EP containing 0.005 – 0.1% (v/v) Tween-20 or DDM) were not detrimental to the native conformation of ApxI or ApxII (Fig. 5C and D). Immobilized sensor chip surfaces were prepared using the Biacore Amine Coupling Kit. Briefly, 70 μL of freshly mixed solution I (200 mM EDC and 50 mM NHS in water) was injected (10 μL/min) to activate surface-exposed carboxymethyl groups to

reactive esters. Next, 70 μL of ApxI or ApxII (20 μg/mL in 10 mM sodium acetate pH 4.5) was injected to generate amine-coupled toxin surfaces (3000 - 7000 RU). Finally, 70 μL of solution II (1 M ethanolamine pH 8.5) was injected to deactivate excess reactive groups and remove any non-specifically bound ligand. Corresponding reference surfaces were prepared in a similar manner without any ligand addition. To assess binding specificity, core OS variants (130 μM WT or 5.1) or BSA (negative control) were injected over immobilized surfaces at 10 μL/min using 'KINJECT' mode (5 min association + 5 min dissociation). To assess overall binding affinities, core OS WT (0 - 130 μM) was injected over immobilized surface at 5 μL/min using 'KINJECT' mode (40 min association + 10 min dissociation). For all SPR assays, surfaces were regenerated between sample injections at 50 μL/min using two 30 sec pulses of solution I (0.5 M NaCl, 0.1% (v/v) DDM in HBS-EP) and solution II (HBS-EP), followed by 'EXTRACLEAN' and 'RINSE' procedures. All binding data was doubled-referenced (Myszka, 1999) and is representative of duplicate injections acquired from two independent trials.

Statistical analysis

Data are reported as mean values ± SEM. Statistical analysis of the data from haemolysis assay was performed using a general linear model with bacterial type and time as factors. Statistical analysis of cytolysis assay, quantitative RT-PCR and ELISA binding assay was performed by using Wilcoxon's rank sum test. $P<0.05$ was considered significant.

RESULTS

Haemolysis and cytolysis assays

Since the virulence of *A. pleuropneumoniae* is often associated with the presence of the secreted Apx toxins, we first sought to evaluate the haemolytic and cytotoxic activities of the core LPS mutant 5.1 which was previously found to be highly attenuated *in vivo* (Rioux *et al.*, 1999). The haemolytic activity of the parent strain and mutant 5.1 was compared using cell-free supernatants (CFS) of bacterial cultures collected at different time points during growth. The efficiency of haemolysis of horse red blood cells by CFS was found to be similar for both the wild type strain and mutant 5.1 (Fig. 2A). There also was no

difference observed with sheep red blood cells (data not shown). We further evaluated the cytotoxic effect of both strains on porcine alveolar macrophages (PAMs) after a 4 h incubation with exponential phase CFS. Interestingly, the core LPS mutant 5.1 showed significantly reduced cytototoxic activity compared to the wild type parent strain ($P<0.05$) (Fig. 2B), yet no decrease in growth (Fig. 2A). Since the ApxI and ApxII toxins are known to be responsible for the haemolytic/cytolytic phenotype of *A. pleuropneumoniae* serotype 1, we focused our attention to gene expression and secretion of these toxins in the mutant strain.

Expression and secretion of Apx toxins

Since *rfaH*, a gene involved in LPS outer core elongation (Sanderson and Stocker, 1981), was previously found to act as a transcriptional activator of the *E. coli* haemolysin *hlyCABD* operon (Bailey *et al.*, 1992) and subsequently increase the toxin efficacy, we first evaluated the effect of the mutation in the outer core biosynthesis gene *galU* on the gene expression of the ApxI and ApxII toxins. Real-time absolute quantification of the expression of the *apxICA* and *apxIICA* genes encoding the structural toxins, as well as the *apxIBD* gene encoding the type I secretion system that actively secretes both ApxI and ApxII, was performed. The overall gene expression of ApxI and ApxII toxins and their secretion system demonstrated similar levels of transcription between the wild type and the core LPS mutant 5.1 strains (data not shown).

To investigate possible changes in Apx toxin production or secretion that might account for the weak cytolytic activity of mutant 5.1, we looked at the amount of toxins in bacterial pellets and in CFS. Western blot analysis using rabbit polyclonal antibodies directed against ApxI or ApxII was performed to evaluate the amount of toxin in each sample. Total cell lysate extracts were assessed to estimate the amount of intracellular and membrane-associated toxins. As shown in Figure 3, no difference was noted in the amount of both ApxI and ApxII in cell lysates originating from the wild type and the mutant strain. Extracellular levels of the secreted toxins in TCA-concentrated culture supernatants were also compared. Despite its lower cytotoxicity, the amount of both ApxI and ApxII toxins detected in core LPS mutant 5.1 supernatants was higher than that of the wild type (Fig. 3A and B). The amount of ApxI detected in the immunoblots (Fig. 3A) was low when

compared to that of ApxII (Fig. 3B). This differential detection may be due to lower reactivity of the anti-ApxI antibody or to a lower expression of *apxIA* as detected by RT-PCR (data not shown). Since the mutant 5.1 is less cytotoxic, we anticipated that there would be reduced expression or secretion of ApxI and/or ApxII. The fact that more toxins were detected in CFS from mutant 5.1 indicates that the low cytotoxicity of this strain is not due to decreased amounts of secreted ApxI or ApxII, but rather a defect in functional properties of the toxins caused by the truncation of the LPS outer core. We further investigated the possibility of LPS/Apx interactions that might have a direct effect on the toxins.

Co-immunoprecipitation of LPS with Apx toxins

To initially test for possible associations between LPS and the Apx toxins, we performed co-immunoprecipitation (CO-IP) experiments with toxin-containing bacterial CFS from the wild type and the mutant strain. Since the O-chain of LPS is present in both strains (Ramjeet *et al.*, 2005), monoclonal antibody directed against the O-antigen of *A. pleuropneumoniae* serotype 1 was bound to agarose beads to pull down any LPS-associated molecules in the supernatants. As expected, Western blot analysis of the pulled-down samples with anti-ApxI/II antibodies revealed physical associations between LPS and both Apx toxins (Fig. 4). Notably, lower toxin levels (ApxI and ApxII) were found associated with LPS molecules in CFS of the mutant 5.1 strain. Since the antibody directed against the O-antigen binds both the wild type and mutant LPS and since no decreased amounts of toxins was observed in CFS from mutant 5.1 (Fig. 3), this result suggests that truncated LPS from mutant 5.1 bind the ApxI/II toxins with weaker affinity. Despite, the association observed between LPS and the Apx toxins in the CO-IP experiment, other components present in CFS such as outer membrane vesicles (OMVs) might contribute to the colocalization of both molecules (Balsalobre *et al.*, 2006). Thus, *in vitro* analysis with purified LPS and toxins was pursued to provide direct evidence of physical binding interactions.

Expression and purification of recombinant Apx toxins

Recombinant ApxI and ApxII were purified following overexpression of the genes *apxICA* and *apxIICA* in an expression vector. The C gene was expressed along with the structural A gene as it is necessary for the maturation of the protoxin in an active form (Thumbikat *et al.*, 2003; Bei *et al.*, 2005). Since insufficient amounts of proteins were recovered in the soluble fraction of bacterial lysates, recombinant toxins were purified from inclusion bodies as denaturated proteins, renaturated in an arginine-containing buffer, and exchanged against phosphate buffer. To ensure proper refolding of the toxins before ELISA or SPR binding studies, we performed far-UV CD spectroscopy to look at the secondary structure of the purified proteins following the renaturation step. The CD spectra of rApxI and rApxII show secondary structures with α-helix and β-strand contents (Table 3; Fig. 5A and B). The spectrum of rApxI shows a higher content in β-strands probably due to its higher number of the glycine-rich Ca^{2+}-binding nonapeptide repeats (Frey *et al.*, 1991; Jansen *et al.*, 1994), which adopt a parallel β-roll structure. Secondary structure approaching that of ApxII was previously described for active HlyA upon CD analysis (Bakas *et al.*, 1998; Soloaga *et al.*, 1998). Thermal denaturation experiments showed that both rApxI and rApxII toxins had indeed properly refolded, as evidenced by a progressive loss of their secondary structure with increasing temperature (Fig. 5A and B). Both purified toxins were also functionally active as they exhibited normal haemolytic activities (data not shown).

ELISA analysis of LPS/Apx interaction

To confirm the preliminary CO-IP results obtained, binding interactions between the recombinant Apx toxins and purified LPS were evaluated using an ELISA-based assay. The ELISA results correlated with the CO-IP outcomes, thus advancing our *in vitro* detection of direct binding interactions between Apx toxins and LPS (Fig. 6A), as opposed to indirect associations via other components present in CFS such as OMVs (Balsalobre *et al.*, 2006). As expected, a significantly weaker interaction was observed between purified LPS from the mutant 5.1 and both toxins when compared to the wild type LPS ($P<0.01$). Notably, decreased binding with the truncated mutant 5.1 suggests that the outer core is an important mediator of the binding interaction between ApxI/II and LPS. However, the results also showed that binding of mutant LPS to the toxins, especially to ApxII, was not completely

abolished (Fig. 6A). To further investigate the suspected role of the core OS in the LPS/Apx interaction, we performed a competition ELISA assay between wild type LPS and the toxins. Purified core OS from the wild type was used as a competitor and added at increasing concentrations ranging from 0 to 100 μg/ml. The results showed a dose-dependant inhibition of the LPS binding to both ApxI and ApxII by the core OS WT suggesting an involvement of the core OS region of LPS in LPS/Apx interactions (Fig. 6B). Therefore, we compared the inhibitory effect of the core OS from the wild type and the mutant 5.1 in a similar competition experiment by adding purified core OS at 100 μg/ml. While a high inhibition of LPS binding to both ApxI and ApxII was observed with the core WT, the core 5.1 could not act as a competitor of the LPS binding to ApxII (Fig. 6C). The binding of LPS to ApxI was however inhibited by the core OS 5.1 but to a lesser extent than by the core WT (Fig. 6C). Along with the ELISA outcomes (Fig. 6A), this competition experiment suggests an important role of the GalNAc-Gal II-Gal I region of the core OS in the LPS/ApxII interaction and at least in part in the LPS/ApxI interaction. Overall, the present ELISA data demonstrate interactions between LPS of *A. pleuropneumoniae* serotype 1 and the Apx toxins that could be competed by core OS. Importantly, we have shown that the GalNAc-Gal II-Gal I region of the outer core is important to the interaction between LPS and the Apx toxins since the binding of the truncated core LPS of mutant 5.1 to both ApxI and ApxII is affected.

Surface plasmon resonance (SPR) analysis of LPS/Apx interaction

To further validate the ELISA results, complementary SPR analyses were performed to examine binding between core OS and the Apx toxins in real-time. Complementary CD analysis performed with ApxI and ApxII demonstrated that the SPR assay conditions (eg. HBS-EP running buffer, 0.1% (v/v) DDM regeneration) were not detrimental to the secondary structure of the toxins (Fig. 5C and D). To examine binding specificity, a fixed concentration of WT or mutant 5.1 core OS was injected over amine-coupled reference, as well as ApxI- and ApxII-coupled surfaces in-tandem. While similar positive signal responses were observed for ApxI and ApxII during the association phase with the core OS WT, equimolar injections of core OS from mutant 5.1 failed to exhibit any detectable association kinetics (Fig. 7A and B); normalizing for differences in molecular mass

between the core OS also did not change the overall outcome. The binding of the wild type core OS to Apx toxins validate the competition ELISA data confirming that the binding of LPS to the toxins involves the core OS region (Fig. 6B). In this regard, decreased binding of the truncated LPS to both ApxI and ApxII in ELISA (Fig. 6A) could be explained by the absence of specific binding responses for core OS 5.1 in the SPR analysis. To examine overall binding affinities, variable concentrations of core OS WT were then injected over the Apx surfaces (Fig. 7C and D) which yielded almost superimposable binding profiles (ie. 6400 RU ApxI versus 7000 RU ApxII). Despite prolonged contact times (ie. 40 min association phase at 5 μL/min), the slow dose-dependent association kinetics observed, failed to reach steady-state plateaus at any of the concentrations injected. Thus, equilibrium analysis (i.e. steady-state binding model) could not be used to fit this experimental data and lower-density Apx surfaces (i.e. 3000 RU) also failed to saturate under similar assay conditions. There also was no noticeable dissociation rate at any of the concentrations tested; thus, kinetic analysis (i.e. simple 1:1 binding model) could not be used to fit the experimental data. Regardless, the slow association phase kinetics and, in particular, the slow dissociation phase kinetics indicate that the core OS WT binding interactions with ApxI or II are very stable. Similar to the ELISA outcomes, the present SPR data provide direct evidence for specific, dose-dependent binding interactions between the core OS of LPS and the ApxI/II toxins. The SPR data further identified that the GalNAc-Gal II-Gal I region (ie. lacking in mutant 5.1) of the LPS outer core is important for core OS/Apx binding.

Cytolysis of PAMs by recombinant Apx toxins

To confirm the biological significance of the LPS/Apx interaction in the cytotoxic activity of the toxins, we performed a kinetic analysis of PAMs cytolysis by recombinant Apx toxins in the presence or absence of LPS. We show that preincubation of ApxI and ApxII with purified wild type LPS increases the cytotoxic activity of both toxins while no significant positive effect of the purified truncated LPS from mutant 5.1 was observed (Fig. 8A and B). This result suggests that LPS molecules can enhance the activity of Apx toxins by potentially interacting with them. Therefore, the low binding of the core truncated LPS

of the mutant 5.1 to both ApxI and ApxII could account for its low cytotoxicity as shown in Figure 2B.

DISCUSSION

The association between LPS molecules and RTX toxins has been established as a critical factor for toxin activity in several studies. In this regard, the involvement of different regions of the LPS in modulating the expression, secretion or activity of RTX toxins was previously reported (Camprubi *et al.*, 1990; Bailey *et al.*, 1992; Stanley *et al.*, 1993) (Wandersman and Letoffe, 1993; Leeds and Welch, 1996; Bauer and Welch, 1997; Herlax *et al.*, 2005). In this study, we showed that *A. pleuropneumoniae* serotype 1 LPS binds to the RTX toxins ApxI and ApxII via the core OS region and that this interaction may be critical for the cytotoxic activity of the bacterium.

To evaluate the importance of specific outer core sugar residues in the LPS/Apx interaction, we used a highly attenuated core LPS mutant 5.1 of *A. pleuropneumoniae* serotype 1 which is affected in the *galU* gene, coding for an UTP-α-D-glucose-1-phosphate uridylyltransferase (Rioux *et al.*, 1999). The absence of the Gal I residue in the mutant 5.1 led to an outer core missing the GalNAc-Gal II-Gal I region beyond the Hep IV residue (Ramjeet *et al.*, 2005) (Fig. 1). Here, we detected an association between LPS and both ApxI and ApxII in CFS of *A. pleuropneumoniae* and further confirmed a direct physical interaction between purified LPS and the recombinant toxins *in vitro*. Notably, when compared to the wild type strain, lower levels of both toxins were found associated with the truncated LPS *in vitro* and in CFS of the mutant 5.1 strain. This demonstrates the importance of the GalNAc-Gal II-Gal I outer core region for the binding of LPS to secreted ApxI and ApxII toxins. In addition to previous reports with *E. coli* deep rough mutants truncated in their core OS region and also lacking their O-antigen (Bailey *et al.*, 1992; Stanley *et al.*, 1993; Wandersman and Letoffe, 1993; Leeds and Welch, 1996; Bauer and Welch, 1997), our study with a defined outer core mutant that still elaborates the O-chain (Rioux *et al.*, 1999) shows unambiguously the involvement of the outer core region in toxin interactions.

Since the absence of core OS residues affected LPS/Apx interactions, we further investigated this issue in the current study. For the first time, we have now demonstrated direct binding interactions between the core OS region of *A. pleuropneumoniae* serotype 1 LPS and the toxins ApxI and ApxII. Indeed, we found that purified core OS inhibited the binding of LPS to both toxins in a dose-dependent manner via ELISA analysis. Real-time binding of purified core OS to Apx toxins was also shown using SPR. Similarly, Ostolaza *et al.* (1991) previously demonstrated the presence of Kdo core sugar residues in active fractions of purified HlyA. Other toxins such as the heat-labile enterotoxin (LT) of *E. coli* were also found to bind to core OS sugars of LPS (Horstman *et al.*, 2004). As opposed to wild type core OS, no binding responses were recorded for the core OS from mutant 5.1. Here again, the GalNAc-Gal II-Gal I portion may contribute to the core OS-mediated binding of LPS to the Apx toxins which could therefore explain the low interaction between the truncated LPS and ApxI/II. Further work could help determining if the GalNAc-Gal II-Gal I trisaccharide binds directly to the toxins. Synthesized mono-, di- or trisaccharide could be used for this purpose. Alternatively, previous studies have demonstrated that the sugar composition has an influence on the conformation of the core OS (Jansson *et al.*, 1989; Bruse *et al.*, 1991). Thus, the loss of the GalNAc-Gal II-Gal I region might lead to a conformation that is less able to bind the toxins.

The possibility also remains that the core OS might not account exclusively for LPS interactions with the toxins. Indeed, slow association kinetics were observed in the SPR assays and ELISA experiments also showed that truncated LPS binding to the toxins was not completely abolished (Fig. 6A). This suggests that additional interactions are likely to occur between the ApxI/II toxins and other regions of LPS. Thus, we can speculate that non-specific hydrophobic interactions between the lipid A and the hydrophobic N-terminal region of Apx toxins are also important. We believe that these hydrophobic interactions along with the core OS-mediated interactions might help stabilize LPS/Apx associations. As for the role of the O-polysaccharide region, its involvement is minimized by the fact that a rough LPS mutant of *A. pleuropneumoniae* serotype 1 (mutant 27.1) (Rioux *et al.*, 1999; Labrie *et al.*, 2002) exhibited wild type cytotoxic activity (data not shown).

Concerning potential LPS-binding sites, it has been demonstrated that surfactant protein D, a collagenous C-type lectin, binds to LPS core OS via residues coordinating calcium

(Wang *et al.*, 2008). In this regard, the glycine-rich Ca^{2+}-binding repeats of the toxins might be good candidates for the binding of the core OS region. Further 3D modelling of the interactions could help address this issue. In addition to the binding of LPS molecules to Apx toxins, we were able to observe ApxI-ApxII interactions by SPR (data not shown). Thus, we can speculate that more complex associations occur where LPS increase the cytotoxic effect of the toxins by bringing them together to the cell surface. Both toxins might also oligomerize to form hybrids pores that would be more stable in cell membrane.

Phenotypic characterization showed a significant decrease in the cytotoxic activity of mutant 5.1 on PAMs. Since no difference was found in gene expression or secretion of both ApxI and ApxII, we assumed that the low cytotoxic activity of mutant 5.1 was due to a defect in the functional or cell-binding properties of the toxins caused by their weak association with the truncated LPS. Several involvements of the LPS/RTX interaction in the activity of the toxins have been reported over the past years. It was demonstrated that RTX toxins have a tendency to self-associate via their hydrophobic residues (Ostolaza *et al.*, 1991; Ostolaza *et al.*, 1997; Soloaga *et al.*, 1998). The fact that our recombinant Apx toxins preparations are excluded from Sephacryl S-200 columns (data not shown), whose exclusion limit for globular proteins corresponds to a molecular weight of 500 kDa, also demonstrates the ability of the Apx toxins to oligomerize. It was proposed that the negative and polar residues within the core-lipid A region of LPS reduced the tendency of RTX toxins to form inactive aggregates (Herlax *et al.*, 2005). LPS was also shown to enhance the stability of the toxin by protecting it from thermal and chemical denaturation, keeping it in an active form (Herlax *et al.*, 2005). Moreover, negative charge of the carboxyl groups present in the core OS of LPS may also act as a reservoir of Ca^{2+} which is necessary for the activity of the toxin (Ostolaza and Goni, 1995). However, the fact that mutant 5.1 still exhibited wild type haemolytic activity suggested that the pore-forming activity of the Apx toxins was not affected. The low cytotoxicity of the mutant could then be attributed to a decreased binding of the toxins to specific cell surface receptors on PAMs. Indeed, it has been shown that β_2 integrins such as LFA-1 (CD11a/CD18) can act as RTX toxins receptors on the surface of leucocytes (Lally *et al.*, 1997). In contrast, no specific receptors are known on the surface of erythrocytes suggesting that the binding of RTX toxins to red blood cells is receptor-independent (Eberspacher *et al.*, 1989; Martin *et al.*, 2004). Since the

binding of LPS was shown to modify the conformation of the toxins (Herlax *et al.*, 2005), we propose that LPS could unmask specific binding sites on ApxI and ApxII and subsequently increase their affinity for their receptors. On the surface of red blood cells, LPS would have minor effect on the binding efficiency of the toxins as no receptors are involved. In addition, the adherence property of LPS might also help the Apx toxins to get in close proximity to their receptors. Alternatively LPS could be important for insertion and oligomerization of Apx toxins in the membrane of PAMs. Those results are of interest in the context of an *A. pleuropneumoniae* infection. Although Apx toxins lyse red blood cells, their potent leukocyte-modulating activity is more likely to be important in pathogenesis. Hence, the low cytotoxic activity of mutant 5.1 could account for its attenuation *in vivo*. Notably, in pigs, this mutant caused significantly less lung lesions (Rioux *et al.*, 1999), which are known to involve mainly the Apx toxins (Choi *et al.*, 2001).

In conclusion, our results demonstrate for the first time that the GalNAc-Gal II-Gal I region of the outer core plays a major role in the physical interaction between *A. pleuropneumoniae* LPS and the toxins ApxI or ApxII, which might subsequently enhance the cytotoxic activity of the bacterium. The direct or indirect involvement of LPS in receptor binding, membrane insertion, oligomerization, and overall activity of both ApxI and ApxII warrants further investigation. To the best of our knowledge, this is the first demonstration of a direct binding interaction between the outer core of LPS and a RTX toxin.

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Table 1. Bacterial strains used in the present study.

Strains	Relevant traits	Reference
Actinobacillus pleuropneumoniae [a] S4074 Nal^r	Serotype 1 (Nal^r), parent strain	(Rioux *et al.*, 1999)
5.1 [b]	LPS core oligosaccharide mutant (*galU*)	(Rioux *et al.*, 1999)

[a] The nalidixic acid resistant (Nal^r) strain was obtained from the reference strain *A. pleuropneumoniae* S4074 serotype 1 (K.R. Mittal, Faculté de médecine vétérinaire, Université de Montréal)

[b] The mutant derived from *A. pleuropneumoniae* serotype 1 S4074 Nal^r

Table 2. Nucleotide sequences of the primer sets used to amplify *apx* genes, the size of the PCR products are represented.

Gene	Oligonucleotide sequence		PCR product (bp)
apxIC	For	5′-TGGTTATGGGCAAGTTCTCC -3′	193
	Rev	5′-CAACTAGCGAGGCAACATCA -3′	
apxIA	For	5′-GGAGGCTTTAGGGATCGAAG -3′	195
	Rev	5′-CCTAATGCTTCCGATGAACG -3′	
apxIB	For	5′-ACGTGGTGCGGACAATCA -3′	229
	Rev	5′-CACTAAATGTGCGCCTAG -3′	
apxID	For	5′-GGTAATTGCTCCGGAAGATG -3′	186
	Rev	5′-CCGAGTTGCGGATGTTCG -3′	
apxIIC	For	5′-CCTGCAATTGAAAATGACCA -3′	197
	Rev	5′-AGAGATGAATCCCCGAATGG -3′	
apxIIA	For	5′-TGATGCTACAGCCGAGACAG -3′	261
	Rev	5′-CCACCATGGAACACATCATC -3′	

Table 3. Secondary structure analysis of Far-UV CD spectra of Apx toxins.

Protein	α-helix (%)		β-strand (%)		Turns (%)	Unordered (%)	Total (%)
	α_R	α_D	β_R	β_D			
ApxI	6	5	25	13	22	28	99
ApxII	30	22	7	6	13	24	102

α_R, regular α-helix; α_D, distorted α-helix; β_R, regular β-strand; β_D, distorted β-strand

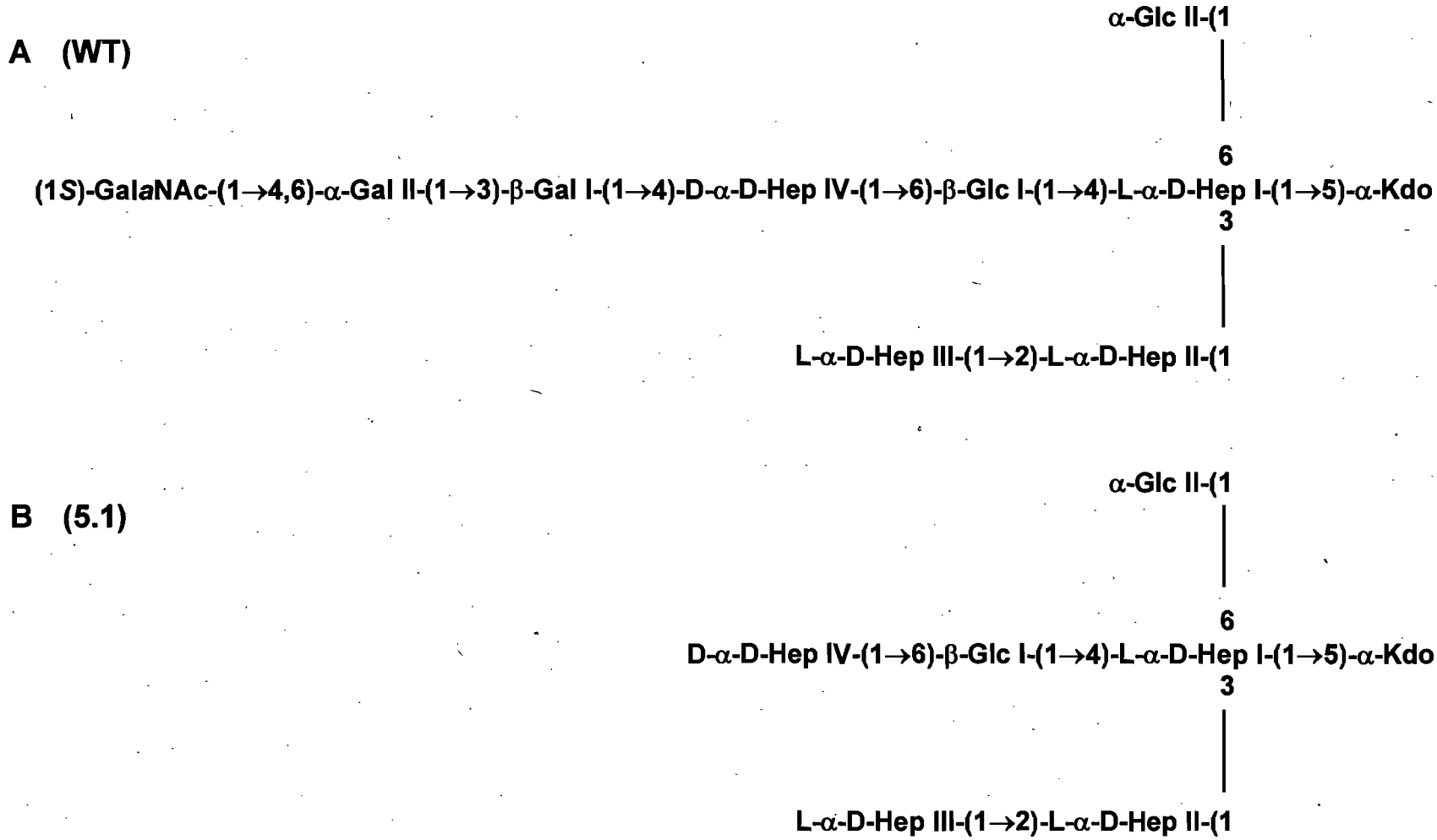

Fig 1. Structural representation of the core oligosaccharide from *A. pleuropneumoniae* serotype 1 parent strain (A) and its isogenic core LPS mutant 5.1 (B) (Ramjeet *et al.*, 2005).

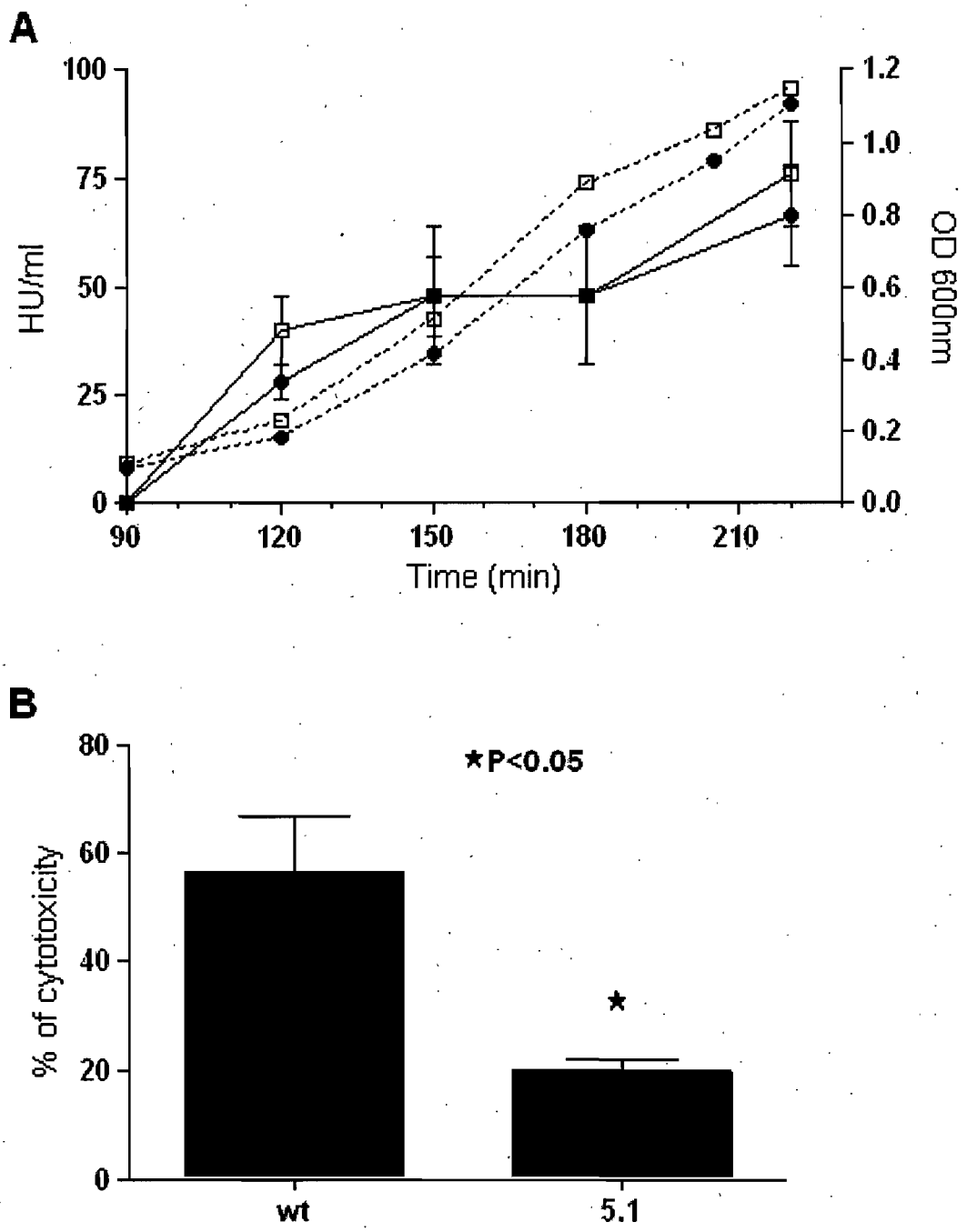

Fig 2. (A) Haemolytic activity (solid lines) of CFS from *A. pleuropneumoniae* wild type strain (filled circle, •) and mutant 5.1 (open square, □) at different time points during growth. Haemolytic activities are expressed in haemolytic units/ml (HU/ml). Similar growth was observed for the wild type and the mutant strains (dashed lines). (B) Cytotoxic effect of exponential phase (OD_{600nm} of 0.7) CFS from the wild type and the mutant 5.1 on PAMs. Data are expressed in percentage calculated from the 100% cytolysis reference with 2% TritonX-100.

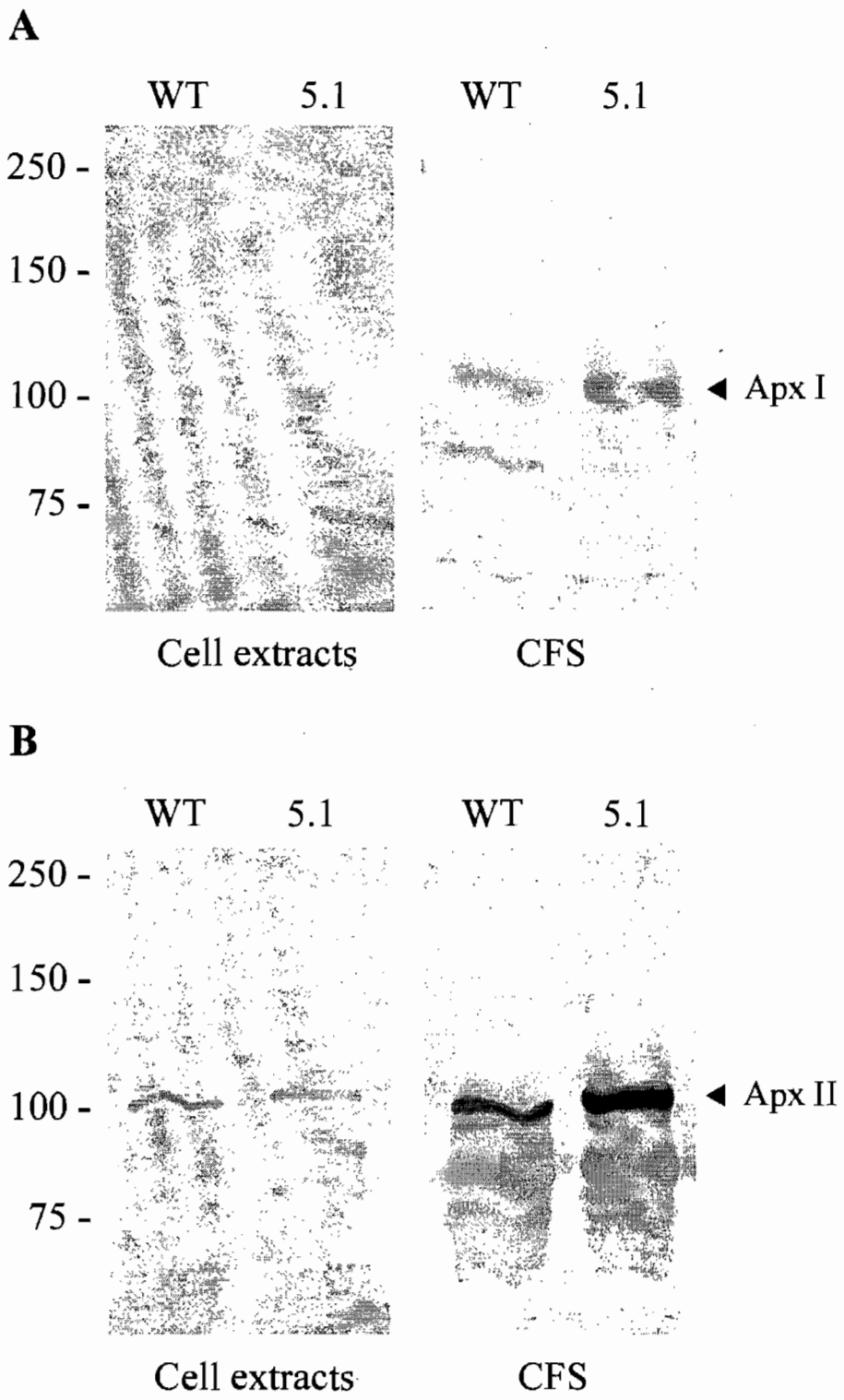

Fig 3. Immunoblots of total cell lysate extracts and 250-fold TCA-concentrated CFS from an exponential phase culture of *A. pleuropneumoniae* wild type strain and mutant 5.1. Immunoblots were probed with monospecific rabbit polyclonal antibodies against ApxI (A) or ApxII (B). Molecular mass markers (in kilodaltons) are indicated on the *left*.

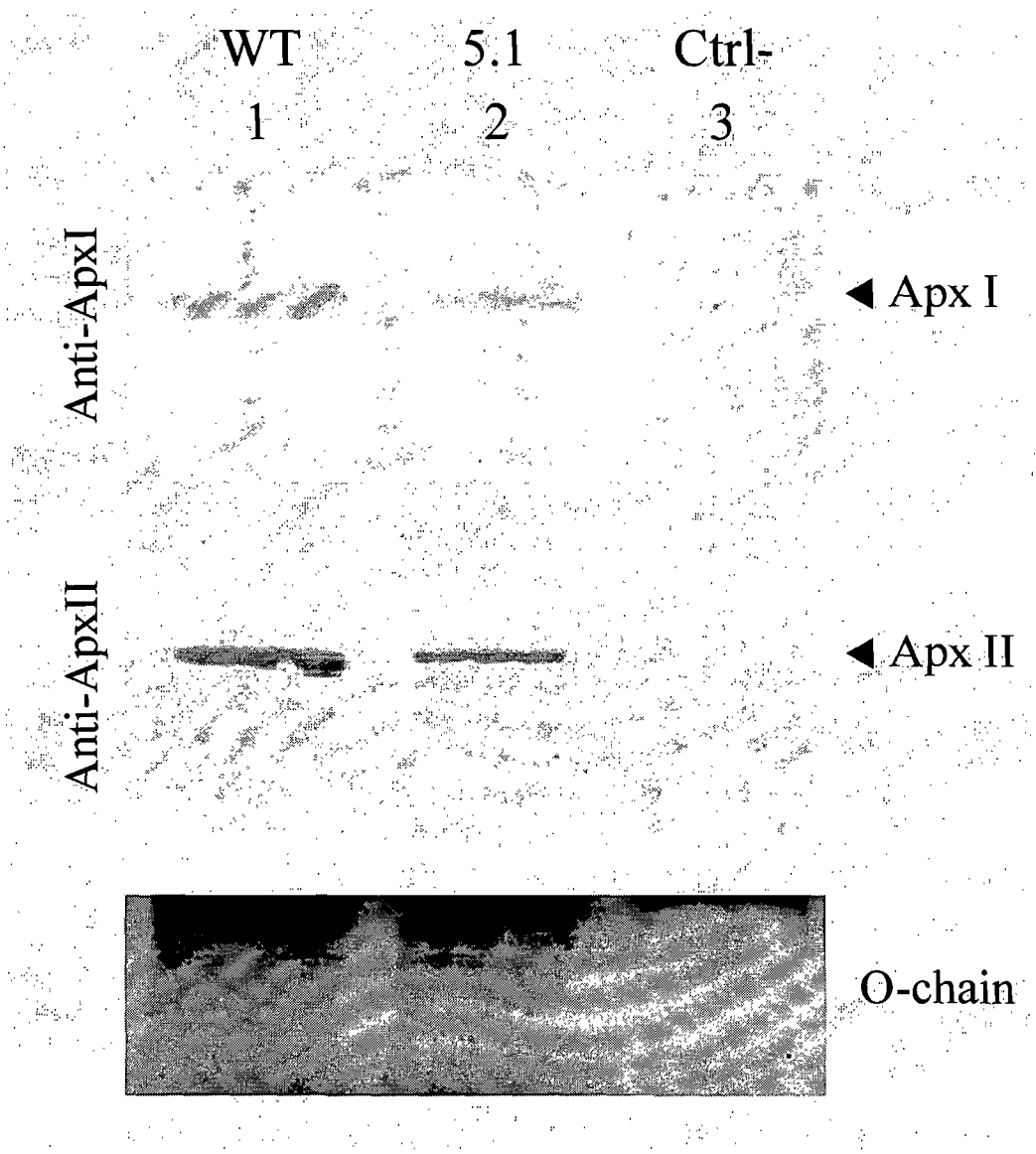

Fig 4. Co-immunoprecipitation of Apx toxins with LPS in exponential phase CFS from *A. pleuropneumoniae* wild type strain (lane 1) and the mutant 5.1 (lane 2). LPS-associated Apx toxins were pulled down by incubating CFS with a monoclonal antibody against *A. pleuropneumoniae* serotype 1 LPS O-antigen (lanes 1 and 2). A co-immunoprecipitation negative control was performed on CFS from the wild type strain without incubation with the anti-LPS antibody (lane 3). Immunoblots were probed with monospecific rabbit polyclonal antibodies against ApxI or ApxII. A silver-stained SDS-PAGE profile of the pulled-down samples shows the presence (lanes 1 and 2) or absence (lane 3) of the high molecular mass O-chains of LPS.

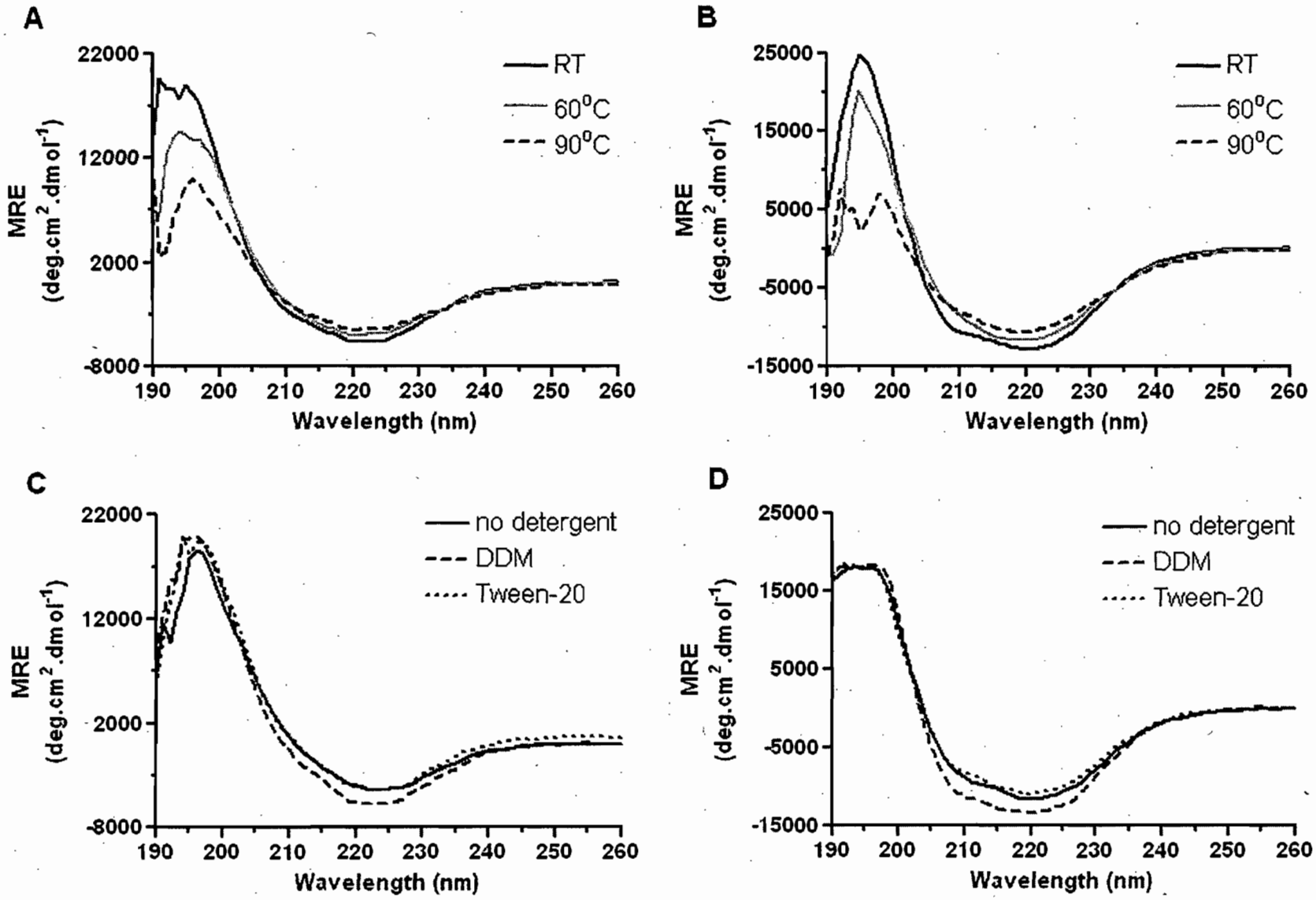

Fig 5. Representative CD analysis of the purified recombinant toxins ApxI (A and C) and ApxII (B and D) upon thermal denaturation or in the presence of detergent. Far-UV CD spectra of rApxI and rApxII at RT, 60°C and 90°C (A and B) and in the presence of 0.1% DDM, 0.1% Tween-20 or without detergent (C and D) are represented. This figure shows no (0.1% Tween-20) or only minor effects (0.1% DDM) of the detergents on the secondary structure of the toxins. MRE, mean residual ellipticity.

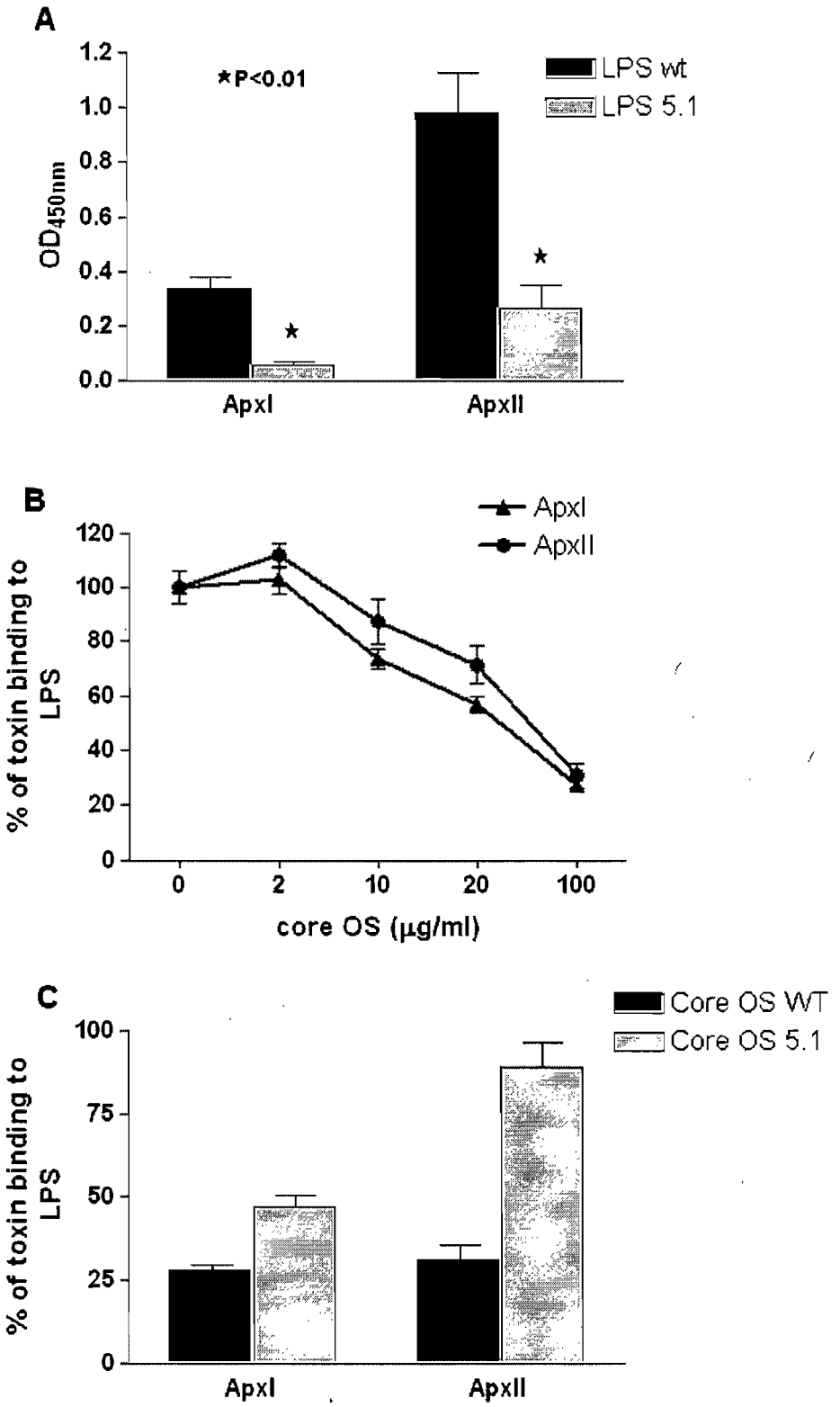

Fig 6. Representative ELISA analysis of ApxI or ApxII binding to coated purified LPS from *A. pleuropneumoniae* wild type strain and the mutant 5.1 (A). The level of binding is represented in absorbance at 450nm. Dose-dependent inhibition of the binding of ApxI or ApxII to wild type LPS by purified core OS from the wild type (B). Comparative inhibitory effect of WT and 5.1 core OS variants on the binding of ApxI or ApxII to wild type LPS (C). The percentage of binding (B and C) was calculated from the 100% reference corresponding to the binding without addition of purified core OS.

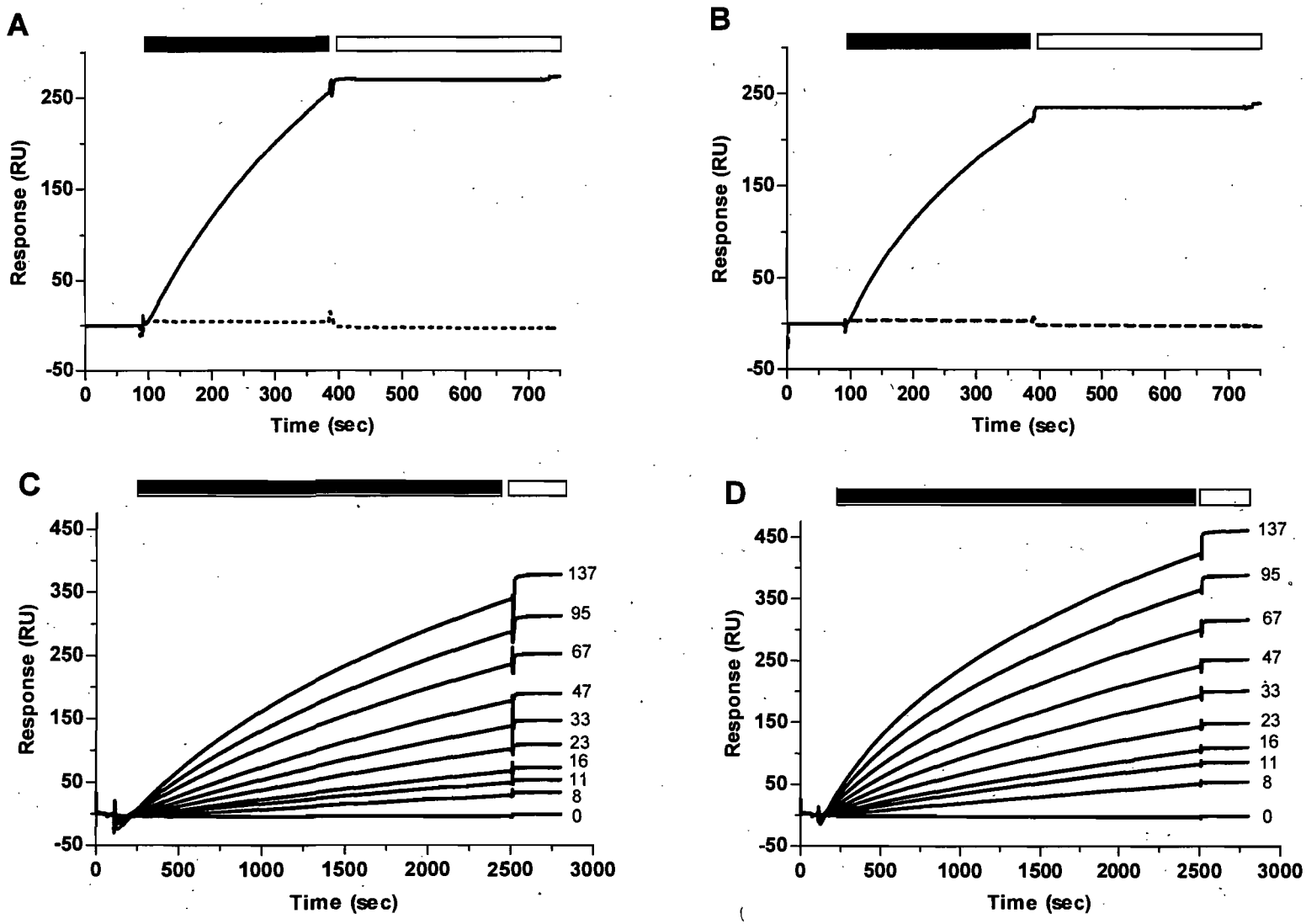

Fig 7 Representative SPR analysis of core OS binding to amine-coupled ApxI and ApxII toxins (association phase, shaded bar; dissociation phase, open bar). To examine binding specificity, WT (solid lines) and 5.1 (dashed lines) core OS variants were injected (130 μM; 5 min association + 5 min dissociation; 10 μl/min) over toxin surfaces (Panel A, 6400 RU ApxI; Panel B 7000 RU ApxII). To examine steady-state binding affinity, WT core OS was injected (0, 8, 11, 16, 23, 33, 47, 67, 95, 137 μM; 40 min association + 10 min dissociation; 5 μl/min) over toxin surfaces (Panel C, 6400 RU ApxI; Panel D, 7000 RU ApxII).

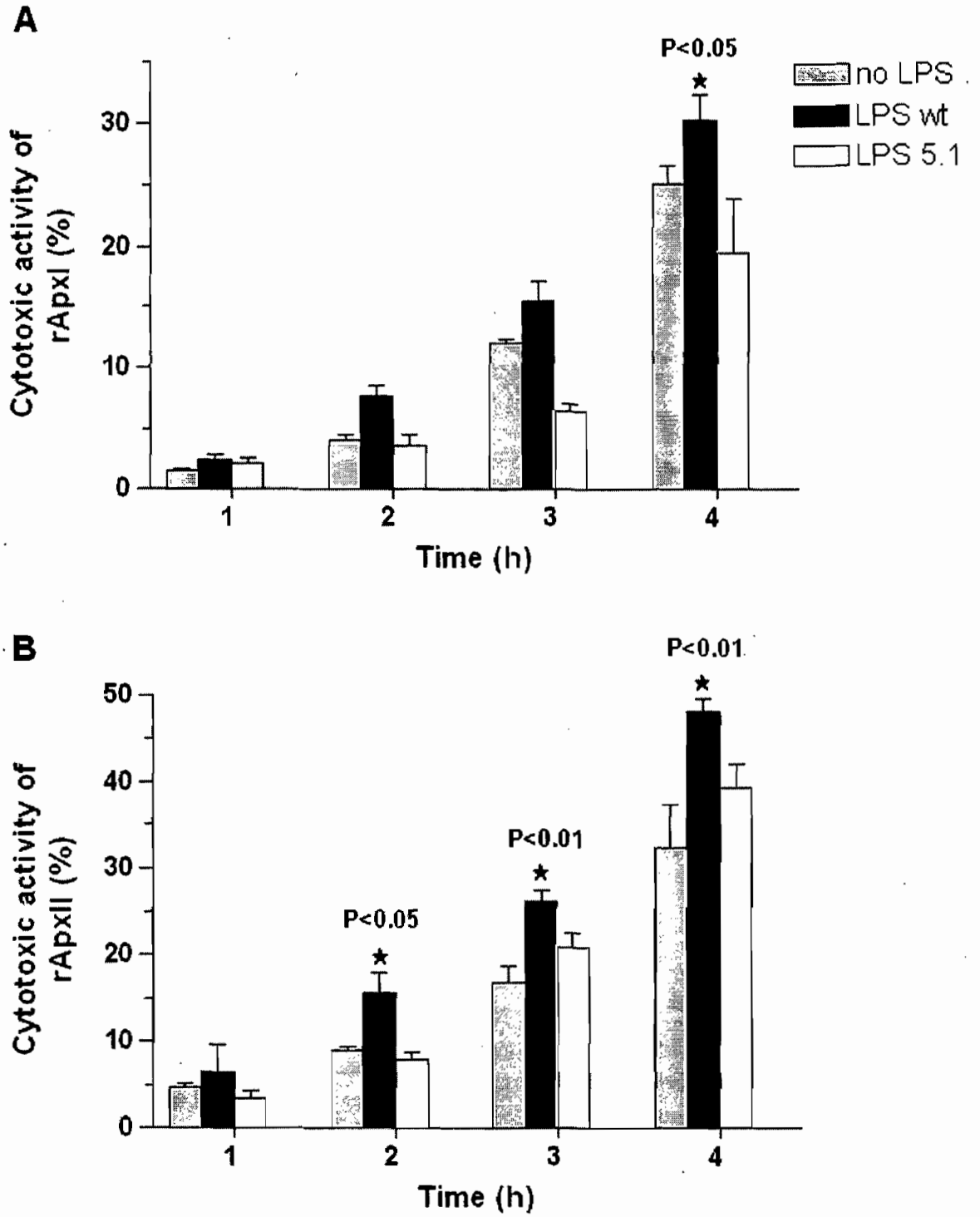

Fig 8. Effect of purified LPS from the wild type strain and the mutant 5.1 on the cytotoxicity of the recombinant toxins ApxI (A) and ApxII (B) to PAMs. Data are expressed in percentage calculated from the 100% cytolysis reference with 2% Triton X-100.

DISCUSSION

Le LPS constitue un facteur de virulence important des bactéries Gram négatives et ses différentes régions jouent chacune un rôle dans la pathogenèse bactérienne. Dans ce projet nous avons montré le rôle essentiel du noyau OS du LPS dans plusieurs mécanismes de virulence d'*A. pleuropneumoniae* grâce à la caractérisation de mutants LPS d'*A. pleuropneumoniae* sérotype 1. Dans l'espoir d'apporter plus d'informations sur le rôle du LPS dans l'interaction de la bactérie avec le système immunitaire inné, nous avons également évalué la réponse cellulaire de macrophages alvéolaires et de cellules épithéliales.

1. Analyse structurale du noyau OS d'*A. pleuropneumoniae* sérotype 1

La structure du noyau OS d'*A. pleuropneumoniae* n'a été déterminée que récemment (Michael *et al.*, 2004). Auparavant, 2 types de noyaux OS, différentiellement répartis chez les sérotypes, avaient été proposés sur la base de leur mobilité électrophorétique (Jacques *et al.*, 1996). L'analyse structurale a montré un noyau interne conservé et un noyau externe plus variable entre les sérotypes (Figure 7). *A. pleuropneumoniae* sérotype 1 possède un noyau de type 1 avec un noyau externe composé du trisaccharide (1*S*)-Gal*a*NAc-(1 → 4,6)-α-Gal-(1 → 3)-β-Gal-, branché au noyau interne en position 4 de l'Hep IV (Figure 7).

Dans cette étude (Article 1), l'analyse de la structure du noyau OS a montré des niveaux de troncation différents entre les 3 mutants « core ». En effet, la structure du noyau chez le mutant CG1 montre la perte du disaccharide terminal GalNAc-Gal II par rapport à la souche sauvage. L'absence du résidu galactose Gal I chez le mutant 5.1 empêche également l'addition du disaccharide terminal GalNAc-Gal II. Le mutant CG3, qui est le plus tronqué, est affecté au niveau de l'ajout du DD-heptose (Hep IV), ce qui se traduit au niveau de la structure par l'absence du tétrasaccharide GalNAc-Gal II-Gal I-Hep IV. Ces résultats nous ont permis de confirmer les différences de migration électrophorétique du noyau-lipide A précédemment observées chez ces mutants. En effet, le noyau du mutant CG3, qui est le plus tronqué selon l'analyse structurale, migre le plus rapidement, suivi de celui du mutant 5.1 et du mutant CG1 (Figure 2; article 1).

La détermination des sucres absents a aussi apporté des informations supplémentaires sur les différents gènes inactivés par l'insertion du transposon mini-Tn*10*. En effet, très peu de données sont disponibles sur les gènes contrôlant la biosynthèse du LPS d'*A.*

pleuropneumoniae. Les gènes mutés chez les différentes souches n'avaient pu être identifiés que par homologie de séquences avec les gènes d'autres bactéries. Le gène affecté chez le mutant CG1 code pour une protéine ayant respectivement 25% et 29% d'homologie avec la galactosyltransférase WlaC et la N-acétylgalactosamine transférase WlaE de *Campylobacter jejuni* (Fry *et al.*, 1998). Ainsi, la perte du disaccharide GalNAc-Gal II est due à un défaut au niveau de l'ajout du résidu Gal II, suggèrant fortement que CG1 est muté au niveau d'un gène homologue à *wlaC* qui code pour une galactosyltransférase. Le gène affecté chez le mutant CG3 code pour une protéine présentant 62% d'homologie avec la D-glycéro-D-manno-heptosyl transférase LbgB d'*Haemophilus ducreyi* (Stevens *et al.*, 1997). Une homologie de 24% est également retrouvée avec la 1,2-N-acétylglucosamine transférase WaaK d'*E. coli* (Heinrichs *et al.*, 1998a). L'analyse structurale montre que le mutant CG3 est affecté au niveau de l'ajout du DD-heptose (Hep IV), ce qui confirme que le gène muté est bien un homologue de *lbgB*. Le mutant 5.1 est affecté au niveau du gène *galU*, qui code pour une UTP-α-D-glucose-1-phosphate uridylyltransférase, une enzyme impliquée dans la synthèse de l'UDP-glucose (Weissborn *et al.*, 1994). La protéine GalU est également retrouvée chez *H. ducreyi*, *H. influenzae*, *E. coli* et *Shigella flexneri* et présente respectivement 87%, 83%, 71% et 77% d'homologie avec GalU d'*A. pleuropneumoniae*. L'UDP-glucose à un rôle central dans la synthèse des LPS car il intervient comme donneur de glucosyl. En effet, il a été démontré que les mutants *galU* chez *E. coli* et *Salmonella* possédaient un noyau OS dépourvu d'hexose (Sanderson & Stocker, 1981; Schnaitman & Klena, 1993). En ce qui concerne la structure du mutant 5.1, l'absence de l'hexose Gal I concorde avec l'absence d'UDP-glucose causée par la mutation de *galU*. Cependant, la structure du noyau OS de ce mutant montre que les résidus glucose Glc I et Glc II sont toujours présents au niveau du noyau interne. Ceci suggère la présence d'une autre voie de biosynthèse de l'UDP-glucose. De plus, la migration électrophorétique de la région noyau-lipide A du mutant 5.1 montre la présence de 2 bandes dont l'une correspondrait au phénotype sauvage, confirmant de nouveau l'hypothèse d'une autre voie de biosynthèse.

La troncation séquentielle des sucres chez les différents mutants « core » s'est avérée intéressante dans la suite de notre étude car cela nous a permis d'évaluer l'importance des différentes régions du noyau externe dans la virulence d'*A. pleuropneumoniae* sérotype 1.

2. Sensibilité d'*A. pleuropneumoniae* sérotype 1 aux peptides antimicrobiens

Compte tenu du rôle potentiel du noyau OS dans la résistance aux AMPs (voir section 3.2.2), nous avons évalué la sensibilité des mutants « core » *d'A. pleuropneumoniae* sérotype 1 à des peptides cationiques de diverses origines (Article 1). Nos résultats montrent que les 3 mutants « core » 5.1, CG1 et CG3 sont plus sensibles aux AMPs (Tableau 5; article 1). Par contre, le mutant rugueux 27.1 et le mutant acapsulé 33.2 sont aussi résistants que la souche sauvage, ce qui exclut un possible rôle de l'antigène O ou de la capsule dans la résistance d'*A. pleuropneumoniae* sérotype 1 aux peptides cationiques. On observe également que le profil de susceptibilité est indépendant de l'origine et de la structure des peptides. Ces résultats démontrent le rôle important du noyau OS du LPS dans la résistance d'*A. pleuropneumoniae* sérotype 1 aux AMPs, comme il a été observé chez d'autres bactéries. Ainsi, l'étude des mutants *galU* de *V. cholerae* et *lpsB* de *S. meliloti* a démontré que le noyau OS, et non l'antigène O, était important dans la résistance aux AMPs (Campbell *et al.*, 2002; Nesper *et al.*, 2001).

Les LPS sont les principales cibles de ces AMPs qui forment, dans la plupart des cas, des pores dans la membrane externe pour accéder à la membrane plasmique (Ding *et al.*, 2003). Plusieurs mécanismes de résistance des bactéries aux AMPs ont été proposés. Ils reposent essentiellement sur la charge, l'accessibilité et la stabilité de la surface membranaire. En effet, la fixation des peptides cationiques à la surface des bactéries est favorisée par les interactions électrostatiques avec les charges négatives du LPS. De plus, la formation de pores nécessite l'insertion des peptides dans la membrane externe et dépend de plusieurs facteurs, comme la fluidité et la stabilité membranaire. Ainsi, la stabilité de la membrane externe est principalement liée au bon arrangement des LPS, notamment par leur interaction avec les cations divalents comme le Mg^{2+} (Stefan *et al.*, 2004).

Il a été démontré que la diminution des charges négatives, qui seraient présentes au niveau du noyau OS et du lipide A du LPS, permet de réduire la fixation des molécules chargées positivement et ainsi de diminuer la sensibilité aux peptides cationiques (Groisman *et al.*, 1997; Gunn & Miller, 1996). À cet effet, plusieurs modifications du LPS comme l'ajout d'aminoarabinose ou de phosphoéthanolamine (Raetz *et al.*, 2007; Trent, 2004) ou encore la

réduction des phosphates (Karbarz *et al.*, 2003) peuvent contribuer à la neutralisation des charges négatives au niveau de la surface bactérienne. D'autre part, des études ont démontré le rôle important du Mg^{2+} dans la stabilité de la membrane externe en pontant les charges négatives des LPS mais aussi en interférant avec la fixation des peptides cationiques par répulsion électrostatique (Matsuzaki *et al.*, 1999). Dans ce cas de figure, la réduction des charges négatives pourrait au contraire augmenter la sensibilité aux AMPs en réduisant la fixation des ions Mg^{2+}.

En ce qui concerne la plus grande sensibilité des 3 mutants « core » d'*A. pleuropneumoniae* sérotype 1 aux peptides testés, la perte des sucres au niveau du noyau externe n'entraîne pas forcément une réduction de la charge négative globale de la bactérie. En effet, les charges négatives du LPS sont souvent liées aux phosphates qui se retrouvent généralement au niveau du lipide A ou associés aux heptoses du noyau interne. La structure du noyau OS d'*A. pleuropneumoniae* sérotype 1 a confirmé l'absence de phosphates au niveau du noyau externe (Michael *et al.*, 2004) et la composition en phosphates des LPS O-désacylés est la même pour les mutants et la souche sauvage (Tableau 4; article 1). Ainsi, une modification de charge de la membrane externe ne semble pas être à l'origine de la sensibilité des mutants « core » aux AMPs. En revanche, il est possible que le noyau externe réduise l'accessibilité des peptides aux charges négatives du lipide A par encombrement stérique. La perte de résidus chez ces mutants pourrait ainsi augmenter la fixation ou l'insertion des peptides. L'étude de mutants du noyau externe chez *Y. enterocolitica* illustre également cette hypothèse (Skurnik *et al.*, 1999). Il s'agit là d'un phénomène intéressant dans la mesure où le noyau externe contrecarre l'action des peptides antimicrobiens alors que le noyau interne et le lipide A, de par leurs charges négatives, favorisent au contraire leur fixation. Ceci pourrait expliquer la nécessité pour les bactéries de modifier leur LPS au niveau du noyau interne et du lipide A, en ajoutant par exemple des résidus aminoarabinose ou phosphoéthanolamine (Raetz *et al.*, 2007; Trent, 2004) alors que la modification du noyau externe n'est pas connue comme un mécanisme de résistance aux AMPs. Dans cette optique, l'étude de la sensibilité de mutants LPS d'*E. coli* à la lactoferricine (voir section 3.2.2) a montré qu'un mutant du noyau externe était plus sensible alors que des mutants du noyau interne, qui avaient perdu leurs résidus heptoses associés aux phosphates, étaient plus résistants que la souche sauvage. La sensibilité du mutant du noyau externe pourrait s'expliquer par une meilleure accessibilité de la

lactoferricine aux charges négatives (Farnaud *et al.*, 2004). Cependant, concernant le mutant CG1, la mesure du potentiel zeta a montré une diminution de la charge négative de sa surface membranaire par rapport aux mutants 5.1 et CG3 et à la souche sauvage (Labrie *et al.*, 2002). Cette modification de la charge chez ce mutant est probablement due à un changement du profil membranaire causé indirectement par la perte de la région GalNAc-Gal II du noyau externe. Il est également possible d'envisager que la diminution de la charge négative diminue la fixation des ions Mg^{2+} à la surface de ce mutant, ce qui augmente sa sensibilité aux AMPs.

Au-delà de la charge et de l'accessibilité de la surface, la stabilité de la membrane externe est un facteur qui peut également jouer un rôle dans le profil de résistance aux AMPs. En effet, le mécanisme d'action de ces peptides se déroule généralement en 2 étapes : la fixation médiée par les interactions électrostatiques entre les charges et l'insertion ou la désorganisation de la membrane externe, pour lesquelles interviennent essentiellement des interactions hydrophobes avec les lipides membranaires. Ainsi, certaines bactéries comme *Salmonella* modifient leur lipide A en ajoutant des chaînes d'acyles supplémentaires pour renforcer les interactions hydrophobes et réduire la fluidité membranaire (Guo *et al.*, 1998). De même, des mutants d'*E. coli* penta- et tétra-acylés sont plus sensibles à l'activité bactéricide de la lactoferricine que la souche sauvage hexa-acylé (Farnaud *et al.*, 2004). Le noyau OS peut également être impliqué indirectement dans la résistance aux AMPs en agissant sur la stabilité de la membrane externe. Ainsi, il a été proposé que la troncation du LPS déstabilise certaines protéines de la membrane externe qui seraient impliquées dans la résistance aux AMPs (Loutet *et al.*, 2006). Le profil protéique des vésicules de la membrane externe chez les différents mutants LPS d'*A. pleuropneumoniae* montre en effet des différences par rapport à la souche sauvage (Annexe 2), ce qui pourrait aller dans le sens de cette hypothèse. D'autre part, plusieurs études montrent que les mutants « core » sont non seulement plus sensibles aux peptides cationiques mais également aux détergents ou aux antibiotiques hydrophobes comme la novobiocine (Yethon *et al.*, 1998; Yethon *et al.*, 2000). Ceci suggère que leur sensibilité accrue au AMPs n'implique pas un mécanisme spécifique à ces peptides comme par exemple une modification de la charge mais serait plutôt due à un défaut global au niveau de la stabilité et de la perméabilité membranaire. Ainsi, le fait que les mutants « core » d'*A. pleuropneumoniae* sérotype 1 aient été criblés initialement pour leur

sensibilité à la novobiocine (voir section 2.6) pourrait expliquer en partie leur plus grande susceptibilité aux AMPs.

3. Stimulation du système immunitaire inné par *A. pleuropneumoniae* sérotype 1

La synthèse de cytokines inflammatoires par l'hôte joue un rôle important dans la l'inflammation associée à la pleuropneumonie porcine. Le LPS, connu comme un des principaux stimulateurs de l'inflammation, contribuerait ainsi à la pathogenèse *d'A. pleuropneumoniae* en stimulant la production de cytokines proinflammatoires. Nous avons évalué la capacité des mutants LPS d'*A. pleuropneumoniae* à stimuler la synthèse par des macrophages alvéolaires porcins (PAMs), de TNF-α, d'IL-6, d'IL-1β, de MCP-1 et d'IL-8 (Article 1). Les cinétiques de stimulation montrent que les LPS purifiés de la souche sauvage et des mutants stimulent de la même façon la synthèse des cytokines proinflammatoires (Figures 4 et 5; article 1). Ceci suggère que la capsule, l'antigène O et le noyau OS ne seraient pas impliqués dans cette stimulation. L'inhibition de la stimulation de la synthèse de cytokines par la polymyxine B, un inhibiteur des LPS, montre cependant que la production de cytokines est due aux LPS (Annexe 3). Ce résultat n'est pas surprenant si l'on considère que la partie endotoxique du LPS est le lipide A (Muroi & Tanamoto, 2002) et que les mutants LPS ne sont pas affectés au niveau de cette région.

Pour évaluer un possible effet stimulateur d'autres constituants de la surface, comme par exemple des protéines de la membrane externe, nous avons effectué une stimulation des PAMs par des bactéries entières. Les profils protéiques différents observés au niveau des vésicules de la membrane externe des mutants (Annexe 2) suggèrent en effet des variations de stimulation qui seraient dues à l'absence ou à la présence d'une ou plusieurs protéines. Cependant, comme pour les LPS purifiés, la stimulation avec des bactéries entières n'a montré aucune différence de synthèse de cytokines entre les mutants et la souche sauvage, minimisant ainsi une implication des protéines de surface. Il est important de noter que nous avons utilisé des bactéries tuées à la chaleur, en raison de la forte cytotoxicité d'*A. pleuropneumoniae*. Cela pose donc le problème de la dénaturation des protéines. En effet, il est possible que l'inactivation des protéines affecte leur capacité à stimuler les PAMs, ce qui ne nous permet

pas d'exclure totalement une implication des protéines de la membrane externe. Il serait intéressant dans cette optique d'évaluer le profil de synthèse de cytokines par des PAMs stimulés par des extraits de membrane externe.

Cette étude nous a également permis de détecter une production importante d'IL-8 par rapport aux autres cytokines. Cette observation est intéressante du point de vue de la pathogenèse d'*A. pleuropneumoniae* car elle est en corrélation avec l'infiltration massive des neutrophiles dans les poumons lors d'une infection (Baarsch *et al.*, 2000). En effet, l'IL-8 est le principal chémoattractant des neutrophiles (Baggiolini & Clark-Lewis, 1992; Lin *et al.*, 1994) et est généralement synthétisé au début de l'inflammation par des macrophages mais également par des cellules épithéliales et endothéliales, pour attirer les neutrophiles au site de l'infection.

Considérant le rôle important de l'épithélium respiratoire en tant que site initial de colonisation des pathogènes respiratoires, nous avons évalué la réponse cytokinaire de deux lignées de cellules épithéliales porcines, suite à une stimulation par des LPS purifiés ou des bactéries entières (Article 2). Les NPTr (Newborn Pig Trachea) sont des cellules épithéliales de trachée (Ferrari *et al.*, 2003) alors que les SJPL (St. Jude Porcine Lung) sont des cellules épithéliales de poumons (Seo *et al.*, 2001). Comme pour les PAMs, aucune différence n'a été observée dans le pouvoir stimulateur de la souche sauvage et des mutants LPS *d'A. pleuropneumoniae* sérotype 1. Cependant, des différences sont retrouvées dans le profil de synthèse des cytokines entre ces 2 lignées cellulaires. Parmi les cytokines testées (TNF-α, d'IL-6, d'IL-1β, de MCP-1 et d'IL-8), aucune production par les SJPL n'a été détectée même après 48 h de stimulation alors que la synthèse d'IL-8 est induite chez les NPTr (Figure 7; article 2). Un criblage du protéome de ces cellules suite à une incubation avec *A. pleuropneumoniae* sérotype 1 a montré une expression différentielle de protéines impliquées dans la voie NF-κB telles que IKKα et IKKβ. D'autre part, la stimulation des NPTr en présence d'un inhibiteur de NF-κB (CAPE) inhibe l'induction de la synthèse d'IL-8. Ces résultats suggèrent que la différence d'induction observée entre ces 2 lignées cellulaires implique des variations au niveau de l'activation de NF-κB. En effet, l'analyse de l'expression des sous-unités de NF-κB dans des conditions de stimulation a montré que la sous unité p50 était induite uniquement chez les SJPL et p65 uniquement chez les NPTr (Figure 6; article 2). L'absence de p65 chez les SJPL favoriserait la formation d'homodimères inactifs p50/p50 de

NF-κB (voir section 3.2.1), entraînant ainsi l'absence d'expression de cytokines. De plus, des études démontrent que l'affinité de p50 pour la séquence promotrice de l'IL-8 humaine est faible comparée à celle de p65 (Kunsch *et al.*, 1994). En effet, la séquence du site de fixation de NF-κB au niveau du promoteur de l'IL-8 humaine (5'-GTGGAATTTCC-3') est plus proche de la séquence consensus de fixation de p65 (5'-GGGRNTTTCC-3') que de celle de p50 (5'-GGGGATYCCC-3'), expliquant la différence d'affinité (Kunsch *et al.*, 1992).

Lors d'une infection, plusieurs voies, dont celle des TLRs, peuvent mener à l'activation de NF-κB (Nishikori, 2005). Les LPS, par exemple, en interagissant avec les TLR-4, induisent une cascade de transduction de signaux dans la cellule hôte qui mène à l'activation de NF-κB et à l'expression de gènes de l'inflammation (voir section 3.2.1). L'inhibition de la voie des TLRs par un inhibiteur des IRAK 1/4 montre que, suite à une stimulation de 3 h avec des bactéries vivantes, l'activation de NF-κB chez les NPTr est TLR-indépendant. Ceci suggère que la voie de stimulation LPS/TLR-4 jouerait un rôle minime dans l'activation des NPTr, comme en témoigne leur faible production d'IL-8 comparée à celle des PAMs, suite à une stimulation par des LPS purifiés ou des bactéries tuées (Figure 7; article 2). La capacité à stimuler les NPTr serait plutôt attribuée à d'autres composants bactériens de nature protéique qui seraient inactivés lors du traitement à la chaleur. Il serait donc intéressant de comparer la synthèse d'IL-8 par les NPTr stimulés par des bactéries vivantes et des bactéries tuées pour évaluer l'implication des protéines. Ces composants protéiques pourraient être les toxines Apx qui sont sécrétées par *A. pleuropneumoniae*. En effet, au-delà de leur rôle cytolytique, la plupart des toxines RTX ont un effet immunomodulateur lorsqu'elles sont présentes à faible concentration. L'hémolysine HlyA de *E. coli* stimule la synthèse d'IL-1 et d'IL-8 par des monocytes ou des cellules épithéliales (May *et al.*, 1996; Uhlen *et al.*, 2000). La leucotoxine LktA de *M. haemolytica* induit l'expression d'IL-1β et de TNF-α par des macrophages alvéolaires et des neutrophiles (Leite *et al.*, 2003; Yoo *et al.*, 1995). La leucotoxine LtxA d'*A. actinomycetemcomitans* induit la production et la sécrétion d'IL-1β par des macrophages (Kelk *et al.*, 2005). Et l'adénylate cyclase CyaA de *B. pertussis* induit la synthèse d'IL-6 par des cellules épithéliales de trachée (Bassinet *et al.*, 2004). En ce qui concerne les NPTr, l'incubation avec des bactéries vivantes pendant une durée suffisamment courte pour éviter la cytotoxicité cellulaire suggère la présence de faibles quantités de toxines Apx qui induiraient alors l'activation de NF-κB par un mécanisme TLR-indépendant.

La faible production d'IL-8 par les NPTr par rapport aux PAMs peut également s'expliquer par l'absence de sécrétion de TNF-α et d'IL-1β. En effet, ces 2 cytokines sont d'importants stimulateurs de la production d'IL-8 par les macrophages, les cellules endothéliales et les cellules épithéliales (Faccioli *et al.*, 1990; Lukacs *et al.*, 1995). Il a été démontré que l'augmentation de TNF-α dans le fluide bronchoalvéolaire, suite à une stimulation par le LPS, était suivie d'un influx de neutrophiles au niveau des voies respiratoires. De plus, la suppression de la réponse TNF inhibe la migration des neutrophiles (Nelson *et al.*, 1989). Des anticorps neutralisants contre IL-1β atténuent aussi leur recrutement (Horai *et al.*, 2000). La forte production d'IL-8 par les PAMs serait liée à une autoactivation due à la synthèse de TNF-α et d'IL-1β. Pour vérifier l'effet stimulateur de ces 2 cytokines, on pourrait évaluer la synthèse d'IL-8 dans une expérience de stimulation des NPTr en présence d'IL-1β et/ou de TNF-α recombinante ou en présence de surnageant de culture de PAMs préstimulés. Ceci nous permettrait de mettre en évidence une synergie d'activation entre les NPTr et les PAMs, *in vitro*.

Malgré les données intéressantes recueillies grâce à nos expériences *in vitro*, la situation dans l'hôte est indéniablement différente. L'activation des cellules lors d'une infection *in vivo* dépend non seulement de la présence de la bactérie mais également de l'environnement et de l'interaction entre les différents constituants du système immunitaire. Ainsi, il est possible que, de par leur promiscuité au niveau des voies respiratoires, les macrophages alvéolaires activent de manière paracrine les cellules épithéliales pulmonaires en produisant du TNF-α et de l'IL-1β.

En résumé, cette étude a confirmé le rôle immunostimulateur du LPS et plus précisément du lipide A et a également suggéré une implication non négligeable d'autres constituants de la surface d'*A. pleuropneumoniae* dans l'induction de l'inflammation.

4. Interaction LPS/Apx et cytotoxicité d'*A. pleuropneumoniae* sérotype 1

Au-delà de leur rôle dans l'interaction avec différents composants du système immunitaire de l'hôte, les LPS interagissent avec d'autres composants bactériens comme les protéines de la membrane externe ou encore des toxines sécrétées. Une association entre les LPS et les toxines RTX a ainsi été proposée dans plusieurs études et jouerait un rôle important dans

l'activité des toxines. D'autre part, différentes régions du LPS telles que le lipide A (Herlax *et al.*, 2005), le noyau OS (Bauer & Welch, 1997) et l'antigène O (Camprubi *et al.*, 1990), jouent un rôle plus ou moins important dans la synergie entre les LPS et les toxines RTX. Compte tenu du rôle important des toxines Apx dans la pathogenèse d'*A. pleuropneumoniae* et de la forte atténuation du mutant 5.1 (Rioux *et al.*, 1999), nous nous sommes interrogés sur une éventuelle interaction entre le LPS et les toxines Apx et sur un possible rôle du noyau OS dans l'activité cytotoxique d'*A. pleuropneumoniae.*

Dans cette étude (Article 3), nous avons démontré une diminution significative de la cytotoxicité du mutant « core » 5.1 et nous avons mis en évidence une interaction entre le LPS et les toxines ApxI et ApxII dans le surnageant d'*A. pleuropneumoniae* sérotype 1. Nous avons également montré *in vitro* une interaction entre le noyau OS du LPS et les toxines, suggérant que le LPS se lie aux toxines Apx via sa région oligosaccharidique. Ainsi, la présence de Kdo dans les fractions actives d'HlyA (Ostolaza *et al.*, 1991) suggère également une association entre le noyau et les RTX. La toxine LT d'*E. coli* se fixe aussi aux résidus oligosaccharidiques du LPS (Horstman *et al.*, 2004). Par ailleurs, il est intéressant de noter que la partie GalNAc-Gal II-Gal I du noyau externe, qui est tronquée chez le mutant 5.1, est essentielle à la fixation du noyau OS aux toxines car en son absence, aucune interaction n'est observée. Cependant, la contribution de ce trisaccharide dans la fixation reste à déterminer. À cet effet, l'analyse de la fixation de mono-, di- et trisaccharide pourrait nous permettre de vérifier si le trisaccharide ou un de ses sucres se fixe directement aux toxines. D'autre part, la structure du noyau OS pourrait indirectement influencer la fixation des toxines, car il a été démontré que la composition en sucres influençait la conformation du noyau (Bruse *et al.*, 1991; Jansson *et al.*, 1989). Dans notre cas de figure, la perte du trisaccharide GalNAc-Gal II-Gal I chez le mutant 5.1, induirait une modification de la conformation du noyau OS, qui serait alors incapable de se fixer aux toxines.

Les cinétiques d'associations montrent cependant que l'affinité de fixation du noyau OS de la souche sauvage aux toxines est faible. Ceci suggère donc que le noyau ne médie pas à lui seul l'interaction LPS/toxine. En effet, on constate que la fixation du LPS tronqué du mutant 5.1 aux toxines n'est pas complètement abolie (Figure 6A; article 3). Cette interaction résiduelle est certainement liée à d'autres régions du LPS. La forte hydrophobicité des toxines RTX due à leur domaine N-terminal et leur chaînes d'acyles (voir section 2.3) suggère par

exemple l'existence d'interaction hydrophobes entre le lipide A et les toxines Apx, comme il a été proposé précédemment pour HlyA (Herlax *et al.*, 2005). D'autre part, la stochiométrie de l'association suggère que plusieurs molécules de noyau se fixeraient à une toxine. La polyvalence de cette interaction permettrait donc de compenser la faible affinité. En ce qui concerne l'antigène O, son rôle est minimisé par le fait que notre mutant rugueux 27.1 possède une activité cytotoxique comparable à celle de la souche sauvage (Annexe 4). Par ailleurs, le fait que le LPS du mutant « core » 5.1 possède encore son antigène O (Rioux *et al.*, 1999) nous a permis d'évaluer sans ambiguïté le rôle du noyau OS dans la cytotoxicité et l'interaction LPS/Apx contrairement aux études avec les mutants « deep rough » d'*E. coli* qui eux, ne le possèdent plus. En effet, le site d'attachement de l'antigène O a été identifié au niveau du noyau interne en position 7 de l'Hep III chez *A. pleuropneumoniae* sérotype 5a (Michael *et al.*, 2004). Ceci expliquerait donc que la troncation du noyau externe chez le mutant 5.1 n'affecte pas l'attachement de l'antigène O. Chez *E. coli* la situation est différente : l'antigène O est attaché au niveau du noyau externe (Figure 6) et les mutants « deep rough » tronqués au niveau du noyau ne possèdent généralement pas d'antigène O. L'étude du rôle du noyau dans le profil cytotoxique de ces mutants ne permet donc pas d'éliminer une possible influence de l'antigène O (Bailey *et al.*, 1992; Bauer & Welch, 1997; Leeds & Welch, 1996; Stanley *et al.*, 1993; Wandersman & Letoffe, 1993).

Étant donné que les toxines ApxI et ApxII sont hémolytiques et cytotoxiques, nous avons évalué l'hémolyse et la cytolyse chez le mutant 5.1. Nos résultats montrent que le mutant 5.1 n'est pas affecté au niveau de son activité hémolytique mais qu'il est significativement moins cytotoxique que la souche sauvage pour les PAMs. Ceci nous permet donc de penser qu'il existe un lien entre l'interaction LPS/Apx et la cytotoxicité d'*A. pleuropneumoniae*. Cependant, plusieurs études montrent que la baisse de cytotoxicité des mutants « core » n'est pas liée à un défaut fonctionnel de la toxine mais plutôt à un défaut d'expression ou de sécrétion des toxines par ces mutants. Ainsi, il a été démontré que les mutants deep rough *rfaH* d'*E. coli* qui sont moins hémolytiques étaient affectés au niveau de l'expression d'HlyA. Ceci s'explique par le fait que RfaH est non seulement impliqué dans l'élongation du noyau externe (Sanderson & Stocker, 1981) mais agit aussi comme un facteur de transcription de l'opéron *hlyCABD* (Bailey *et al.*, 1992; Leeds & Welch, 1996). D'autre part, il a été démontré que les mutants *rfaH* et *galU* étaient affectés au niveau de la sécrétion d'HlyA dû à un défaut

d'insertion de TolC dans la membrane externe (Wandersman & Letoffe, 1993). Ainsi, l'absence de certains sucres au niveau du LPS produit des changements dans le profil de la membrane externe, ce qui par conséquent affecte la sécrétion des toxines. Dans ces cas de figure, la troncation du LPS a un effet indirect sur l'activité cytotoxique de la bactérie et l'interaction LPS/toxine ne jouerait peu ou pas de rôle. En revanche, d'autres mutants « core » d'*E. coli* comme *rfaJ* et *rfaP* ne montrent qu'une baisse de leur activité hémolytique (Bauer & Welch, 1997; Stanley *et al.*, 1993), suggérant que les résidus absents pourraient être impliqués dans une interaction LPS/HlyA qui serait nécessaire pour l'activité hémolytique de la bactérie.

Dans notre étude, aucune baisse de l'expression et de la sécrétion des toxines ApxI et ApxII n'a été observée pour le mutant 5.1. Ces résultats suggèrent que la faible cytotoxicité de ce mutant serait associée à un défaut dans la fonctionnalité des toxines dû à leur faible interaction avec le LPS tronqué. Plusieurs rôles de l'interaction LPS/RTX dans l'activité des toxines ont été proposés. Les charges négatives et les résidus polaires de la région noyau-lipide A du LPS réduiraient la formation d'agrégats inactifs de toxines (Herlax *et al.*, 2005). En effet, il a été démontré que les toxines RTX ont tendance à agréger notamment par l'intermédiaire de leur parties hydrophobes (Ostolaza *et al.*, 1991; Ostolaza *et al.*, 1997; Soloaga *et al.*, 1998). Nos expériences de filtration sur gel montrent également que les toxines Apx autoagrègent car elles sont exclues d'une colonne de Séphacryl S-200 qui possède une limite d'exclusion de 500 kDa pour les protéines globulaires. D'autre part, le LPS jouerait un rôle dans la stabilité de la toxine en la protégeant de la dénaturation thermique et chimique (Herlax *et al.*, 2005). Il a aussi été proposé que les charges négatives des groupes carboxyles du noyau OS serviraient de réservoir de Ca^{2+} nécessaire à l'activité de la toxine (Ostolaza & Goni, 1995).

Cependant, dans notre cas, le fait que le mutant 5.1 possède une activité hémolytique comparable à celle de la souche sauvage suggère que l'activité lytique des toxines Apx n'est pas affectée. La faible cytotoxicité de ce mutant pourrait être attribuée à une faible fixation des toxines Apx à leurs récepteurs spécifiques présents à la surface des PAMs. En effet, il a été démontré que les intégrines de type β2, présentes à la surface des leucocytes, agissent comme récepteurs des toxines RTX. Ainsi, LFA-1 (CD11a/CD18) a été identifié comme le récepteur des leucotoxines LtxA et LktA et de l'hémolysine HlyA (Lally *et al.*, 1997; Leite *et al.*, 2003) alors que l'adénylate cyclase CyaA utilise l'intégrine (CD11a/CD18) comme récepteur (Guermonprez *et al.*, 2001). À l'inverse, les globules rouges ne possèdent pas de β2 intégrine à

leur surface et la fixation des toxines RTX aux érythrocytes semble être récepteur-indépendante (Eberspacher *et al.*, 1989; Martin *et al.*, 2004). Étant donné que l'interaction LPS/toxine peut modifier la conformation de la toxine (Herlax *et al.*, 2005), on propose que l'interaction entre le LPS et ApxI/II exposerait les sites de fixation de ces toxines à leur récepteur, ce qui augmenterait leur affinité pour les PAMs. L'absence de récepteurs spécifiques à la surface des érythrocytes minimiserait le rôle du LPS dans l'activité hémolytique des toxines Apx et expliquerait pourquoi le mutant 5.1 n'est pas affecté au niveau de son activité hémolytique. D'autres études sont cependant nécessaires pour comprendre le rôle de l'interaction LPS/Apx dans la reconnaissance et la fixation des toxines Apx à leur récepteur.

Nos résultats sont intéressants dans le cadre de la pathogenèse d'*A. pleuropneumoniae* car les toxines Apx sont très cytotoxiques pour les macrophages alvéolaires *in vivo* et sont en grande partie responsables des lésions pulmonaires observées lors de la pleuropneumonie porcine (Choi *et al.*, 2001). Il a été démontré que le mutant 5.1 causait significativement moins de lésions pulmonaires que la souche sauvage (Rioux *et al.*, 1999). La faible cytotoxicité de ce mutant pour les PAMs due à une faible interaction LPS/Apx pourrait donc en être la cause et expliquer en partie son atténuation.

En conclusion, cette étude (Article 3) nous a permis de démontrer la contribution majeure de la région GalNAc-Gal II-Gal I du noyau externe dans l'interaction entre le LPS d'*A. pleuropneumoniae* sérotype 1 et les toxines ApxI et ApxII. Nous pensons que cette interaction serait importante pour la cytotoxicité de la bactérie en favorisant la fixation des toxines Apx à leurs cellules cibles. Cependant, d'autres études seraient nécessaires pour déterminer le rôle du LPS dans la reconnaissance et la fixation des toxines RTX à leurs récepteurs, dans leur oligomérisation et dans leur insertion dans les membranes. Il serait aussi intéressant de déterminer les sites de fixation du LPS au niveau de ces toxines. En effet, dans la mesure où l'interaction entre le LPS et les toxines RTX a souvent été considérée comme non spécifique, aucun site spécifique de fixation du LPS n'a été proposé à ce jour. Dans cette étude, l'interaction entre le noyau OS et les toxines Apx suggèrent l'existence d'un site de fixation pour les sucres. Ainsi, il a été démontré que la protéine de surfactant SP-D, se liait au noyau du LPS au niveau de résidus fixant le calcium (Wang *et al.*, 2008). Les séquences consensus riches en glycine qui fixent le calcium pourraient donc représenter un site de fixation potentiel

du LPS au niveau des toxines RTX. Des études de modélisation de l'interaction LPS/toxine permettraient d'apporter plus d'informations à ce sujet. D'autre part, au-delà de l'association entre le LPS et les toxines Apx, nous avons observé une interaction entre les toxines ApxI et ApxII en résonance plasmonique de surface. Ceci suggère que des associations plus complexes peuvent exister et que le LPS pourrait éventuellement augmenter l'effet cytolytique des toxines en les amenant en même temps à la surface. On peut également envisager la formation de pores hybrides qui seraient plus stables dans les membranes.

Il est important de noter que, dans cette étude, nous avons montré une interaction entre les toxines ApxI et ApxII et le noyau OS du sérotype 1 d'*A. pleuropneumoniae*. Étant donné que la perte du trisaccharide GalNAc-Gal II-Gal I chez le mutant 5.1 abolit la fixation du noyau OS aux toxines, il serait intéressant d'étudier cette interaction chez d'autres sérotypes *d'A. pleuropneumoniae* qui possèdent un noyau externe différent (Michael *et al.*, 2004). Il serait également intéressant d'étudier cette interaction chez les autres mutants « core » CG1 et CG3 d'*A. pleuropneumoniae* sérotype 1, pour évaluer l'importance des résidus du noyau externe dans la cytotoxicité d'*A. pleuropneumoniae*.

La faible cytotoxicité du mutant 5.1 pourrait être intéressante dans la vaccination contre *A. pleuropneumoniae*. En effet, les toxines Apx sont très immunogènes et sont présentes dans la majorité des vaccins contre *A. pleuropneumoniae* (Annexe 1). Cependant, la forte cytotoxicité de ces toxines a souvent nécessité leur utilisation comme toxoïde dans les vaccins sous unitaires (Tumamao *et al.*, 2004; van den Bosch & Frey, 2003) ou encore leur inactivation dans les vaccins vivants atténués (Bei *et al.*, 2007; Lin *et al.*, 2007). D'autre part, le problème que pose l'inactivation de ces toxines est la perte de leur pouvoir immunogène et donc de leur effet protecteur. Ainsi, l'utilisation d'un mutant *galU* qui serait moins cytotoxique tout en produisant des toxines Apx immunogènes permettrait d'augmenter l'efficacité vaccinale.

5. Virulence et pathogenèse d'*A. pleuropneumoniae* sérotype 1

Le LPS est un facteur de virulence important impliqué dans de nombreux mécanismes de pathogenèse d'*A. pleuropneumoniae*, comme il a été discuté ci-dessus. La disponibilité de mutants LPS affectés au niveau de leur antigène O ou de leur noyau OS nous a ainsi permis d'évaluer le rôle de ces 2 régions dans la pathogenèse de la bactérie. Cependant, quelles que

soient les propriétés du LPS *in vitro*, sa contribution *in vivo* dans la capacité de la bactérie à provoquer la maladie reste la plus importante. Nos précédentes infections expérimentales de porcs avec le mutant rugueux 27.1 d'*A. pleuropneumoniae* sérotype 1 (voir section 2.6) ont démontré que l'antigène O n'était pas essentiel pour la virulence de la bactérie (Labrie *et al.*, 2002). De plus, l'étude de l'adhérence de ce mutant à des sections de trachée de porc a également montré que l'antigène O ne jouait aucun rôle dans l'adhérence de la bactérie. Dans ce projet, l'étude du mutant rugueux 27.1 démontre que l'antigène O n'intervient ni dans la résistance aux peptides antimicrobiens, ni dans l'activité cytotoxique de la bactérie (Annexe 4).

À l'inverse, la faible adhérence du mutant 5.1 et l'atténuation de sa virulence avait permis de suggérer un rôle crucial du noyau OS dans la pathogenèse d'*A. pleuropneumoniae* (Rioux *et al.*, 1999). Ces résultats combinés à ceux obtenus pour les mutants rugueux montrent un lien entre l'adhérence et la virulence. En effet, le LPS est connu comme l'adhésine majeure d'*A. pleuropneumonie* permettant la colonisation du tractus respiratoire (Bélanger *et al.*, 1990; Paradis *et al.*, 1994). Étant donné que la colonisation est une étape essentielle de l'infection, le noyau OS, en favorisant l'adhérence, serait important pour la virulence d'*A. pleuropneumoniae*. Cependant, alors que le mutant « core » CG3 est moins adhérent, le mutant CG1 a une adhérence comparable à celle de la souche sauvage (Galarneau *et al.*, 2000). Considérant l'importance de l'adhérence dans la pathogenèse d'*A. pleuropneumoniae*, nous avons évalué la virulence des 2 mutants « core » CG1 et CG3 (Article 1). Nos résultats montrent encore un lien entre l'adhérence et la virulence car CG3 est atténué alors que CG1 est aussi virulent que la souche sauvage (Tableau 6; article 1). En s'intéressant à la structure du noyau chez ces mutants, on observe que les 2 mutants les plus tronqués (5.1 et CG3) sont affectés au niveau de l'adhérence et de la virulence. On en déduit donc que la région Gal I-Hep IV du noyau externe jouerait un rôle important dans la virulence d'*A. pleuropneumoniae* en favorisant la colonisation d'*A. pleuropneumoniae* sérotype 1. À l'inverse, la région terminale GalNAc-Gal II du noyau externe ne serait pas impliquée dans la colonisation et la virulence.

La plus grande sensibilité des mutants 5.1 et CG3 aux AMPs est en corrélation avec leur atténuation *in vivo*. Cependant, le mutant CG1 est également plus sensible aux AMPs alors que sa virulence n'est pas affectée. Ceci suggère que la résistance aux peptides cationiques est

importante pour la viabilité de la bactérie *in vitro* mais que la propriété d'adhérence d'*A. pleuropneumoniae* serait plus importante *in vivo*. Il est possible que la résistance aux AMPs joue un rôle dans le stade chronique de la maladie en favorisant le maintien de la bactérie. Dans cette optique, on observe une légère baisse dans l'isolement du mutant CG1 au niveau des cavités nasales 15 jours post-infection.

En comparant les 2 mutants atténués 5.1 et CG3, on observe que 5.1 semble le plus atténué alors qu'il est moins tronqué. En effet, les infections expérimentales montrent que le mutant 5.1 cause moins de mortalité, moins de lésions pulmonaires et moins de fièvre que le mutant CG3 (Rioux *et al.*, 1999). Le faible taux de lésions pulmonaires observé avec le mutant 5.1 est certainement dû à sa faible cytotoxicité comme il a été démontré ci-dessus. D'autre part, le mutant 5.1 semble être plus sensible que CG3 à la polymyxine B, à la protamine et au mastoparan avec des MIC respectives de 0.125, 62.5 et 25 µg/ml contre 0.25, 125 et 50 µg/ml pour le mutant CG3 (Tableau 5; article 1). Ces résultats suggèrent que l'atténuation de la bactérie n'est pas forcément lié au niveau de troncation de son noyau OS.

L'ensemble de cette étude démontre que le noyau OS et non l'antigène O du LPS joue un rôle crucial dans la pathogenèse d'*A. pleuropneumoniae*. On a montré que la région Gal I-Hep IV du noyau externe du LPS est importante pour la virulence d'*A. pleuropneumoniae* sérotype 1 en favorisant la colonisation du tractus respiratoire porcin. Cependant, nous montrons que d'autres sucres du noyau OS, qui ne semblent pas jouer de rôle *in vivo*, sont important pour la bactérie *in vitro*. En effet, la région GalNAc-Gal II absente chez le mutant CG1 joue aussi un rôle dans la résistance aux AMPs. D'autre part, nous avons aussi démontré que le trisaccharide GalNAc-Gal II-Gal I est important dans l'interaction entre le LPS et les toxines Apx et pour l'activité cytotoxique de la bactérie.

On en déduit que la pathogenèse d'*A. pleuropneumoniae* implique des mécanismes complexes où les différentes propriétés de virulence de la bactérie auraient une importance particulière à une certaine étape de l'infection ou selon le statut immunitaire de l'hôte.

CONCLUSION

Ce projet a permis de démontrer le rôle crucial du noyau OS du LPS dans la pathogenèse d'*A. pleuropneumoniae*. En effet, nous avons montré dans cette étude que cette région du LPS est impliquée dans de nombreux mécanismes de virulence de la bactérie comme la colonisation, la résistance aux peptides antimicrobiens et l'activité cytotoxique. Nous avons également observé pour la première fois une interaction entre les toxines hémolytiques et cytotoxiques ApxI et ApxII et le LPS d'*A. pleuropneumoniae* sérotype 1, via le noyau OS. Cette étude représente donc la première démonstration d'une interaction directe entre la partie OS du LPS et une toxine RTX. L'analyse de la structure du noyau OS des mutants « core » s'est avérée très intéressante dans l'ensemble de ce projet, dans la mesure où la connaissance de la composition en sucres du noyau OS des mutants nous a permis d'observer une importance différentielle de chaque région du noyau externe dans la pathogenèse d'*A. pleuropneumoniae*. D'autre part, cette étude suggère que le LPS contribue en grande partie à l'inflammation observée lors de la pleuropneumonie porcine en stimulant la synthèse de cytokines proinflammatoires par les PAMs. Cependant, l'étude de cellules épithéliales porcines de trachée et de poumon suggèrent que l'induction de l'inflammation chez le porc par *A. pleuropneumoniae* implique des mécanismes plus complexes où interviennent probablement d'autres constituants de la bactérie.

En conclusion, ce projet nous a apporté des informations supplémentaires sur le rôle du LPS dans l'interaction d'*A. pleuropneumoniae* avec son hôte et ouvre également la voie à d'autres études, notamment au niveau de la stimulation du système immunitaire par la bactérie et au niveau de l'interaction entre le LPS et les toxines Apx.

PERSPECTIVES

Analyse structurale du LPS d'*A. pleuropneumoniae* sérotype 1

Notre étude a montré que contrairement à la plupart des mutants « deep rough » d'*E. coli*, nos mutants « core » d'*A. pleuropneumoniae* sérotype 1 possèdent encore leur antigène O. D'autre part, le site d'attachement de l'antigène O a été identifié au niveau du noyau interne en position 7 de l'Hep III chez le sérotype 5a. Il serait donc intéressant de localiser le point d'attachement de l'antigène O au niveau du noyau interne du sérotype 1 en résonance magnétique nucléaire (RMN). Cependant, en raison de la nature polymérique de l'antigène O, il est difficile d'obtenir une fraction ne possédant qu'une seule unité répétée de sucre, ce qui faciliterait l'analyse. À cet effet, le mutant rugueux 27.1 ou un autre mutant de l'antigène O, qui produirait une seule unité de la chaîne O pourraient être de bons candidats.

A. pleuropneumoniae sérotype 1 et système immunitaire inné

Nous avons montré grâce à l'utilisation des mutant LPS que l'antigène O et le noyau OS du LPS n'avaient aucun rôle direct sur la capacité d'*A. pleuropneumoniae* sérotype 1 à stimuler la synthèse de cytokines inflammatoires par les PAMs ou les cellules épithéliales porcines (SJPL et NPTr). Cependant, d'autres composants protéiques seraient susceptibles d'avoir un effet stimulateur et l'utilisation de bactéries tuées à la chaleur dans nos expériences ne permet pas d'éliminer cette hypothèse. En revanche, la forte cytotoxicité d'*A. pleuropneumoniae* sérotype 1 limite l'utilisation des bactéries vivantes.

Cette étude nous montre également que, contrairement aux PAMs activés qui expriment toutes les cytokines testées, les cellules épithéliales se comportent différemment. En effet, aucune synthèse de cytokine n'a été détectée chez les SJPL alors que les NPTr ne semblent produire que l'IL-8 en réponse à une stimulation pas les LPS ou les bactéries entières. De plus, l'activation de NF-κB chez les NPTr semble être TLR-indépendante. Ainsi d'autres expériences permettraient de mieux évaluer l'activation de ces cellules :

- L'incubation des cellules avec des bactéries vivantes, pendant un temps suffisamment court pour éviter la cytotoxicité, permettrait une détection des ARNm des cytokines en

RT-PCR. Ceci pourrait éventuellement révéler l'expression d'autres cytokines chez les SJPL et les NPTr.

- L'utilisation de mutants pour les toxines Apx permettrait de prolonger le temps de stimulation des cellules par des bactéries vivantes.
- La stimulation des cellules par des extraits de membrane externe pourrait permettre de mettre en évidence un rôle stimulateur des OMPs et éventuellement de détecter une différence de stimulation entre les mutants LPS qui présentent des profils protéiques différents au niveau de leur membrane.
- L'incubation des cellules avec des toxines Apx purifiées ou des surnageants de bactéries permettrait de mettre en évidence un éventuel rôle immunomodulateur de ces toxines.

Des différences sont aussi retrouvées entre les deux lignées de cellules épithéliales au niveau des sous unités de NF-κB induites. Ces résultats ouvrent la voie à de nouvelles perspectives d'études qui peuvent nous amener à comprendre d'autres voies de signalisation menant à l'activation de ces cellules :

- Au delà de la voie classique p50/p65, il serait intéressant d'évaluer l'activation de la voie alternative de NF-κB en étudiant les autres sous unités, soit RelB, c-Rel et p52.
- Étant donné qu'on ne détecte l'induction de p50 uniquement chez les SJPL et l'induction de p65 uniquement chez les NPTr, il serait intéressant de vérifier la présence des gènes codant pour ces sous unités et également d'évaluer leur expression chez ces cellules.

L'activation de NF-κB est également modulée par d'autres cytokines comme TNF-α et IL-1β qui agissent via leurs récepteurs présent à la surface des cellules. Dans un contexte *in vivo*, ceci suggère des mécanismes d'auto-activation ou d'activation paracrine par des cellules se retrouvant dans le même environnement :

- Il serait donc intéressant d'étudier l'activation des PAMs ou des cellules épithéliales suite à une incubation avec ces cytokines seules ou en combinaison avec du LPS.

- La co-culture des PAMs et des cellules épithéliales ou encore l'incubation des cellules épithéliales avec du surnageant de culture de PAMs préstimulés permettraient de mettre en évidence une synergie d'activation entre ces cellules.

Interaction LPS/Apx et cytotoxicité d'*A. pleuropneumoniae* sérotype 1

Dans cette étude, nous avons montré une interaction entre le LPS d'*A. pleuropneumoniae* sérotype 1 et les toxines ApxI et ApxII via le noyau OS. Nous montrons le rôle fondamental du trisaccharide GalNAc-Gal II-Gal I du noyau externe dans ces interactions. D'autres études sont cependant nécessaires pour élucider les bases de l'interaction LPS/Apx :

- Dans la mesure où la présence des résidus GalNAc-Gal II-Gal I semble être primordiale pour l'interaction LPS/Apx, l'étude de la fixation de mono-, di-, et trisaccharide aux toxines Apx permettrait de mettre en évidence le rôle direct de ces sucres dans l'interaction.
- Il serait aussi intéressant de réaliser la même étude pour les mutants « core » CG1 et CG3 qui ne possèdent pas respectivement le disaccharide GalNAc-Gal II et le tetrasaccharide GalNAc-Gal II-Gal I-Hep IV au niveau de leur noyau OS
- Concernant les régions riches en glycine comme sites de fixation potentiels du LPS au niveau des toxines Apx, une étude de mutagenèse dirigée dans ce domaine pourrait permettre de déterminer les résidus important dans l'interaction LPS/Apx. D'autre part, une modélisation tridimensionnelle de cette interaction permettrait de mettre en évidence d'autres sites de fixation potentiels.

Cette étude suggère également un rôle important de l'interaction LPS/Apx dans l'activité cytotoxique de ces toxines. Nous pensons que le LPS en interagissant avec les toxines, faciliterait leur reconnaissance par leurs récepteurs membranaires. Les études précédentes montrent en effet que les intégrines de type β2 à la surface des leucocytes agiraient comme récepteurs des toxines RTX. D'autres études sont cependant nécessaires pour comprendre les mécanismes par lesquels le LPS influence la fonctionnalité des toxines Apx :

- Il serait intéressant d'étudier l'effet du LPS sur la fixation des toxines Apx aux intégrines de type β2.

- On pourrait également étudier l'insertion et l'oligomérisation de ces toxines dans des liposomes contenant des récepteurs et évaluer le rôle du LPS.
- Dans le but d'identifier des récepteurs potentiels, on pourrait cribler des extraits de membranes cellulaires par capture en SPR couplée à l'identification par spectrométrie de masse.

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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 *ΔapxIIAΔureCΔdmsA-ΔhybBΔaspAΔfur* 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 non-replicating 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 investi-

gation 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 ™, Biokema (capsular antigens and Apx toxins)	NA	1, 2, 7, 9	SC	endobr	9	Pig	(Chiers *et al.*, 1998)
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)

Subunit vaccine	Formulation and adjuvant	Vaccine serotype	Route of immunization	Challenge		Animal model	References
				Route	Serotype		
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 lipoprotein PalA and/or ApxI + ApxII)	Diluvac Forte adjuvant formulation	2, 5b	IM	Aerosol	1	Pig	(Van Den Bosch and Frey, 2003)
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 supernatant of *A. pleuropneumoniae*	Emulsigen-Plus	2 and 9	IM	Endobr	2	Pig	(Goethe *et al.*, 2000)

Subunit vaccine	Formulation and adjuvant	Vaccine serotype	Route of immunization	Challenge		Animal model	References
				Route	Serotype		
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 + B subunit of the *E. coli* heat-labile enterotoxin EtxB)	Freund's complete adjuvant	1, 10	SC	NA	NA	Mouse	(Bagdasarian *et al.*, 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)

Subunit vaccine	Formulation and adjuvant	Vaccine serotype	Route of immunization	Challenge		Animal model	References
				Route	Serotype		
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)
Serotype 7 strain AP76	Δ*exbB* and Δ*ureC* single mutants and Δ*exbB*Δ*ureC* 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	

Wild type *A. pleuropneumoniae* strain	Live vaccine	Route of immunization	Challenge		Animal model	References
			Route	Serotype		
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)
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	*ΔapxIIΔureCΔdmsAΔhybBΔaspAΔfur* 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)
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)

Protein	OrfID	Function	Method of identification	References
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).

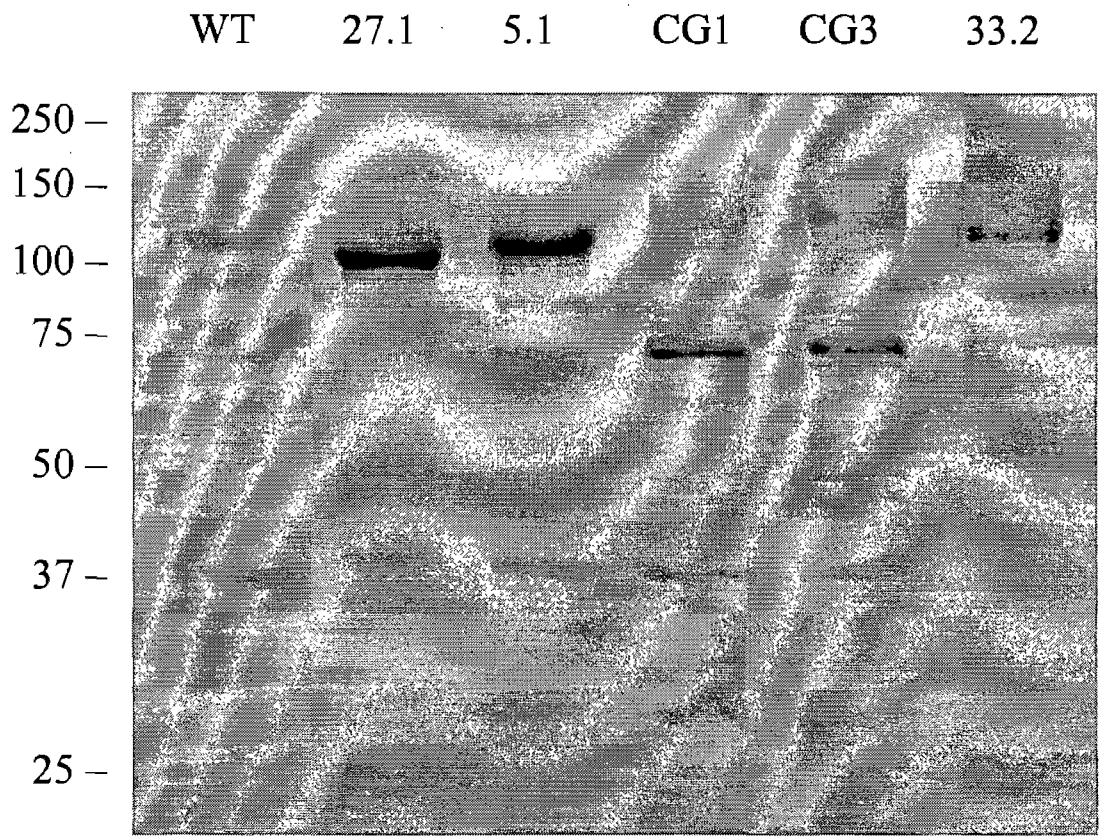
IVET = *in vivo* expression technology, STM = signature tagged mutagenesis, SCOTS = selective capture of transcribed sequences

Table 4. Genes identified by more than one gene expression methodology

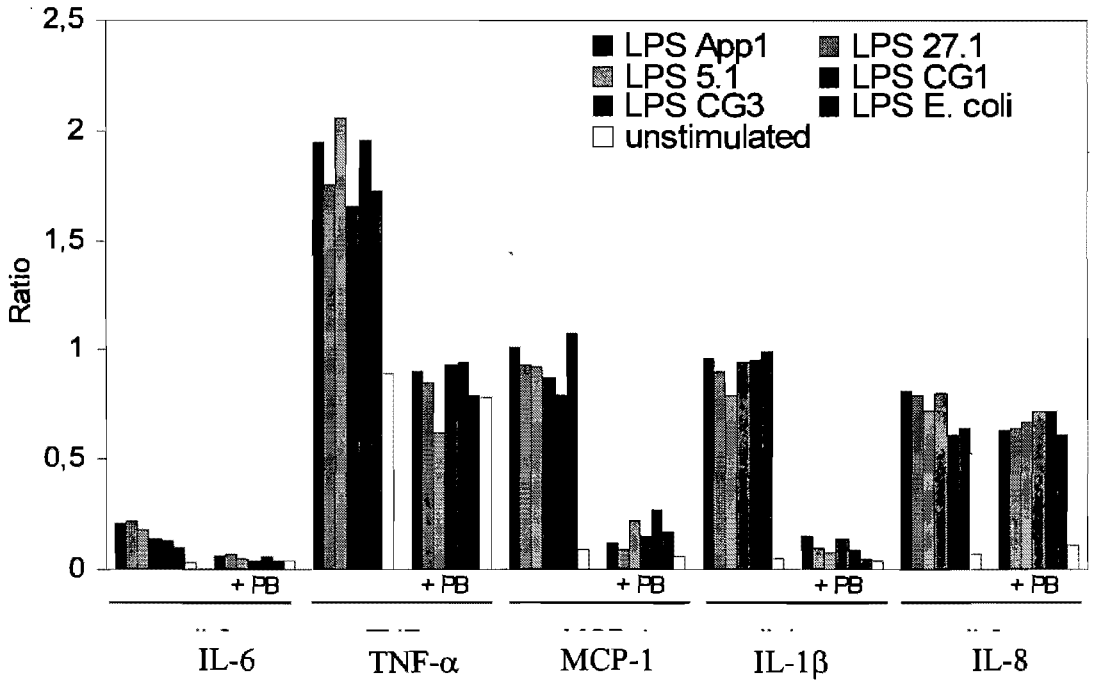
Gene	OrfID	Function	Method of identification	References
prfC	ap0033	peptide chain release factor 3	IVET, STM	(Fuller *et al.*, 1999; Sheehan *et al.*, 2003)
typA	ap0056	GTP-binding protein	SCOTS, microarray[a]	(Baltes *et al.*, 2007; Deslandes *et al.*, 2007)
tonB2	ap0082	protein TonB2	STM, microarray	(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)
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)

Gene	OrfID	Function	Method of identification	References
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)

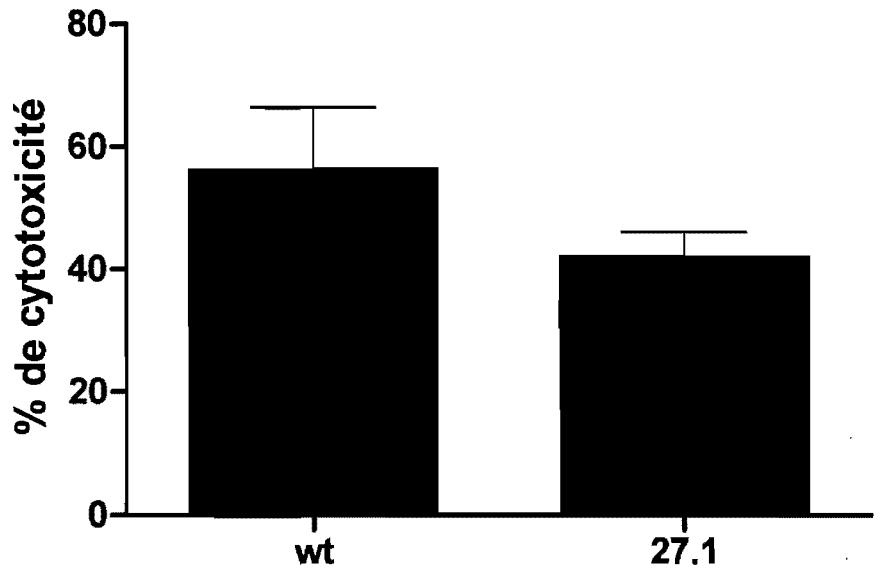
[a] Transcriptional profiling under iron-restricted conditions (Deslandes *et al.*, 2007).



Annexe 2. Profil protéique de vésicules de membrane externe (OMVs) d'*A. pleuropneumoniae* sérotype 1. Les OMVs ont été purifiés à partir de surnageants de culture « overnight » de la souche sauvage et des mutants par ultrafiltration (« cutoff de 300 kDa). Suite à un dosage des protéines totales dans les OMVs, la même quantité de chaque échantillon a été séparée par SDS-PAGE (gel à 7.5%) et coloré au bleu de Coomassie. Les poids moléculaires (en kilodaltons) sont indiqués à gauche. Ce gel montre les variations dans le profil protéique des OMVs provenant des différentes souches



Annexe 3. Effet de la polymyxine B sur l'expression de cytokines proinflammatoires par des PAMs stimulés par des LPS. Les macrophages ont été stimulés pendant 24 h par 1 µg de LPS purifiés provenant de la souche sauvage et des mutants en présence (+ PB) ou en absence de 10 µg/ml de polymyxine B. Les données sont représentées en ratio de cytokine/β_2-microglobuline cytokine pour IL-6, TNF-α et MCP-1 et en ratio de cytokine/GAPDH pour IL-1β et IL-8. Un contrôle négatif (unstimulated) et un contrôle positif (LPS E. coli) ont été ajoutés. Cette figure montre l'inhibition de l'expression des cytokines par la polymyxine B. L'absence d'inhibition pour IL-8 est certainement due à une trop forte expression et nécessiterait donc une concentration plus élevée de polymyxine B.



Annexe 4. Effet cytotoxique d'*A. pleuropneumoniae* sérotype 1 et de son mutant rugueux 27.1 sur des macrophages alvéolaires porcins. Les PAMs ont été incubés avec des surnageants de culture en phase exponentielle (DO_{600nm} de 0.7). La cytotoxicité a été évaluée après 4 h d'incubation en dosant la lactate déshydrogénase (LDH) dans le milieu de culture. La cytotoxicité est représentée en pourcentage en utilisant comme référence 100% une cytolyse complète avec du Triton X-100 à 2%. Aucune différence significative de cytotoxicité n'est détectée entre la souche sauvage et le mutant rugueux 27.1.