Université de Montréal

Rôle des lipopolysaccharides dans l'adhérence d'*Actinobacillus pleuropneumoniae* aux cellules des voies respiratoires porcines et caractérisation préliminaire des récepteurs

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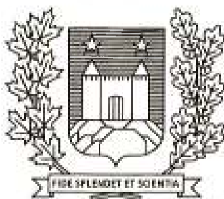
Thèse présentée à la Faculté des études supérieures
en vue de l'obtention du grade de
Philosophiæ Doctor (Ph.D.)
microbiologie et immunologie



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Faculté des études supérieures
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Université de Montréal
Faculté des études supérieures

Cette thèse intitulée:

Rôle des lipopolysaccharides dans l'adhérence d'*Actinobacillus pleuropneumoniae* aux cellules des voies respiratoires porcines et caractérisation préliminaire des récepteurs

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Thèse acceptée le: 29.06.1998

SOMMAIRE

Actinobacillus pleuropneumoniae est l'agent étiologique de la pleuropneumonie fibrinohémorragique et nécrosante porcine, il est répandu mondialement et cause d'énormes pertes économiques à l'industrie porcine. La pathogenèse de la pleuropneumonie porcine due à *A. pleuropneumoniae* ainsi que le rôle des différents facteurs de virulence impliqués dans la colonisation de ce microorganisme ne sont pas encore parfaitement élucidés. Cependant, sachant que les lipopolysaccharides (LPS) constitueraient l'adhésine principale d'*A. pleuropneumoniae*, nous avons voulu approfondir notre compréhension de l'interaction adhésine-récepteur chez *A. pleuropneumoniae*.

Tout d'abord, pour étudier l'adhérence des LPS d'*A. pleuropneumoniae* aux cellules des voies respiratoires du porc, nous avons développé un système utilisant des coupes congelées de trachée de porcelets. Avec ce modèle, nous avons pu démontrer qu'*A. pleuropneumoniae* adhère à l'épithélium de la trachée de porcelets. Par hydrolyse acide et fractionnement des LPS en présence de désoxycholate, nous avons démontré que c'est la partie polysaccharidique du LPS qui est impliquée dans cette reconnaissance.

Par cytofluorométrie ainsi que par microscopie électronique, nous avons confirmé l'accessibilité de l'antigène-O (Ag-O) d'*A. pleuropneumoniae*, malgré la présence d'une capsule chez ce microorganisme. Ensuite, nous avons démontré que des anticorps dirigés contre les LPS, plus précisément contre l'Ag-O des LPS, peuvent bloquer l'adhérence d'*A. pleuropneumoniae* dans notre modèle de coupes congelées. Ainsi de tels anticorps pourraient avoir un effet protecteur chez l'animal.

Au cours de ce projet, nous avons observé sur gel que la région du noyau oligosaccharidique (OS)-lipide A migrait différemment selon la souche étudiée. Nous avons réussi à regrouper les divers sérotypes d'*A. pleuropneumoniae*, en deux types de noyau OS-lipide A selon leur mobilité électrophorétique et leur antigénicité. Ainsi les sérotypes 1, 6, 9 et 11 appartiennent au type I, tandis que les autres, i.e. les sérotypes 2, 3, 4, 5a, 5b, 7, 8, 10 et 12, appartiennent au type II.

De plus, nous avons cherché à évaluer l'effet de conditions de croissance réduites en fer sur la production des polysaccharides de surface. Selon que la bactérie ait été placée dans des conditions riches ou réduites en fer, nous

n'avons pas observé, par cytofluorométrie et par microscopie électronique, de différence dans la production des polysaccharides capsulaires. Par contre, nous avons observé que chez la souche de référence d'*A. pleuropneumoniae* sérotype 1, il y aurait deux sous-populations de cellules selon la capacité des cellules à lier des anticorps dirigés contre l'Ag-O. De plus, selon les conditions de culture riches ou réduites en fer, la proportion des cellules fortement marquées augmente lorsque les conditions de culture sont réduites en fer. Ce phénomène pourrait permettre à la bactérie d'augmenter la quantité de ses adhésines chez l'hôte et ainsi augmenter ses chances d'adhérer et d'infecter le système respiratoire du porc.

Enfin, nous avons désiré identifier des récepteurs cellulaires reconnus par le LPS d'*A. pleuropneumoniae*. Nous avons, en ce sens, démontré que les LPS d'*A. pleuropneumoniae* se lient aux histones, des protéines cationiques ainsi qu'à des protéines d'environ 38,5 kDa présentes dans une préparation de cellules de trachée et de macrophages alvéolaires.

MOTS CLÉS: *Actinobacillus pleuropneumoniae*, adhérence, lipopolysaccharides, cellules du tractus respiratoire, récepteurs, polysaccharides capsulaires.

TABLE DES MATIÈRES

SOMMAIRE iii

TABLE DES MATIÈRES vi

LISTE DES TABLEAUX x

LISTE DES FIGURES xi

LISTE DES ABRÉVIATIONS xviii

REMERCIEMENTS xix

DÉDICACE xxi

I. INTRODUCTION 1

II. REVUE DE LA LITTÉRATURE 5

1. *Actinobacillus pleuropneumoniae.* 6
   - 1.1 Historique et taxonomie 6
   - 1.2 Caractéristiques 8
   - 1.3 Facteurs de virulence 8
     - 1.3.1 Capsule 9
     - 1.3.2 Protéines de la membrane externe 11
       - 1.3.2.1 Protéines de la membrane externe communes à tous les sérotypes 12
       - 1.3.2.2 Protéine liant la transferrine porcine 13
     - 1.3.3 Produits de sécrétion 16
       - 1.3.3.1 Toxines Apx 16
       - 1.3.3.2 Facteur de perméabilité 20
       - 1.3.3.3 Protéases 20
       - 1.3.3.4 Autres produits de sécrétion 21
     - 1.3.4 Liaison à la fibronectine 22
2. Lipopolysaccharides 23
   - 2.1 Historique 23
   - 2.2 Nature et structure des lipopolysaccharides 24
     - 2.2.1 Lipide A 25
     - 2.2.2 Noyau oligosaccharidique 27
     - 2.2.3 Chaîne O 29

2.2.4 Influences des conditions de croissance sur la production des lipopolysaccharides 33
2.2.5 Structure tridimensionnelle des lipopolysaccharides 36
2.3 Immunogénicité des lipopolysaccharides 38
2.3.1 Choc septique 38
2.3.2 Anticorps anti-lipopolysaccharides 41
2.3.3 Lipopolysaccharides et maladies auto-immunitaires....42
2.3.4 Autres effets des endotoxines 42
2.4 Techniques d'extraction des lipopolysaccharides 43

3. Interactions adhésine-récepteur 45
3.1 Adhérence 45
3.2 Adhésines d'*A. pleuropneumoniae* 46
3.2.1 Fimbriae, pili ou appendices filamenteuses....46
3.2.2 Hémagglutinines....47
3.2.3 Lipopolysaccharides d'*A. pleuropneumoniae* 47
3.3 Les lipopolysaccharides comme adhésines....48
3.3.1 Accessibilité des lipopolysaccharides à la surface bactérienne 48
3.3.2 Les lipopolysaccharides comme adhésines chez différentes espèces bactériennes....49
3.4 Récepteurs de lipopolysaccharides 50

III. MATÉRIEL, MÉTHODES ET RÉSULATS 57

ARTICLE 1:
Sonia-Élaine Paradis, Daniel Dubreuil, Stéphane Rioux, Marcelo Gottschalk, and Mario Jacques. 1994. High-molecular-mass lipopolysaccharides are involved in *Actinobacillus pleuropneumoniae* adherence to porcine respiratory tract cells. Infect. Immun. **62:** 3311-3319. 58

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IV. DISCUSSION 102

V. CONCLUSION..113

VI. BIBLIOGRAPHIE 115

VII. ANNEXES xxii

ACTE DE COLLOQUE 1:
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ARTICLE 6:

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LISTE DES TABLEAUX

REVUE DE LA LITTÉRATURE

Tableau 1.
Lipopolysaccharides d'*Actinobacillus pleuropneumoniae* 31

Tableau 2.
Exemple de bactérie produisant plus d'un type de LOS/LPS.............................34

Tableau 3.
Récepteurs et molécules liant les LPS, présents chez les mammifères, selon leur masse moléculaire 51

Tableau 4.
Autres récepteurs et molécules liant les LPS, présents chez les mammifères, dont la masse moléculaire n'est pas précisée..55

Tableau 5.
Récepteurs et molécules liant les LPS présents chez d'autres organismes 55

ARTICLE 6

Table 1.
Members of the *Pasteurellaceae* family described in the present review and the diseases they cause in their respective host.. lxix

Table 2.
Adhesin-receptor systems found in HAP organisms... lxx

LISTE DES FIGURES

REVUE DE LA LITTÉRATURE

Figure 1.
Les pourcentages d'hybridation génomique entre les différents groupes de bactéries 7

Figure 2.
Représentation moléculaire de l'enveloppe d'une bactérie Gram-négatif 25

Figure 3.
Structure chimique schématisée des LPS de *Salmonella* 27

Figure 4.
Modèles de la forme lisse des LPS de *Salmonella* 38

Figure 5.
Voies probables des interactions entre les facteurs humoraux et les cytokines dans la pathogénie de l'inflammation systémique sévère 40

ARTICLE 1

Figure 1.
Transmission electron micrographs of whole cells of *A. pleuropneumoniae* serotype 1 isolate probed with monoclonal antibodies against serotype 1 (A) or 2 (B) O antigen, and goat anti-mouse IgG-gold particles (10 nm). Bar, 200 nm 60

Figure 2.
Flow cytometry analysis of *A. pleuropneumoniae* serotype 1 isolate. (A) Whole cells were labeled with the anti-mouse IgG fluorescein isothiocyanate (FITC)-conjugated antibody (left peak) and with monoclonal antibodies against serotype 1 O antigen plus the anti-mouse IgG FITC-conjugated antibody (right peak). (B) Whole cells were labeled with the anti-mouse IgG FITC-conjugated antibody and with monoclonal antibodies against serotype 2 O antigen plus the anti-mouse IgG FITC-conjugated antibody (left peaks). The horizontal bars indicate fluorescent cells 61

Figure 3.
Binding of *A. pleuropneumoniae* serotype 2 extracted LPS to porcine lung and tracheal frozen sections. Lung sections A and B (original magnification, 250X) and tracheal sections C and D (original magnification, 400X) were incubated with (A and C) or without (B and D) LPS. Sections were then incubated with a serotype-specific rabbit antiserum, and the results of the reaction were revealed with an alkaline phosphatase conjugate and a chromogenic substrate. Arrowheads and arrow denote areas of intense reaction....................................62

Figure 4.
Inhibition of adherence of an *A. pleuropneumoniae* serotype 1 isolate and a serotype 2 reference strain to porcine tracheal frozen sections by homologous polysaccharides and lipid A obtained after acid hydrolysis of *A. pleuropneumoniae* extracted LPS 62

Figure 5.
Fractionation of extracted LPS from *A. pleuropneumoniae* serotype 1 (A) or serotype 2 (B) on Sephacryl S-300SF column in the presence of 0.25% deoxycholate. Fractions (milliliters) were analysed for KDO content (squares) and for neutral sugars content (diamonds). Fractions were grouped into five pools (A to E)...63

Figure 6.
Silver-stained SDS-PAGE profiles of *A. pleuropneumoniae* serotype 1 (A) or serotype 2 (B) extracted LPS fractionated on a gel filtration column. Pools of fractions (A to E) are as in Fig. 5. The positions of low-molecular-mass markers (in kilodaltons) are indicated on the left 64

Figure 7.
Inhibition of adherence of an *A. pleuropneumoniae* serotype 1 isolate to porcine tracheal frozen sections by pooled fractions obtained after gel filtration chromatography of extracted LPS from *A. pleuropneumoniae* serotype 1 64

Figure 8.
Transmission electron micrographs of negatively stained *A. pleuropneumoniae* serotype 2 LPS. (A) Extracted LPS preparation. (B to D) LPS preparations obtained after chromatography on a Sephacryl S-300SF column: (B) pool A, (C) pool C, or (D) pool E. Bars, 200 nm 65

ARTICLE 2

Figure 1.
Silver-stained Tricine-SDS-PAGE profiles of whole-cell, proteinase-K-treated preparations of *A. pleuropneumoniae* reference strains representing serotype 1 (lane 2; representative of core type I), 2 (lane 3; representative of core type II), 3 (lane 4), 4 (lane 5), 5a (lane 6), 5b (lane 7), 6 (lane 8), 7 (lane 9), 8 (lane 10), 9 (lane 11), 10 (lane 12), 11 (lane 13), and 12 (lane 14). For comparison, LPS (2 μg) from *S. typhimurium* TV119 (lane 1; Ra mutant) and *S. typhimurium* SL1181 (lane 15; Re mutant) are shown 70

Figure 2.
Western blot analysis of whole-cell, proteinase-K-treated preparations of *A. pleuropneumoniae* probed with serum from a pig experimentally infected with (A) *A. pleuropneumoniae* serotype 1 reference strain (core type I) or (B) *A. pleuropneumoniae* serotype 2 reference strain (core type II). (A) *A. pleuropneumoniae* serotypes 1 (lane 1); 6 (lane 2); 9 (lane 3); 11 (lane 4), and 2 (lane 5). (B) *A. pleuropneumoniae* serotypes 1 (lane 1); 2 (lane 2); 3 (lane 3); 4 (lane 4); 5b (strain 81-750, lane 5), and 6 (lane 6). The positions of prestained low-molecular-mass markers (in kilodaltons) are indicated on the left. Arrows indicate the core-lipid A region 71

Figure 3.
Western blot analysis of whole-cell, proteinase-K-treated preparations of *A. pleuropneumoniae* probed with serum from a pig experimentally infected with (A) *A. pleuropneumoniae* serotype 6 reference strain (core type I) or (B) *A. pleuropneumoniae* serotype 3 reference strain (core type II). *A. pleuropneumoniae* serotypes 3 (lane 1); 6 (lane 2); and 8 (lane 3) are shown. The positions of prestained low-molecular-mass markers (in kilodaltons) are indicated on the left. Arrows indicate the core-lipid A region..71

Figure 4.
Western blot analysis of whole-cell, proteinase-K-treated preparations of *A. pleuropneumoniae* serotype 1 reference strain probed with monoclonal antibody 5.1 G8F10 specific to serotype 1 O-antigen (lane 1) and *A. pleuropneumoniae* serotype 2 reference strain probed with monoclonal antibody 102-G02 specific to serotype 2 O-antigen (lane 2). The positions of prestained low-molecular-mass markers (in kilodaltons) are indicated on the left......................................72

ARTICLE 3

Figure 1.
Flow cytometry analysis of *A. pleuropneumoniae* serotype 1 reference strain 4074 grown under iron-sufficient (A) or iron-restricted conditions (B). Cells were labelled with mAb against serotype 1 capsular antigen and anti-mouse IgG FITC-conjugated antibody. In both panels, the left peak represents control cells incubated with the anti-mouse IgG FITC-conjugated antibody. The left horizontal bars indicate non-fluorescent cells (1) and the right horizontal bars indicate fluorescent cells (2) 76

Figure 2.
Electrophoretic and Western blot analyses of whole-cell, proteinase K-treated preparations of *A. pleuropneumoniae* serotype 1 reference strain 4074 (lanes 1 and 2) and field isolate FMV87-682 (lanes 3 and 4) grown under iron-sufficient (lanes 1 and 3) or iron-restricted conditions (lanes 2 and 4). Preparations were silver-stained (A) or transferred to a nitrocellulose membrane and then probed with the mAb 5.1 G8F10 (B). The positions of molecular-mass markers (in kilodaltons) are indicated on the left 76

Figure 3.
Flow cytometry analysis of *A. pleuropneumoniae* serotype 1 field isolate FMV87-682 grown under iron-sufficient (A) or iron-restricted conditions (B), and serotype 1 reference strain 4074 grown under iron-sufficient (C) or iron-restricted conditions (D). Cells were labelled with mAb against serotype 1 O-antigen and anti-mouse IgG FITC-conjugated antibody. In all panels, the left peak represents control cells incubated with the anti-mouse IgG FITC-conjugated antibody. The left horizontal bars indicate non-fluorescent cells (1) and the right horizontal bars indicate fluorescent cells (2). Transmission electron micrographs of whole cells of *A. pleuropneumoniae* serotype 1 reference strain probed with mAb against serotype 1 O-antigen (E and F). Bars, 200 nm..........77

ARTICLE 4

Figure 1.
Inhibition of adherence of *A. pleuropneumoniae* strain 4074 (hatched bars) and strain 4226 (spotted bars) to porcine tracheal frozen sections by different concentrations of Fab fragments obtained from mAb 5·1 G8F10 (specific for serotype 1 O-Ag) (a) or mAb 102-G02 (specific for serotype 2 O-Ag) (b). Results shown are the mean of at least two experiments with SD bars. The columns marqued (*) were significantly different from the controls incubated without Fab fragments ($P = 0{\cdot}0001$)..........99

Figure 2.
Electrophoretic and Far-Western analyses of porcine respiratory tract cells preparation. Preparations were Coomassie brilliant blue-stained (a) or transferred to a nitrocellulose membrane, incubated with *A. pleuropneumoniae* serotype 2 reference strain cells and probed with homologous rabbit antiserum (b). Controls were made by incubating the preparations on the nitrocellulose membranes with the antiserum against *A. pleuropneumoniae* serotype 2 (data not shown). Tracheal cells collected from 3-day old Duroc piglets (lanes 1); Hamphsire Duroc piglets (lanes 2); Landrace piglets (lanes 3); or Landrace Duroc piglets (lanes 4). Tracheal cells collected from 7-8-week old piglets (lanes 5), and from adult pigs at slaughter (lanes 6). Alveolar macrophages from 4-week old piglets (lanes 7). The position of molecular mass markers (in kilodaltons) are indicated on the left. Arrows indicate the apparent molecular mass of the putative receptors..........100

Figure 3.
Far-Western analyses of porcine tracheal cells preparation. The nitrocellulose membranes were incubated with cells of *A. pleuropneumoniae* serotype 1 reference strain (lane 1) and isolate FMV87-682 (lane 2); or cells of *A. pleuropneumoniae* serotype 2 reference strain (lane 4) and isolate 84-4397 (lane 5). They were probed with homologous rabbit antiserum. Nitrocellulose membranes were incubated with extracted LPS from serotype 1 reference strain and probed with mAb 5·1 G8F10 (lane 7). Controls were made by incubating the preparations on the nitrocellulose membranes with the same antibody (lanes 3, 6, and 8). The position of molecular mass markers (in kilodaltons) are indicated

on the left. Arrows indicate the apparent molecular mass of the putative receptors....101

ACTE DE COLLOQUE 1

Figure 1.
(A) Transmission electron micrograph of thin sections of *A. pleuropneumoniae* 4074 cells immunostabilized with monoclonal antibody (1.5 C5F4) against serotype 1 capsular antigen and stained with ruthenium red. (B) Transmission electron micrograph of whole cells of *A. pleuropneumoniae* 4074 cells probed with monoclonal antibody (5.1 G8F10) against serotype 1 O-antigen and goat anti-mouse IgG-gold particles (10 nm). Bar, 200 nm.... xxv

ACTE DE COLLOQUE 2

Figure 1.
Fractionation of extracted LPS from *A. pleuropneumoniae* serotype 1 on a Sephacryl S-300SF column in the presence of 0,25% deoxycholate. Fractions (in ml) were analysed for KDO (squares) and for neutral sugars (diamonds). xxviii

Figure 2.
Transmission electron micrographs of negatively stained *A. pleuropneumoniae* serotype 1 LPS. LPS preparations obtained after chromatography on a Sephacryl S-300SF column: (A) fractions 104 to 128; (B) fractions 224 to 236; (C) fractions 248 to 332. Bars, 200 nm....xxviii

ARTICLE 5

Figure 1.
Electron transmission micrographs of whole cells of *A. pleuropneumoniae* serotype 1. (A) Control cells that were not stained, nor incubated with pig Hb. (B) Unstained cells that were incubated during 30 min with pig Hb (1 mg/ml). Bar, 200 nm....xxxiii

Figure 2.
EDS spectrum of whole cells of *A. pleuropneumoniae* serotype 1 shown in Figure 1. Elemental analysis of control cells (A) or cells incubated with pig Hb (B). A 8-fold increase in iron (Fe) was observed in cells previously incubated with pig Hb xxxiv

Figure 3.
Flow cytometry analysis of whole cells of *A. pleuropneumoniae* serotype 2 not incubated with pig Hb (A) or incubated with pig Hb (B) were labeled with the anti-mouse IgG fluorescein isothiocyanate (FITC)-conjugated antibody (left peak, control) or with monoclonal antibodies against serotype 2 O-antigen plus

the anti-mouse IgG FITC-conjugated antibody (right peak). Whole cells of *A. pleuropneumoniae* serotype 1 not incubated with pig Hb (C) or incubated with pig Hb (D) were labeled with the anti-mouse IgG FITC-conjugated antibody (left peak, control) or with monoclonal antibodies against serotype 1 capsular-antigen plus the anti-mouse IgG FITC-conjugated antibody (right peak). The horizontal bars labelled '1' indicate non specific fluorescence while bars labelled '2' indicate specific fluorescence xxxv

Figure 4.
Transmission electron micrographs of negatively stained *A. pleuropneumoniae* serotype 2 extracted LPS. In the absence of Hb, the LPS appeared as ribbon-like structures with frequent branching (A). When LPS was incubated with Hb (1 mg/ml) for 1h, the typical structure was broken down into shorter molecular aggregates or completely disaggregated in small particules (B). After 18h of incubation with pig Hb, micelles were observed (C). A hexagonal lattice was observed when LPS from *A.pleuropneumoniae* was incubated with polymyxin B (1 mg/ml) (D). Bar, 200 nm xxxvi

Figure 5.
Western Blot analysis of fractions obtained following sucrose density centrifugation. *A. pleuropneumoniae* serotype 2 extracted LPS (A), pig Hb (C), or *A. pleuropneumoniae* serotype 2 extracted LPS incubated with pig Hb (B, D) were layered over a 5-20% continuous sucrose gradient. After centrifugation, 8 fractions (of 1.5 ml) were recovered and analyzed by SDS-PAGE and Western blot; fractions 1 and 8 correspond to the bottom and the top of the tubes, respectively. Nitrocellulose membranes were either incubated with a serum from a pig experimentally infected with *A. pleuropneumoniae* (A, B) or with a rabbit antiserum against pig Hb (C, D) xxxvii

Figure 6.
Dansylcadaverine displacement assay: representative emission spectra. The maximum fluorescence of DC was achieved by using 20 μg/ml of *A. pleuropneumoniae* lipid A. The addition of different concentration of pig Hb (A) or polymyxin B (B) to the mixture of lipid A and DC resulted in an attenuation of fluorescence intensity which is indicative of the displacement of bound DC to lipid A by these compounds. DC alone (50μM) was used as control xxxviii

Figure 7.
Inhibition of extracted LPS or bacterial cells activation of cLAL by pig Hb or polymyxin B. A decrease was obtained in cLAL activation from the addition of 10-fold diluted pig Hb (starting concentration 1mg/ml) (A) or polymyxin B (starting concentration 1 mg/ml) (B) to a constant concentration of extracted LPS from serotype 1 or serotype 2 (1 μg/ml) or bacterial cells of serotype 1 or serotype 2 (1 mg/ml). Two independent experiments were performed, *P<0,05.
.... xxxix

Figure 8.
Effect of pig Hb (pHb), human Hb (hHb), or polymyxin B (PX) on LPS biological activity of *S. minnesota* smooth or Re595 or *A. pleuropneumoniae* serotype 1 in the cLAL test. The addition of pHb (1 mg/ml) or hHb (1 mg/ml) or PX (1 mg/ml)

to extracted LPS (1 μg/ml) resulted in a significant decrease of cLAL activation, *P<0,05. xl

Figure 9. Effect of pig Hb on LPS biological activity of *A. pleuropneumoniae* serotype 2 in inducing NO_2^- production in J774 cells. The addition of a constant concentration of pig Hb (1mg/ml) to 10-fold diluted extracted LPS (starting concentration 1 μg/ml) resulted in a significant decrease of NO_2^- production, *P<0,05. xl

LISTE DES ABRÉVIATIONS

ADN: acide désoxyribonucléique
Ag-K: antigène capsulaire (K)
Ag-O: antigène somatique (O)
Apx: *Actinobacillus pleuropneumoniae* RTX
BHI: infusion cœur-cervelle
CPS: polysaccharides capsulaires
DNase: désoxyribonucléase
EDTA: tétraacétate d'éthylènediamine
ELISA: enzyme-linked immunosorbent assay
Hb: hémoglobine
IgA, IgG ou IgM: immunoglobuline A, G ou M
kb: kilobase
kDa: kiloDalton
KDO: acide 3-désoxy-D-manno-2-octulosonique
LOS: lipooligosaccharides
LPS: lipopolysaccharides
mAb: anticorps monoclonal
MET: microscopie électronique à transmission
Mr: masse moléculaire apparente
NAD: nicotiamide adénine dinucléotide
noyau OS: noyau oligosaccharidique ("core")
OMP: protéines de la membrane externe
PBS: tampon phosphate salin
PBS-BSA-T20: tampon phosphate salin contenant de l'albumine sérique de bœuf et du Tween-20
RNase: ribonucléase
RTX: pour "repeats in the structural toxin"
SDS-PAGE: électrophorèse sur gel de polyacrylamide contenant du dodécyle sulfate de sodium
TBS: tampon tris salin
TEMED: tétraméthyle d'éthylènediamine
TSDS-PAGE: tricine SDS-PAGE

REMERCIEMENTS

Je tiens à remercier sincèrement:

•Le Dr Mario Jacques, mon directeur de thèse, pour sa confiance et son appui inconditionnel. Je lui suis reconnaissante de m'avoir initiée à cette grande famille qu'est le monde scientifique ainsi que pour sa grande disponibilité et son aide financière.

•Le Dr J. Daniel Dubreuil, mon co-directeur, pour son support scientifique, ses conseils et ses encouragements.

•Le Dr Marcelo Gottschalk pour sa collaboration scientifique et ses encouragements.

•Le Dr Réal Lallier, directeur du groupe de recherche, pour son engagement, son appui et ses encouragements ainsi que son aide financière.

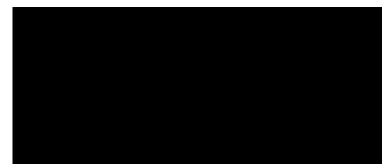
•Les membres du jury pour l'investissement de leur temps, aussi précieux soit il, à l'évaluation de ma thèse.

•Les nombreux membres du laboratoire du Dr Mario Jacques, dont j'ai eu la chance de côtoyer durant différentes périodes de temps et plus spécialement à Stéphane Rioux, pour son amitié et les nombreux souvenirs de complicité que je garderai.

•Madame Thérèse Gendron-Bernard, secrétaire du GREMIP, pour sa disponibilité et ses sourires.

•Les Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR) ainsi que l'Université de Montréal pour leur appui financier.

Mes remerciements vont de plus à tous les membres de ma famille, Stéphane, Hélène et Fidèle, Suzanne, Patrick et Myriam, Marc-François et Karine, Patrick et Isabelle pour tous les encouragements et les preuves d'amour.



Merci à vous tous

"Dans l'esprit de ceux qui suivent un <<exploit>> , il est suffisant de franchir le poteau d'arrivée pour avoir réussi. En général, pour le grand public, seul le résultat compte et, si celui-ci est atteint, on crie: bravo! Mais l'homme qui a réalisé la performance ne se contente pas d'un jugement aussi simple. Pour lui, si le résultat conserve son importance, la façon dont il a été obtenu en a bien davantage."

Willy de Roos, 1996

Je dédie cette thèse à Stéphane, mon mari, qui m'a épaulé tout au long de mes études ainsi qu'à Hélène et Fidèle, mes parents, qui m'ont incité à m'épanouir et à réaliser mes rêves au fil des ans.

I. INTRODUCTION

Actinobacillus pleuropneumoniae est l'agent étiologique de la pleuropneumonie fibrinohémorragique et nécrosante porcine (Nicolet, 1992). Cette maladie respiratoire est répandue mondialement et cause d'énormes pertes économiques à l'industrie porcine. La pathogenèse de la pleuropneumonie porcine due à *A. pleuropneumoniae* ainsi que le rôle des différents facteurs de virulence impliqués dans la colonisation de ce microorganisme ne sont pas encore parfaitement élucidés. Plusieurs toxines RTX ainsi que des molécules de surface telles que les polysaccharides capsulaires, les lipopolysaccharides (LPS) et certaines protéines de la membrane externe ont été décrites et impliquées dans le développement de la maladie (Inzana, 1991; Frey *et al.*, 1993a; Frey *et al.*, 1993b; Tascón *et al.*, 1996). Cependant, ce sont les LPS qui constitueraient l'adhésine principale d'*A. pleuropneumoniae* comme démontré à l'aide d'anneaux de trachée porcine maintenus en culture (Bélanger *et al.*, 1990). À partir de cette observation, nous avons élaboré un projet d'étude basé sur les prémisses voulant que l'adhérence des microorganismes aux tissus de l'hôte soit l'étape initiale dans le développement d'une infection et que l'inhibition de l'adhérence d'un microorganisme constitue une stratégie alternative de prévention et de traitement pour les maladies infectieuses (Ofek et Beachey, 1980; Beachey, 1981; Ofek *et al.*, 1996).

Les souches d'*A. pleuropneumoniae* sont principalement classées en douze sérotypes capsulaires, plusieurs de ces sérotypes possèdent, en plus, un type de LPS qui leur est propre. Certains épitopes communs sont toutefois retrouvés entre les LPS de différents sérotypes (Perry *et al.*, 1990). Les LPS sont des molécules amphiphiles caractéristiques des bactéries Gram-négatif. Ils sont composés essentiellement de trois parties: le lipide A; le noyau qui est un oligosaccharide se liant au lipide A par une molécule de KDO; et l'antigène-O (Ag-O) qui est quant à lui formé d'unités saccharidiques répétitives. Selon la présence et le nombre d'unités répétitives de l'antigène, les LPS sont soit rugueux, semi-rugueux (e.g. le sérotype 1 d'*A. pleuropneumoniae*) ou lisse (e.g. le sérotype 2 d'*A. pleuropneumoniae*) (Hitchcock *et al.*, 1986; Byrd et Kadis, 1989; Bélanger *et al.*, 1990). Nous avons décidé d'utiliser, en parallèle, les sérotypes 1 et 2, car ils appartiennent à des types de LPS différents et ils ont tous les deux une importance géographique; au Québec, les sérotypes 1 et 5 prédominent, tandis que le sérotype 2 prédomine dans plusieurs pays européens (Mittal *et al.*, 1992).

Le mécanisme d'action des LPS, ou endotoxine, dans le processus des infections à bactéries Gram-négatif pouvant mener au développement du syndrome du choc septique a été, et est encore, très étudié. Ainsi l'interaction des LPS avec différentes cellules telles que les monocytes/macrophages, neutrophiles, cellules endothéliales et musculaires lisses, est largement documentée et plusieurs récepteurs ont été identifiés (Hewett et Roth, 1993; Raetz, 1993; Rietschel *et al.*, 1994; Schletter *et al.*, 1995b). Par contre, il en est tout autrement pour l'identification des récepteurs de LPS retrouvés à la surface des muqueuses et des cellules épithéliales dont se servent les bactéries pour s'établir et éventuellement coloniser des tissus. Une des premières études, sinon la première démontrant que les LPS peuvent être employés par des bactéries comme adhésine remonte à 1982 (Izhar *et al.*, 1982). Ce n'est que dans les années 90 que la majorité des travaux démontrant cette aptitude chez d'autres bactéries surgiront, aussi n'est-il pas si surprenant qu'il y ait encore très peu d'informations sur ce sujet (Jacques, 1996).

C'est en voulant approfondir notre compréhension de l'interaction adhésine-récepteur chez *A. pleuropneumoniae* que les différents objectifs de ce projet d'étude ont été élaborés.

Les objectifs principaux étaient: (i) d'établir un système miniaturisé pour étudier l'adhérence des LPS d'*A. pleuropneumoniae* aux cellules des voies respiratoires du porc; (ii) de déterminer quelle partie de la molécule de LPS est impliquée dans ce système de reconnaissance; (iii) de déterminer si des anticorps dirigés contre le LPS peuvent bloquer l'adhérence; (iv) d'identifier des récepteurs cellulaires reconnus par le LPS d'*A. pleuropneumoniae*.

S'articulant autour de ces derniers, des objectifs supplémentaires se sont ajoutés. Ainsi, d'une part, comme *A. pleuropneumoniae* produits de nombreux sérotypes capsulaires et somatiques, nous avons été intéressés de vérifier si la région du noyau oligosaccharidique-lipide A de ses LPS était elle aussi soumise à une certaine diversité. D'autre part, sachant que la quantité de fer disponible pour la croissance d'*A. pleuropneumoniae* a une influence sur l'expression de certaines protéines de la membrane externe dont le récepteur de la transferrine porcine (Deneer et Potter, 1989a; Niven *et al.*, 1989; Gonzalez *et al.*, 1990; Ricard *et al.*, 1991; D'Silva *et al.*, 1995), nous avons voulu évaluer l'effet de concentrations

réduites en fer sur la production des polysaccharides de surface d'*A. pleuropneumoniae* i.e. sur les polysaccharides capsulaires ainsi que sur les LPS.

II. REVUE DE LA LITTÉRATURE

1. *Actinobacillus pleuropneumoniae*

1.1 Historique et taxonomie

L'agent étiologique de la pleuropneumonie porcine fut isolé par différents chercheurs à travers le monde de façon quasi simultanée. Chacun d'eux suggérant une classification de cette bactérie nouvellement isolée selon cinq caractéristiques, disponibles avant la publication de la 8e édition du Bergey's en 1974, qui définissaient le genre *Haemophilus* soit: la morphologie, la coloration de Gram, la dépendance au facteur X (hémine ou certaines porphyrines) et/ou au facteur V (NAD: ß-nicotinamide adénine dinucléotide), l'hémolyse et la croissance en présence de 5-10% CO_2 (Zinneman, 1981).

Durant les années 1960, Matthews et Pattison (1961) de la Grande-Bretagne classèrent la bactérie *Haemophilus parainfluenzae*, suivant sa ressemblance aux organismes du genre *Haemophilus* associés à la pneumonie et/ou à la pleurésie et la définirent comme dépendante de la NAD, soit du facteur de croissance V, mais non-dépendante du facteur X, donc différente de *Haemophilus influenzae suis* qui nécessite les deux, ainsi que sérologiquement différente de *H. parainfluenzae* d'origine humaine. De même, Olander aux États-Unis (1963) ainsi que Nicolet en Suisse (1966; 1968) attribuèrent le nom d'*Haemophilus parahaemolyticus* à cette bactérie, dû à la ressemblance biochimique qu'ils démontrèrent avec la bactérie d'origine humaine du même nom. Ils la caractérisèrent comme étant NAD dépendante et hémolytique. Parallèlement, Shope (1964) en Argentine nomme cette bactérie NAD dépendante *Haemophilus pleuropneumoniae*.

Des rapports indiquèrent par la suite sa présence dans plusieurs pays à travers le monde, mais au Canada, le premier véritable rapport de cas nous parvint de la Saskatchewan qu'en 1974 (Schiefer *et al.*, 1974). Au Québec, c'est en 1978-1979 que les pertes causées par la pleuropneumonie porcine commencèrent à être plus considérables (Higgins *et al.*, 1982).

Grâce aux travaux de Kilian (1978) sur le genre *Haemophilus*, l'agent causal de la pleuropneumonie porcine fut regroupé sous le vocable proposé par Shope soit

H. pleuropneumoniae. Ses travaux démontrèrent la grande similitude entre ces souches isolées de porcs atteints de la pleuropneumonie porcine ainsi que l'existence de différences phénotypiques importantes entre les souches d'origine porcine et celles d'origine humaine; entre autres: le %G+C, la fermentation des sucres et la pathogénicité. Enfin, ces travaux de recherche indiquèrent que l'hémolyse n'est pas une caractéristique constante puisque d'une part; le pouvoir hémolytique d'une souche varie selon l'origine animale du sang utilisé (Kilian, 1976) et d'autre part; une souche hémolytique peut, après seulement quelques passages *in vitro*, perdre son pouvoir hémolytique.

Finalement, Pohl *et al.* (1983) transférèrent l'agent causal de la pleuropneumonie porcine au genre *Actinobacillus*, appartenant aussi à la famille des *Pasteurellaceae* tout comme les genres *Haemophilus*, *Pasteurella* et les organismes apparentés à *Pasteurella haemolytica* causant aussi des pleuropneumonies porcines, décrits par Bertshinger et Seifert (1978) d'après les résultats d'hybridation génomique (ADN-ADN) qu'ils obtinrent (fig. 1).

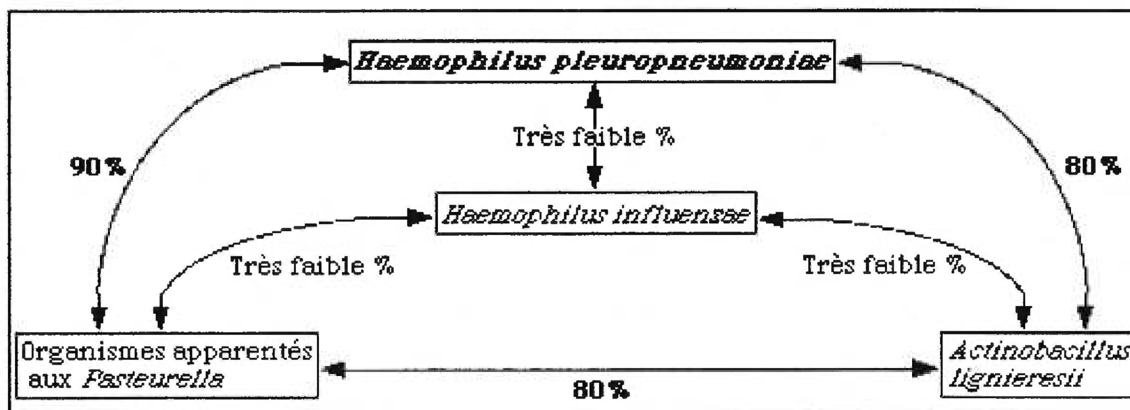


Figure 1. Les pourcentages d'hybridation génomique entre les différents groupes de bactéries.

Les organismes appartenant à l'espèce *Actinobacillus pleuropneumoniae* furent divisés en deux biovars selon leur besoin en NAD. On classsa les bactéries NAD-dépendantes dans le biovar 1, représenté par la souche de Shope 4074, et les bactéries NAD-indépendantes dans le biovar 2, représenté par la souche de Bertschinger 2008/76, (Pohl *et al.*, 1983). Le biovar ou biotype 2 comporterait quant à lui deux sérotypes (Fodor *et al.*, 1989). Cependant, Niven et Lévesque (1988) démontrèrent par la suite que le biotype 2 d'*A. pleuropneumoniae* est

incapable de synthétiser du NAD *de novo*. Ils suggérèrent d'élargir la définition du facteur V pour y inclure la NAm (nicotinamide), un précurseur nucléotidique, en plus du NAD, de la NMN (nicotinamide adénine mononucléotide) et de la NR (nicotinamide riboside). De cette façon, il serait alors possible de décrire toutes les souches d'*A. pleuropneumoniae* (biotypes 1 et 2) comme dépendantes du facteur de croissance V et d'éliminer les biovars.

1.2 Caractéristiques

A. pleuropneumoniae est un coccobacille pléomorphe Gram-négatif, anaérobie facultatif, non sporulant, encapsulé, non motile, ß-hémolytique sur gélose au sang et uréase positif (Fedorka-Cray *et al.*, 1993). *A. pleuropneumoniae* produit le phénomène de CAMP (décrit par Christie, Atkins, et Munch-Petersen) i.e. une augmentation de la zone hémolytique à l'intérieur de la lyse partielle due à une strie de *Staphylococcus aureus* ß-toxigénique (Kilian, 1976).

A. pleuropneumoniae est un parasite du tractus respiratoire ayant une affinité quasi exclusive pour l'espèce porcine. Sa principale voie de transmission est aérienne et s'effectue principalement par des contacts directs entre porcs ou, sur de courtes distances, par l'entremise de gouttelettes. La survie d' *A. pleuropneumoniae* dans l'environnement est considérée de courte durée, par contre si ce microorganisme est protégé par des sécrétions mucosales ou autres sécrétions organiques, sa survie peut se prolonger jusqu'à quelques jours (Nicolet, 1992).

Récemment, *A. pleuropneumoniae* a été isolé d'une infection mixte de l'oreille moyenne chez des porcs. Les lésions retrouvées sembleraient avoir été causées par les deux microorganismes présents soit *Pasteurella multocida* et *A. pleuropneumoniae*. Cette étude est la première à suggérer un nouveau rôle pathologique pour *A. pleuropneumoniae* (Duff *et al.*, 1996).

1.3 Facteurs de virulence

A. pleuropneumoniae possède de nombreux facteurs de virulence tel qu'une capsule, des protéines de la membrane externe, des toxines et autres produits de sécrétion. Il possède de plus des adhésines et/ou lipopolysaccharides (LPS) dont

il sera question à la section 3 (Inzana, 1991; Tascón *et al.*, 1996; Haesebrouck *et al.*, 1997).

1.3.1 Capsule

La capsule d'*A. pleuropneumoniae* est de nature polysaccharidique variable. Les antigènes capsulaires (Ag-K), identifiés selon la composition chimique de l'unité oligosaccharidique constitutrice, ont été classifiés principalement en douze sérotypes (Mittal *et al.*, 1992). Le sérotype 5 a, de plus, été séparé en deux sous-groupes (les sérotypes 5a et 5b) aussi selon une variante au niveau de l'unité saccharidique constitutrice (Nielsen, 1986).

Souvent représentées comme recouvrant de façon homogène la paroi cellulaire des bactéries, les capsules d'*A. pleuropneumoniae* peuvent se présenter sous différents aspects variant d'une couche uniforme à une couche inégale exposant même par endroits la membrane externe (Steffens *et al.*, 1990). De plus, elles possèdent un arrangement beaucoup plus aléatoire que pour d'autres bactéries Gram-négatif (Graham *et al.*, 1991).

À l'aide de polysaccharides capsulaires (CPS) purifiés, il a été démontré que les CPS sont les déterminants antigéniques spécifiques des sérotypes d'*A. pleuropneumoniae* (Inzana et Mathison, 1987). De plus, la diversité structurale entre les CPS des différents sérotypes est telle que la production d'anticorps polyclonaux ou monoclonaux homologues et spécifiques contre ces CPS devraient fournir de bons outils de sérotypage (Perry *et al.*, 1990).

On retrouverait trois types de structures au niveau des CPS d'*A. pleuropneumoniae*: a) des séquences normales d'unités saccharidiques liées glycosidiquement, chez les sérotypes 5a (Altman *et al.*, 1987c), 5b (Altman *et al.*, 1992) et 10 (Beynon *et al.*, 1991e); b) des polymères de type acide téïchoique, dans lesquels des unités glycosyl-glycitol sont jointes par des liens phosphate diester, chez les sérotypes 2 (Altman *et al.*, 1987a), 3 (Altman *et al.*, 1987b), 6 (Altman *et al.*, 1988b), 7 (Beynon *et al.*, 1991b), 8 (Altman *et al.*, 1990a), 9 (Beynon *et al.*, 1992a) et 11 (Beynon *et al.*, 1993); et c) des polymères d'unités oligosaccharidiques répétitives maintenues par des liens phosphate, chez les sérotypes 1 (Altman *et al.*, 1986a), 4 (Altman *et al.*, 1988a) et 12 (Beynon *et al.*,

1991d). Ces derniers polymères seraient relativement instables car les liens phosphate s'hydrolysent facilement, ce qui entraîne la formation de produits de dégradation de faible masse moléculaire (Perry *et al.*, 1990).

Les sérotypes 5a, 5b et 10 comprennent dans leur structure du KDO (Cf. section 2.2.2). Autre particularité dans la structure du sérotype 10, le mannose est le seul hexose présent comparativement à tous les autres sérotypes où le glucose et le galactose sont les deux seuls hexoses retrouvés.

La capsule protège *A. pleuropneumoniae* des systèmes de défense de l'hôte, elle protègerait effectivement *A. pleuropneumoniae* contre la lyse par le complément, l'opsonisation et la phagocytose (Fenwick, 1995). Les CPS sont de par leur nature de faibles immunogènes, par contre une fois que la reconnaissance est établie par le système immunitaire de l'hôte, ils deviennent une cible facile pour les anticorps qui amèneraient alors une protection chez l'hôte (Fenwick, 1995). L'opsonisation serait en effet nécessaire pour la phagocytose d'*A. pleuropneumoniae* par les macrophages alvéolaires et les neutrophiles (Byrd et Kadis, 1992; Beaudet *et al.*, 1994).

La production de capsule des *Haemophilus* est influencée par les conditions de culture (Fenwick, 1995). Chez les *Pasteurella*, placés dans des conditions de culture réduites en fer, la production des CPS serait diminuée, ce qui augmenterait l'affinité des bactéries pour le mucus respiratoire porcin (Jacques *et al.*, 1994). Il avait déjà été démontré que la capsule semblait interférer dans l'adhérence de ce microorganisme au mucus respiratoire porcin (Jacques *et al.*, 1993). Donc la capsule masquerait des adhésines et ainsi diminuerait la capacité d'un microorganisme à coloniser un tissu. Des mutants isogéniques déficients en CPS d'*H. influenzae* démontreraient effectivement une adhérence et une invasion accrue comparativement aux souches parentales encapsulées (Fenwick, 1995). Chez *A. pleuropneumoniae*, la capsule semblerait aussi bloquer l'adhérence du microorganisme au mucus respiratoire porcin (Bélanger *et al.*, 1994). D'un autre côté, les souches acapsulées ou peu capsulées i.e. ayant une capsule de moindre épaisseur sont beaucoup moins virulentes que les souches sauvages, elles seraient éliminées des poumons avant même de pouvoir causer des effets pathologiques (Jensen et Bertram, 1986; Rosendal et MacInnes, 1990; Inzana *et*

al., 1993). La capsule d'*A. pleuropneumoniae* pourrait jouer un rôle dans la production des lésions et dans la virulence bactérienne (Bertram, 1990).

Des différences dans la structure et l'expression du matériel capsulaire entre les sérotypes d'*A. pleuropneumoniae*, pourraient expliquer les différences de virulence entre les sérotypes (Jacques *et al.*, 1988). Ward *et al.* (1996) ont confirmé cette hypothèse par la production de mutants défectueux qu'au niveau de la biosynthèse des CPS, ces mutants acapsulés seraient avirulents.

En observant par microscopie électronique à transmission (MET), les capsules immunostabilisées, à l'aide d'antisérums homologues ou hétérologues, des souches de référence des différents sérotypes d'*A. pleuropneumoniae*, il a été démontré que les capsules seraient plus épaisses chez les cellules cultivées en bouillon par rapport à celles cultivées sur gélose et chez les cellules récoltées au début de la phase de croissance (6h) par rapport à celles récoltées à la fin (18h) (Jacques *et al.*, 1988).

La résistance au système du complément de la souche 4074, d'*A. pleuropneumoniae*, pourrait être liée à l'inhibition de l'action bactéricide des anticorps anti-capsulaires par des anticorps non spécifiques, présents avant immunisation, qui reconnaîtraient des épitopes sur des polypeptides exposés à la surface bactérienne (Udeze et Kadis, 1992b).

1.3.2 Protéines de la membrane externe

La membrane externe des bactéries Gram-négatif est constituée de LPS, de polysaccharides et de protéines de la membrane externe (OMPs pour "outer membrane proteins"). La plupart des différents sérotypes capsulaires d'*A. pleuropneumoniae* présenteraient aussi des différences d'OMPs (Rapp *et al.*, 1986).

Sept patrons de profils protéiques sur SDS-PAGE seraient observables parmi les neuf premiers sérotypes. Les sérotypes 1 et 9 appartiendraient à un patron, les sérotypes 2 et 6 appartiendraient à un deuxième patron et les sérotypes, 3, 4, 5, 7 et 8 formeraient les cinq autres patrons (Rapp *et al.*, 1986).

L'expression de certaines OMPs serait induite sous des conditions de croissance particulières e.g. sous des conditions réduites en fer ou sous des conditions de culture supplémentées en maltose (Deneer et Potter, 1989a; Deneer et Potter, 1989b).

Plusieurs de ces protéines sont reconnues par des sérums de porcs convalescents (MacInnes et Rosendal, 1987), mais l'immunisation de porcs avec des extraits de OMPs brutes (van den Bosch *et al.*, 1992) ou par des OMPs purifiées (Beaudet *et al.*, 1994) n'offre qu'une protection limitée contre une infection expérimentale avec *A. pleuropneumoniae*.

1.3.2.1 Protéines de la membrane externe communes à tous les sérotypes

Une OMP de nature lipoprotéique d'environ 40 kDa, OmlA (pour "outer membrane lipoprotein A"), et probablement commune à tous les sérotypes, a été clonée, caractérisée et utilisée pour immuniser des porcs. Une protection homologue a été observée, mais sans empêcher la formation de lésions pulmonaires (Gerlach *et al.*, 1993). Une OMP de 14 kDa, PalA, qui réagit fortement avec le sérum de porcs infectés expérimentalement ou naturellement a aussi été identifiée chez tous les sérotypes. La séquence d'ADN du gène *palA*, de cette OMP de 14 kDa, révèle une séquence d'acides aminés qui serait fortement similaire à la lipoprotéine PAL (pour "peptidoglycan-associated lipoprotein") d'*Escherichia coli* (Frey *et al.*, 1996).

Une OMP de 48 kDa, nommée ApoA (pour "*Actinobacillus* outer membrane protein A"), a été identifiée chez les douze sérotypes d'*A. pleuropneumoniae* et ne serait pas présente chez d'autres pathogènes porcins Gram-négatif. Le gène de cette protéine, *apoA*, serait présent chez les douze sérotypes d'*A. pleuropneumoniae* ainsi que chez *Actinobacillus suis* et *Pasteurella multocida*. On ne sait pas encore si des anticorps dirigés contre cette protéine participent à une protection croisée contre les infections à *A. pleuropneumoniae* (Cruz *et al.*, 1996). Cette même équipe avait préalablement mis en évidence une OMP commune de 43 kDa (Cruz *et al.*, 1993).

1.3.2.2 Protéines liant la transferrine porcine

Le fer est un facteur de croissance essentiel utilisé pour diverses fonctions cellulaires fondamentales telles que le transport d'électrons de la chaîne respiratoire, le métabolisme énergétique et la biosynthèse de l'ADN (Kirby *et al.*, 1995). Chez les vertébrés, la majorité du fer est intracellulaire, sous-forme de ferritine ou de composés de type hème (Otto *et al.*, 1992; Payne, 1993), et est ainsi inaccessible pour les pathogènes extracellulaires. Même si de petites quantités de fer intracellulaire sont relâchées sous forme d'hémoglobine, d'hème ou autres complexes lors de la lyse de cellules, elles sont éliminées par des mécanismes bien établis. La quantité limitée de fer extracellulaire est liée et transportée par des glycoprotéines comme la transferrine ou la lactoferrine (Williams et Griffiths, 1992) rendant la concentration du fer disponible à 10^{-18} M, une concentration bien en dessous de celle requise pour supporter la croissance bactérienne (Griffiths, 1987). L'habileté d'un microorganisme à compétitionner avec l'hôte pour le fer est donc un important déterminant de sa virulence.

La lactoferrine est principalement retrouvée sur les muqueuses, dans les sécrétions mucosales et aux sites d'infections d'où elle est relâchée par les neutrophiles. La transferrine est principalement retrouvée dans le sérum ainsi que dans les fluides de lavages bronchoalvéolaires, offrant donc une source potentielle de fer dans les poumons pour les pathogènes respiratoires dont diverses espèces de *Pasteurellaceae*. Même si les transferrines diminuent la concentration de fer libre dans le sérum, la forte concentration en transferrine et la forte saturation de ces dernières, procure une réserve adéquate pour permettre la croissance bactérienne. Les pathogènes ont donc développé des mécanismes de hautes affinités pour le fer leurs permettant de survivre (Kirby *et al.*, 1995). Ces adaptations amèneraient des changements phénotypiques au niveau du métabolisme et dans la composition de la membrane externe de la bactérie (Griffiths et Bullen, 1987).

Sous des conditions réduites en fer, *A. pleuropneumoniae* exprimerait des protéines de la membrane externe régulées par le fer (Deneer et Potter, 1989a; Morton et Williams, 1989; Niven *et al.*, 1989).

Le mécanisme d'acquisition du fer le mieux connu est celui médié par les sidérophores, des chélateurs de fer. En conditions réduites en fer, les sidérophores, molécules de faible masse moléculaire ayant une forte affinité pour le fer, sont libérées dans l'environnement par les bactéries. Après avoir solubilisé le fer, les complexes sidérophore-fer interagissent avec des récepteurs présents à la surface de la membrane externe des micoorganismes leurs permettant ainsi d'internaliser le fer (Kirby *et al.*, 1995). Deux classes de sidérophores sont connues, les hydroxamates et les catéchols, selon le groupement chimique impliqué dans la liaison avec le fer (Diarra *et al.*, 1996).

Aucun sidérophore n'avait été retrouvé jusqu'à très récemment chez *A. pleuropneumoniae* (Morton et Williams, 1989; Niven *et al.*, 1989). Cependant, il est maintenant clair que toutes les souches d'*A. pleuropneumoniae* seraient capables d'utiliser certains sidérophores exogènes (i.e. produits par d'autres microorganismes) dont le ferrichrome (un hydroxamate) et le bis-catéchol (Diarra *et al.*, 1996). De plus, les souches de référence des sérotypes 1 et 5 sécrèteraient des chélateurs de fer qui ne seraient toutefois pas reliés aux hydroxamates et catécholates bien connus, tout comme ceux que produiraient *P. multocida* (Diarra *et al.*, 1996).

Certains pathogènes ne synthétisent pas de sidérophores, mais utiliseraient plutôt la transferrine ou la lactoferrine. La plupart des ces espèces bactériennes produiraient deux récepteurs protéiques qui démontrent une spécificité d'espèce très marquée. *A. pleuropneumoniae* exprimerait les deux récepteurs protéiques Tbp1 et Tbp2 (pour "transferrin binding protein 1 or 2") qui lui permettraient de lier spécifiquement et uniquement la transferrine porcine (Morton et Williams, 1989; Gonzalez *et al.*, 1990; Ricard *et al.*, 1991). Cette spécificité pourrait expliquer pourquoi le porc est le seul hôte naturel d'*A. pleuropneumoniae* (Gonzalez *et al.*, 1990). Des Tbps de masse moléculaire légèrement différentes, 60 à 66 kDa, ont été identifiées parmi les différents sérotypes capsulaires d'*A. pleuropneumoniae* (Gerlach *et al.*, 1992c; Heegaard *et al.*, 1994). Les sérums de porcs convalescents d'une infection à *A. pleuropneumoniae* réagissent avec des OMPs régulées par le fer dont certaines auraient été identifiées comme étant des Tbps (Niven *et al.*, 1989; Gerlach *et al.*, 1992c), par contre l'immunisation des porcs avec la Tpb de 60 kDa n'apporterait qu'une protection limitée et homologue (Gerlach *et al.*, 1992a; Rossi-Campos *et al.*, 1992). Selon une autre étude, la présence d'un autre groupe

de protéines, de masse moléculaire plus élevées soit de 93-99 kDa, aussi régulées par le fer et liant la transferrine concorderait mieux avec la capacité d'une souche à utiliser la transferrine porcine comme seule source de fer (D'Silva *et al.*, 1995).

La Tbp1 serait absolument essentielle pour l'acquisition du fer. Le rôle de la Tbp2 est moins clair. Tbp1, qui est homologue aux OMPs TonB-dépendantes de *E. coli*, serait une protéine transmembranaire qui agirait comme un pore bien contrôlé de la membrane externe fonctionnant à l'aide d'un système d'énergie couplé dépendant de TonB. Le complexe Tbp1/2 lierait la transferrine, un changement conformationnel de la transferrine dû à la transduction d'énergie par TonB permettrait au fer d'être relâché dans l'espace périplasmique où une protéine périplasmique liant le fer Fbp (pour "ferric binding protein") transfererait le fer de Tbp à un complexe membranaire Fbp-perméase qui par translocation introduirait le fer dans le cytoplasme. Fbp fait partie de la même famille que la protéine SfuA de *Serratia marcescens*, ce sont des composantes de systèmes de transport de type ABC (pour "ATP Binding Cassette").

Les gènes des Tbp sont organisés en opéron constitué: a) d'un promoteur TbpR (pour "Tbp regulator"), homologue à la protéine OxyR d'*E. coli* qui est un régulateur positif sensible aux concentrations d'oxygène du milieu; b) d'un répresseur protéique liant le fer (Fur); c) du gène *tbpB*, codant pour Tbp2 et d) du gène *tbpA*, codant pour Tbp1, espacé d'une région intergénique formée de quelques paires de base. La présence du répresseur Fur a été démontré chez *A. pleuropneumoniae* (Gonzalez *et al.*, 1995).

Les transferrines et lactoferrines sont des glycoprotéines monomériques, d'environ 80 kDa, constituées de deux lobes, le lobe C-terminal et le lobe N-terminal pouvant chacun lier un ion ferrique et un cofacteur anionique, maintenus ensemble par un domaine intercalaire de longueur variable selon les différentes transferrines et lactoferrines.

Chez toutes les espèces testées, c'est le lobe C de la transferrine qui interagirait avec la Tbp1, tandis que pour la Tbp2 l'interaction de la transferrine se ferait avec un ou l'autre des lobes. Chez *A. pleuropneumoniae*, la transferrine porcine réagirait qu'avec les lobes C des Tbp1 et Tbp2 (Gonzalez *et al.*, 1995).

Une autre adaptation de plusieurs microorganismes, dont *A. pleuropneumoniae,* leur permettant d'acquérir du fer réside dans l'utilisation de l'hème, même lorsque combiné à l'hémoglobine (Deneer et Potter, 1989a; Niven *et al.*, 1989). L'hémoglobine et l'hème relâchés durant la lyse des érythrocytes sont avidement liés par l'haptoglobine et l'hémopexine de l'hôte. Quoique l'hémoglobine et l'hème soient des sources potentielles de fer pour plusieurs pathogènes, leur forme combinée n'est pas facilement disponible pour toutes les espèces (Schryvers et Lee, 1993).

Certains isolats d'*A. pleuropneumoniae* seraient capables d'utiliser l'hémoglobine de diverses espèces animales ainsi que d'autres composés de type hème comme seule source de fer pour leur croissance. Les LPS d'*A. pleuropneumoniae* et plus particulièrement leur partie lipidique seraient effectivement capables de lier les chaînes α et ß de l'hémoglobine et pourraient donc expliquer, du moins en partie, la façon dont la bactérie acquiert son fer (Bélanger *et al.*, 1994; Bélanger *et al.*, 1995).

1.3.3 Produits de sécrétion

1.3.3.1 Toxines Apx

Les leucocytes procurent une première ligne de défense cellulaire contre les microorganismes envahissants. Ces derniers ont ainsi développé plusieurs stratégies dont les capsules anti-phagocytaires et la sécrétion d'exotoxines qui sont capables d'inhiber l'action antimicrobienne des leucocytes ou de les tuer.

Contrairement à la leucotoxine de *P. haemolytica* spécifique pour les leucocytes bovins, l'activité des toxines d'*A. pleuropneumoniae* ne démontrerait pas de spécificité d'espèces animales.

A. pleuropneumoniae sécréterait des produits capables d'hémolyser des érythrocytes de différentes espèces et de lyser des cellules telles que les macrophages alvéolaires ainsi que les neutrophiles (Rosendal *et al.*, 1988; van Leengoed *et al.*, 1989). Les premiers à les avoir identifiés nommèrent ces produits toxiques par différents noms: a) des hémolysines: HlyI, HlyII (Frey et Nicolet, 1988a; Frey et Nicolet, 1988b; Frey et Nicolet, 1990); b) des cytolysines:

ClyI, ClyII, ClyIII (Kamp et van, 1989; Kamp *et al.*, 1991) et c) une pleurotoxine: PTX (Rycroft *et al.*, 1991). Plusieurs groupes de recherche à travers le monde les ont indépendamment étudiés et il fut finalement démontré que les douze sérotypes avaient la possibilité de sécréter trois protéines (Kamp *et al.*, 1991). Lorsqu'il s'est avéré clair que les différents groupes de recherche travaillaient avec les mêmes protéines, une nomenclature uniforme des exotoxines d'*A. pleuropneumoniae* fut proposée. Les trois toxines furent donc nommées ApxI, ApxII et ApxIII pour "Actinobacillus pleuropneumoniae RTX-toxins" (Frey *et al.*, 1993b).

Les toxines Apx font partie de la famille des toxines RTX (pour "repeats in the structural toxin"), lesquelles sont retrouvées chez différentes bactéries pathogènes. L'hémolysine-A (HlyA) d'*E. coli* fut la première toxine RTX à être identifiée (Felmlee *et al.*, 1985). Chez *P. haemolytica* sérotype A1, on retrouve une leucotoxine (LktA) appartenant aussi aux toxines RTX (Strathdee et Lo, 1987) ainsi que l'exotoxine de *A. suis* (Fedorka-Cray *et al.*, 1994).

Les toxines RTX partagent la même structure, elles possèdent un nombre varié de répétitions riches en glycine (Strathdee et Lo, 1987), au niveau de l'extrémité N-terminale de la molécule, qui seraient capables de chélater les ions calcium et seraient impliquées dans la liaison aux cellules cibles (Ludwig *et al.*, 1988; 1990b; Boehm *et al.*, 1990a). Plus une toxine RTX a des répétitions riches en glycine, plus elle lierait de calcium et plus elle possèderait une forte activité hémolytique (Czuprynski, 1995). Elles possèdent une région hydrophobe au tiers de la protéine qui en s'insérant dans la membrane cytoplasmique des cellules cibles (Ludwig *et al.*, 1991; Oropeza-Wekerle *et al.*, 1992) provoquerait un influx de Ca^{2+} ainsi qu'un efflux de K^{+} et de macromolécules (e.g. ATP). Finalement, l'extrémité C-terminale formerait le signal de sécrétion (Welch, 1991). La région hydrophobe ainsi que l'extrémité C-terminale seraient impliquées dans la sécrétion de la toxine par la bactérie (Stanley *et al.*, 1991; Kenny *et al.*, 1992).

ApxI est une exotoxine de 105 kDa, fortement hémolytique et fortement cytotoxique pour les cellules phagocytaires. Elle est produite et sécrétée par les sérotypes de référence 1, 5a, 5b, 9, 10 et 11. Ces sérotypes possèdent tous l'opéron *apx*I composé des quatre gènes *apx*ICABD (C étant le gène activateur, A le gène de structure, BD les gènes de sécrétion). La toxine ApxIA s'apparenterait

beaucoup plus à la l'hémolysine HlyA d'*E. coli* qu'aux autres Apx. Elle est formée d'un N-terminal amphipatique, de trois domaines fortement hydrophobes et de treize nonapeptides riches en glycine. L'opéron *apx*ICABD est présent chez *Actinobacillus lignieresii*, mais n'est pas exprimé (Frey, 1995).

ApxII est une exotoxine de 103-105 kDa, faiblement hémolytique et faiblement cytotoxique. Elle est produite par tous les sérotypes de référence sauf le sérotype 10. Tous les sérotypes qui produisent ApxII la sécrète, sauf chez le sérotype 3 où la toxine ApxII est principalement localisée au niveau du cytoplasme. Sa séquence d'acides aminés est semblable à celle de ApxI, par contre elle n'est formée que de huit répétitions nonapeptides riches en glycine. La toxine ApxIIA ressemblerait fortement à la leucotoxine LktA de *P. haemolytica*. L'opéron *apx*II est seulement composé des gènes *apx*ICA. La toxine ApxII des sérotypes 1, 5, 9 et 11 emprunte donc les gènes de sécrétion de ApxI. Chez les sérotypes 2, 4, 6, 7, 8 et 12 qui ne produisent pas ApxI, un opéron partiel *apx*IBD est présent, mais chez le sérotype 3 qui ne produit pas non plus ApxI ni ne secrète ApxII, aucun gène de l'opéron *apx*I n'est présent. Les gènes de l'hémolysine d'*A. suis ash*CA seraient identiques à ceux de ApxII (Frey, 1995). De plus, des anticorps capables de neutraliser ApxI seraient retrouvés dans le sérum de porcs immunisés contre *A. suis* (Fenwick *et al.*, 1996).

ApxIII est une exotoxine de 120 kDa, non-hémolytique, mais fortement cytotoxique pour les macrophages et les neutrophiles. Elle est produite et sécrétée par les sérotypes de référence 2, 3, 4, 6 et 8. Sa séquence d'acides aminés est typique des toxines RTX. Tout comme ApxI elle est formée de treize répétitions nonapeptides riches en glycine. Ces sérotypes possèdent tous un opéron complet *apx*IIICABD, tout comme l'opéron *apx*I. La toxine ApxIIIA s'apparenterait beaucoup à ApxI et donc à HlyA (Frey, 1995).

Tout récemment, le gène d'une quatrième toxine RTX a été mis en évidence chez *A. pleuropneumoniae*. Seul le gène de structure, codant pour une protéine de 154 à 186 kDa, serait présent, sous des formes légèrement variantes, mais ce, chez les souches de référence de tous les sérotypes. Elle serait formée de trois domaines hydrophobes au niveau du N-terminal et de trois à quatre nonapeptides riches en glycine. L'expression de cette protéine n'a pas pu être observée en laboratoire, cependant, chez les porcs infectés, des anticorps dirigés contre cette protéine

seraient présents. Il semblerait donc que cette protéine soit exprimée *in vivo*, toutefois sa fonction biologique reste inconnue (Frey *et al.*, 1997).

Les toxines RTX sont des agents modulateurs qui auraient la possibilité d'activer, d'inhiber, ou de tuer les leucocytes selon la concentration de toxine et les conditions présentes (Czuprynski, 1995).

Des études ont démontré que les leucotoxines d'*A. pleuropneumoniae* ont des effets similaires sur les neutrophiles porcins. Les bactéries ou de fortes concentrations de filtrats de culture seraient létales pour les neutrophiles ou inhiberaient leur activité phagocytaire (Devenish *et al.*, 1992). Des concentrations sublétales de toxines stimuleraient la réponse oxydative des neutrophiles et les sensibiliseraient à manifester une réponse oxydative plus forte au phorbol myristate acétate (Dom *et al.*, 1992; Udeze et Kadis, 1992a). Les effets sur les neutrophiles seraient calcium-dépendants et pourraient être neutralisés par du sérum de porcs convalescents d'une infection à *A. pleuropneumoniae* (Byrd et Kadis, 1992; van Leengoed et Dickerson, 1992). Beaucoup moins d'informations sont disponibles sur les effets des toxines d'*A. pleuropenumoniae* sur les phagocytes mononucléaires. L'élimination des bactéries est possible à de faibles ratios bactéries par macrophages, tandis qu'à de hauts ratios bactéries par macrophages des dommages cytotoxiques aux macrophages seraient observés. Les macrophages alvéolaires porcins sembleraient aussi très susceptibles aux activités inhibitrices et létales des leucotoxines d'*A. pleuropneumoniae* (Cruijsen *et al.*, 1992). Cruijsen *et al.* (1992) ont aussi démontré que les macrophages alvéolaires porcins peuvent phagocyter, mais non tuer *A. pleuropneumoniae in vitro*. Les macrophages subiraient des dommages par les actinobacilles intracellulaires encore probablement dus à la production des leucotoxines (Chung *et al.*, 1993).

L'absence des toxines RTX chez *A. pleuropneumoniae* rendrait la bactérie avirulente comme l'ont démontré des infections expérimentales produites chez des porcs à partir de souches mutantes d'*A. pleuropneumoniae* ne produisant plus de toxines (Anderson *et al.*, 1991; Inzana *et al.*, 1991; Gerlach *et al.*, 1992b). Des études génétiques suggèrent qu'au moins une toxine Apx serait requise pour qu'*A. pleuropneumoniae* soit virulent. De façon générale, la présence d'ApxI est associée à de hauts niveaux de virulence, tandis que la présence de seulement

ApxII semble conférer une virulence moyenne. Les souches sécrétant deux toxines différentes seraient significativement plus virulentes que celles n'en produisant qu'une, ce qui indiquerait un effet synergique des toxines. Fait intéressant à noter, *A. pleuropneumoniae* est la seule espèce bactérienne, jusqu'à maintenant, à produire deux toxines RTX différentes (Frey, 1995). De récents travaux ont démontré que les toxines, ApxI, ApxIII et à moindre importance ApxII, sont bel et bien les facteurs bactériens pouvant déclencher le développement de symptômes cliniques et des lésions pulmonaires typiques de la pleuropneumonie porcine (Kamp *et al.*, 1997).

Le phénomène de CAMP est une hémolyse d'érythrocytes ovins due à la synergie du facteur CAMP présent dans la zone de diffusion produite par la sphingomyélase d'une strie de *S. aureus* et d'une toxine provenant de la strie bactérienne perpendiculaire. Ce phénomène est produit par les trois toxines (Frey *et al.*, 1989) ainsi que par un gène de régulation d'*A. pleuropneumoniae* (*hlyX* ou *cfp*). Ce gène produirait une protéine de 29,5 kDa chez *E. coli* capable d'une activité hémolytique et co-hémolytique (Frey, 1995).

1.3.3.2 Facteur de perméabilité

A. pleuropneumoniae sérotypes 1 et 5 produiraient un facteur de perméabilité, non apparenté aux hémolysines connues. Ce facteur de perméabillité non-hémolytique, ni protéolytique et retrouvé dans le surnageant de culture induirait un zone œdémateuse si injectée intradermiquement à des lapins (Lallier *et al.*, 1987). La cytotoxicité de ce facteur de perméabilité pour les macrophages pulmonaires n'a pas été vérifiée.

1.3.3.3 Protéases

A. pleuropneumoniae produirait une protéase spécifiquement capable de cliver les IgA porcines (Kilian *et al.*, 1979; Mulks *et al.*, 1984). Différentes protéases ont été identifiées par gélatine-SDS-PAGE à partir de surnageants de culture, ces protéases seraient capables de dégrader la gélatine, l'hémoglobine porcine, bovine et humaine en plus des IgA porcines. Leur activité serait inhibée par l'EDTA (acide tétraacétique éthylènediamine). De plus, la protéase de masse moléculaire la plus élevée, 200 kDa, serait reconnue par des sérums immuns de

porcs (Negrete-Abascal *et al.*, 1994a; Negrete-Abascal *et al.*, 1994b). Récemment, un gène codant pour un polypeptide de 24 kDa reconnu par du sérum de porcs convalescents a été séquencé et cloné. Des tests biochimiques auraient démontré que le polypeptide recombinant est une métalloprotéase (Garcia C. *et al.*, 1996).

1.3.3.4 Autres produits de sécrétion

La plupart des souches d'*A. pleuropneumoniae* produisent une uréase, qui s'avère utile au niveau du diagnostic, mais dont le rôle dans le développement de la pleuropneumonie porcine n'a pas encore été élucidé. Aucun lien n'a effectivement été trouvé entre l'activité de cette enzyme et le développement aiguë de la pleuropneumonie porcine, il reste à savoir si cette dernière pourrait avoir un effet dans le développement chronique de la maladie (Tascón Cabrero *et al.*, 1997). De plus, il a été démontré que l'expression de l'uréase chez *A. pleuropneumoniae* nécessite au moins 7 gènes. La proportion d'ADN que réserve *A. pleuropneumoniae* à l'expression de cette enzyme sur son petit chromosome semblerait donc trop grande pour que la sélection naturelle cherche à la maintenir si cette activité enzymatique n'avait pas d'importance. Il est donc suggéré que l'uréase ait un rôle dans la survie et/ou la pathogénie d'*A. pleuropneumoniae* (Bossé et MacInnes, 1997).

A. pleuropneumoniae sécrète deux formes de superoxyde dismutase, Cu, Zn SOD et Mn SOD, ces enzymes sont produites par les gènes *sodC* et *sodA*, respectivement. La Cu, Zn SOD pourrait permettre au microorganisme de survivre à la flambée oxydative ("respiratory burst") et ainsi contrecarrer les moyens de défense des cellules hôtes (Langford *et al.*, 1996).

Une substance de nature saccharidique, stable à la chaleur, hémolytique, cytotoxique et anti-phagocytaire a été identifiée dans le surnageant de culture d'*A. pleuropneumoniae* sérotype 2 (Kume *et al.*, 1986).

Enfin, une hémolysine thermolabile de 16-23 kDa produite par *A. pleuropneumoniae* sérotype 1 a été purifiée grâce à une stabilisation préalable, nécessaire due à son instabilité, par de l'albumine sérique bovine ou du dithiothreitol (DTT) (Beaudet *et al.*, 1993). On ne peut toutefois exclure la

possibilité que cette hémolysine soit un produit de dégradation d'une des hémolysines de plus haute masse moléculaire.

1.3.4 Liaison à la fibronectine

Il a été démontré qu'*A. pleuropneumoniae* aurait la capacité de lier la fibronectine porcine, un composé de la matrice extracellulaire. La structure bactérienne responsable de cette interaction n'a pas encore été identifiée (Durán Avelar *et al.*, 1996). Cette propriété s'ajoute aux nombreux facteurs de virulence d'*A. pleuropneumoniae* étudiés.

2. Lipopolysaccharides

2.1 Historique

Vers la fin du XIXe siècle, deux biologistes découvrirent indépendamment des substances bactériennes toxiques dont les propriétés différaient de celles des exotoxines (i.e. des toxines sécrétées) identifiées à cette époque grâce à leur inactivation par la chaleur (Westphal *et al.*, 1977; Rietschel et Brade, 1992). En Allemagne, Richard Pfeiffer, un étudiant de Robert Koch, démontra le premier qu'en plus d'une exotoxine, une seconde substance thermorésistante n'était libérée que lors de la lyse de *Vibrio cholerae*. Il nomma endotoxine cette substance thermorésistante supposant à tort qu'elle se retrouvait à l'intérieur du microorganisme. Tandis qu'en Italie, Eugenio Centanni isola et nomma pyrotoxine une toxine thermostable produite par *Salmonella typhi*, qui déclenchait de la fièvre chez le lapin. Dans les années 1930 et 1940, les progrès de la chimie amenèrent trois scientifiques à analyser des extraits de ces toxines thermorésistantes et à conclure qu'elles étaient composées de polysaccharides, de lipides et de protéines puisqu'à cette époque les techniques de purification existantes permettaient d'obtenir que des extraits d'une pureté relative.

En 1932, le microbiologiste français André Boivin et la roumaine Lydia Mesrobeanu furent les premiers à mettre au point une extraction, à l'acide trichloroacétique, des complexes endotoxiques, qui furent alors nommés antigènes glyco-lipidiques dû à leur composition presque exempte de protéines (Westphal *et al.*, 1977). Cependant, ce ne fut qu'en 1943 que le nom lipopolysaccharide apparu. Murray Shear, des États-Unis, découvrit alors que la substance toxique de *S. marcescens*, ayant aussi des propriétés antitumorales, était essentiellement composée de lipides et de polysaccharides et ainsi la nomma lipopolysaccharide (Rietschel et Brade, 1992).

Toutefois, ce ne fut qu'en 1952, lorsque les allemands Otto Westphal et Otto Lüderitz mirent au point une technique pour obtenir des extraits bactériens purs, que l'identité de ces trois molécules fut établie. Comme les toxines thermorésistantes de *Vibrio*, de *Salmonella*, de *Serratia* et des autres bactéries Gram-négatif ne comportaient que des polysaccharides, des lipides et du

phosphore et qu'elles déclenchaient les mêmes réactions chez l'animal, il fut alors supposé qu'elles avaient des structures chimiques très semblables. Cependant, il fallut patienter environ 25 ans avant de connaître les structures chimiques de ces molécules. Depuis, le terme pyrotoxine fut abandonné, tandis que les termes endotoxine et lipopolysaccharides devinrent des synonymes (Westphal *et al.*, 1977).

Observant que la composition des polysaccharides variait trop pour induire les effets constants d'une endotoxine à l'autre, Westphal et Lüderitz proposèrent déjà en 1954, que les pouvoirs pathogènes et immunostimulants des endotoxines étaient dus au lipide A. Mais, leur hypothèse ne fut définitivement confirmé qu'en 1984 par deux japonais, Tetsuo Shiba et Shoichi Kusumoto, qui réussirent alors à synthétiser le lipide A d'*E. coli*. Le lipide A naturel et sa version synthétique ayant la même structure et la même activité biologique, il fut établi que le principe actif des endotoxines était propre au lipide A. Des études ultérieures démontrèrent de plus que l'intégrité du lipide A était nécessaire pour l'obtention d'une activité biologique optimale des endotoxines (Rietschel et Brade, 1992).

2.2 Nature et structure des lipopolysaccharides

Les LPS sont des macromolécules amphiphiles formant une classe unique de molécules propres aux bactéries Gram-négatif. Les LPS font partie intégrante de la membrane externe s'insérant au niveau du feuillet externe de la membrane externe. Leur distribution asymétrique ainsi que leurs caractéristiques chimiques donnent à la membrane externe plusieurs de ses propriétés uniques en tant que barrière physique. L'association des LPS, qui sont anioniques, aux cations divalents ainsi qu'aux protéines membranaires est critique à cet égard (Hancock, 1991). Toutes les OMPs majeures étudiées ont effectivement démontré une interaction avec les LPS (Hancock *et al.*, 1994).

La membrane externe, sensible aux changements de l'environnement, permet les interactions physiologiques entre la bactérie et les cellules hôtes, dont le transport des nutriments et la non-perméabilité aux composés toxiques (e.g. antibiotiques). L'intégrité de l'architecture membranaire et particulièrement celle

des LPS sont essentielles à la viabilité des bactéries Gram-négatif. Les mutants sans LPS ne sont effectivement pas viables (Rietschel *et al.*, 1994).

Une bactérie contient environ $2 \cdot 10^6$ molécules de lipide A et environ $2 \cdot 10^7$ molécules de glycérophospholipides, mais approximativement le quart des chaînes d'acides gras sont associés aux LPS (fig. 2) (Raetz *et al.*, 1991). Chez une cellule d'*E. coli*, les $3{,}5 \cdot 10^6$ molécules de LPS occupent une surface de 4,9 μm^2 et puisque la surface bactérienne totale d'une cellule d'*E. coli* est de 6,7 μm^2, les LPS en composent les trois quart, la surface restante étant occupée par les protéines (Rietschel *et al.*, 1994).

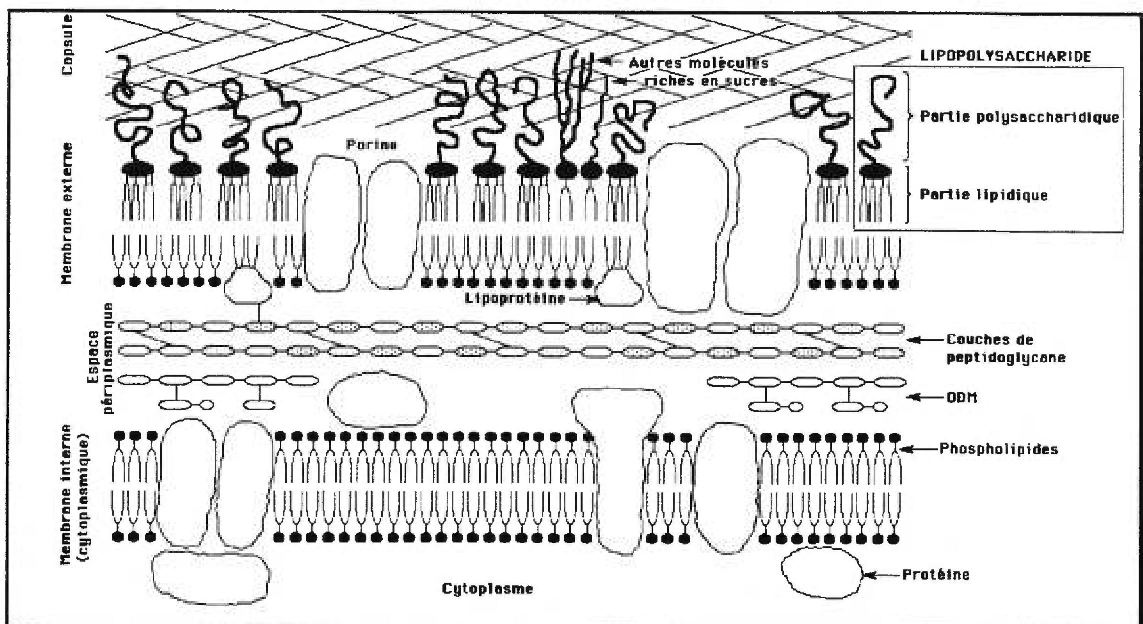


Figure 2. Représentation moléculaire de l'enveloppe d'une bactérie Gram-négatif. Les petits cercles noirs représentent les têtes polaires des glycérophospholipides, les formes ovales noires représentent le disaccharide du lipide A, les ODM représentent les oligosaccharides dérivés de la membrane. Figure modifiée et traduite de Raetz (1993) et de Rietschel et Brade (1992).

Les LPS se composent de trois régions maintenues les unes aux autres par des liaisons covalentes.

2.2.1 Lipide A

Le lipide A est la partie hydrophobe de la molécule. Un LPS actif est composé d'un glucosamine biphosphorylé auquel est relié quatre chaînes d'acides gras,

qui comportent un groupement hydroxyle (OH) sur le troisième atome de carbone, et deux autres acides gras non hydroxylés. Malgré l'arrangement peu variable du lipide A, de légères variations peuvent exister dans sa structure. Ces variations de structure résultent du type d'hexosamine présent, du degré de phosphorylation, de la présence de substituants phosphatés et plus particulièrement, de la nature, de la longueur, du nombre et de la localisation des acides gras. Les six acides gras présents sont distribués selon deux patrons. Le patron asymétrique qui se retrouve par exemple chez *E. coli, H. influenzae et Campylobacter jejuni* présente une acylation sur les deux chaînes d'acides gras C_{14} (i.e. de 14 atomes de carbone) hydroxylés du deuxième glucosamine. Au total quatre chaînes d'acides gras sont reliées au deuxième glucosamine pour deux reliés au premier glucosamine (fig. 3). Le patron symétrique qui se retrouve entre autres chez *Neisseria meningitidis* et *Chromobacterium violaceum* présente une acylation sur une des deux chaînes d'acides gras de C_{14} ou moins hydroxylés de chacun des deux glucosamines. Au total trois chaînes d'acides gras sont reliées à chacun des glucosamines (Rietschel *et al.*, 1994).

Lors de la synthèse du lipide A, plusieurs précurseurs sont aussi produits. Le lipide X et le lipide IV_A sont des précurseurs de la synthèse du lipide A (Raetz, 1990) et sont fréquemment utilisés dans des tests d'inhibition d'activités biologiques permettant ainsi d'identifier les régions du lipide A impliquées. Le lipide X est un précurseur monosaccharidique précoce (un diacyl glucosamine phosphaté), tandis que le lipide IV_A est un précurseur disaccharidique plus tardif (un tétraacyl diglucosamine biphosphaté) (Qureshi et Takayama, 1990). D'autres lipides A sont aussi intéressants dans ce genre d'études car ils ne sont pas conventionnels e.g. le lipide A de *Bacteroides fragilis* ainsi que celui de *Helicobacter pylori* seraient constitués d'un diglucosamine monophosphorylé (Mattsby-Baltzer *et al.*, 1992) et le lipide A de *Rhodobacter capsulatus* qui ne possède que cinq chaînes d'acides gras (Seydel *et al.*, 1994). Récemment, il a été démontré que le lipide A de *Francisella tularensis* n'interagirait pas avec les récepteurs de LPS, la structure de ce lipide A reste par contre à être élucidée (Ancuta *et al.*, 1996).

Le lipide A d'*A. pleuropneumoniae* est composé de 2-amino-2-désoxy-D-glucose (un disaccharide de glucosamine) et possède principalement des acides 3-hydroxytétradécanoïque (C_{14}). Des acides gras C_{12}, C_{16} et même C_{18} sont aussi

retrouvés en moindre proportion parmi les LPS des différents sérotypes (Cf. les références présentées au tableau 1).

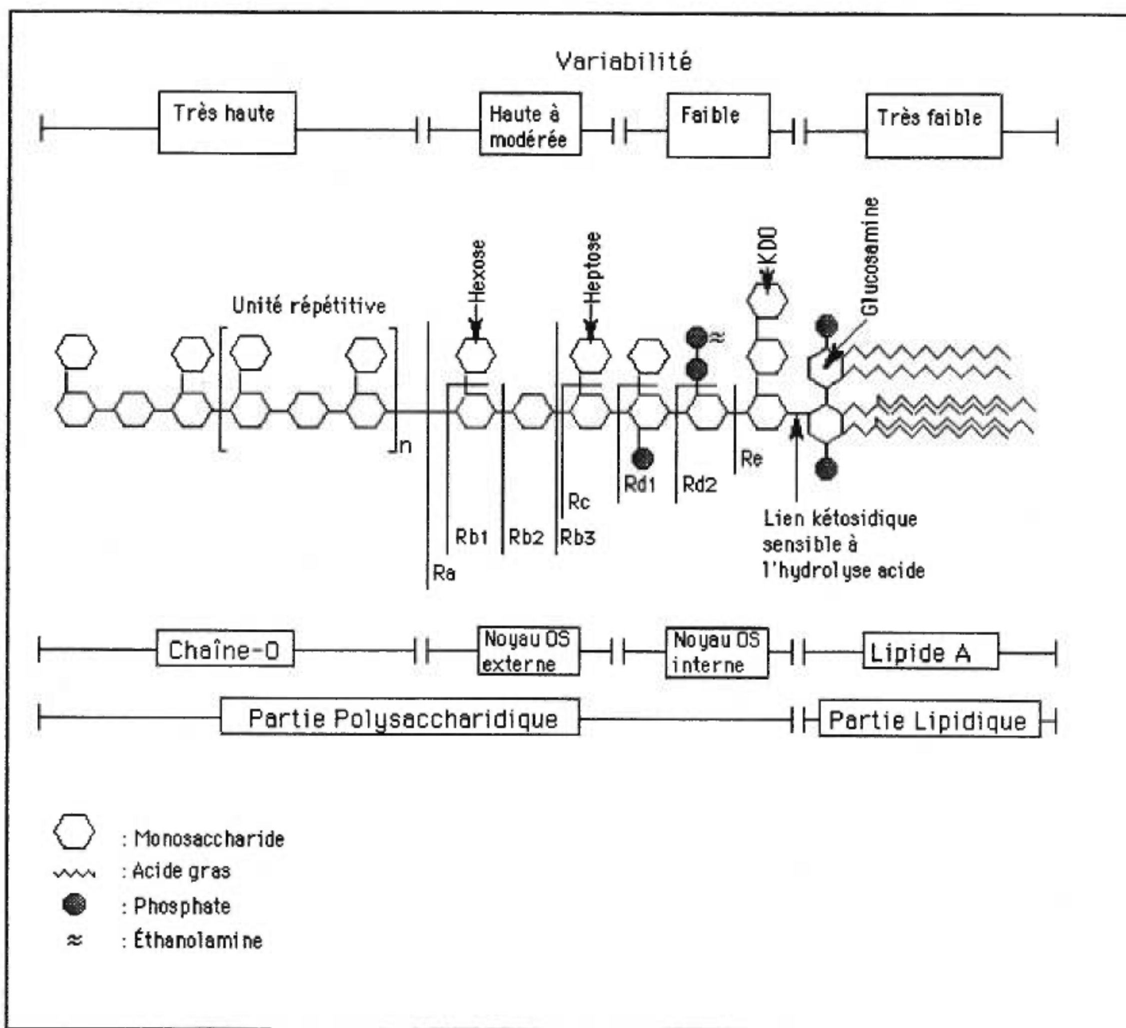


Figure 3. Structure chimique schématisée des LPS de *Salmonella*. Les divisions des différentes régions des LPS, les taux de variation de ces différentes régions et les différents types de mutants au niveau du noyau OS sont représentés. Figure modifiée et traduite de NNalue *et al.* (1992) et de Schletter *et al.* (1995b).

2.2.2 Noyau oligosaccharidique

Le noyau oligosaccharidique (noyau OS ou "core") est composé d'un nombre limité de sucres, il contient typiquement 10 à 15 résidus hétérogènes. Il se subdivise en deux régions, le noyau OS interne, proximal au lipide A, et le noyau OS externe, distal au lipide A. Le noyau OS externe est composé de quelques hexoses (cinq chez les entérobactéries), tandis que le noyau OS interne

contient des heptoses (trois chez les entérobactéries), du KDO (acide 2-céto-3-désoxy-octonique ou plus précisément acide 3-désoxy-D-manno-2-octulosonique (Vaara et Nikaido, 1984) et des phosphates (fig. 3) (Poxton, 1995). Le KDO est un sucre de 8 atomes de carbone essentiel au noyau OS interne pour établir le lien avec le lipide A. Ce lien cétosidique est sensible à l'hydrolyse acide, cette propriété permet de séparer la portion lipidique de la portion saccharidique des LPS (fig. 3).

Longtemps, on a cru que le KDO n'était retrouvé que dans les molécules de LPS, mais il a été démontré que ce sucre pouvait aussi se retrouver dans la composition d'autres polysaccharides. Chez *E. coli*, les polysaccharides capsulaires de certaines souches présentant de minces capsules en contiennent (Jann et Jann, 1985). De même, on en retrouve dans les polysaccharides capsulaires d'*A. pleuropneumoniae* sérotypes 5a (Altman *et al.*, 1987c), 5b (Altman *et al.*, 1992) et 10 (Beynon *et al.*, 1991e).

Chez les *Salmonella*, différents mutants au niveau de la composition du noyau OS (appelés "deep-rough") ont été identifiés (fig. 3). Ces mutants permettent de déterminer l'importance de chacun des sucres composant le noyau OS et ainsi sont eux aussi des outils pratiques dans les tests d'inhibition d'activités biologiques. Les LPS des mutants de type Ra sont formés d'un lipide A et d'un noyau OS complet, les LPS des mutants Re sont formés d'un lipide A et de KDO seulement, tandis que les LPS des mutants Rd$_{1\text{à}3}$, Rc et Rb$_{1\text{et}2}$ sont formés d'un lipide A, de KDO et de un à plusieurs saccharides. Un lipide A mature et au moins un KDO constitueraient la structure minimale retrouvée naturellement et requise pour la viabilité bactérienne (Rietschel *et al.*, 1994; Poxton, 1995). Les LPS de *Bacteroides*, microorganismes anaérobies, ne possèdent cependant pas de KDO, ni de L-glycéro-D-mannoheptoses (heptoses) (Kasper, 1976).

Une microhétérogénéité au niveau de la structure du noyau OS est observable (Reeves, 1994). *E. coli* posséderait cinq types de noyau OS (Reeves, 1994; Rietschel *et al.*, 1994), de même que *Proteus* (Rietschel *et al.*, 1994) et *Citrobacter* en compterait trois (Rietschel *et al.*, 1994). Il y a lieu de soupçonner une diversité des noyaux OS aussi chez *Pseudomonas aeruginosa* (De Kievit et Lam, 1994), chez *P. multocida* (Rimler, 1990) ainsi que chez *A. pleuropneumoniae* puisque des réactions croisées au niveau de la région noyau OS-lipide A ont été observées

parmi les différents sérotypes (Radacovici *et al.*, 1992). De plus, des différences dans l'exposition des antigènes du noyau OS d'*A. pleuropneumoniae* seraient détectables selon la courbe de croissance (Fenwick *et al.*, 1986a).

2.2.3 Chaîne O

L'Ag-O ou somatique consiste en une chaîne polysaccharidique formée d'unités saccharidiques répétitives formant la région hydrophile des LPS (Hancock *et al.*, 1994). Chacune des unités étant elles-même constituées de 1 à 7 sucres (Hitchcock *et al.*, 1986). Le nombre de répétition permet de distinguer différents types de LPS. Le LPS de type lisse ou S (pour "smooth", selon l'apparence des colonies sur géloses) possède un nombre élevé d'unités saccharidiques, tandis que le LPS de type rugueux ou R (pour "rough", encore selon l'apparence des colonies sur géloses) se caractérise par l'absence totale de la chaîne O. Chez les entérobactéries, ces mutants de type rugueux apparaissent en laboratoire, à partir des souches parentales de type lisse, suite à de nombreux repiquages (Reeves, 1994). Finalement, s'il y a présence d'une unité saccharidique mais que celle-ci n'est pas répétée, ou très peu, alors les LPS sont qualifiés de semi-rugueux (Byrd et Kadis, 1989). Les lipooligosaccharides (LOS) sont des LPS de type rugueux retrouvés de façon naturelle principalement chez les espèces des genres *Neisseria*, *Haemophilus*, *Bordetella* et *Chlamydia* (Reeves, 1994).

La chaîne O est, sans contredit, la région des LPS présentant le plus de variations. Chez *E. coli*, on dénombre plus de 160 sérotypes somatiques (Poxton, 1995). Cette diversité de spécificité des chaînes O à même une espèce bactérienne est due à des variations au niveau de la composition des sucres, de la séquence des sucres et de leurs liaisons ainsi que de la substitution des monomères par d'autres résidus saccharidiques ou non-saccharidiques (Whitfield, 1995). Contrairement à ce que l'on pourrait croire, les unités de la chaîne O ne seraient pas transférées directement à une molécule de LPS en croissance. La chaîne O serait plutôt synthétisée séparément sur un transporteur lipidique, le undécaprénol phosphate, et une fois complète la chaîne O serait transférée et liée de façon covalente à un noyau OS-lipide A préformé et faisant face au périplasme. Cette liaison entraînerait la fin de la synthèse de la chaîne O. La molécule de LPS complète serait ensuite transloquée à la surface bactérienne par un mécanisme encore inconnu (Whitfield, 1995). Deux modèles sont

proposés pour tenter d'expliquer la synthèse des LPS. Les deux voies utiliseraient des précurseurs et intermédiaires communs et seraient initier par des réactions similaires, par contre ces deux voies n'utiliseraient pas le même site cellulaire pour les réactions de polymérisation et ne polymériseraient pas les chaînes O dans la même direction. Les gènes *Rfb* coderaient pour les enzymes nécessaires à la formation de l'Ag-O, tandis que les gènes *Rfc* seraient des médiateurs de la polymérisation. La voie *Rol(Cld)* (régulateur de la longueur des chaînes O) et *Rfc*-dépendante polymériserait les chaînes O à l'extrémité réductrice de celles-ci, tandis que la voie *Rol* et *Rfc*-indépendante polymériserait les chaînes O à leur extrémité non-réductrice. Cette seconde voie serait limitée à la synthèse d'homopolymères et nécessiterait un transporteur de type ABC-2 (Whitfield, 1995).

Chez *A. pleuropneumoniae,* il y aurait dix types d'Ag-O pour treize types d'Ag-K (Perry *et al.*, 1990). Particulièrement, les chaînes O de deux des sérotypes somatiques, soit O5 et O10, sont constituées d'un homopolymère et des réactions croisées au niveau des chaînes O sont observées entre les sérotypes somatiques O1(O11) et O9, O3(O8) et O6, et O4 et O7 (tableau 1).

Les LPS sont maintenant reconnus comme existant sous forme de populations hétérogènes chez une même souche bactérienne. Les molécules peuvent en effet varier dans la longueur de la chaîne O ainsi que le patron de substitution (Fenwick *et al.*, 1986b).

Des groupes de recherche ont démontré qu'il existerait des différences antigéniques à l'intérieur du sérotype 1 d'*A. pleuropneumoniae* dues aux lipopolysaccharides. Jolie *et al.* (1994; 1995) ont proposé deux sous-groupes principaux au sérotype 1 d'*A. pleuropneumoniae*. Ils observèrent, qu'après avoir immunisé des porcs avec une souche de sous-sérotype 1A, ces porcs étaient partiellement protégés contre des infections expérimentales par les sous-sérotypes 1A et 1B; mais qu'après avoir immunisé des porcs avec une souche de sous-sérotype 1B, ces porcs étaient partiellement protégés contre des infections expérimentales par le sous-sérotype 1B seulement. Les auteurs conclurent que *A. pleuropneumoniae* sérotype 1B ne possèderait pas les antigènes nécessaires pour protéger les porcs contre des infections de sérotype 1A (Jolie *et al.*, 1995). Antigéniquement, le sous-sérotype 1B ressemblerait plus au sérotype 9 d'*A.*

Tableau 1. Lipopolysaccharides d'*Actinobacillus pleuropneumoniae*

Pays où les sérotypes sont dominants (Mittal *et al.*, 1992)	**Sérotypes et Souches de référence**	**Nomen-clature proposée en 1992** (Beynon *et al.*, 1992b)	**Structures des unités répétitives constituant l'antigène O des lipopolysaccharides** (pour une revue: Perry, *et al.* (1990))	**Réactions sérologiques croisées avec les Ag-O des sérotypes suivants** (Perry *et al.*, 1990)	**Profil électrophorétique des souches**
Argentine, Australie, Canada, Chili, États-Unis, Hongrie, Mexique, Pologne	1 Shope 4074 ATCC 27088	K1:O1	→6)-α-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→ 3 ↑ 1 β-D-Glc*p*NAc (Altman *et al.*, 1986b)	9 et 11	Lisse (Altman *et al.*, 1986b; Radacovici *et al.*, 1994) Semi-rugueux (Byrd et Kadis, 1989; Bélanger *et al.*, 1990)
Allemagne, Corée, Danemark, Hongrie, Japon, Norvège, Pays-Bas, Suède, Suisse, Tchéchoslovaquie	2 4226 ATCC 27089	K2:O2	→2)-α-D-Gal*p*-(1→3)-β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-β-D-Gal*p*NAc-(1→2)-α-L-Rha*p*-(1→ 6 | Ac (0,63) (Altman *et al.*, 1987d)	Aucune	Lisse (Altman *et al.*, 1987d; Byrd et Kadis, 1989; Bélanger *et al.*, 1990)
Belgique, Irlande, Grande-Bretagne	3 1421 ATCC 27090	K3:O3	→3)-α-D-Glc*p*-(1→2)-β-D-Gal*f*-(1→6)-α-D-Gal*p*-(1→6)-β-D-Glc*p*-(1→3)-β-D-Gal*f*-(1→ (Altman *et al.*, 1988c)	6 et 8	Lisse (Altman *et al.*, 1988c) Rugueux (Byrd et Kadis, 1989)
Espagne	4 M62 ATCC33378	K4:O4	→3)-β-D-Gal*p*-(1→4)-β-D-Gal*p*NAc-(1→4)-α-L-Rha*p*-(1→ 3 ↑ 1 β-D-Glc*p* (Altman *et al.*, 1989a)	7	Lisse (Altman *et al.*, 1989a; Byrd et Kadis, 1989)
Brésil, Canada, Chili, Corée, États-Unis, Italie	5 a = K17 ATCC 33377 b = L20	K5a:O5 ou K5b:O5	→ 6)-β-D-Gal*p*-(1→ (Altman *et al.*, 1990b)	Aucune	Lisse (Altman *et al.*, 1990b) Semi-rugueux (Byrd et Kadis, 1989; Radacovici *et al.*, 1992)

-	6 Femo ATCC 33590	K6:O6	→3)-α-D-Glc*p*-(1→2)-β-D-Gal*f*-(1→6)-α-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→3)-β-D-Gal*f*-(1→ (Altman *et al.*, 1989b)	3 te 8	Lisse (Altman *et al.*, 1989b) Rugueux (Byrd et Kadis, 1989)
-	7 WF83 53	K7:O7	→ 3)-β-D-Gal*p*-(1→4)-β-D-Gal*p*NAc-(1→4)-α-L-Rha*p*-(1→ 3 ↑ 1 β-D-Gal*p* (Beynon *et al.*, 1991a)	4	Lisse (Byrd et Kadis, 1989; Beynon *et al.*, 1991a)
-	8 405	**K8:O3**	→3)-α-D-Glc*p*-(1→2)-β-D-Gal*f*-(1→6)-α-D-Gal*p*-(1→6)-β-D-Glc*p*-(1→3)-β-D-Gal*f*-(1→ (Altman, *et al.*, 1990a)	3 et 6	Lisse (Altman *et al.*, 1990a)
-	9 13261 NRCC 4264	K9:O9	→6)-α-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→ 3 ↑ 1 β-D-Glc*p*NAc (0,25) (Beynon *et al.*, 1992b)	1 et 11	Lisse (Beynon *et al.*, 1992b)
-	10 13039 NRCC 4265	K10:O10	→ 2)-β-D-Gal*f*-(1→ (Perry, 1990)	Aucune	Lisse (Perry, 1990)
-	11 56513 456153 NRCC 4330	**K11:O1**	→6)-α-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→ 3 ↑ 1 β-D-Glc*p*NAc (Beynon *et al.*, 1992b)	1 et 9	Lisse (Beynon *et al.*, 1992b)
-	12 1096 8329 NRCC4267	K12:O12	→ 5)-β-D-Gal*f*-(1→6)-β-D-Gal*f*-(1→ 6 ↑ 1 α-D-Gal*p* (Beynon *et al.*, 1991c)	Aucune	Lisse (Beynon *et al.*, 1991c)

Légende:

Glc: glucose; Gal: galactose; Rha: rhamnose; Ac: acétyle; *p*: pyranosyle; *f*: furanosyle; Glc*p*NAc: 2-acétamido-2-désoxy-D-glucose; Gal*p*NAc: 2-acétamido-2-désoxy-D-galactose

pleuropneumoniae car il serait reconnu beaucoup plus fortement en dot-blot par un antisérum dirigé contre *A. pleuropneumoniae* sérotype 9 que par un antisérum dirigé contre *A. pleuropneumoniae* sérotype 11, tandis que le sous-sérotype 1A (auquel appartiendrait la souche de référence 4074) serait plus apparenté au sérotype 11 d'*A. pleuropneumoniae* (Jolie *et al.*, 1995).

Une publication un peu moins récente, démontrait elle aussi que les souches d'*A. pleuropneumoniae* sérotype 1 pouvaient être subdivisées en deux groupes selon la présence (sous-sérotype 1b) ou l'absence (sous-sérotype 1a) d'un antigène polysaccharidique thermostable (i.e. résistant à une température de 121°C pendant une heure) détecté par coagglutination et par le test d'anneau de précipitation (Mittal *et al.*, 1987). Malencontreusement, la souche de référence 4074 a été classée de façon indépendante en 1994 par l'équipe de Jolie *et al.* dans le sous-sérotype 1A (Jolie *et al.*, 1994), tandis qu'elle avait déjà été placée en 1987 dans le sous-sérotype 1b par l'équipe de Mittal *et al.* (Mittal *et al.*, 1987).

À la lumière des résultats de ces deux équipes, il serait intéressant de faire une corrélation entre l'absence de l'antigène polysaccharidique thermorésistant de Mittal *et al.* (1987) et la non-protection des porcs immunisés avec le sous-sérotype 1B de Jolie *et al.* (1994) contre des infections dues au sous-sérotype 1A ainsi qu'entre la présence de l'antigène polysaccharidique thermorésistant de Mittal *et al.* (1987) et la protection des porcs immunisés avec le sous-sérotype 1A de Jolie *et al.* (1994) contre des infections dues aux sous-sérotypes 1A et 1B.

Ce phénomène de variation n'est pas unique à *A. pleuropneumoniae* car plusieurs équipes à travers le monde ont effectivement décrit plus d'un type d'Ag-O exprimé simultanément chez certaines espèces bactériennes. Certaines de ces variations seraient présentes sur une même molécule de LPS ou par la coexistence de deux types de molécules de LPS et seraient soit créées par un système unique d'assemblage des LPS ou par plus d'un système fonctionnant en parallèle. Quelques exemples sont présentés au tableau 2.

2.2.4 Influences des conditions de croissance sur la production des lipopolysaccharides

La production des polysaccharides constituant les LPS, au niveau de la chaîne O

Tableau 2. Exemple de bactérie produisant plus d'un type de LOS/LPS

Espèce bactérienne	**1er type**	**2e type**	**Commentaires et références**
Bordetella pertussis	Deux types de LOS antigéniquement et chimiquement différents peuvent être synthétisés simultanément		(Le Dur *et al.*, 1980; Peppler 1984)
Burkholderia pseudomallei	O-PSI: polymère linéaire de masse moléculaire élevée	O-PSII: polymère linéaire formé d'un disaccharide (glucose et talose)	(*Perry et al.*, 1995)
Haemophilus influenzae	Deux types de LOS antigéniquement différents peuvent être synthétisés simultanément		(Patrick *et al.*, 1989)
Klebsiella pneumoniae serotype O1	D-galactan I (disaccharide de galactose) serait relié directement au noyau OS	D-galactan II (disaccharide de galactose) serait relié à l'extrémité distale du D-galactan I	(Whitfield *et al.*, 1991)
	D-galactan I (disaccharide de galactose) est relié au noyau OS par le galactan II	D-galactan II (disaccharide de galactose)	Sur la même molécule (Kol *et al.*, 1992)
Klebsiella pneumoniae serotype O2(2a,2b)	2a = antigène O de D-galactan I	2b = n'est pas un antigène O	(Whitfield *et al.*, 1992)
Klebsiella pneumoniae serotype O2(2a,2c):	2a = antigène O de D-galactan I	2c = un disaccharide composé de glucose et de galactose	(Whitfield *et al.*, 1992)
Klebsiella pneumoniae serotype O2(2a,2f,2g):	2a = antigène O de D-galactan I	D-galactan I avec un résidu galactose comme ramification	(Kelly *et al.*, 1995)
Neisseria gonorrhoeae	Deux types de LOS antigéniquement différents peuvent être synthétisés simultanément et en proportion variante chez différentes cellules d'une même colonie/La chaîne oligosaccharidique peut présenter une hétérogénicité physique		(Schneider *et al.*, 1984; Mandrell *et al.*, 1986; Griffiss *et al.*, 1987)

Neisseria meningitidis	Deux types de LOS antigéniquement différents peuvent être synthétisés simultanément / La chaîne oligosaccharidique peut présenter une hétérogénicité physique (Mr différent sur SDS-PAGE)		(Tsai *et al.*, 1983; Schneider *et al.*, 1984; Griffiss *et al.*, 1987; Tsai *et al.*, 1987; Kim *et al.*, 1988)
Pasteurella multocida	Reconnu par lectine RCA, Bandes majeures à 11 et 7,4 kDa	Non reconnus par lectine RCA, Bande majeure à 3,7 kDa Non virulent, Adhérence diminuée	Deux phénotypes présents chez la même souche (Coy *et al.* 1997)
Pseudomonas aeruginosa	Bande A = antigène commun	Bande B = antigène O	Sur la même molécule (Pier et Goldberg, 1995b)
	A synthétisé par mécanisme du transporteur ABC	B synthétisé par un mécanisme *Rfc*-dépendant	Sur deux molécules différentes (Lam *et al.*, 1995). Lam *et al.* tentent de convaincre Pier et Goldberg (Pier et Goldberg, 1995a)
	Chaîne A: courtes chaînes O de polysaccharides neutres (pas de groupements phosphates)	Chaîne B: longues chaînes O de polysaccharides contenant un bon nombre de groupements phosphate et de sucres aminés	(Makin et Beveridge, 1996)
Mutant *Salmonella*	Antigène O4 appartenant aux *Salmonella* du groupe B	Antigène O9 appartenant aux *Salmonella* du groupe D	(Johnson *et al.*, 1992)
Salmonella virchow	Même antigénicité, mais masse moléculaire différente (migration double)		Hétérogénéité due à l'utilisation probable de deux voies biochimiques pour l'assemblage des chaînes O des LPS (Chart et Rowe, 1995)

comme au niveau du noyau OS, serait influençée par les conditions environnementales. Par exemple, les LPS d'*Aeromonas hydrophila* subiraient des changements phénotypiques induient par la température. Les Ag-O:34 des LPS seraient exprimés lorsque les bactéries sont cultivées à une température de 20°C, mais ne seraient pas exprimés à une température de 37°C (Merino *et al.*, 1992).

Chez *E. coli*, une diminution de la concentration en magnésium entraînerait une augmentation de l'expression du noyau OS et une diminution de l'expression des chaînes O; une diminution de la concentration en azote ou une augmentation de la concentration en carbone provoquerait une augmentation de l'expression des chaînes O; mais une diminution de la concentration en fer n'entraînerait que des changements mineurs (Nelson *et al.*, 1991).

Chez *P. aeruginosa*, une diminution du nombre des longues chaînes O serait observée à des températures de croissance élevées (près de la limite maximale pouvant supporter la croissance du microorganisme); à de faibles pH; à de faibles concentrations en phosphate; ou à de fortes concentrations en NaCl, $MgCl_2$, glycérol ou sucrose (McGroarty et Rivera, 1990).

Chez *P. haemolytica* sérotype A1, l'agent étiologique de la pleuropneumonie fibrineuse des bovins, des variations au niveau des LPS sont aussi observées selon les conditions de culture (Davies *et al.*, 1992).

Les LOS de certaines espèces d'*Haemophilus* subiraient même des variations antigéniques et de composition. Ces variations de phase seraient induites *in vivo* comme *in vitro*. Il a été démontré que ces variations de LOS seraient reliées à des variations de virulence, les mutants LOS pourraient donc être un moyen de défense pour ces microorganismes vis-à-vis du système immunitaire de l'hôte (Fenwick, 1995).

2.2.5 Structure tridimensionnelle des lipopolysaccharides

Les quatre régions que l'on distingue au niveau de la structure chimique primaire des LPS soit le lipide A, le noyau OS interne, le noyau OS externe et la chaîne O peuvent aussi être reconnues par leurs propriétés conformationnelles (forme globale et flexibilité). La chaîne O est la partie la plus flexible d'une

molécule complète de LPS. Cette flexibilité n'est pas seulement due à la liaison de cette dernière avec le noyau OS, mais doit être due à une propriété intrinsèque de la chaîne O elle-même. Des sites tels que les liaisons rhamnose-galactose (de tels liens sont présents chez les sérotypes O2, O4 et O7 d'*A. pleuropneumoniae,* Cf. tableau 1) et galactose-mannose sont spécifiquement appelés à provoquer des repliements. La modélisation moléculaire d'une molécule de LPS ayant une chaîne O composée de quatre unités saccharidiques répétitives prévoit que la molécule de LPS allongée atteindrait une longueur de 9,6 nm (fig. 4A). Cette conformation étant énergétiquement défavorable, la molécule de LPS adopterait plutôt une conformation repliée où la chaîne O viendrait s'étendre par dessus le sommet des molécules avoisinantes avec un angle presque droit par rapport au feuillet lipidique de la membrane externe (fig 4B). La taille de cette molécule de LPS ayant une conformation énergétiquement favorable se situerait alors entre 5 et 5,5 nm. Les molécules de LPS n'adopteraient cependant pas toutes la même conformation sur une cellule bactérienne (Kastowsky *et al.*, 1992).

L'évolution a peut-être favorisé chez les bactéries Gram-négatif la synthèse des LPS de courtes chaînes et des LPS de longues chaînes, l'hétérogénéité et l'enlacement des longues chaînes O créeraient ainsi une barrière plus stable et moins perméable pour les produits toxiques (Peterson *et al.*, 1986; Kastowsky *et al.*, 1992).

Les souches sauvages de *Salmonella* et de *E. coli* démontrent une distribution bimodale dans la longueur des chaînes O sur SDS-PAGE, i.e. que deux groupes de longueurs de chaîne O sont prédominantes. Les premières sont de masse moléculaire élevée (20-40 unités répétitives) et les secondes sont de faible masse moléculaire (1-8 unités répétitives) (Rietschel *et al.*, 1993).

Chez *A. pleuropneumoniae,* Byrd et Kadis (1989) ont observé que les LPS du sérotype 1 (caractérisés comme semi-rugueux) présentent sur SDS-PAGE une coloration typique du noyau OS-lipide A et une large bande tout en haut du gel i.e. au niveau des masses moléculaires élevées.

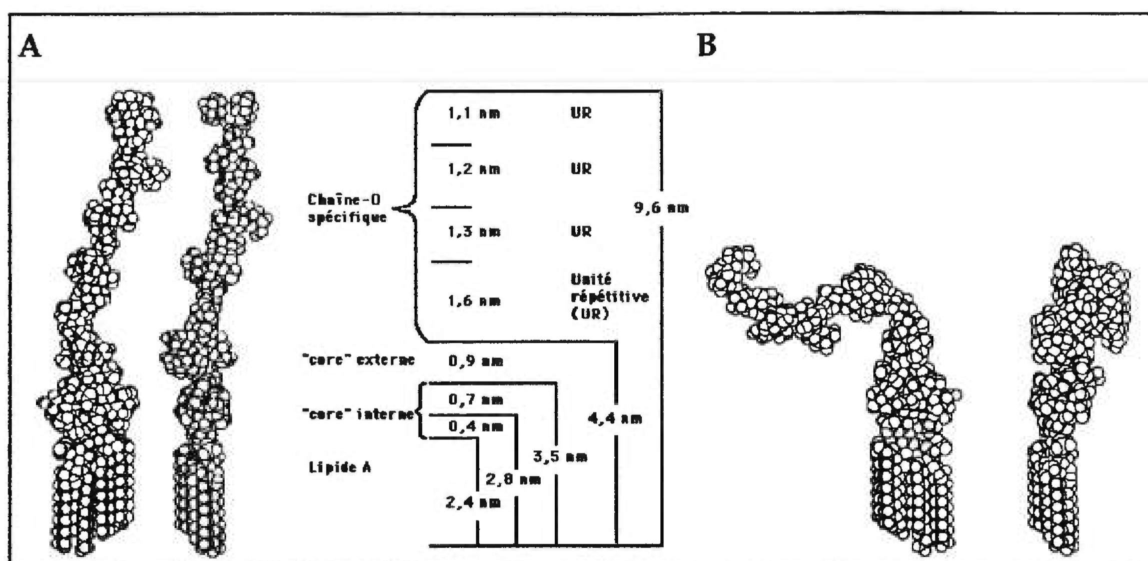


Figure 4. Modèles de la forme lisse des LPS de *Salmonella*. Dimensions d'un modèle de LPS présentant une chaîne O composée de quatre unités répétitives sous sa forme la plus allongée. La longueur de la chaîne O est ici maximale, tandis que les autres dimensions sont des valeurs typiques (A). Vues de côté d'une conformation énergétiquement favorable représentant la chaîne O à un angle presque droit par rapport à la membrane. Dans l'arrangement membranaire, la chaîne O viendrait s'étendre par dessus le sommet des molécules avoisinantes (B). Figure modifiée et traduite de Kastowsky *et al.*, (1992).

2.3 Immunogénicité des lipopolysaccharides

2.3.1 Choc septique

L'importance relative des endotoxines comme facteur de virulence est paradoxale. Peu de molécules possèdent autant de potentiel pathogénique pour induire une réponse inflammatoire systémique. D'une part, les endotoxines ont la capacité intrinsèque d'activer les défenses non-spécifiques de l'hôte, ce qui, par exemple, peut permettre à ce dernier de combattre une infection aigüe due à des bactéries Gram-négatif. D'autre part, la réponse inflammatoire et l'antibiothérapie peuvent contribuer à la sévérité clinique de l'infection, car en provoquant la mort massive des microorganismes, un relâchement rapide d'une grande quantité d'endotoxines s'ensuit (Fenwick, 1995). Les endotoxines n'agissent effectivement sur l'organisme que lorsqu'elles sont libérées par les bactéries, lors de leur lyse ou lors de leur mutiplication. Une fois libérées, les endotoxines induisent la sécrétion de médiateurs par des cellules de l'hôte, qui

agissent localement ou qui circulent dans le sang et déclenchent des réactions à distance. Les processus par lesquels l'hôte métabolise, détoxifie et régularise la réponse aux endotoxines ne font que commencer à être élucidés (Glauser *et al.*, 1991; Stone, 1994; Fenwick, 1995).

Les effets complexes des endotoxines chez les vertébrés incluent l'activation de masse de l'immunité non spécifique (fig. 5). D'un côté, l'activation des protéines plasmatiques dont: a) l'activation de la cascade du complément par la voie alterne et l'inhibition des protéines de régulation servant à prévenir la destruction des cellules de l'hôte par une activité excessive du complément provoquent des dommages tissulaires dus à la formation de complexes d'attaque des membranes et de l'œdème dû aux peptides chimiotactiques relâchés; b) l'activation du facteur Hageman (XII) donc de la cascade de la coagulation sanguine par la voie intrinsèque provoque la coagulation intravasculaire diffuse et le collapsus cardiovasculaire; c) l'activation du système des kinines provoque de la douleur, l'œdème et de l'insuffisance pulmonaire; d) l'activation du système fibrinolytique provoque une hémorragie diffuse.

D'une part, il y a l'activation du système de défense cellulaire dont les monocytes/macrophages, les cellules endothéliales et les granulocytes qui produisent selon leur fonction des cytokines (IL-1, IL-6, IL-8, TNFα), des métabolites lipidiques (prostaglandine E2, thromboxane A2, et leukotriènes, le facteur agrégeant les plaquettes), des produits toxiques dérivés de l'oxygène (O_2^-, ·OH, H_2O_2, NO), des molécules d'adhésion (intégrines, ICAMs, ELAM, endothéline, sélectine-E) et/ou des protéases (élastase, collagénase). Ces produits provoquent la fièvre, l'hypotension, la perméabilité vasculaire, la migration transendothéliale des neutrophiles, l'œdème, la perforation des parois vasculaires, les lésions pulmonaires et autres dommages tissulaires. D'autre part, il y a l'activation des protéines sériques et celle des cellules du système de défense non spécifique interagissent entre elles et s'amplifient. Dans le cas de l'inflammation systémique sévère (choc septique), ces effets résultent en une dysfonction tissulaire multiple entraînant la mort de l'individu.

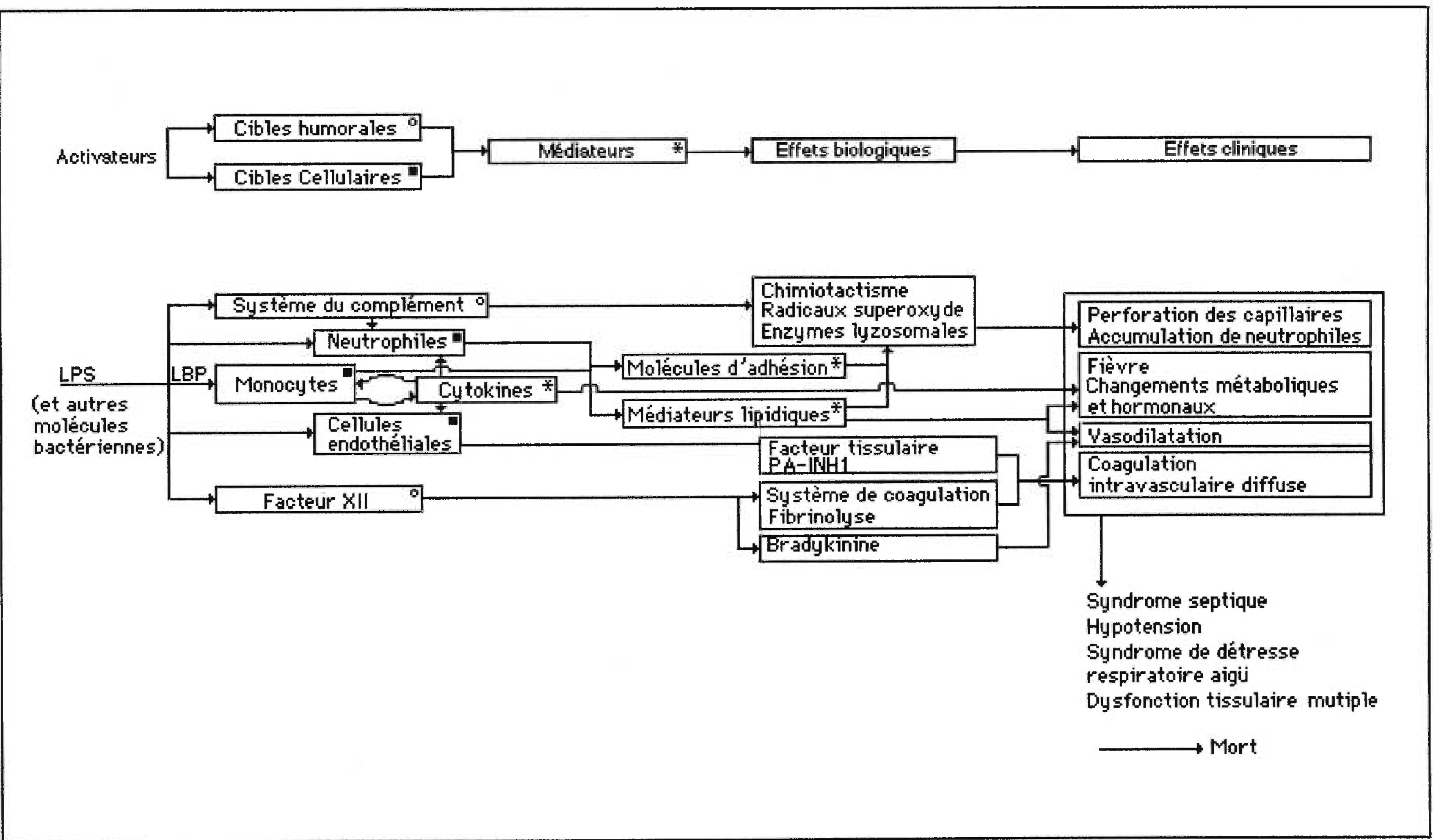


Figure 5. Voies probables des interactions entre les facteurs humoraux et les cytokines dans la pathogénie de l'inflammation systémique sévère. Figure traduite de Glauser, *et al.* (1991).

2.3.2 Anticorps anti-lipopolysaccharides

Les molécules de LPS sont des antigènes T-indépendants (Jacobs et Morrison, 1975) n'élicitant qu'une réponse primaire d'anticorps IgM de faible affinité et très peu de réponse mémoire. Ce sont des mitogènes, i.e. des molécules capables d'induire la formation d'anticorps par les cellules B, sans l'intervention de cellules présentatrices d'antigènes ni de cellules T auxiliaires ("T_H"). Les unités répétitives des molécules de LPS peuvent se lier de façon multivalente aux cellules B et stimuler la sécrétion d'immunoglobulines de type IgM par ces dernières (l'activation polyclonale des cellules B murines, i.e. qu'il peut y avoir stimulation de plusieurs clones de lymphocytes B). La production d'immunoglobulines de type IgG est aussi possible par l'association des molécules de LPS à des protéines bactériennes (ayant un rôle de transporteurs). Des réponses primaire et secondaire de type T-dépendantes peuvent ainsi être induites.

Des anticorps monoclonaux dirigés contre les LPS ont été développés en vue de traiter les individus atteints d'infections graves à bactéries Gram-négatif e.g. des anticorps monoclonaux humains, HA-1A, d'isotype IgM et des anticorps monoclonaux murins, E5, aussi d'isotype IgM (Fujihara *et al.*, 1993). La plupart des anticorps monoclonaux dirigés spécifiquement contre la région noyau OS-lipide A ne reconnaissent pas facilement les LPS de type lisse, ce qui remet en question leur efficacité clinique (Field *et al.*, 1995).

Un anticorps anti-idiotype Ab2 qui reflèterait l'image interne d'un anticorps murin spécifique pour le noyau OS interne du LPS a été développé et utilisé pour immuniser des souris. Les souris auraient généré des anticorps primaires IgG, après rappel, elles auraient maintenu des titres anti-LPS-Re élevés et seraient protégées contre les propriétés létales des endotoxines. Les anticorps sériques spécifiques reconnaîtraient les LPS de type lisse dans un surnageant de culture de bactéries Gram-négatif traitées aux antibiotiques. Cette approche semble donc prometteuse (Field *et al.*, 1995).

Des anticorps dirigés contre les LPS seraient présents en bonne quantité dans le sérum de porcs convalescents (Fenwick et Osburn, 1986a). Par contre, les porcs immunisés avec des LPS purifiés ne seraient protégés que contre une infection

homologue (Inzana, 1991), de même, pour les porcs immunisés avec des LPS détoxifiés (Rioux *et al.*, 1996). Les LPS amèneraient donc une protection spécifique de sérotype (Fenwick et Osburn, 1986b; Inzana *et al.*, 1988).

Des anticorps monoclonaux dirigés contre les LPS d'*A. pleuropneumoniae* ont été développés (Giese *et al.*, 1993; Lairini *et al.*, 1995; Lacouture *et al.*, 1996) et ont rendu possibles de nombreuses applications, entre autres au niveau du diagnostic.

2.3.3 Lipopolysaccharides et maladies auto-immunes

La structure moléculaire des LPS de *C. jejuni* imite celle des gangliosides et amènerait la production d'anticorps dirigés contre les gangliosides, ces anticorps seraient reconnus comme jouant un rôle central dans la pathogénie du syndrome auto-immunitaire et neurodégénératif de Guillain-Barré (Aspinall *et al.*, 1994) ainsi que dans celui du syndrome de Miller Fisher, une variante du premier (Neisser *et al.*, 1997).

Les chaînes O des LPS de *H. pylori* ont une composition moléculaire similaire aux antigènes sanguins Lewis x et Lewis y. Des anticorps dirigés contre ces antigènes sanguins auraient comme cibles les ATPases gastriques, ce qui bloqueraient la pompe à protons (H^+) et à potassium (K^+). Les anticorps anti-Lewis x reconnaîtraient de plus les PMNs, lesquels causent une fois adhérés aux tissus des dommages locaux et une inflammation. Le complément pourrait aussi avoir un rôle à jouer dans les dommages gastriques amenés par les anticorps auto-immuns (Appelmelk *et al.*, 1996).

2.3.4 Autres effets des endotoxines

Les endotoxines provoquent encore bien d'autres effets tels la réaction de Schwartzman, l'induction de la tolérance, l'induction du facteur stimulant des colonies (qui active la formation des granulocytes), la gélation des amébocytes du *Limulus* et possèdent une activité adjuvante.

2.4 Techniques de purification des lipopolysaccharides

Les LPS constituent 3 à 8% de la masse sèche des bactéries (Hancock *et al.*, 1994). Plusieurs méthodes d'extraction ont été décrites pour ces molécules. Wesphal et Jann (1965) introduisent une technique d'extraction des LPS à partir des cellules bactériennes mises en présence d'une solution aqueuse de phénol chauffée à 68°C. Une fois refroidie, c'est dans la phase aqueuse que se retrouvent les LPS. Cette technique permettrait surtout l'extraction des LPS de type lisse. Pour remédier à cette situation, Galanos *et al.* (1969) développent une méthode qui permettrait de récupérer uniquement les LPS de type rugueux comportant plus de 70% de lipides. Cette méthode solubiliserait ces derniers dans une solution de phénol aqueux à laquelle sont ajoutés du chloroforme et de l'éther de pétrole. Cette technique a été amélioré par Austin *et al.* (1990). Rimsay *et al.* (1981) appliquent la technique classique de Wesphal et Jann à la fraction insoluble obtenue par l'extraction de Galanos *et al.* (1969), ce qui leur permettraient d'extraire les LPS de type lisse, en plus des LPS de type rugueux déjà extraits par la fraction soluble.

Darveau et Hancock (1983) mettent au point une méthode basée sur la digestion enzymatique (ribonucléase, désoxyribonucléase, pronase) de cellules bactériennes lysées mécaniquement suivie d'une précipitation des LPS par un mélange de $MgCl_2$-éthanol 95%. Cette nouvelle méthodologie permettrait d'extraire les LPS des deux types chez *Pseudomonas* et ce avec un rendement beaucoup plus élevé qu'à l'aide des techniques précédentes.

Hitchcock et Brown (1983) ont eux aussi décrit une méthode pour l'isolement des LPS de type lisse et rugueux qui ne requiert pas de solvants. Cette préparation permet l'analyse rapide des LPS sur gel de polyacrylamide. La visualisation des LPS est rendue possible grâce à une coloration au nitrate d'argent mise au point par Tsai et Frasch (1982). Le nitrate d'argent permet de visualiser les aldéhydes, résultant de l'oxydation au périodate des sucres du noyau OS des LPS. La réussite de l'électrophorèse et de la visualisation des LPS, demandent trois facteurs. Premièrement, les LPS doivent contenir suffisamment de lipides pour l'obtention de complexes LPS-SDS chargés négativement nécessaires à la migration durant l'électrophorèse. Deuxièment, les LPS doivent être précipités par la solution aqueuse d'isopropanol et d'acide acétique.

Troisièmement, le noyau OS des LPS doit contenir au moins une paire de groupements hydroxylés substitués qui sont clivés par l'oxydation au périodate et qui génèrent des aldéhydes pouvant réagir avec le nitrate d'argent. La coloration au nitrate d'argent offre des informations qualitatives et semi-quantitatives sur la distribution des différentes classes de LPS dans un échantillon. L'intensité de la coloration au nitrate d'argent reflète le nombre de molécules de LPS, mais non le nombre de résidus de sucre par molécule avant le clivage (Sprott *et al.*, 1994).

Le LPS de type lisse possède un nombre élevé d'unités produisant, sur un SDS-PAGE coloré au nitrate d'argent, une apparence d'échelle caractéristique. Chacune des bandes formant l'échelle étant différente de la précédente par l'addition d'un monomère de chaîne O (Sprott *et al.*, 1994). Ce patron est observable chez les sérotypes 2, 4 et 7 d'*A. pleuropneumoniae*. Lorsqu'il y a absence totale de chaîne O, seule la région du noyau OS-lipide A apparaît, ce qui est propre aux LPS rugueux des sérotypes 3 et 6 d'*A. pleuropneumoniae*. Par contre, s'il y a absence de patron en échelle, mais présence d'autres bandes en plus de la coloration du noyau OS-lipide A, les LPS sont qualifiés de semi-rugueux, ce qui est observé chez les sérotypes 1 et 5 d'*A. pleuropneumoniae* (Byrd et Kadis, 1989).

Lesse *et al.* (1990) ont décrit l'électrophorèse sur gels de Tricine-SDS-PAGE qui permet une meilleure résolution des LPS de faibles masses moléculaires. Le choix de la technique de purification des LPS chez les bactéries Gram-négatif dépend grandement de la présence ou de l'absence des chaînes O ainsi que des tests dans lesquels les LPS seront utilisés. Les LPS lisses sont plus facilement extraits par des méthodes aqueuses, tandis que les LPS rugueux demandent souvent des solvants beaucoup plus puissants. De même, les LPS isolés par l'extraction phénol-eau conservent mieux la réactivité avec des anticorps en Western blot que ceux isolés par la technique de Darveau et Hancock (Sprott *et al.*, 1994). Pour cette raison plusieurs chercheurs ont développé, selon le type de bactérie Gram-négatif et le genre d'étude qui les intéressaient, leurs propres modifications au niveau de la purification des LPS, mais aussi au niveau de l'électrophorèse, de la coloration et/ou de la détection des LPS (Johnson et Perry, 1976; Al-Hendy *et al.*, 1991; Blake Jr et Russell, 1993; Kittelberger et Hilbink, 1993; Davies *et al.*, 1994; Guard-Petter *et al.*, 1995; Hardy *et al.*, 1997; Valverde *et al.*, 1997).

3. Interactions adhésine-récepteur

3.1 Adhérence

Plusieurs microorganismes peuvent se retrouver dans une variété de niches écologiques, tandis que d'autres sont restreints à des microenvironnements spécifiques. La susceptibilité d'un hôte, le tropisme tissulaire et les cellules cibles d'un microorganisme sont dus, en partie, à la complémentarité entre les adhésines microbiennes et la structure des récepteurs présents à la surface des cellules de l'hôte. Ce processus dynamique a pour objectif de diriger le microbe vers une cible bien précise. Une régulation de la synthèse des adhésines permettrait donc aux microorganismes de coloniser les différents tissus rencontrés à partir de leurs portes d'entrée jusqu'à leurs cibles finales (Hoepelman et Tuomanen, 1992; van Alphen, 1995). D'autres mécanismes, tels que les interactions électrostatiques et hydrophobes ne sont pas négligeables (Makin et Beveridge, 1996). Pour adhérer irréversiblement à une cellule, une bactérie doit pouvoir s'approcher de façon à initier un contact intime entre ses adhésines et les récepteurs. Puisque les deux surfaces biologiques possèdent toutes deux des charges négatives, ces forces répulsives doivent être combattues. Ce serait les interactions hydrophobes qui permettraient aux bactéries de les surmonter (Doyle et Sonnenfeld, 1989; Plotkowski *et al.*, 1993).

Le système respiratoire possède plusieurs mécanismes de défense pour éviter toute contamination. Le réflexe de la toux permet de rejeter les grosses particules s'étant introduites dans les voies respiratoires avant qu'elles ne fassent obstruction. L'arbre trachéobronchique, dépourvu de microflore normale, est doté d'un ascenseur mucociliaire lui permettant d'éliminer les microorganismes inhalés. Cet ascenseur qui est tapissé de cils et recouvert d'une couche de mucus, emprisonne les microorganismes et autres petites particules. Par le mouvement des cils, le matériel emprisonné est entraîné vers le haut du tractus respiratoire pour y être expulsé. Si l'équilibre mouvement ciliaire-mucus est rompu, certains microorganismes en profiteront pour coloniser les lieux, d'autres réussiront eux-mêmes à provoquer un déséquilibre qui leur permettra de s'établir (Freter, 1984; Plotkowski *et al.*, 1993; Widdicombe, 1995). Une fois que le pathogène respiratoire atteint le niveau des alvéoles pulmonaires, il devra surmonter

d'autres mécanismes de défense de l'hôte incluant le surfactant pulmonaire et les macrophages alvéolaires avant de produire la pneumonie (Confer *et al.*, 1995).

De façon générale l'adhérence survient via des adhésines fimbriaires qui possèdent une affinité élevée pour les glycolipides ou glycoprotéines présents au niveau des muqueuses (Confer *et al.*, 1995). Cependant de nombreuses adhésines non-fimbrillaires sont rencontrées e.g. des hémagglutinines, des exotoxines, des lectines, des protéines non associées à des pili ou des exopolysaccharides. Ces adhésines impliquent autant de récepteurs différents qu'elles sont diversifiées (Widdicombe, 1995). De plus, presque tous les pathogènes possèdent plus d'une adhésine, ce qui leurs permet d'interagir avec plus d'un récepteur (Hoepelman et Tuomanen, 1992).

3.2 Adhésines d'*A. pleuropneumoniae*

3.2.1 Fimbriae, pili ou appendices filamenteux

La présence de fimbriae chez *A. pleuropneumoniae* est controversée. Certaines équipes ont rapporté ne pas en avoir observé (Rapp *et al.*, 1986; Jacques *et al.*, 1988). Par contre, d'autres équipes en font mention, mais en rapportent la présence de façon inconstante.

Des structures s'apparentant à des pili auraient été observées sur des souches virulentes ayant été cultivées sur milieu solide et seraient constituées de sous-unités protéiques de piline de 20 kDa (Tomcik *et al.*, 1988).

La moitié des isolats provenant de porcs infectés présenterait des fimbriae. *In vitro*, les fimbriae de *A. pleuropneumonie* ne seraient observables que sur culture primaire ou au deuxième passage si les souches sont cultivées sur gélose au sang. Les fimbriae ont été mis en évidence par coloration négative et observés par MET. Les fimbriae seraient péritriches, chaque cellule en compterait en moyenne 85. Leurs dimensions se situeraient autour de 0,5 à 2 nm X 60 à 450 nm (Utrera et Pijoan, 1990; 1991).

Une autre équipe aurait visualisé les fimbriae par balayage, car il leur aurait été impossible d'en observer par colorarion négative en MET (Dom *et al.*, 1994).

Nombreux, les fimbriae auraient une distribution péritriche et des dimensions se situant autour de 1 nm X 100 à 300 nm.

Récemment, des appendices extracellulaires ont été observés à l'aide de coloration négative en MET par une équipe mexicaine (Garibay *et al.*, 1996). La moitié des souches fraîchement isolées de poumons porcins sur géloses au sang présenteraient des fimbriae et 25 à 80% des cellules de chaque souche seraient piliées. La distribution de ces derniers serait péritriche ou aléatoire et leur dimensions très variables de 2 à 7 µm X 0,5 à 6 nm (moyenne de 4 µm X 0,5 à 4 nm). Les auteurs suggèrent qu'*A. pleuropneumoniae* exprimerait deux types de structure piliées comme chez *E. coli* (Kahn et Gromkova, 1991): une rigide d'un diamètre de 5 à 7 nm et l'autre, impliquée dans l'adhérence, d'un diamètre de 2 nm. Il se pourrait aussi qu'*A. pleuropneumoniae* possède des pili sexuels (Frost, 1992).

3.2.2 Hémagglutinines

Sept patrons d'hémagglutination (composés des résultats d'agglutination d'une souche vis-à-vis différentes espèces d'érythrocytes) ont été observés parmi plusieurs souches de champ appartenant aux dix premiers sérotypes d'*A. pleuropneumoniae*, mais aucune corrélation n'associe un patron avec un ou plusieurs sérotypes (Jacques *et al.*, 1988). Aucun des isolats d'*A. pleuropneumoniae* n'a hémagglutiné les érythrocytes de porcs.

3.2.3 Lipopolysaccharides d'*A. pleuropneumoniae*

Les LPS permettraient l'adhérence aux anneaux de trachée porcine maintenus en culture d'organe et cette adhérence serait influencée par le type de LPS car les souches possédant des LPS lisses adhéreraient mieux que les souches possédant des LPS semi-rugueux (Bélanger *et al.*, 1990). Les LPS d'*A. pleuropneumoniae* lieraient aussi les protéines du mucus respiratoire porcin (Bélanger *et al.*, 1994). Cette adhérence des LPS au mucus semblerait bloquée par la présence d'une capsule masquante (Bélanger *et al.*, 1992).

Les bactéries auraient, de plus, la possibilité d'adhérer aux cellules épithéliales de trachée porcine ainsi qu'aux coupes congelées de poumons porcins et ici

encore la présence de matériel capsulaire semblerait diminuer l'adhérence des souches (Jacques *et al.*, 1991). Enfin, il a été démontré qu'*A. pleuropneumoniae* adhérerait *in vivo* à l'épithélium alvéolaire porcin et aux cils des bronchioles terminales (Dom *et al.*, 1994). Ces deux dernières études d'adhérence démontrent bien l'affinité du pathogène pour les tissus respiratoires porcins, mais ne nous donne aucune information sur la ou les structure(s) impliquée(s).

3.3 Les lipopolysaccharides comme adhésine

3.3.1 Accessibilité des lipopolysaccharides à la surface bactérienne

L'accessibilité d'une adhésine est une caractéristique essentielle pour rendre possible les interactions entre le microorganisme et les cellules de l'hôte.

Des anticorps marqués à l'or colloïdal et dirigés contre les Ag-O peuvent être observés, par MET, à des distances de plus de 20 nm du feuillet lipidique de la membrane externe. Selon les dimensions d'un modèle moléculaire allongé (Cf. section 2.2.5), des molécules de LPS comptant plus de 11 unités saccharidiques répétitives pourraient s'étendre sur cette distance (Kastowsky *et al.*, 1992).

Le fait que les polysaccharides formant les LPS soit reconnus par certains bactériophages est aussi en accord avec leur accessibilité. Différentes régions des LPS d'*A. hydrophila* servent de récepteurs pour des bactériophages, le bactériophage PM1 reconnait l'Ag-O:34 (Merino *et al.*, 1990a), tandis que le bactériophage PM2 en reconnait le noyau OS (Merino *et al.*, 1990b). De plus, les bactériophages FC3-1 (Tomas et Jofre, 1985) et FC3-2 (Tomas *et al.*, 1987) reconnaissent l'Ag-O1 de *K. pneumoniae*, pour ne dénombrer que ces derniers.

L'exposition à la surface bactérienne des chaînes O des LPS de *K. pneumoniae* a même été démontrée chez certaines souches encapsulées (Tomas *et al.*, 1988; 1991). Ces LPS, de masse moléculaire élevée, exposés à la surface amèneraient de plus une protection immunologique chez des souris infectées expérimentalement (Tomas *et al.*, 1991). L'exposition des LPS à la surface de souches encapsulées chez *P. haemolytica* a aussi été démontrée (Wilson *et al.*, 1992).

Étant exposés à la surface cellulaire, les LPS sont impliqués dans les interactions de l'organisme avec son environnement dont l'adhérence aux cellules hôtes. Chez *A. pleuropneumoniae* des vésicules de la membrane externe ("blebs") traversent les polysaccharides capsulaires et exposent ainsi les LPS à la surface cellulaire (Bélanger *et al.*, 1995).

3.3.2 Les lipopolysaccharides comme adhésines chez différentes espèces bactériennes

L'utilisation des LPS comme adhésines n'est pas unique à *A. pleuropneumoniae*. Le rôle d'adhésine pour les LOS/LPS de différentes bactéries a depuis été observé par de nombreuses équipes de recherche. Un éventail de ces bactéries et des différents épithéliums, tissus ou lignées cellulaires auxquels elles adhèrent sont présentés dans une revue récemment rédigé par Jacques (1996).

Pour certaines bactéries, le rôle d'adhésine des LPS semblerait, vraisemblablement, nécessiter la présence de l'Ag-O e.g. chez *Aeromonas sobria* (Francki et Chang, 1994), *C. jejuni* (McSweegan et Walker, 1986), *P. aeruginosa* (Fletcher *et al.*, 1993; Gupta *et al.*, 1994; Zaidi *et al.*, 1995), *S. typhi* (Mroczenski-Wildey *et al.*, 1989), *S. marcescens* (Palomar *et al.*, 1995). Chez *Shigella dysenteriae* sérotype 1, il a été démontré que les LPS lisses ainsi que les polysaccharides obtenus de ces LPS par hydrolyse acide agglutineraient les érythrocytes contrairement aux LPS rugueux. Ces derniers se lieraient à des récepteurs contenant de l'acide sialique puisque l'agglutination serait inhibée par cette substance et par la fétuine (une protéine contenant de l'acide sialique). De plus, des extraits de LPS lisses inhiberaient l'adhérence de *S. dysenteriae* sérotype 1 aux cellules intestinales Henle 407. Les chaînes O semblent donc essentielles puisque les mutants rugueux de *S. dysenteriae* seraient incapables d'agglutiner les érythrocytes et n'adhéreraient que faiblement aux cellules intestinales (Quadri *et al.*, 1991). Dernièrement, Merino *et al.* (1996a; 1996b) ont aussi démontré le rôle de l'Ag-O:34 comme adhésine de *A. hydrophila* dans la colonisation intestinale aviaire. L'adhérence de différentes souches d'*A. hydrophila* aux cellules Hep-2 serait diminuée d'environ 60% lorsque les Ag-O ne sont pas exprimés (1996a; Merino *et al.*, 1996b).

Pour d'autres bactéries le rôle d'adhésine des LOS/LPS semblerait aussi médié par des polysaccharidiques, mais non par ceux de l'Ag-O e.g. *E. coli* (Cohen *et al.*, 1985), *Haemophilus ducreyi* (Campagnari *et al.*, 1994), *H. pylori* (Valkonen *et al.*, 1994), *Neisseria gonorrhoeae* (Porat *et al.*, 1995a; Schwan *et al.*, 1995), *P. aeruginosa* (Fletcher *et al.*, 1993; Gupta *et al.*, 1994; Zaidi *et al.*, 1995; Zaidi *et al.*, 1996).

La partie polysaccharidique des LOS/LPS semble donc primordiale pour permettre l'adhérence bactérienne. Pour ce qui est des LPS de *P. multocida* (Jacques *et al.*, 1993), de *Shigella flexneri* (Izhar *et al.*, 1982) et de *V. cholerae* (Chitnis *et al.*, 1982), la partie impliquée au niveau de l'adhérence n'a pas encore été précisée.

Finalement, même si les LOS/LPS ne sont pas encore reconnus comme une adhésine chez certaines espèces, une corrélation, suite à une infection expérimentale, aurait été démontrée entre la colonisation et la présence de LOS de masses moléculaires plus élevées chez *H. influenzae* (Weiser, 1993); ainsi qu'entre la colonisation et la présence des Ag-O des LPS de *Salmonella typhimurium* (Craven, 1994) et de *K. pneumoniae* (Camprubi *et al.*, 1993).

3.4 Récepteurs de lipopolysaccharides

De nombreux récepteurs et/ou molécules liant les LPS ont été identifiés sur diverses cellules animales. Les récepteurs les mieux connus sont ceux impliqués dans la réponse immunitaire provoquée par les LPS, mais de plus en plus d'études démontrent les multiples interactions dans lesquelles prennent part les LPS. La correspondance entre certains récepteurs décrits par plusieurs chercheurs est souvent difficile à éclaircir car chacun utilise selon ses besoins des types cellulaires différents, provenant d'espèces différentes et évaluent les interactions dans des systèmes d'électrophorèse et/ou de révélation différents (Lei et Chen, 1992; Kielian et Blecha, 1995). Les tableaux 3 à 5 présentent un bon aperçu de ce qui ce retrouve dans la littérature.

Tableau 3. Récepteurs et molécules liant les LPS, présents chez les mammifères, selon leur masse moléculaire

Récepteurs et molécules liant les LPS	**Localisation**	**Partie du LPS impliquée**	**Références**
Lipoglycoprotéine (265 kDa)	membrane d'érythrocytes humains	indéterminée	(Springer *et al.*, 1974; Lei et Chen, 1992)
Microtubule-associated protein-2 (MAP-2) (>200 kDa)	cerveau murin	Lipide A et peut être d'autres régions	(Ding *et al.*, 1992)
Récepteur Mannose (175 kDa)	macrophages alvéolaires humains		(Stephenson et Shepherd, 1987; Lei et Chen, 1992)
Glycoprotéine (150 à 250 kDa)	mucus intestinal murin	Partie polysaccharidique	(Cohen *et al.*, 1985)
96 kDa	érythrocytes murins	LPS Re	(Kirikae *et al.*, 1988; Lei et Chen, 1992)
Acétyle-LDL (Scavenger 95 kDa)	hépatocytes, cellules de Kupffer, cellules endothéliales sinusoidales	Lipide A de LPS libres ou LPS présentés par lipoprotéines	(Wright, 1991; Cross et Opal, 1995; Shnyra et Lindberg, 1995)
95 kDa	lignée cellulaire de type macrophage RAW264.7	Lipide IV_A	(Hampton *et al.*, 1988; Wright, 1991; Lei et Chen, 1992)
Famille des intégrines CD18 (95 kDa)		Lipide A en présence de cations divalents	(Wright, 1991; Lei et Chen, 1992; Kielian et Blecha, 1995)
CD11a(LFA-1: 180kDa)/CD18	leucocytes		
CD11b(CR3 ou MAC-1: 160 kDa)/CD18	phagocytes		
CD11c(p150-95: 150 kDa)/CD18	phagocytes mononuclaires et neutrophiles		
Lactoferrine (80 kDa)	granules des neutrophiles, sécrétions mucosales	Lipide A	(Appelmelk *et al.*, 1994)

80 kDa	monocytes, cellules endothéliales	Lipide A en présence de CD14 et de LBP	(Schletter *et al.*, 1995a)
80 kDa (73 kDa)	splénocytes de souris normales C3HeB/FeJ, splénocytes de souris C3H/HeJ (non sensible aux LPS), lymphocytes spléniques B/lymphocytes T, macrophages, lignée cellulaire Pré-B murine: 70Z/3, lignées cellulaires: YAC-1, EL4T, cellules lymphoides de divers mammifères	Lipide A	(Lei et Morrison, 1988b; Lei et Morrison, 1988a; Lei *et al.*, 1991; Wright, 1991; Lei et Chen, 1992; Kielian et Blecha, 1995)
73 kDa (80 kDa)	monocytes, lymphocytes B et T, neutrophiles, plaquettes		(Halling *et al.*, 1992; Lei et Chen, 1992)
70 kDa	splénocytes murins, lymphocytes B murins	$(GlcNAc)_2$ du lipide A	(Dziarski, 1991; Lei et Chen, 1992)
Albumine (70 kDa)	associé aux cellules soit par le sérum in vivo ou par le sérum contenu dans le milieu de culture	$(GlcNAc)_2$ du lipide A	(Dziarski, 1994)
70 kDa (=80 kDa?)	lignée cellulaire hepatique HepG2	Région du noyau OS des LOS de gonocoques	(Porat *et al.*, 1995a)
65 kDa (=80 kDa?)	lignée cellulaire de type macrophage J774.1	Lipide A (et lipide X)	(Hara-Kuge *et al.*, 1990; Lei et Chen, 1992; Kielian et Blecha, 1995)
65 kDa	monocytes, cellules endothéliales	Lipide A	(Schletter *et al.*, 1995a)
Septine (60-70 kDa)	sérum		(Wright, 1994; Kielian et Blecha, 1995)
LBP (58-60 kDa) Fragment N-terminal de 197 aa	sérum (synthétisé par les hépatocytes)	Lipide A (et lipide IV_A, mais non lipide X)	(Wright, 1991; Lei et Chen, 1992; Han *et al.*, 1994; Gazzano-Santoro *et al.*, 1995; Kielian et Blecha, 1995)
mCD14 (55 kDa) Fragment N-terminal de 151 aa	monocytes/macrophages, neutrophiles	Lipide A en présence de LBP	(Lei et Chen, 1992; Kielian et Blecha, 1995; Viriyakosol et Kirkland, 1996)
55 kDa (=mCD14?)	lignée cellulaire de type macrophage J774.1	Lipide A (et lipide X)	(Hara-Kuge *et al.*, 1990; Lei et Chen, 1992; Kielian et Blecha, 1995)

BPI (55 kDa) = CAP57 ou BP55 Fragment N-terminal de 23 kDa	granules des neutrophiles	Lipide A (et lipide IV_A, mais non lipide X)	(Capodici *et al.*, 1994; Gazzano-Santoro *et al.*, 1995)
β-tubuline (55 kDa)	cerveau murin	Lipide A et peut être d'autres régions	(Ding *et al.*, 1992)
sCD14 (50-53 kDa) Fragment N-terminal de 152 aa	sérum (libéré à partir des mCD14)	Lipide A en présence de LBP	(Lei et Chen, 1992; Juan *et al.*, 1995; Kielian et Blecha, 1995)
Acyloxyacyl hydro-lase (52-60 kDa)	leucocytes	chaînes acyles du lipide A	(Munford et Hall, 1989)
52 kDa	lignée cellulaire hépatique HepG2	LOS de *N. gonorrhaea* et LPS de *E. coli*	(Porat *et al.*, 1995a)
51 kDa			(Roeder *et al.*, 1989)
50 kDa	lymphocytes humains, monocytes humains		(Halling *et al.*, 1992; Lei *et al.*, 1993; Kielian et Blecha, 1995)
47 kDa	hépatocytes de rats	Région heptose-KDO	(Parent, 1989b; Parent, 1989a; Parent, 1990; Lei et Chen, 1992)
46 kDa	lignée cellulaire hépatique HepG2	LOS de *N. gonorrhaea* et LPS de *E. coli*	(Porat *et al.*, 1995a)
SP-D (sous-unités de 42-44 kDa)	espace alvéolaire	glucose terminal du noyau OS et/ou residus heptoses	(Kuan *et al.*, 1992)
38 kDa	splénocytes murins, lignée cellulaire de type macrophage J774.1, lignée cellulaire de cellules pré-B murines 70Z/3, lymphocytes murins, macrophages murins	KDO (inhibé par Re)	(Lei et Morrison, 1991; Lei et Chen, 1992; Lei *et al.*, 1993; Kielian et Blecha, 1995)
38 kDa	lymphocytes humains, monocytes humains		(Halling *et al.*, 1992)
CAP37 (37 kDa) = azurocidine ou BP30 Peptide 20-44	granules des neutrophiles humains	Lipide A	(Pereira *et al.*, 1993)
MBP (mannan-binding protein) (31 kDa)	sérum de lapin, humain, de rat et bovin	noyau oligosacchridique contenant du L-glycéro-D-manno-heptose et N-acetylglucosamine	(Kawasaki *et al.*, 1989)
Histone H1 (doublet à 31 kDa)	lignée cellulaire de type macrophage RAW264.7	Lipide IV_A	(Hampton *et al.*, 1988; Lei et Chen, 1992)

31 kDa	lymphocytes humains, monocytes humains		(Halling *et al.*, 1992; Kielian et Blecha, 1995)
30 kDa	lignée cellulaire hépatique HepG2	LOS de *N. gonorrhaea* et LPS de *E. coli*	(Porat *et al.*, 1995a)
RaRF (28 à 70 kDa)	sérum murin	spécifique pour Ra	(Ihara *et al.*, 1982; Kawakami *et al.*, 1984)
SP-A (28-35 kDa selon le degré de glycosylation)	espace alvéolaire	Lipide A	(van Iwaarden *et al.*, 1994)
28 kDa	sérum murin	Région du noyau OS interne	(Brade et Brade, 1985; Brade *et al.*, 1988)
28 kDa	lignée cellulaire Pré-B murine 70Z/3 (probabalement une protéine sérique adsorbée)	Lipide A	(Kirkland *et al.*, 1990; Lei et Chen, 1992)
Galectine-3 Région N-terminale	monocytes humains	noyau OS interne des LPS de *S. minnesota* R7	(Couturier *et al.*, 1991; Mey *et al.*, 1996)
Galectine-3 Région C-terminale		β-galactosides de la chaîne-O des LPS de *K. pneumoniae*	(Mandrell *et al.*, 1994; Mey *et al.*, 1996)
Galectine-3 (26,2 à 30,3 kDa)		noyau OS interne des LPS de *P. aeruginosa*	(Barondes *et al.*, 1994; Hazlett, 1996)
25 kDa	lignée cellulaire Pré-B murine 70Z/3	Lipide A	(Kirkland *et al.*, 1990; Lei et Chen, 1992; Kielian et Blecha, 1995)
20 kDa (18 kDa)	lymphocytes humains, monocytes humains		(Halling *et al.*, 1992; Lei, *et al.*, 1993; Kielian et Blecha, 1995)
18 kDa	lignée cellulaire pré-B murine 70Z/3	Lipide A	(Kirkland *et al.*, 1990; Kielian et Blecha, 1995)
CAP18 (18 kDa) Fragment C-terminal de 37 aa (= CAP7)	granules des leucocytes humains et de lapin	Lipide A	(Hirata *et al.*, 1994; Larrick *et al.*, 1995)
Lysozyme (13 kDa)	sérum sécrétions exocrines granules des neutrophiles	Lipide A	(Ohno *et al.*, 1991; Takada *et al.*, 1994; Takada *et al.*, 1995)
Chaînes α et β de l'hémoglobine (10 et 11 kDa)	sang	Lipide A	(Bélanger *et al.*, 1994; Kaca *et al.*, 1994; Bélanger *et al.*, 1995)

Tableau 4. Autres récepteurs et molécules liant les LPS, présents chez les mammifères, dont la masse moléculaire n'est pas précisée

Récepteurs et molécules liant les LPS	**Localisation**	**Partie du LPS impliquée**	**Références**
Récepteur Mannose	macrophages péritonéaux murins		(Raichvarg *et al.*, 1982; Lei et Chen, 1992)
Récepteur de type lectine (sérum dépendant)	macrophages péritonéaux de lapins et murins, monocytes sanguins humains	LPS de *B. pertussis*	(Haeffner-Cavaillon *et al.*, 1982; Haeffner-Cavaillon *et al.*, 1985; Lei et Chen, 1992)
Récepteur asialo-glycoprotéine	lignée cellulaire hépatique HepG2	LOS de gonocoques	(Porat *et al.*, 1995b)
Récepteur asialo-glycolipide GM1	épithélium de la cornée	LPS de *P. aeruginosa*	(Gupta *et al.*, 1994)
Laminine	matrice extracellulaire	noyau OS de *H. pylori*	(Valkonen *et al.*, 1994)
Autres LBP	granules		(Ohno, 1992)
HDL	sérum		(Munford *et al.*, 1981)(Flegel, *et al.*, 1993)

Tableau 5. Récepteurs et molécules liant les LPS présents chez d'autres organismes

Récepteurs et molécules liant les LPS	**Localisation**	**Partie du LPS impliquée**	**Références**
Dansylcadavérine	molécule synthétique	Lipide A	(David *et al.*, 1992)
Facteur C (protéine procoagulante) 123 kDa	*Tachypleus tridentatus*	Lipide A	(Roth et Tobias, 1993)
Facteur anti-LPS (LALF: protéine anti-coagulante) 15 kDa	*Tachypleus tridentatus*	Lipide A	(Roth et Tobias, 1993)
LAL LBP (inhibe les protéases)	*Tachypleus tridentatus*	Lipide A	(Roth et Tobias, 1993)

LAL 82 kDa	amébocytes des *Limulus polyphemus*	Lipide A	(Warren *et al.*, 1992)
LAL 50 kDa	hémolymphe des *Limulus polyphemus*	Lipide A	(Warren *et al.*, 1992)
Bombyx mori LPS binding protein (BmLBP) 40 et 43 kDa	*Bombyx mori*	Lipide A	(Koizumi *et al.*, 1997)
Mellitine	venin d'abeille	Lipide A	(David, 1992)
Polymyxine B	*Bacillus polymyxa*	Région lipide A-KDO	(Morrison et Jacobs, 1976)
La sous-unité S2 de la toxine pertussique	*Bordetella pertussis*		(Lei et Morrison, 1993a; Lei et Morrison, 1993b)

III. MATÉRIEL, MÉTHODES ET RÉSULTATS

Article 1

High-molecular-mass lipopolysaccharides are involved in *Actinobacillus pleuropneumoniae* adherence to porcine respiratory tract cells

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Infection and Immunity **62:** 3311-3319, 1994

Ce premier article introduit le système miniaturisé développé pour l'étude de l'adhérence, soit la technique des coupes congelées, et permet d'incriminer la partie polysaccharidique des LPS d'*A. pleuropneumoniae* comme structure responsable de l'adhérence.

INFECTION AND IMMUNITY, Aug. 1994, p. 3311–3319
0019-9567/94/$04.00+0
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Vol. 62, No. 8

High-Molecular-Mass Lipopolysaccharides Are Involved in *Actinobacillus pleuropneumoniae* Adherence to Porcine Respiratory Tract Cells

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Received 27 December 1993/Returned for modification 9 February 1994/Accepted 10 May 1994

Actinobacillus pleuropneumoniae **is the causative agent of porcine pleuropneumonia. The major adhesin of *A. pleuropneumoniae* has been identified as the lipopolysaccharides (LPSs) (M. Bélanger, D. Dubreuil, J. Harel, C. Girard, and M. Jacques, Infect. Immun. 58:3523–3530, 1990). Using immunoelectron microscopy and flow cytometry, we showed in the present study that LPSs were well exposed at the surface of this encapsulated microorganism. Immunolocalization with porcine lung and tracheal frozen sections showed that extracted LPS bound to the lung mesenchyme and vascular endothelium and to the tracheal epithelium, respectively. Inhibition of adherence of *A. pleuropneumoniae* with extracted LPS was also performed with lung and tracheal frozen sections. Acid hydrolysis of LPS revealed that the active component of LPS was not lipid A but the polysaccharides. LPSs from *A. pleuropneumoniae* serotypes 1 and 2 were separated by chromatography on Sephacryl S-300 SF, in the presence of sodium deoxycholate, according to their molecular masses. The adherence-inhibitory activity was found in the high-molecular-mass fractions. These high-molecular-mass fractions contained 2-keto-3-deoxyoctulosonic acid and neutral sugars, and they were recognized by a monoclonal antibody directed against *A. pleuropneumoniae* O antigen but not recognized by a monoclonal antibody against capsular antigen.**

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia (28), a disease found worldwide that causes tremendous economic loss to the swine industry. Twelve serotypes of *A. pleuropneumoniae*, based on capsular antigens, have been recognized (29). Furthermore, the serotypes have different lipopolysaccharide (LPS) compositions, except that serotypes 1, 9, and 11, serotypes 3, 6, and 8, and serotypes 4 and 7 have common epitopes (31). In Québec, serotypes 1 and 5 are predominant, while serotype 2 is predominant in most European countries (27). The pathogenesis of porcine pleuropneumonia is not well understood. Several cytotoxic and hemolytic activities have been described (10–12, 22), but the virulence factors involved in colonization of the respiratory tract remain largely unknown.

The initial event in bacterial colonization is the adherence of microorganisms to the epithelial cells and/or mucus layer of the mucosal surfaces, which involves specific interactions between bacterial adhesins and host receptors (2, 30). We previously demonstrated that LPSs were the major adhesin of *A. pleuropneumoniae* involved in adherence to porcine tracheal rings maintained in culture (3). LPSs are complex molecules composed of three well-defined regions: the lipid A; the core, an oligosaccharide containing 2-keto-3-deoxyoctulosonic acid (KDO); and the O antigen, a chain of polysaccharides consisting of repeating units (18). Depending on the presence and the number of O-antigen repeating units, LPS can be rough, semirough (e.g., in *A. pleuropneumoniae* serotype 1), or smooth (e.g., in *A. pleuropneumoniae* serotype 2) (3, 7, 18). The purpose of the present study was to show the accessibility of LPS at the surface of this encapsulated microorganism, to localize the preferential binding sites of *A. pleuropneumoniae* LPS on porcine tissue sections, and to determine which region of this complex molecule is involved in adherence. We used strains of *A. pleuropneumoniae* showing two different LPS profiles, either semirough or smooth.

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MATERIALS AND METHODS

Bacterial isolates. *A. pleuropneumoniae* reference strains representing serotypes 1 (strain 4074) and 2 (strain 4226) were provided by A. Gunnarson, National Veterinary Institute, Uppsala, Sweden. A serotype 1 field isolate of *A. pleuropneumoniae* (FMV-87-682) was obtained from the clinical diagnostic laboratory, Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, Québec, Canada.

Growth conditions. Bacteria were grown on brain heart infusion (Difco Laboratories, Detroit, Mich.) agar plates supplemented with 40 μg of NAD per ml. Plates were incubated at 37°C in a 5% CO_2 atmosphere for 18 to 24 h.

Electron microscopy. (i) Immunolabeling. Bacteria were harvested in phosphate-buffered saline (PBS) (0.01 M, pH 7.4). Single drops of bacterial suspension were placed on Formvar-coated grids and were allowed to partially air dry. The grids were then placed sequentially on drops of PBS containing 1% (wt/vol) egg albumin (for 5 min) and mouse monoclonal antibodies against *A. pleuropneumoniae* serotype 1 O antigen (5.1 G8F10) or *A. pleuropneumoniae* serotype 2 O antigen (102-G02) (13) (for 30 min); both monoclonal antibodies were kindly supplied by Eva I. Stenbaek, Department of Biochemistry and Immunology, National Veterinary Laboratory, Copenhagen V, Denmark. The grids were then washed in distilled water and placed on drops of colloidal gold particles (10 nm) conjugated to goat anti-mouse immunoglobulin G

(IgG) (Sigma Chemical Co., St. Louis, Mo.) for 30 min. After a final wash in distilled water, the grids were stained with 0.2% (wt/vol) phosphotungstic acid (pH 7.1) and were examined with a Philips 201 electron microscope at an accelerating voltage of 60 kV.

(ii) LPS negative staining. LPS preparations were examined by negative staining. A drop of each LPS preparation, sonicated for 5 min, was placed on 200-mesh Formvar-coated grids. A drop of 1% (wt/vol) phosphotungstate was then applied to the grids, which were then examined as described above.

Flow cytometry. Washed overnight cultures of *A. pleuropneumoniae* were resuspended in PBS to an A_{540} of 0.2, equivalent to approximately 10^8 CFU/ml. Suspensions (1 ml) were centrifuged at 10,000 × *g* for 2 min, and the pellets were resuspended in 1 ml of a monoclonal antibody against serotype 1 or 2 O antigen and incubated for 60 min at room temperature. Samples were washed twice in PBS, and then 0.5 ml of sheep fluorescein isothiocyanate-conjugated anti-mouse IgG (Boehringer Mannheim, Laval, Québec, Canada) diluted 1:50 in PBS was added. After a further incubation of 60 min at room temperature, samples were washed twice in PBS and fixed with 2% paraformaldehyde. Cells were kept in the dark at 4°C until analyzed by flow cytometry. The flow analysis was performed with a FACStar flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) equipped with a water-cooled 2-W argon ion laser operating at 488 nm and a 200-mW light output. Multiparametric data were acquired for 10,000 events and analyzed by using Consort 30 software. The flow system was equipped with a 75-μm nozzle tip, and the analysis was performed at a flow rate of 500 events per s. Green (fluorescein isothiocyanate) fluorescence was collected in log scale, using a 530/30-nm filter. Data were analyzed in monoparametric histograms.

Extraction and isolation of LPSs. LPSs from *A. pleuropneumoniae* serotypes 1 and 2 were extracted and isolated by the method of Darveau and Hancock (8). Briefly, disrupted cells were treated with DNase, RNase, pronase, and sodium dodecyl sulfate (SDS) and were subjected to $MgCl_2$ precipitation and high-speed centrifugation. These LPS preparations contained less than 1% protein as determined by a dye-binding assay (Bio-Rad Laboratories, Richmond, Calif.), and no bands were detected after silver staining of SDS-polyacrylamide gels.

LPS hydrolysis. Ten milligrams (dry weight) of LPS was hydrolyzed at 100°C for 2 h in 1 ml of 1% (vol/vol) acetic acid previously saturated with nitrogen. Lipid A (insoluble) and polysaccharides (soluble) were separated by centrifugation at 12,000 × *g* for 10 min after neutralization with 5 N NaOH (1). Lipid A was washed and resolubilized in 1 ml of EDTA (20 mM) (26).

LPS fractionation by chromatography. LPSs of *A. pleuropneumoniae* serotypes 1 and 2 were separated in the presence of sodium deoxycholate by chromatography on a Sephacryl S-300SF (Pharmacia, Baie d'Urfé, Québec, Canada) column according to the procedure described by Peterson and McGroarty (33). Briefly, 20 mg of extracted LPS was resuspended in 3 ml of 0.25% (wt/vol) sodium deoxycholate–0.2 M NaCl–1 mM EDTA–10 mM Tris (pH 8.0), sonicated for 5 min, and subjected to gel filtration chromatography on a column (60 by 2.5 cm) of Sephacryl S-300SF. Elution was performed at a flow rate of 0.9 ml/min in deoxycholate-containing buffer. Fractions of 12 ml were collected. After extensive dialysis against column buffer (three times, 18 liters each) without deoxycholate at 37°C for 2 days and then against distilled water (three times, 18 liters each) at 4°C for another 2 days, the fractions were assayed for neutral sugar (9) and KDO (17) contents and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

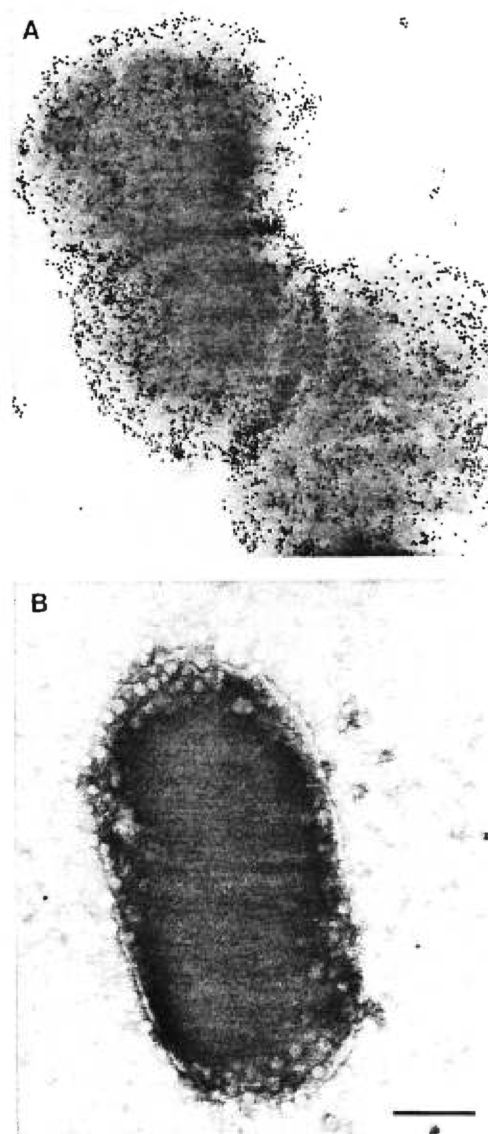

FIG. 1. Transmission electron micrographs of whole cells of an *A. pleuropneumoniae* serotype 1 isolate probed with monoclonal antibodies against serotype 1 (A) or 2 (B) O antigen and goat anti-mouse IgG–gold particles (10 nm). Bar, 200 nm.

LPS profiles. A volume of 500 μl of each fraction was lyophilized, suspended in 100 μl of solubilization buffer containing 10% (vol/vol) glycerol, 5% (vol/vol) β-mercaptoethanol, 2% (wt/vol) SDS, 0.0625 M Tris-hydrochloride (pH 6.8),

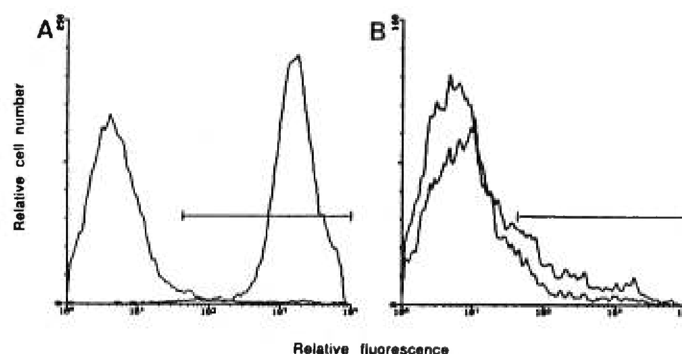

FIG. 2. Flow cytometry analysis of an *A. pleuropneumoniae* serotype 1 isolate. (A) Whole cells were labeled with the anti-mouse IgG fluorescein isothiocyanate (FITC)-conjugated antibody (left peak) and with monoclonal antibodies against serotype 1 O antigen plus the anti-mouse IgG FITC-conjugated antibody (right peak). (B) Whole cells were labeled with the anti-mouse IgG FITC-conjugated antibody and with monoclonal antibodies against serotype 2 O antigen plus the anti-mouse IgG FITC-conjugated antibody (left peaks). The horizontal bars indicate fluorescent cells.

and 0.025% (vol/vol) bromophenol blue, and heated for 20 min at 100°C. Volumes of 7 μl were loaded, and samples were separated by discontinuous SDS-PAGE by using a stacking gel of 4.5% (wt/vol) polyacrylamide and a separating gel of 15% (wt/vol) polyacrylamide (24). Samples were electrophoresed at 100 V (stacking gel) and 200 V (separating gel) with a Mini-Protean II apparatus (Bio-Rad). Gels were stained with the silver-staining procedure of Tsai and Frasch (36).

Dot blot. Ten-microliter aliquots of *A. pleuropneumoniae* serotype 1 LPS fractions were placed on a nitrocellulose membrane. All incubations were performed at room temperature and were followed by four 3-min washes with a Tris-saline buffer (10 mM Tris, 150 mM NaCl, pH 7.4). The membranes were first incubated for 1 h with 2% casein (a blocking solution) and then were incubated for 2 h with monoclonal antibodies against capsular polysaccharides of *A. pleuropneumoniae* serotype 1 (1.5 C5F4) or against LPS of *A. pleuropneumoniae* serotype 1. The membranes then were incubated for 1 h with a goat anti-mouse IgG (heavy plus light chains) horseradish peroxidase conjugate (Bio-Rad). Results of the reaction were revealed by addition of 4-chloro-1-naphthol and hydrogen peroxide (Sigma). As controls, bacterial suspensions of *A. pleuropneumoniae* serotypes 1 and 2 and extracted LPSs of both serotypes were dotted onto the membranes.

Frozen sections and adherence assay. Adherence to frozen sections was selected because we needed a system in which minute amounts of fractionated LPS could be tested for inhibitory activity. Lung and tracheal samples, obtained from newborn piglets, were washed in PBS, embedded in O.C.T. compound (Miles Laboratories, Inc., Elkhart, Ind.), frozen, and stored at −70°C until used (20). Frozen sections (4 to 6 μm thick) were cut in a cryostat microtome, mounted on glass slides, fixed in methanol for 1 min, and air dried (6). Bacteria were diluted in PBS containing 1% (wt/vol) bovine serum albumin and 0.01% (vol/vol) Tween 20 (PBS-BSA-T20) (37) to give an A_{540} of 0.2. A volume (100 μl) of the bacterial suspension was pipetted onto tissue sections on glass slides and incubated in a moist chamber at 37°C for 2 h. After intensive washing in distilled water, sections were stained with the Diff-Quik stain (Baxter Healthcare Corporation, McGraw Park, Ill.) according to the manufacturer's instructions. Upon microscopic examination, the number of bacterial cells attached to the tracheal epithelium was determined at a magnification of ×1,000.

Adherence inhibition assay. Frozen sections were preincubated with 25 μl of extracted LPS (2 mg/ml) and 75 μl of PBS-BSA-T20 in a moist chamber at 37°C for 30 min. Sections were then incubated with 25 μl of extracted LPS preparations and 75 μl of bacterial suspension in a moist chamber at 37°C for 2 h, washed, and stained as described above. Controls were made by substituting PBS-BSA-T20 for extracted LPS.

Localization of LPS-binding sites. A volume (100 μl) of extracted LPS (0.1 mg/ml) was deposited onto lung and tracheal frozen sections. The sections were incubated in a moist chamber at 37°C for 2 h and washed extensively. LPSs were detected by using either rabbit antisera raised against *A. pleuropneumoniae* serotypes 1 and 2 or monoclonal antibodies against *A. pleuropneumoniae* serotypes 1 and 2 and the AS/AP Immunostaining Kits (Bio/Can Scientific, Mississauga, Ontario, Canada). The slides were counterstained with Mayer's hematoxylin. Visualization of the preferential sites of attachment was done by microscopic examination at magnifications of ×100 and ×400. Controls were made either by substituting PBS-BSA-T20 for extracted LPS or by using normal rabbit serum or monoclonal antibodies against *A. pleuropneumoniae* serotype 1 capsular antigen.

RESULTS

Previous work in our laboratory identified LPS as the major adhesin of *A. pleuropneumoniae* involved in adherence to porcine upper respiratory tract cells. The aim of the present study was to show the accessibility of LPS at the surface of this encapsulated microorganism, to localize the preferential binding sites of *A. pleuropneumoniae* LPS on porcine lung and tracheal tissue sections, and to determine which region of this complex molecule is involved in adherence. We first determined the accessibility of LPS at the surface of this encapsulated microorganism, an essential prerequisite for a bacterial adhesin. Cells of *A. pleuropneumoniae* serotype 1 incubated with monoclonal antibodies against serotype 1 O antigen were heavily labeled with gold particles in immunoelectron microscopy (Fig. 1A), and were highly fluorescent when analyzed by

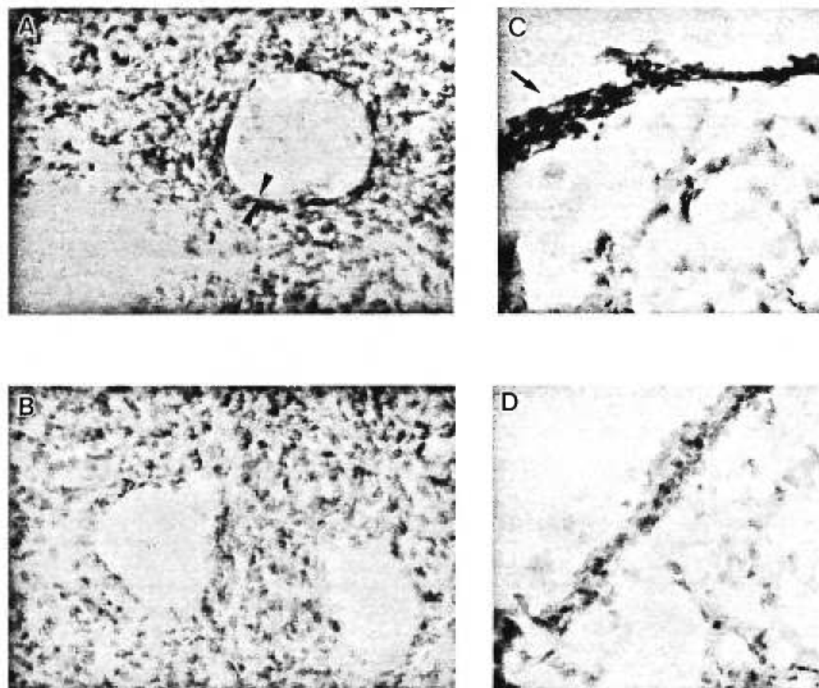

FIG. 3. Binding of *A. pleuropneumoniae* serotype 2 extracted LPS to porcine lung and tracheal frozen sections. Lung sections A and B (original magnification, ×250) and tracheal sections C and D (original magnification, ×400) were incubated with (A and C) or without (B and D) LPS. Sections were then incubated with a serotype-specific rabbit antiserum, and the results of the reaction were revealed with an alkaline phosphatase conjugate and a chromogenic substrate. Arrowheads and arrow denote areas of intense reaction.

flow cytometry (Fig. 2A), indicating that LPSs were surface exposed. Cells of *A. pleuropneumoniae* serotype 1 incubated with an irrelevant monoclonal antibody (against serotype 2 O antigen) were not labeled with gold particles (Fig. 1B) and were not fluorescent (Fig. 2B). It is important to note that when cells of *A. pleuropneumoniae* serotype 1 were incubated with monoclonal antibodies against serotype 1 capsular antigen, they were also heavily labeled with gold particles and highly fluorescent by flow cytometry, confirming the presence of capsular material on these cells. Similar results were obtained when cells of *A. pleuropneumoniae* serotype 2 were incubated with monoclonal antibodies against serotype 2 O antigen (data not shown).

Porcine lung and tracheal frozen sections were incubated in the presence of either *A. pleuropneumoniae* serotype 1 or 2 extracted LPS. Detection of bound LPS was done by immunostaining, using a serotype-specific rabbit antiserum or monoclonal antibodies directed against *A. pleuropneumoniae* serotype 1 or 2 O antigen, an alkaline phosphatase conjugate, and a chromogenic substrate. Microscopic examination showed an important brown deposit localized at the lung vascular endothelium (Fig. 3A, arrowheads) and the tracheal epithelium (Fig. 3C, arrow). In addition, the lung mesenchyme showed diffuse staining of a lesser intensity. Controls in which LPSs were omitted (Fig. 3B and D) did not show any reaction.

Inhibition of adherence of *A. pleuropneumoniae* serotypes 1 and 2 to tracheal frozen sections was observed with extracted LPS (Fig. 4). In order to determine which region of the LPS molecule was essential for inhibition of adherence, extracted LPSs were hydrolyzed. After acid hydrolysis of *A. pleuropneumoniae* serotype 1 and 2 extracted LPSs, the inhibiting activity

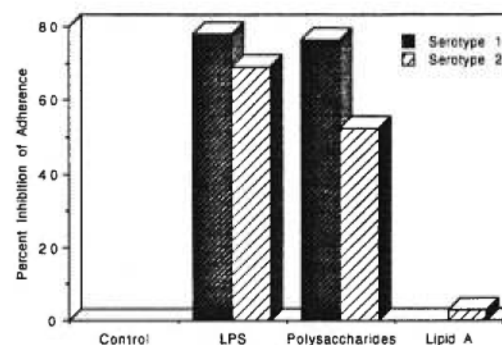

FIG. 4. Inhibition of adherence of an *A. pleuropneumoniae* serotype 1 isolate and a serotype 2 reference strain to porcine tracheal frozen sections by homologous polysaccharides and lipid A obtained after acid hydrolysis of *A. pleuropneumoniae* extracted LPS.

was associated with the polysaccharide moiety of LPS, while the resolubilized lipid A was totally devoid of activity (Fig. 4).

We then separated LPS molecules according to their molecular masses by chromatography in the presence of deoxycholate. *A. pleuropneumoniae* fractionated LPSs were divided into five major pools that each contained KDO and neutral sugars (Fig. 5). Silver staining of these fractionated LPSs after SDS-PAGE revealed molecules of decreasing molecular masses (Fig. 6). Material from pool A of serotype 1 and 2 LPSs remained at the top of the polyacrylamide gel. Pools B, C, and D were composed of LPSs with decreasing chain lengths, whereas pool E contained LPS molecules with no or small numbers of O-antigen repeating units (Fig. 6). Dot blotting was used to ascertain the LPS nature of the different pools of LPS. *A. pleuropneumoniae* serotype 1 extracted LPS and all fractions reacted with the monoclonal antibody directed against *A. pleuropneumoniae* serotype 1 O antigen and not with the monoclonal antibody directed against *A. pleuropneumoniae* serotype 1 capsular polysaccharides.

Inhibition of adherence was then done with the different pools. Pool A, containing the high-molecular-mass fractions of *A. pleuropneumoniae* serotype 1 LPS, clearly showed more inhibition activity than the other pools (Fig. 7), an activity comparable to that obtained with control, unfractionated LPS. Similar results were obtained with fractions of *A. pleuropneumoniae* serotype 2 LPS (data not shown).

Finally, fractionated LPSs were examined by transmission electron microscopy after negative staining. Molecular aggregates of extracted LPS as well as fractions corresponding to pools B to D appeared as ribbons approximately 12 nm wide (Fig. 8A and C). High-molecular-mass fractions of pool A contained thin filaments approximately 2 nm wide (Fig. 8B), whereas vesicles 55 to 75 nm in diameter were observed in the low-molecular-mass fractions of pool E (Fig. 8D).

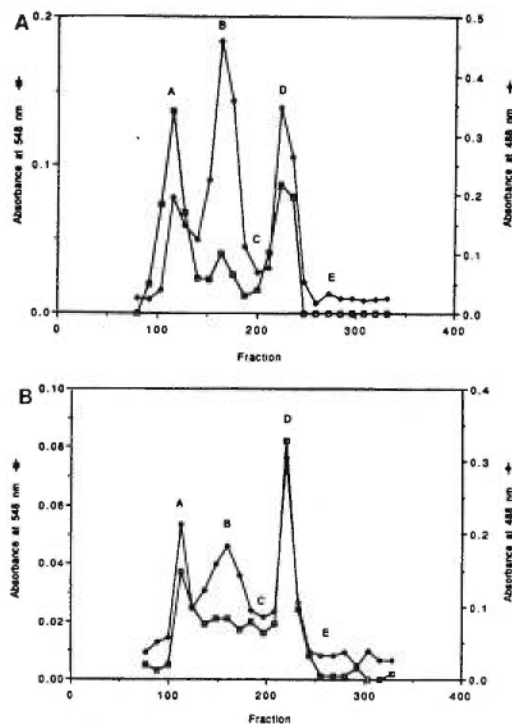

FIG. 5. Fractionation of extracted LPS from *A. pleuropneumoniae* serotype 1 (A) or serotype 2 (B) on a Sephacryl S-300SF column in the presence of 0.25% deoxycholate. Fractions (milliliters) were analyzed for KDO content (squares) and for neutral sugar content (diamonds). Fractions were grouped into five pools (A to E).

DISCUSSION

The initiating event in the pathogenesis of most bacterial pulmonary infections is most probably the establishment of the organisms in the upper respiratory tract. Adherence is a complex interaction between the bacterium and the target cell which enables colonization to occur and allows the bacterium to exert its pathogenic and immunogenic effects. We have previously shown the involvement of LPS in adherence of *A. pleuropneumoniae* to porcine respiratory tract cells and mucus (3, 4). Cells of *A. pleuropneumoniae* serotype 1 and 2 reference strains have been shown to be covered by a capsule layer of approximately 220 and 85 nm, respectively, as determined by electron microscopy after immunostabilization (21). The results of the present study, using immunoelectron microscopy and flow cytometry, indicate that LPSs are indeed accessible at the surface of this encapsulated microorganism, which is an essential prerequisite for any bacterial adhesin. Surface exposure of LPS has been reported for other heavily encapsulated organisms, including *Klebsiella pneumoniae* (35).

We used frozen sections to further study the involvement of LPS in the adherence of *A. pleuropneumoniae* to porcine respiratory tract cells. This adhesion assay combined with immunostaining showed that *A. pleuropneumoniae* extracted LPS adhered to the vascular endothelium and the mesenchyme of porcine lung and to the tracheal epithelium. These observations support the idea that *A. pleuropneumoniae* LPSs play an important role in adherence. Our in vitro model for adhesion of *A. pleuropneumoniae* with tracheal frozen sections was more convenient for the quantitative evaluation of attached bacteria than that with lung frozen sections because it allowed us to count bacterial cells over a definite area (i.e., the epithelial surface). Adherence inhibition experiments showed that extracted LPSs were able to prevent the adherence of *A. pleuropneumoniae*, which confirmed our previous work with porcine tracheal rings maintained in culture (3).

Acid hydrolysis of LPS clearly indicated that the polysaccharides were the essential LPS components responsible for adherence inhibition, since the lipid A by itself was not able to prevent attachment of *A. pleuropneumoniae*. Most biological effects of LPS are associated with lipid A (5, 34). It has been established that molecular structures present in endotoxin specifically recognized by different cells may be located in the hydrophobic (lipid A) or in the hydrophilic (polysaccharide) regions of the macromolecule (15). Although most LPS receptors are known to recognize the lipid A (15, 38), a lectin-like binding site for endotoxin has been identified on activated T cells (25) and on macrophages (14, 16). We do not know at this time what are the cellular receptors for *A. pleuropneumoniae* LPS.

Gel chromatography on Sephacryl S-300SF identified the high-molecular-mass fractions as the most effective molecules in adherence inhibition. These high-molecular-mass fractions were not detected by SDS-PAGE and silver staining. However, the KDO and Dubois assays revealed the presence of KDO and neutral sugars, as for the other fractions. Furthermore,

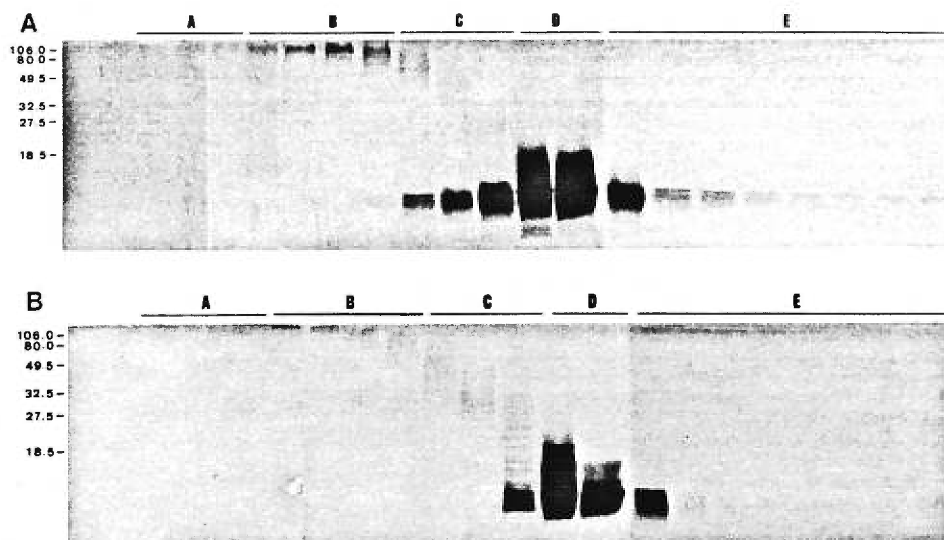

FIG. 6. Silver-stained SDS-PAGE profiles of *A. pleuropneumoniae* serotype 1 (A) or serotype 2 (B) extracted LPS fractionated on a gel filtration column. Pools of fractions (A to E) are as in Fig. 5. The positions of low-molecular-mass markers (in kilodaltons) are indicated on the left.

monoclonal antibodies against *A. pleuropneumoniae* O antigen reacted with all these fractions. The lack of silver-stained bands in the higher-molecular-mass fractions may be due to the inability of the material to penetrate in the separating gel and/or to the absence of reactive groups involved in the staining reaction, as reported by Peterson and McGroarty, who observed the same phenomenon with *Escherichia coli* O111:B4 LPS (33). Interestingly, the gel filtration chromatography demonstrated that *A. pleuropneumoniae* serotype 2 smooth LPS is composed of O-antigen repeating units distributed more uniformly than the O-antigen repeating units of *A. pleuropneumoniae* serotype 1 semirough LPS. SDS-PAGE

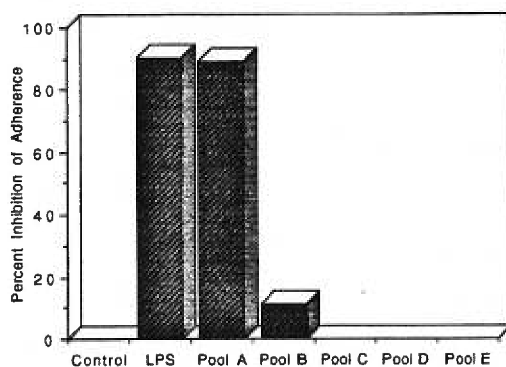

FIG. 7. Inhibition of adherence of an *A. pleuropneumoniae* serotype 1 isolate to porcine tracheal frozen sections by pooled fractions obtained after gel filtration chromatography of extracted LPS from *A. pleuropneumoniae* serotype 1.

profiles showed a very constant decrease in molecular mass for *A. pleuropneumoniae* serotype 2 fractionated LPS. This was not observed for *A. pleuropneumoniae* serotype 1 fractionated LPS, in which the distribution of O-chain lengths was bimodal.

The electron microscopic examination showed a filamentous form for the higher-molecular-mass fractions of LPS. These filaments had a smaller diameter and longer length than the ribbons observed in the extracted LPS preparation and did not adopt a vesicle conformation as observed with the lower-molecular-mass fractions. Kato (23) reported that negatively stained smooth LPSs from *K. pneumoniae* O3, *E. coli* O9 and O127, and *Salmonella minnesota* have common structural features consisting principally of ribbon-like structures, which branch freely and often form loops, and of spheres; both structures are covered with fine hairy structures. Kato also suggested that the surface projections covering the ribbon-like structures and the spherical structures would be polysaccharides. These observations suggest that LPS aggregates adopt different shapes depending on the O-antigen chain length and the lipid content. Similar results with LPS of *E. coli* O111:B4 have been described (32).

Fractionation of *A. pleuropneumoniae* LPS showed that low-molecular-mass fractions corresponding to the core-lipid A region did not inhibit adherence but that the high-molecular-mass fractions were responsible for adherence inhibition. This was observed with the semirough (serotype 1) as well as the smooth (serotype 2) LPSs of *A. pleuropneumoniae*. Our results suggest that longer polysaccharide chains embodied the adhesin and that these longer chains, possibly because of their conformation, are more able to efficiently inhibit adherence. Interestingly, we have shown in a previous study that adherence of *A. pleuropneumoniae* isolates to porcine tracheal rings was related to their LPS profile; isolates with a smooth profile adhered in higher numbers than isolates with a semirough profile (3). Taken together, our results clearly suggest that

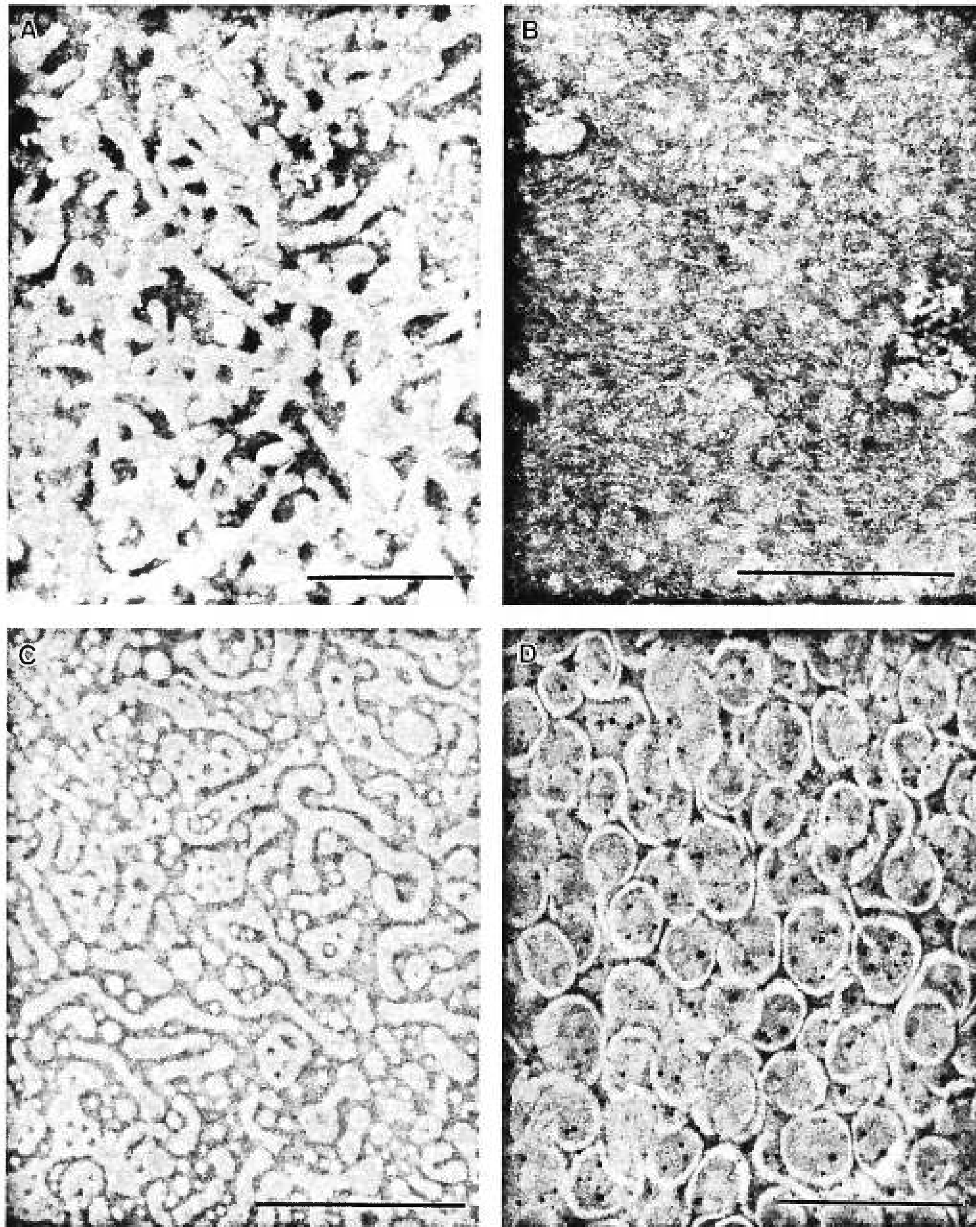

FIG. 8. Transmission electron micrographs of negatively stained *A. pleuropneumoniae* serotype 2 LPS. (A) Extracted LPS preparation. (B to D) LPS preparations obtained after chromatography on a Sephacryl S-300SF column. (B) Pool A; (C) pool C; (D) pool E. Bars, 200 nm.

long-chain LPSs play a predominant role in adherence of *A. pleuropneumoniae*. Since many bacterial adhesins also act as biological effector molecules (19), eukaryotic cells would be expected to experience the well-known effects of endotoxin in conjunction with the presentation of an LPS adhesin. Further studies are needed to identify the cellular receptors and to determine whether *A. pleuropneumoniae* LPS does elicit a response in the target cells.

ACKNOWLEDGMENTS

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC A3428 to M.J.), from Association Pulmonaire du Québec, and from a research partnership program involving the Natural Sciences and Engineering Research Council of Canada, Agriculture Canada, and Fédération des Producteurs de Porcs du Québec. Sonia-Élaine Paradis is the recipient of a studentship from Fonds pour la Formation de Chercheurs et l'Aide à la Recherche.

We are grateful to Bernadette Foiry for performing the electron microscopy, to Serge Sénéchal for his expertise in flow cytometry, and to Caroline Bégin for technical assistance.

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Article 2

Identification of two core types in lipopolysaccharides of *Actinobacillus pleuropneumoniae* representing serotypes 1 to 12

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Canadian Journal of Microbiology. **42:** 855-858, 1996

Dans cet article, la présence de deux types de noyau OS-lipide A chez les LPS d'*A. pleuropneumoniae* est établie. Ces résultats amènent ainsi des informations supplémentaires quant à la caractérisation des adhésines lipopolysaccharidiques d'*A. pleuropneumoniae.*

Dans ce travail, j'ai contribué à la mise au point des gels Tricine SDS-PAGE et j'ai participé à l'analyse des résultats et à la rédaction du manuscrit.

Identification of two core types in lipopolysaccharides of *Actinobacillus pleuropneumoniae* representing serotypes 1 to 12

Mario Jacques, Stéphane Rioux, Sonia-Élaine Paradis, Caroline Bégin, and Marcelo Gottschalk

Abstract: Lipopolysaccharides (LPS) of *Actinobacillus pleuropneumoniae* were separated by Tricine–SDS–polyacrylamide gel electrophoresis, which has been shown to improve resolution of low-molecular-mass fast migrating bands. Strains representing the 12 serotypes of *A. pleuropneumoniae* can be divided in two groups according to the gel mobility of the core – lipid A region of their LPS. The first electromorphic core type (core type I), found in serotypes 1, 6, 9, and 11, had a migration slower than *Salmonella typhimurium* Ra LPS. The second electromorphic core type (core type II), found in the remaining serotypes (i.e., 2, 3, 4, 5, 7, 8, 10, and 12) had a migration similar to *S. typhimurium* Ra LPS. Furthermore, we observed that these two core types were antigenically different. Western blot analyses indicated that core – lipid A region of LPS from electromorphic core type I strains reacted when probed with serum from a pig experimentally infected with a core type I strain but not when probed with serum from a pig experimentally infected with a core type II strain. Conversely, core – lipid A region of LPS from electromorphic core type II strains reacted only when probed with serum from a pig experimentally infected with a core type II strain. Our results, based on both electrophoretic mobility and antigenicity, suggest the presence of two LPS core types in *A. pleuropneumoniae*.

Key words: *Actinobacillus pleuropneumoniae*, lipopolysaccharides, core.

Résumé : Les lipopolysaccharides (LPS) d'*Actinobacillus pleuropneumoniae* ont été séparés par électrophorèse sur gel de polyacrylamide en présence de Tricine et de SDS, une méthode qui permet une meilleure résolution des molécules de faible masse moléculaire. Les souches représentant les 12 sérotypes d'*A. pleuropneumoniae* peuvent être divisées en deux groupes selon la mobilité sur gel de la région noyau oligosaccharidique – lipide A de leurs LPS. Le premier type électrophorétique (type I) migre plus lentement que le LPS de *Salmonella typhimurium* Ra et est retrouvé chez les sérotypes 1, 6, 9 et 11. Le second type électrophorétique (type II) a une migration similaire au LPS de *S. typhimurium* Ra et est retrouvé chez les autres sérotypes. De plus, nous avons observé que ces deux types électrophorétiques étaient aussi antigéniquement différents. Des analyses par immunobuvardage ont montré que la région noyau oligosaccharidique – lipide A des LPS du type I réagissait avec un antisérum provenant d'un porc infecté expérimentallement avec une souche du type I mais ne réagissait pas avec un antisérum provenant d'un porc infecté expérimentallement avec une souche du type II. De la même manière, la région noyau oligosaccharidique – lipide A des LPS du type II réagissait uniquement avec un antisérum provenant d'un porc infecté expérimentallement avec une souche du type II. Nos résultats, basés sur la mobilité électrophorétique et l'antigénicité, suggèrent la présence de deux types de noyau oligosaccharidique chez les LPS d'*A. pleuropneumoniae*.

Mots clés : Actinobacillus pleuropneumoniae, lipopolysaccharides, noyau oligosaccharidique.

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia (Nicolet 1992), a disease found worldwide that causes tremendous economic loss to the swine industry. Twelve serotypes of *A. pleuropneumoniae* based on capsular antigens have been recognized (Nielsen 1986*b*), with serotype 5 divided into 5a and 5b (Nielsen 1986*a*). The chemical structures of the capsular polysaccharides, as well as the O-polysaccharides, of the 12 serotypes of *A. pleuropneumoniae* have been determined (Perry et al. 1990).

Lipopolysaccharides (LPS) are among the important virulence factors of *A. pleuropneumoniae*. We previously demonstrated that high-molecular-mass LPS were the major adhesin of *A. pleuropneumoniae* involved in adherence to porcine respiratory tract cells (Bélanger et al. 1990; Paradis et al. 1994). More recently, we observed that *A. pleuropneumoniae* LPS were binding pig hemoglobin and that the core – lipid A region was involved (Bélanger et al. 1995). The purpose of the present study was to characterize, using gel electrophoresis and Western blotting, the core – lipid A region of *A. pleuropneumoniae* LPS representing the 12 serotypes.

Received December 18, 1995. Revision received March 26, 1996. Accepted April 29, 1996.

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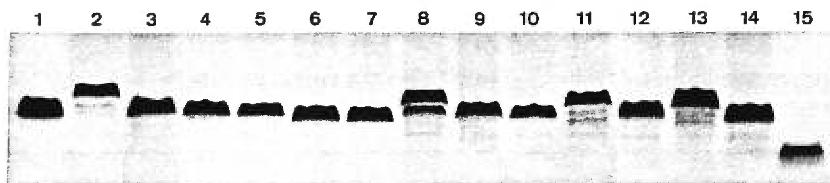
Fig. 1. Silver-stained Tricine–SDS–PAGE profiles of whole-cell, proteinase-K-treated preparations of *A. pleuropneumoniae* reference strains representing serotypes 1 (lane 2; representative of core type I), 2 (lane 3; representative of core type II), 3 (lane 4), 4 (lane 5), 5a (lane 6), 5b (lane 7), 6 (lane 8), 7 (lane 9), 8 (lane 10), 9 (lane 11), 10 (lane 12), 11 (lane 13), and 12 (lane 14). For comparison, LPS (2 μg) from *S. typhimurium* TV119 (lane 1; Ra mutant) and *S. typhimurium* SL1181 (lane 15; Re mutant) are shown.



Reference strains representing serotypes 1–12 of *A. pleuropneumoniae* were grown on PPLO agar base (Difco Laboratories, Detroit, Mich.) plates supplemented with 10% yeast extract, 0.1% dextrose, and 5% horse serum, at 37°C in a 5% CO_2 atmosphere for 18–24 h. We used Tricine–SDS–polyacrylamide gel electrophoresis (TSDS–PAGE), a method which has been shown to increase the resolution of lipooligosaccharides and the core region of LPS (Lesse et al. 1990; Sprott et al. 1994). The separating gel was prepared at a final concentration of 18% acrylamide, 0.36% bisacrylamide, and 10.5% glycerol. The gel was polymerized by the addition of 40 μL of 10% ammonium persulfate and 4 μL of TEMED (per 20 mL of gel). The stacking gel was prepared at a final concentration of 4.5% acrylamide and 0.08% bisacrylamide, and polymerized by the addition of 30 μL of 10% ammonium persulfate and 10 μL of TEMED (per 10 mL of gel). The buffers were as follows: anode buffer (0.2 M Tris–HCl, pH 8.9), cathode buffer (0.1 M Tris–HCl, 0.1 M Tricine, 0.1% SDS, pH 8.25), and 4× gel buffer stock solution (4.0 M Tris, 0.4% SDS, pH 8.45). Agar-grown bacteria (2 mg) were suspended in 100 μL of solubilization buffer (5.9 mL of 0.06 M Tris–HCl – 1 mM EDTA – 2% SDS (pH 6.8), 4 mL glycerol, 0.8 mL 2-mercaptoethanol, and 0.4 mL saturated solution of bromophenol blue), and then boiled for 20 min. An equal volume of proteinase K (1 mg/mL; Sigma Chemical Co., St Louis, Mo.) prepared in 50 mM Tris–HCl (pH 8.0) containing 1 mM $CaCl_2$ was added to the samples, and the mixtures were incubated for 60 min at 60°C. Samples containing 10 μg KDO/mL (Daniels et al. 1994) were electrophoresed for 3 h at 38 mA using a Mini-Protean II apparatus (Bio-Rad Laboratories, Richmond, Calif.). Gels were fixed 30 min in 40% ethanol – 5% acetic acid solution and stained with the silver-staining procedure of Tsai and Frasch (1982). Extracted LPS from *Salmonella typhimurium* TV119 (Ra mutant) and SL1181 (Re mutant) (Sigma) were used as standards.

Separation and visualization of components of the core – lipid A region of *A. pleuropneumoniae* LPS was achieved by using gels loaded with 8 μL (Fig. 1). Reference strains could be divided in two electromorphic core types according to their mobility. Reference strains of serotypes 1, 6, 9, and 11 form the first electromorphic core type (core type 1; Fig. 1, lanes 2, 8, 11, and 13), composed of three distinct bands, which migration of the major band was slower than *S. typhimurium* Ra LPS. Reference strains of the other serotypes (i.e., 2, 3, 4, 5a, 5b, 7, 8, 10, and 12) exhibited another electromorphic core type (core type II; Fig. 1, lanes 3–7, 9, 10, 12, and 14), composed of one band, which migration was similar to *S. typhimurium* Ra LPS.

Although the structural diversity of the core region is low compared with that of the O-chain, different core types have been observed with other bacterial species. In *Escherichia coli*, for example, five core types have been identified (R1–R4 and K-12) (Reeves 1994; Rietschel et al. 1994). Five core types have been also recognized for *Proteus* and three have been recognized for *Citrobacter* (Rietschel et al. 1994). Results from a recent study by de Kievit and Lam (1994), using monoclonal antibodies, strongly suggest that a structural diversity exists within the outer core region of *Pseudomonas aeruginosa* LPS. Finally, in *Pasteurella multocida*, a member of the *Pasteurellaceae* family as is *A. pleuropneumoniae*, the LPS representing the 16 somatic serotypes could be divided into two groups on the basis of the leading band mobility (Rimler 1990).

We previously observed antigenic cross-reactions between the core – lipid A region of different serotypes of *A. pleuropneumoniae* that complicated the serodiagnosis of the infection (Radacovici et al. 1992). We, thus, decided to determine whether these cross-reactions were associated with the two electromorphic core types.

Western blot (immunoblot) experiments were performed by the method of Towbin et al. (1979) as described previously (Li et al. 1992). The samples separated by electrophoresis were transferred to nitrocellulose membranes (0.2 μm; Bio-Rad) and incubated with pig antisera overnight at 4°C. These antisera were obtained from pigs experimentally infected with the serotype 1, 2, 3, or 6 reference strain of *A. pleuropneumoniae* as previously described (Radacovici et al. 1992).

Western blot analyses indicated that core – lipid A region of LPS from electromorphic core type I strains reacted when probed with serum from a pig experimentally infected with a core type I strain (serotype 1 antiserum in Fig. 2A, lanes 1–4; serotype 6 antiserum in Fig. 3A, lane 2) but not when probed with serum from a pig experimentally infected with a core type II strain (serotype 2 antiserum in Fig. 2B, lanes 1 and 6; serotype 3 antiserum in Fig. 3B, lane 2). Conversely, core – lipid A region of LPS from electromorphic core type II strains reacted only when probed with serum from a pig experimentally infected with a core type II strain (Fig. 2A, lane 5; Fig. 2B, lanes 2–5; Figs. 3A and 3B, lanes 1 and 3). These results indicate that the two electromorphic core types are, in addition, antigenically different. These cross-reactions are different from

Fig. 2. Western blot analysis of whole-cell, proteinase-K-treated preparations of *A. pleuropneumoniae* probed with serum from a pig experimentally infected with (A) *A. pleuropneumoniae* serotype 1 reference strain (core type I) or (B) *A. pleuropneumoniae* serotype 2 reference strain (core type II). (A) *Actinobacillus pleuropneumoniae* serotypes 1 (lane 1), 6 (lane 2), 9 (lane 3), 11 (lane 4), and 2 (lane 5). (B) *Actinobacillus pleuropneumoniae* serotypes 1 (lane 1), 2 (lane 2), 3 (lane 3), 4 (lane 4), 5b (strain 81–750, lane 5), and 6 (lane 6). The positions of prestained low-molecular-mass markers (in kilodaltons) are indicated on the left. Arrows indicate the core – lipid A region.

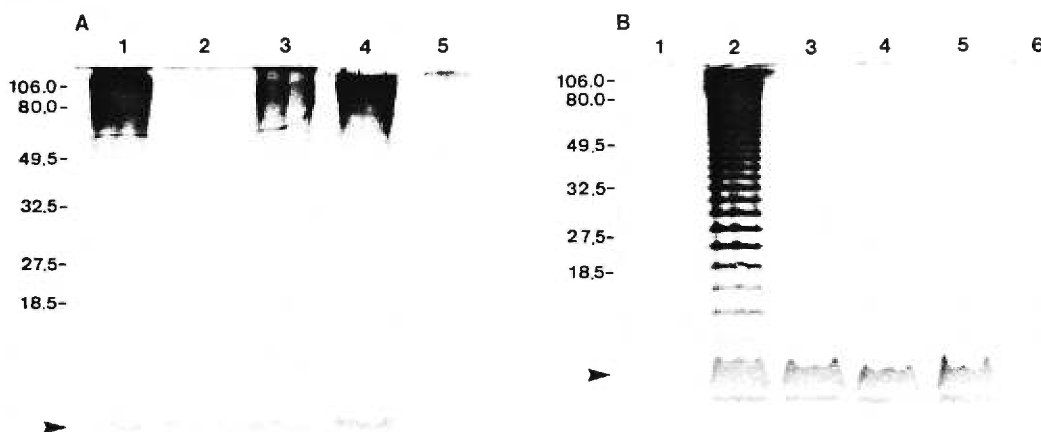
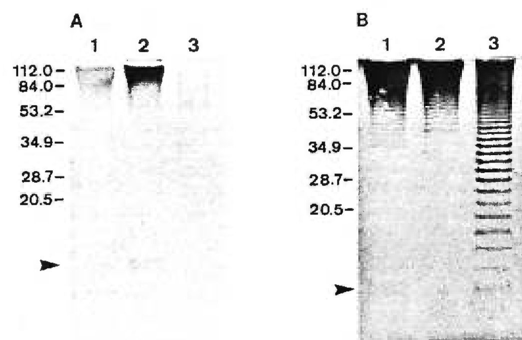


Fig. 3. Western blot analysis of whole-cell, proteinase-K-treated preparations of *A. pleuropneumoniae* probed with serum from a pig experimentally infected with (A) *A. pleuropneumoniae* serotype 6 reference strain (core type I) or (B) *A. pleuropneumoniae* serotype 3 reference strain (core type II). *Actinobacillus pleuropneumoniae* serotypes 3 (lane 1), 6 (lane 2), and 8 (lane 3) are shown. The positions of prestained low-molecular-mass markers (in kilodaltons) are indicated on the left. Arrows indicate the core – lipid A region.



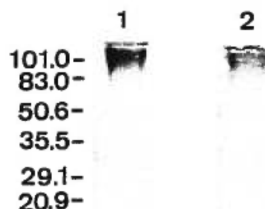
the serological cross-reactions observed between the O-polysaccharides of serotypes 1, 9, and 11 (which can also be seen in the upper part of Fig. 2A, lanes 1, 3, and 4); 4 and 7; and 3, 6, and 8 (which can also be seen in the upper part of Fig. 3), respectively (Perry et al. 1990).

It is interesting to note that, as expected, the core – lipid A region of serotypes 1 and 2 did not react when probed with monoclonal antibodies 5.1G8F10 or 102-G02 specific to serotypes 1 and 2 O-antigen (Paradis et al. 1994), respectively (Fig. 4, lanes 1 and 2). However, there is a possibility that the major bands in the core – lipid A region may be in fact core – lipid A plus one O repeating unit. It has been observed previously with *Pseudomonas aeruginosa* that monoclonal antibodies specific for O-antigen do not necessarily recognize core with one O-antigen unit attached (Lam et al. 1992).

Our results, based on both electrophoretic mobility and antigenicity, suggest the presence of two LPS core types in *A. pleuropneumoniae*. Differences in the electrophoretic mobilities and antigenic profiles probably reflect differences in composition and (or) structure of the outer core usually composed of hexoses, since the inner core is generally highly conserved. The composition of the core oligosaccharide has been determined for some serotypes of *A. pleuropneumoniae*. For example, the core oligosaccharide of serotype 1 LPS was found to be composed of D-galactose, D-glucose, L-glycero-D-mannoheptose, D-glycero-D-mannoheptose, and 2-amino-2-deoxy-D-glucose in the molar ratio of 3:3:0.5:1:3 (Altman et al. 1986), while the core oligosaccharide of serotype 2 LPS was found to be composed of D-galactose, D-glucose, L-glycero-D-mannoheptose, and D-glycero-D-mannoheptose in the molar ratio of 0.2:1:1:0.6 (Altman et al. 1987). However, the conclusive identification of the two core types in *A. pleuropneumoniae* LPS would be obtained by the use of core-specific antiserum.

It has been suggested that the core region plays an important role in the tertiary and (or) quaternary conformation of LPS, by strengthening the interaction of LPS with their different target molecules, as well as contributing to endotoxin activity

Fig. 4. Western blot analysis of whole-cell, proteinase-K-treated preparations of *A. pleuropneumoniae* serotype 1 reference strain probed with monoclonal antibody 5.1G8F10 specific to serotype 1 O-antigen (lane 1) and *A. pleuropneumoniae* serotype 2 reference strain probed with monoclonal antibody 102-G02 specific to serotype 2 O-antigen (lane 2). The positions of prestained low-molecular-mass markers (in kilodaltons) are indicated on the left.



(Cavaillon and Haeffner-Cavaillon 1992). We do not know, at the present time, whether the core type has any influence on the virulence of *A. pleuropneumoniae*.

Acknowledgments

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada (A3428 to M.J.). S.R. and S.-É.P. are both recipient of a studentship from Fonds pour la formation de chercheurs et l'aide à la recherche.

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Article 3

Examination of surface polysaccharides of *Actinobacillus pleuropneumoniae* serotype 1 grown under iron-restricted conditions

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FEMS Microbiology Letters **137:** 201-206, 1996

Les résultats, inclus dans cet article, évaluent l'effet de faibles concentrations de fer, soit une condition semblable à celle rencontrée *in vivo*, sur la production des polysaccharides de surface d'*A. pleuropneumoniae* dont les adhésines lipopolysaccharidiques.

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FEMS Microbiology Letters 137 (1996) 201–206

FEMS MICROBIOLOGY LETTERS

Examination of surface polysaccharides of *Actinobacillus pleuropneumoniae* serotype 1 grown under iron-restricted conditions

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Received 13 October 1995; revised 17 January 1996; accepted 21 January 1996

Abstract

We investigated the expression of important *Actinobacillus pleuropneumoniae* surface polysaccharides, namely, capsular polysaccharides (CPS) and lipopolysaccharides (LPS), after growth under iron-restricted conditions. Iron restriction did not seem to affect the production of CPS, as determined by labelling with a monoclonal antibody (mAb) against the serotype 1 K-antigen and flow cytometry analysis, and also as determined by electron microscopy. SDS-PAGE revealed that the LPS profiles of these cells were also unaffected by iron restriction. Using flow cytometry analysis, however, we observed that binding of mAb against serotype 1 O-antigen was altered in cells of *A. pleuropneumoniae* serotype 1 reference strain (4074) grown under iron-restricted conditions. This strain exhibited two subpopulations with distinct patterns of reactivity with the mAb against the O-antigen. When strain 4074 was grown under iron-restricted conditions, a shift from one cell subpopulation (moderately fluorescent) to another cell subpopulation (highly fluorescent, thus binding more antibodies) was observed. Our results indicate that growth of *A. pleuropneumoniae* serotype 1 under iron-restricted conditions did not seem to affect CPS production, but might alter, at least for the reference strain, the expression of LPS.

Keywords: *Actinobacillus pleuropneumoniae*; Iron restriction; Capsule; Lipopolysaccharide

1. Introduction

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia [1], a disease found worldwide that causes tremendous economic loss to the swine industry. Twelve serotypes of *A. pleuropneumoniae* based on capsular antigens have been recognized [2]. In Québec, serotype 1 is by far the most predominant [3]. The pathogenesis of porcine pleuropneumonia is not well understood but RTX-toxins as well as surface molecules such as capsular polysaccharides (CPS), lipopolysaccharides (LPS), and outer membrane proteins (OMPs), are thought to play an important role [4,5]. We previously demonstrated that LPS were the major adhesin of *A. pleuropneumoniae* involved in adherence to porcine respiratory tract cells [6,7], and that LPS were involved in binding pig hemoglobin [8].

Several studies have investigated the expression of iron-regulated OMPs by *A. pleuropneumoniae*

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PII S0378-1097(96)00055-9

including porcine transferrin receptors [9–13], but none has dealt with the expression of surface polysaccharides during growth under iron-restricted conditions. Thus, the purpose of the present study was to evaluate the expression of surface polysaccharides of *A. pleuropneumoniae* grown under iron-restricted conditions.

2. Materials and methods

2.1. Bacterial isolates and growth conditions

The field isolate (FMV87-682, serotype 1) and the reference strain representing serotype 1 (strain 4074) of *A. pleuropneumoniae*, used in this study, have been described previously [6]. Bacteria were grown on BHI (Difco Laboratories, Detroit, MI) agar plates supplemented with 15 $\mu g\ ml^{-1}$ NAD (iron-sufficient conditions). To obtain bacterial growth under conditions of iron restriction, organisms were inoculated onto plates supplemented with 160 μM 2,2′-dipyridyl (Sigma Chemical Co., St. Louis, MO) [9,14]. Plates were incubated at 37°C in a 5% CO_2 atmosphere for 18 h.

2.2. Transmission electron microscopy

2.2.1. Thin sections

Capsular material of *A. pleuropneumoniae* strains was either immunostabilized and stained with ruthenium red before examination by transmission electron microscopy (TEM) or prepared for TEM following polycationic ferritin labelling as described by Jacques et al. [15].

2.2.2. Immunolabelling

Bacterial cells were placed on Formvar-coated grids wich were placed sequentially on drops of phosphate-buffered saline (PBS: 0.01 M, pH 7.4) containing 1% (w/v) egg albumin for 5 min and mouse monoclonal antibodies (mAb) against *A. pleuropneumoniae* serotype 1 O-antigen (5.1 G8F10) (kindly supplied by Eva I. Stenbaek, Department of Biochemistry and Immunology, National Veterinary Laboratory, Copenhagen V, Denmark) or *A. pleuropneumoniae* serotype 1 K-antigen (LA 40#1.M) (kindly supplied by Marcelo Gottschalk, Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, Québec, Canada) for 30 min. They were then washed in distilled water and placed on drops of colloidal gold particles (10 nm) conjugated to goat anti-mouse IgG (Sigma) for 30 min. After a final wash in distilled water, they were stained with 0.2% (wt/vol) phosphotungstate (pH 7.1) and were then examined with a Philips 201 electron microscope at an accelerating voltage of 60 kV.

2.3. Flow cytometry

Cells of *A. pleuropneumoniae* were incubated with either mAb against serotype 1 O-antigen or serotype 1 K-antigen, or mAb E5 directed against bacterial lipid A (kindly supplied by P. Dessain, Pfizer Canada, Inc., Pointe-Claire, Québec, Canada) and analyzed by flow cytometry as described previously [7]. The flow analysis was performed with a FACStar flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with a water-cooled 2 W argon ion laser operating at 488 nm and a 200 mW light output.

2.4. Electrophoresis and Western blot analysis

LPS profiles of whole-cell proteinase K-treated preparations of *A. pleuropneumoniae* were determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5% polyacrylamide separating gel [6]. Gels were either stained with silver nitrate as described by Tsai and Frasch [16] or transferred to nitrocellulose for Western blotting (immunobloting). Western blots were carried out as described by Towbin et al. [17]. The membrane was first blocked with 2% casein in Tris-saline buffer (TSB; 10 mM Tris, 150 mM NaCl, pH 7.4), then incubated overnight with the mAb against serotype 1 O-antigen in 2% casein-TSB and finally incubated 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Richmond, CA) again in 2% casein-TSB. The membrane was washed three times in TSB between each incubation. The reaction was revealed by addition of H_2O_2 and 4-chloro-1-naphthol (Sigma) as the chromogenic substrate.

3. Results and discussion

We first examined the production of capsular material by cells of both strains of *A. pleuropneumoniae* grown under iron-restricted conditions. Cells of *A. pleuropneumoniae* were incubated with mAb LA 40#1.M against serotype 1 K-antigen, and analyzed by flow cytometry. Fig. 1 shows representative flow cytometry profiles of whole cells of *A. pleuropneumoniae* serotype 1 reference strain 4074 grown under iron-sufficient (Fig. 1A) or iron-restricted conditions (Fig. 1B). The expression of capsular material did not seem to be affected by growth under iron-restricted conditions, since the fluorescence patterns obtained were similar for both growth conditions. When cells of both strains of *A. pleuropneumoniae* were exposed to whole-cell homologous rabbit antiserum and stained with ruthenium red for TEM, a layer of capsular material of approximately 150–180 nm was observed after growth under iron-sufficient or iron-restricted conditions (data not shown). No differences in capsular material thickness between cells grown under iron-sufficient or iron-restricted conditions were observed when cells were labelled with polycationic ferritin, another classical way to stabilize the capsular polysaccharides before TEM (data not shown). Interestingly, with *Pasteurella multocida*, another important swine-pathogenic member of the Pasteurellaceae, iron deprivation markedly reduced the amount of capsular material covering the cells [18]. It is of interest to note that cells of *A. pleuropneumoniae* grown under these iron-restricted conditions expressed iron-regulated proteins, as reported by others [9,12], when detected by Coomassie brillant blue staining of SDS-PAGE profiles and by Western blot using serum from a pig experimentally infected with the reference strain of *A. pleuropneumoniae* serotype 1 (data not shown), indicating that the iron deprivation was indeed achieved.

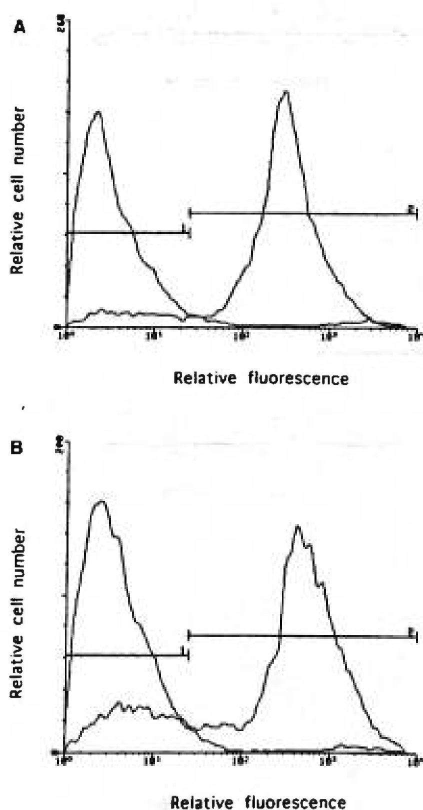


Fig. 1. Flow cytometry analysis of *A. pleuropneumoniae* serotype 1 reference strain 4074 grown under iron-sufficient (A) or iron-restricted conditions (B). Cells were labelled with Mab against serotype 1 capsular antigen and anti-mouse IgG FITC-conjugated antibody. In both panels, the left peak represents control cells incubated with the anti-mouse IgG FITC-conjugated antibody. The left horizontal bars indicate non-fluorescent cells (1) and the right horizontal bars indicate fluorescent cells (2).

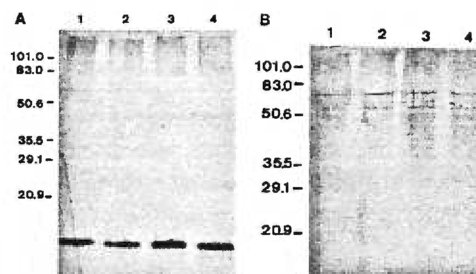
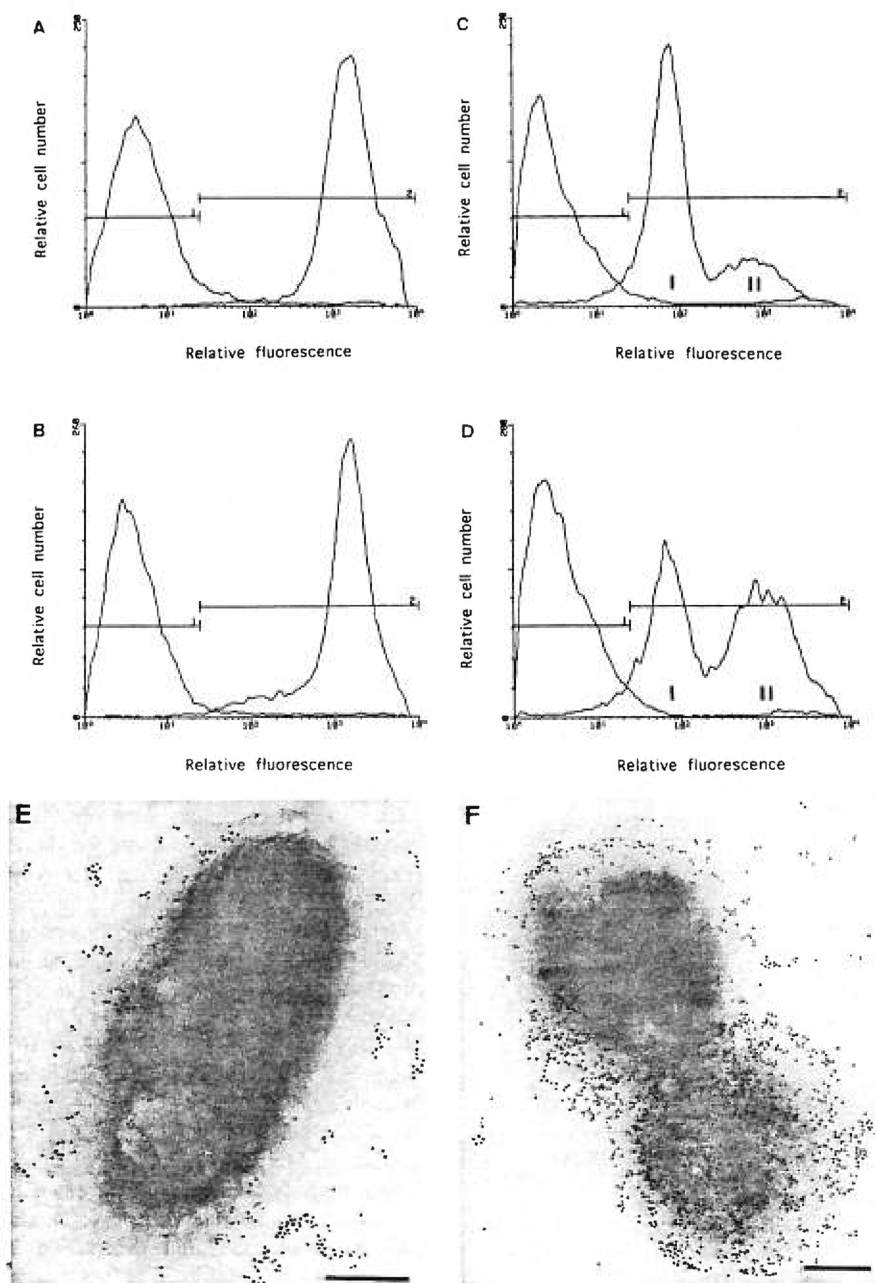


Fig. 2. Electrophoretic and Western blot analyses of whole-cell, proteinase K-treated preparations of A. pleuropneumoniae serotype 1 reference strain 4074 (lanes 1 and 2) and field isolate FMV87-682 (lanes 3 and 4) grown under iron-sufficient (lanes 1 and 3) or iron-restricted conditions (lanes 2 and 4). Preparations were silver-stained (A) or transferred to a nitrocellulose membrane and then probed with the mAb 5.1 G8F10 (B). The positions of molecular mass markers (in kDa) are indicated on the left.


A
B
C
D
E
F
Relative cell number
Relative fluorescence
I
II

We then determined the LPS profiles of cells of *A. pleuropneumoniae* by SDS-PAGE. As seen in Fig. 2A, the profile of cells grown under iron-sufficient conditions (lanes 1 and 3) was similar to that of cells grown under iron-restricted conditions (lanes 2 and 4) for the two serotype 1 strains studied. Furthermore, when transferred to a nitrocellulose membrane these preparations had the same reactivity pattern when probed with mAb 5.1 G8F10 directed specifically against the serotype 1 O-antigen (Fig. 2B)

Cells of *A. pleuropneumoniae* were incubated with mAb 5.1 G8F10 and analyzed by flow cytometry. Interestingly, the fluorescence patterns produced by the mAb against the O-antigen were not identical for the two strains of *A. pleuropneumoniae* serotype 1 tested. The field strain FMV87-682 showed only one peak of high fluorescence intensity after growth under iron-sufficient conditions (Fig. 3A), as we reported before [7], and also after growth under iron-restricted conditions (Fig. 3B). The reference strain 4074, however, demonstrated a biphasic fluorescence pattern (Fig. 3C and D). When cells of the reference strain were grown under iron-sufficient conditions, 77% of the fluorescence produced appears in a low-intensity peak (peak I), while the remaining 23% of fluorescence is found in a second, higher intensity peak (peak II) (Fig. 3C). When cells of the reference strain were grown under iron-restricted conditions, a shift of cells from the low-intensity peak I to the high-intensity peak II (binding more antibodies) was observed (Fig. 3D), and approximately 50% of the fluorescence was associated with each subpopulations of cells. This shift was reversible when the cells of the reference strain were grown under iron-replete conditions, i.e. when the cells were grown on agar plates containing in addition to the iron chelator an iron source (50 μM $FeCl_3$) to overcome the iron depletion conditions (data not shown). It is noteworthy that when cells of the reference strain were incubated with mAb E5, directed against another region of the LPS molecules (the lipid A), only one peak of high fluorescence intensity was observed (data not shown).

Immunoelectron microscopy likewise identified bacterial subpopulations exhibiting distinct patterns of reactivity with the mAb 5.1 G8F10. Some cells of *A. pleuropneumoniae* serotype 1 reference strain were moderately labelled with gold particles (Fig. 3E) while others were heavily labelled (Fig. 3F); these cells most probably correspond to cells found in peaks I and II observed by flow cytometry, respectively.

The results obtained with the three mAbs indicate the presence of two distinct cell subpopulations in *A. pleuropneumoniae* reference strain 4074 on the basis of differential binding of mAb 5.1 G8F10 against the O-antigen. The presence of these two cell subpopulations is probably not due to differences in capsulation since labelling with a mAb against the K-antigen resulted in only one population (peak), nor to different amounts of LPS since labelling with a mAb against the lipid A resulted in only one population. One possible explanation for the presence of the oberved two subpopulations might be the bimodal distribution of O-chain lengths in *A. pleuropneumoniae* LPS [7]; one subpopulation of cells expresses LPS with longer O-chains than the other subpopulation, and can thus bind more antibodies at their surface. Another possibility is that the observed cell subpopulations correspond to the two serotype 1 LPS subtypes (1A and 1B) recently proposed by Jolie et al. [19].

We have previously shown the accessibility of LPS at the cell surface of *A. pleuropneumoniae* [7], which is an essential prerequisite for a bacterial adhesin. The results of the present study indicate that although capsule production was not affected, iron deprivation might influence the expression of LPS resulting, at least for serotype 1 reference strain, in an increased accessibility at the cell surface. A vari-

Fig. 3. Flow cytometry analysis of *A. pleuropneumoniae* serotype 1 field isolate FMV87-682 grown under iron-sufficient (A) or iron-restricted conditions (B), and serotype 1 reference strain 4074 grown under iron-sufficient (C) or iron-restricted conditions (D). Cells were labelled with Mab against serotype 1 O-antigen and anti-mouse IgG FITC-conjugated antibody. In all panels, the left peak represents control cells incubated with the anti-mouse IgG FITC-conjugated antibody. The left horizontal bars indicate non-fluorescent cells (1) and the right horizontal bars indicate fluorescent cells (2). Transmission electron micrographs of whole cells of *A. pleuropneumoniae* serotype 1 reference strain probed with Mab against serotype 1 O-antigen (E and F). Bars, 200 nm.

ety of growth conditions have been shown to alter the expression of LPS in other Gram-negative bacteria including *Escherichia coli* [20] and *Pseudomonas aeruginosa* [21]. In vivo, where free iron concentration is extremely low, the increased exposition of LPS at the cell surface might be beneficial for *A. pleuropneumoniae* considering the involvement of its LPS in adherence and hemoglobin-binding.

Acknowledgements

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada (A3428 to M.J.). S.-É.P. is the recipient of a studentship from Fonds pour la Formation de Chercheurs et l'Aide à la Recherche. We are grateful to Bernadette Foiry and Caroline Bégin for technical assistance, and to Serge Sénéchal for expertise in flow cytometry.

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Article 4

Inhibition of adherence of *Actinobacillus pleuropneumoniae* to porcine respiratory tract cells by monoclonal antibodies directed against LPS and partial characterization of the LPS receptors

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Soumis au journal Microbiology

Ce dernier article, présenté dans cette thèse, complète le principal bloc d'objectifs de mon projet de doctorat. Les résultats obtenus mettent en évidence le pouvoir inhibiteur d'anticorps spécifiquement dirigés contre l'Ag-O des LPS d'*A. pleuropneumoniae* et apportent de premières informations quant à des récepteurs potentiels qui seraient présents au niveau des voies respiratoires.

Inhibition of Adherence of *Actinobacillus pleuropneumoniae* to Porcine Respiratory Tract Cells by Monoclonal Antibodies Directed Against LPS and Partial Characterization of the LPS Receptors

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Key words: - *Actinobacillus pleuropneumoniae*
- Adherence
- Lipopolysaccharides
- Respiratory tract cells
- Receptors

Running title: LPS of *A. pleuropneumoniae*

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Abbreviation: O-Ag, O-antigen

SUMMARY

Actinobacillus pleuropneumoniae is the causative agent of porcine fibrinohemorrhagic necrotizing pleuropneumonia. We have previously identified the lipopolysaccharides (LPS) as the major adhesin of *A. pleuropneumoniae* and demonstrated that the high-molecular-mass LPS were involved in adherence of *A. pleuropneumoniae* to porcine respiratory tract cells. In the present study, adherence of *A. pleuropneumoniae* to tracheal frozen sections was inhibited by homologous monovalent Fab fragments produced from monoclonal antibodies 5.1 G8F10 and 102-G02 directed, respectively, against the *A. pleuropneumoniae* serotype 1 or serotype 2 O-antigens. These results confirm the important role played by LPS in adherence of *A. pleuropneumoniae* and suggest that these adhesins might represent good vaccine candidates. We also investigated the presence of *A. pleuropneumoniae* receptors in tracheal cells preparation from crossbred piglets of four different breeds. By Far-Western binding assays, we identified proteins recognized by whole cells of *A. pleuropneumoniae* reference strains for serotype 1 and 2, and local isolates belonging to the same serotypes, and also recognized by extracted LPS from both reference strains. We confirmed the proteinaceous nature of these LPS-binding molecules by their staining with Coomassie brilliant blue, sensitivity to proteinase K digestion, resistance to sodium m-periodate oxidation, and their inability to stain with glycoproteins specific reagents. Four low molecular mass bands (14-17 kDa) seemed to correspond to histones. We also identified proteins at Mr 38 500 that could represent putative receptors for *A. pleuropneumoniae* LPS in swine respiratory tract cells.

INTRODUCTION

Actinobacillus pleuropneumoniae is the causative agent of porcine fibrinohemorragic necrotizing pleuropneumonia (Nicolet, 1992) a worldwide spread disease causing tremendous economic loss to the swine industry. Twelve serotypes of *A. pleuropneumoniae* based on capsular antigens have been recognized. In Québec, serotypes 1 and 5 are predominant while serotype 2 is dominant in most European countries (Mittal *et al.*, 1992). The mechanism of pathogenicity of porcine pleuropneumonia is not completely understood. Several bacterial cytotoxic and hemolytic activities have been described (Frey,

1995). However, the virulence factors involved in *A. pleuropneumoniae* colonization of the respiratory tract still remain largely unknown.

We have previously reported that lipopolysaccharides (LPS) were the major adhesin of *A. pleuropneumoniae* mediating its adherence to porcine tracheal rings maintained in culture (Bélanger *et al.*, 1990). The LPS have been shown to play an important role in the adherence of various Gram negative bacteria to host cells (Jacques, 1996). The LPS are complex molecules composed of three well-defined regions: the lipid A; the core region, an oligosaccharide containing KDO; and the O-antigen (O-Ag), a polysaccharide chain consisting of repeated units (Hitchcock *et al.*, 1986). Depending on the presence of O-Ag and the number repeating units, the LPS can be rough, semi-rough (e.g. *A. pleuropneumoniae* serotype 1) or smooth (e.g. *A. pleuropneumoniae* serotype 2) (Hitchcock *et al.*, 1986; Byrd & Kadis, 1989; Bélanger *et al.*, 1990). Each serotype of *A. pleuropneumoniae* has a different LPS composition but common epitopes are shared by the O-polysaccharides and are responsible for cross-reactions encountered between serotypes 1, 9, and 11; 3, 6, and 8; and 4 and 7 (Perry *et al.*, 1990). In a recent study, we reported electromorphic and antigenic differences in the core region of *A. pleuropneumoniae* LPS (Jacques *et al.*, 1996). Despite the heterogeneity of *A. pleuropneumoniae* major adhesin, strains of different serotypes are capable of adherence to porcine respiratory tract cells (Bélanger *et al.*, 1990; Jacques *et al.*, 1991; Paradis *et al.*, 1994), which is the initial event in bacterial colonization (Ofek & Doyle, 1994).

We also previously showed that the polysaccharidic portion of the LPS inhibited the adherence of *A. pleuropneumoniae* to porcine frozen tracheal sections (Paradis *et al.*, 1994). In addition, *A. pleuropneumoniae* LPS was shown to be well exposed at the surface of this encapsulated microorganism (Paradis *et al.*, 1994; Paradis *et al.*, 1996). It might thus be possible to block adherence (Kahane & Ofek, 1996) with antibodies against *A. pleuropneumoniae* LPS.

Adherence of microorganisms to the epithelial cells and/or mucus layer of the mucosal surfaces involves specific interactions between bacterial adhesins and host receptors (Ofek & Doyle, 1994). Recently, our laboratory has demonstrated the binding of *A. pleuropneumoniae* LPS to proteins within porcine respiratory tract secretions (Bélanger *et al.*, 1994). Many LPS receptors have also been

identified on the membrane of different mammalian cells. These receptors, identified from various sources, are very different in nature and their interactions differ too, as they specifically bind to different regions of the LPS molecules. Most identified proteinaceous LPS receptors bind lipid A (Morrison *et al.*, 1993), but lectin-like receptors have also been identified (Lei & Chen, 1992). Studies reported to date have dealt almost exclusively with receptors on leukocytes. However, when LPS act as an adhesin, mucosal and epithelial receptors should receive a particular attention (Jacques, 1996).
The aims of the present study was i) to determine whether antibodies raised against *A. pleuropneumoniae* LPS were able to inhibit the adherence of the organism to porcine tracheal cells; and ii) to identify and characterize the porcine tracheal epithelial cells putative receptors recognized by *A. pleuropneumoniae* lipopolysaccharidic adhesins.

METHODS

Bacterial isolates and growth conditions. *A. pleuropneumoniae* reference strains representing serotypes 1 (4074) and 2 (4226) were provided by A. Gunnarson, National Veterinary Institute, Uppsala, Sweden. Three serotype 1 (Q87-586, FMV87-682, and 78-4118-88) and three serotype 2 (84-4397, Q87-981, and JG-141) field isolates of *A. pleuropneumoniae,* were used in previous studies (Bélanger *et al.*, 1990). Bacteria were grown on BHI (Difco Laboratories) agar plates supplemented with 15 μg NAD ml^{-1} or BHI broth supplemented with 5 μg NAD ml^{-1}. *Escherichia coli* P91-19-172-O45 strain, kindly provided by J. M. Fairbrother (Faculté de médecine vétérinaire, Université de Montréal), was grown on blood agar plates. Cultures were incubated at 37 °C in a 5 % CO2 atmosphere for 18-24 h.

Monoclonal and polyclonal antibodies. Mouse mAb against *A. pleuropneumoniae* serotype 1 O-Ag (5·1 G8F10) (Lairini *et al.*, 1995) or *A. pleuropneumoniae* serotype 2 O-Ag (102-G02) (Giese *et al.*, 1993) were used. Rabbit antisera raised against whole cells of *A. pleuropneumoniae* serotypes 1 or 2 were also used. The rabbit antiserum against the strain of *E. coli* P91-19-172-O45 was kindly provided by J. M. Fairbrother.

Fab fragments preparation. The ImmunoPure® Fab Preparation Kit (Pierce Chemical) was used to obtained monovalent Fab fragments from mAbs 5·1G8F10 and 102-G02. Preparations were carried out as suggested by the manufacturer. Briefly, mAbs were digested with papain to split IgG in Fab and Fc fragments. Then, the crude digested materials were added to an affinity column coated with protein A which binds Fc fragments and non-digested IgG. The eluates which contain the Fab fragments were recovered and dialysed against PBS (0·01 M, pH 7·4). The protein concentration was determined by the Lowry assay as modified by Markwell *et al.* (1978), and adjusted to 70 μg protein ml^{-1}.

Inhibition of adherence to frozen tracheal sections by Fab fragments. Frozen sections were prepared as described previously (Paradis *et al.*, 1994). Trachea samples, obtained from newborn piglets, were washed in PBS, embedded in O.C.T. compound (Miles Laboratories), frozen and stored at -70 °C until used. The frozen sections (4-6 μm thick) were cut in a cryostat microtome, mounted on glass slides, fixed in methanol for 1 min, and air dried. Bacteria were washed three times in PBS and then diluted in PBS containing 1 % (w/v) bovine serum albumin and 0·01 % (v/v) Tween-20 (PBS-BSA-T20) (Paradis *et al.*, 1994) to give an OD_{540} of 0·2 (LKB Biochrom Novaspec II spectrophotometer; 1 cm light pathlenght). The bacterial suspensions were preincubated with different dilutions of the homologous Fab fragments preparation in a moist chamber at 37 °C for 15 min with gentle agitation. Controls were made by using the heterologous Fab fragments preparation. A volume of 100 μl of each bacterial suspensions, that have been preincubated with different dilutions of Fab fragments, were used to overlay two separated tissue sections fixed on glass slides. The slides were incubated in a moist chamber at 37 °C for 2 h. After extensive washing in distilled water, the sections were stained with the Diff-Quik stain (Baxter Healthcare) according to the manufacturer's instructions. Upon microscopic examination, the number of bacterial cells attached to the tracheal epithelium was determined at X 1000 magnification. All experiments were performed at least twice, and the values were given as the mean ± standard deviation. The significance of the inhibition of adherence was determined with the Student's t-test.

Extraction and isolation of lipopolysaccharides. Lipopolysaccharides from *A. pleuropneumoniae* serotypes 1 and 2 were extracted and isolated by the method of Darveau & Hancock with some modifications (Sprott *et al.*, 1994). Briefly, disrupted cells were treated with deoxyribonuclease, ribonuclease, pronase, and sodium dodecyl sulfate and were subjected to $MgCl_2$ precipitation and high-speed centrifugation. These prepared LPS contained less than 1 % of proteins as determined by UV absorbance and the Bradford assay. No bands were detected after silver staining of a SDS-PAGE.

Porcine tracheal cells preparation. Tracheas, obtained from newborn piglets to adult pigs, were washed in PBS, cut longitudinally, and the epithelial cells were gently scraped and resuspended in fresh PBS. The cells were washed three times in PBS after which, they were heated for 20 min at 100 °C in a solubilization buffer containing 10 % (v/v) glycerol, 5 % (v/v) ß-mercaptoethanol, 2 % (w/v) SDS, 0·0625 M Tris-hydrochloride (pH 6·8), and 0·025 % (v/v) bromophenol blue.

Porcine alveolar macrophages. Pulmonary alveolar macrophages were collected from 4-week old specific-pathogen-free piglets. After euthanasia with barbiturates, the lungs were removed aseptically. They were filled to maximum capacity (600 ml) with PBS containing 1 % penicillin-streptomycin (Gibco BRL) and 100 μg gentamycine (Gibco BRL). The PBS was then emptied into a sterile container held on ice. This procedure was repeated twice using fresh PBS (Mengeling *et al.*, 1995). Following centrifugation at 480 *g* for 10 min, the cell pellet which contains alveolar macrophages was resuspended in the solubilizing buffer and boiled 20 min at 100 °C.

Electrophoretic analysis. Solubilized cells preparations were separated by discontinuous SDS-PAGE (Laemmli, 1970). Samples were electrophoresed at 100 V (stacking gel) and 200 V (separating gel) using a Mini-Protean II apparatus (Bio-Rad Laboratories). Low range molecular mass prestained standards were obtained from Bio-Rad. Gels were stained with Coomassie brillant blue R-250.

Far-Western binding assay. Adherence to replicas were carried out as described by Prakobphol *et al.* (1987). Tracheal cells or macrophages samples separated by electrophoresis were transferred (MilliblotTM-Graphite electroblotter systems;

Millipore) to nitrocellulose membrane (pore size, 0·2 μm; Bio-Rad) for 18 min at 220 mA. Incubations were performed at room temperature, all followed by four 3-min washes with a Tris-buffered saline (TBS: 10 mM Tris [pH 7·4], 150 mM NaCl). The nitrocellulose membranes were first blocked with 2 % skim milk in TBS for 1 h, and then incubated for 1 h with bacterial cells resuspended in PBS to an OD_{540} of 1·0 or with extracted LPS (2 mg ml^{-1}), both containing 2 % of skim milk. The nitrocellulose membranes were incubated with diluted antibodies in the blocking solution overnight at 4 °C, and bound antibodies were detected by an incubation for 1 h with goat anti-rabbit or goat anti-mouse IgG (H+L) horseradish peroxidase-conjugate (Bio-Rad). The reaction was revealed by addition of hydrogen peroxide and 4-chloro-1-naphthol (Sigma) as the chromogenic substrate.

Proteinase K digestion. A volume of the piglet tracheal cells preparation in the solubilization buffer to which we added the same volume of a proteinase K solution (1 mg ml^{-1}) was incubated 1 h at 60 °C (Li *et al.*, 1992). The protein profile and the binding activity were then established as mentioned above.

Periodate oxydation. The nitrocellulose membranes with transferred tracheal cells or macrophages samples were treated overnight at 4 °C with 100 mM sodium m-periodate (in 100 mM sodium acetate buffer [pH 4·5]) to detect periodate-sensitive material (Li *et al.*, 1992).

Glycoproteins detection. The DIG glycan detection kit (Boehringer Mannheim Canada) was used to detect the presence of glycoproteins in the piglet tracheal cells preparation using the samples separated by SDS-PAGE and transferred to nitrocellulose membranes. Detection was carried out as described in the manufacturer's instructions.

Inhibition assays. Polymyxin B sulfate was obtained from Sigma, added at final concentrations of 1 and 10 mg ml^{-1} to bacterial suspensions, and incubated 30 min at room temperature before performing the Far-Western binding assay described above. Nitrocellulose membranes with the transferred piglet tracheal cells samples were incubated in presence of the bacteria treated with polymyxin B sulfate for 1 h after the blocking step. In another set of experiments, nitrocellulose membranes with the transferred piglet tracheal cells samples were

incubated for 1 h with 1 and 10 mg ml^{-1} of glucose, galactose, rhamnose, lactose, N-acetylglucosamine, N-acetylgalactosamine, glucosamine, glucoheptose or mannoheptulose, or with 1 mg ml^{-1} KDO (3-deoxy-2-octulosonate), all obtained from Sigma. The membranes were then incubated in presence of the bacteria in presence of the same concentrations of sugars for 1 h after the blocking step.

N-terminal amino acid sequencing. Tracheal cells proteins were electroblotted onto a ProBlott transfer membrane (Applied Biosystems) by using the method of LeGendre & Matsudaira (1988). The proteins were stained with Coomassie brillant blue R-250. Selected proteins were subjected to N-terminal amino acid sequencing. Automated Edman degradation was performed with a model 470A Applied Biosystems gas phase sequencer equipped with a model 120A on-line phenylthiohydantoin analyzer (Applied Biosystems) by using the general protocol of Hewick *et al.* (1981). Samples were applied to trifluoroacetic acid-treated cartridge filters coated with 1·5 mg of Polybrene and 0·1 mg of NaCl (Biobrene Plus; Applied Biosystems). The standard 03RPTH program was used for sequencing. The identities of the phenylthiohydantoin amino acid derivatives were determined by comparison with standards (phenylthiohydantoin analyzer standards; Applied Biosystems) that were analysed on-line prior to each sequence analysis.

RESULTS AND DISCUSSION

Inhibition of adherence of *A. pleuropneumoniae* by Fab fragments

We have previously shown that extracted LPS inhibited the adherence of *A. pleuropneumoniae* to porcine tracheal frozen sections (Paradis *et al.*, 1994) . We were interested to see whether antibodies against LPS would be able to block also the adherence of *A. pleuropneumoniae,* and thus confirm that LPS were involved in adherence. Since agglutination was observed when *A. pleuropneumoniae* serotype 1 (4074) or serotype 2 reference strains (4226) were incubated with their homologous mAbs directed against the O-Ag (mAb 5·1 G8F10 and mAb 102-G02, respectively), we prepared Fab fragments from these mAbs to evaluate their potential to inhibit adherence of this microorganism to porcine tracheal frozen sections. A concentration-dependent reduction of adherence was observed with homologous Fab fragments (Fig. 1). Adherence of

A. pleuropneumoniae strain 4074 (serotype 1) to the tracheal cells was significantly inhibited ($P = 0{\cdot}0001$) by Fab fragments 5·1 G8F10 against serotype 1 O-Ag (Fig. 1a), while adherence of *A. pleuropneumoniae* strain 4226 (serotype 2) was significantly inhibited by Fab fragments 102-G02 against serotype 2 O-Ag (Fig. 1b). Controls were made by using the heterologous Fab fragments, therefore we used Fab fragments 5·1 G8F10 with *A. pleuropneumoniae* strain 4226 and Fab fragments 102-G02 with *A. pleuropneumoniae* strain 4074. No significant inhibition of bacterial adherence to the tracheal cells was observed even with the highest concentration of heterologous Fab fragments used (Fig. 1a and 1b). These results indicate that the inhibition of adherence of *A. pleuropneumoniae* to tracheal frozen sections could effectively be achieved by antibodies directed against LPS. Since these antibodies were raised against the O-Ag of each LPS serotype, it is not surprising that these inhibitions were serotype-specific.

The inhibition of adherence by monoclonal antibodies against LPS that we observed adds to accumulating data incriminating LPS as an important adhesin of *A. pleuropneumoniae* (Bélanger *et al.*, 1990; Bélanger *et al.*, 1994; Paradis *et al.*, 1994; Rioux *et al.*, 1997a). These results are in agreement with our previous observations which strongly suggest that the polysaccharide portion of the LPS was the region within the LPS molecule involved in the adherence of *A. pleuropneumoniae* (Paradis *et al.*, 1994). In addition, these results indicate that it might be possible to protect animals by inducing a good immune response against LPS. We recently obtained evidence to support this hypothesis. Infact, we showed that LPS-based vaccines were able to protect mice (Rioux *et al.*, 1997b) and pigs (Rioux *et al.*, 1998) against challenge with a virulent strain of *A. pleuropneumoniae*.

Identification of putative *A. pleuropneumoniae* LPS receptors on porcine tracheal cells and alveolar macrophages preparations

The recognition of target tissue receptors by an organism is a specific and complex phenomenon, which may include a multi-step process of bacteria-receptor interactions leading to a successful colonization and interaction (Clark & Bavoil, 1994). Gel electrophoresis of host cells components followed by blotting of the gel and detection with suitable reagents is an extremely useful technique for the identification and characterization of cell receptors specific for

antibodies, lectins, growth factors, or for other molecules of biological interest, as well as intact bacterial cells. It is also employed to an increasing extent for the investigation of receptors for bacterial adhesins (Clark & Bavoil, 1994; Doyle & Ofek, 1995).

Epithelial cell lines are not differentiated, and the availability and density of receptors specific to particular pathogens (and their adhesins) do not necessarily correspond to natural environment (Doyle & Ofek, 1995). Therefore, in this work we investigated the presence of *A. pleuropneumoniae* receptors in tracheal cells harvested by scraping the porcine tracheal mucosal surface.

The protein profiles of tracheal cells collected from four different breeds of crossbred piglets were compared to see if individual differences were important. The tracheal protein profiles of Duroc, Hampshire Duroc, Landrace, and Landrace Duroc 3-day old piglets were found to be identical (Fig. 2; panel a, lanes 1-4). At least two individuals of each crossbred piglets were tested. Once transferred to nitrocellulose membranes, these host cells components were incubated in the presence of *A. pleuropneumoniae* strain 4074 or 4226, and probed with an antiserum against *A. pleuropneumoniae* serotype 1 reference strain or serotype 2 reference strain, respectively. Seven major bands reacted with both reference strains. There was a triplet at Mr 38 500, a doublet at Mr 17 000, and single bands at Mr 16 000 and Mr 14 000 (Fig. 2; panel b). We also compared tracheal cells preparation from newborn piglets (3-day old) to preparations from 7-8-week old piglets and from adult pigs at slaughter. The proteins profile of adult pigs was not identical to the profile of the younger pigs (Fig. 2; panel a, lanes 1-5 vs lane 6), however, the same reacting bands were observed after incubation with cells (Fig. 2; panel b, lanes 1-6). In addition to the two reference strains of *A. pleuropneumoniae*, we have tested six different field isolates of *A. pleuropneumoniae*, three representing serotype 1 (Q87-586 [Fig 3; lane 2], FMV87-682, and 78-4118-88) and three representing serotype 2 (84-4397 [Fig 3; lane 5], Q87-981, and JG-141), and found that they were all binding to the same seven putative receptors. Most importantly, the same seven major reacting bands were revealed when the nitrocellulose membranes were incubated in the presence of *A. pleuropneumoniae* extracted LPS (Fig. 3; lane 7).

Other laboratories using a similar overlay assay procedure have also identified multiple LPS-interactive proteins. Gupta *et al.* (1997) have recently demonstrated that *Pseudomonas aeruginosa* LPS was binding to human corneal epithelium proteins of different molecular masses ranging from 18 to 66 kDa, whereas Halling *et al.* (1992) have previously demonstrated that *E. coli* O111:B4 LPS could bind different LPS-binding proteins present on human monocytes and lymphocytes. As a control, we have tested a strain of *E. coli* causing post-weaning diarrhea in piglets. For this adherence assay, the nitrocellulose membrane was probed with a rabbit antiserum raised against this particular strain. A very faint reaction could be observed with the seven putative receptors when a highly concentrated suspension of *E. coli* was used (OD_{540} of 1·5 compared to an OD_{540} of 1·0 for *A. pleuropneumopniae*). This is not totally surprising considering the relatedness existing among LPS molecules from different species. The three main regions of the LPS molecule exhibit different degrees of variability. While, the lipid A is the most conserved region among Gram negative bacteria, the O-Ag is the most variable part (Rietschel *et al.*, 1994).

Porcine alveolar macrophages were submitted to the same Far-Western binding assay as described above for porcine tracheal cells and *A. pleuropneumoniae* strains 4074 and 4226. Because, the cellular composition of alveolar macrophages collected by lung lavage (> 90 % when examined by light microscopy) is more homogenous than the epithelial cells scraped from trachea, and because the majority of the reports on LPS receptors have been produced using leukocytes, and more particularly macrophages, we were interested in examining and comparing *A. pleuropneumoniae* LPS binding proteins.

Again, seven putative bands were revealed with relative molecular masses corresponding to the ones detected in tracheal cells preparation (Fig. 2; panel b; lane 7). We also probed the epithelial cells and alveolar macrophages preparations with the antibody MY4, which recognizes CD14 on porcine monocytes (Ziegler-Heitbrock *et al.*, 1994), and with an anti-ß-tubulin. CD14 is an antigen which is used to identify monocytes, and is a well known LPS-binding protein of 54 kDa, whereas ß-tubulin, a 55 kDa protein, as been proposed as an intracellular target for LPS (Ding *et al.*, 1992). Antibodies against both molecules did not react with the putative receptors, as expected by their

differences in molecular mass. These results also most probably eliminate the possibility that the reactive bands are degradation products of these molecules.

Preliminary characterization of the putative receptors

When preincubated with polymyxin B the adherence of *A. pleuropneumoniae* whole cells or extracted LPS was inhibited for serotype 1 and did not seem affected for serotype 2; in both cases a considerable high background was generated. These results would suggest that the core-lipid A region, at least in serotype 1, is involved in binding to the putative receptors. Polymyxin B is a cationic antibiotic which possibly binds nonselectively to both the KDO and phosphate groups of LPS according to Schindler & Osborn (1979) and which probably involve ionic and hydrophobic interactions with the lipid A-KDO region of the LPS molecule according to Morrison & Jacobs (1976). The fact that *A. pleuropneumoniae* serotype 1 LPS are semi-rough whereas the serotype 2 LPS are smooth probably explains why the serotype 2 is less affected by polymyxin B. The chain length of O-Ag from smooth type LPS probably have a better protecting effect in intact cells or in micelles of extracted LPS. The difference in the core composition between serotype 1 (Altman *et al.*, 1986) and 2 (Altman *et al.*, 1987) might also have a role in the susceptibility of these two LPS types to polymyxin B. Some sugars composing the O-Ag and core region including glucose, galactose, rhamnose, lactose, N-acetylglucosamine, N-acetylgalactosamine, glucosamine, glucoheptose, mannoheptulose or KDO) were tested individually and did not affect binding.

We confirmed the proteinaceous nature of these putative receptors following staining with Coomassie brilliant-blue, their sensitivity to proteinase K digestion, their resistance to sodium m-periodate oxidation, and their inability to stain with glycoproteins reagents (data not shown).

N-terminal amino acid sequencing of each putative receptors from the tracheal cells preparation were performed. Sequences were compared by use of the SwissProt database. The doublet at 17 kDa showed homology with human histone, the top band (XXXXXTAXKSTGGKAPXKQLA) has 87·5 % identity, from amino acid 6 to 21, with histone H3·1/H3·2, and the lower band (PEPAKSAPAPKKGSXKAVTKAQKK) has 100 % identity, from amino acid 1 to

24, with histone H2B1. The proteins at Mr 14 000 and Mr 16 000 were blocked at their N-terminal and no sequence could be determined despite differents attempts such as trifluoro acetic acid treatment and heating at 65 °C (Wellner *et al.*, 1990), cyanogen bromide clivage and endoproteinases digestion with glu-C and lys-C (Gagnon & Christie, 1983), in-gel trysin digestion (Rosenfeld *et al.*, 1992), and reduction and alkylation of cysteine with N-isopropyliodoacetamide (Krutzsch & Inman, 1993). Nevertheless, these bands could also possibly be histones knowing that the pattern 17/16/14 kDa resembles that expected for histones binding (Smith *et al.*, 1994; Thiriet & Albert, 1995; Righetti *et al.*, 1996) and that the doublet at Mr 17 000 have sequence homology with histone H3·1/H3·2 and H2B1 as mentioned above. We can strongly suspect that the proteins at Mr 16 000 and Mr 14 000 are histone H2A and H4, respectively as demonstrated by Watson *et al.* (1995). Histones which are cationic molecules will bind to anionic molecules and have already been reported as LPS-binding proteins. Hampton *et al.* (1988) have identified histone H1, as a binding doublet for lipid IVA, which appeared in the nuclear fraction following subcellular fractionation of RAW 264·7 macrophage-like cultured cells. Interestingly, Holers & Kotzin (1985) have reported cell-surface histone molecules on monocytes. More recently, Watson *et al.* (1995) have shown that histones are detectable on the surface of a human T-cell line and phytohemagglutinin-activated human peripheral blood lymphocytes, and Bolton & Perry (1997) concluded that histone H1, capable of binding LPS, is a 30 kDa membrane-bound protein on the surface of neuronal cell bodies. The band at Mr 14 000 could also be lysozyme, a major cationic enzyme present at relatively high concentrations in several mammalian materials, such as in epithelia and macrophages (Ganz & Weiss, 1997), and which is known to bind smooth and rough types of LPS and also lipid A (Takada *et al.*, 1995). The interactions between lysozyme and LPS are of two types. The first would be related to an ionic interaction with the surface of the LPS micellar structure; the second would involves more hydrophobic interactions which are dependent on the reaction temperature (Takada *et al.*, 1995).

Unfortunately, we did not obtain any N-terminal amino acid sequence for the proteins at Mr 38 500 despit many attempts. Gupta *et al.* (1997) have recently demonstrated that *Pseudomonas aeruginosa* LPS was binding to a 31-32 kDa protein present in human corneal epithelium which was identified as galectin-3;

a known LPS-binding protein (Mey *et al.*, 1996). Halling *et al.* (1992) have demonstrated that LPS-binding proteins were present on human monocytes and lymphocytes. They were particularly interested in a 73 kDa, but also noted the presence of three other major LPS-binding proteins of 50, 31 and 18 kDa, and of a minor one of 38 kDa minor one. Of interest, they also revealed several additional LPS-binding proteins of lower-molecular masses. The same laboratory also identified a 38 kDa LPS-binding protein, on mouse splenocytes, which would have a binding specificity for KDO residues (Halling *et al.*, 1992; Lei *et al.*, 1993). Finally, pulmonary surfactant proteins SP-A (28-36 kDa) and SP-D (42-44 kDa) have LPS binding properties for lipid A (Possmayer, 1988) and for core terminal glucose and/or heptose residues (Kuan *et al.*, 1992), respectively.

Taken together our data indicate that mAbs against LPS significatively inhibit adherence of *A. pleuropneumoniae* to porcine tracheal frozen sections, and support the idea that LPS represent an important adhesin in *A. pleuropneumoniae*. Furthermore, a class of proteins of Mr 38 500, present in swine respiratory tract cells, has been identified as putative receptors for *A. pleuropneumoniae* LPS. Although many proteins with a molecular mass around 38-39 kDa and exhibiting LPS-binding activity have been reported in the literature, we do not know at the present time whether the putative 38·5 kDa receptors we have identified correspond to one of these molecules; the pulmonary surfactant proteins represent likely candidates however.

ACKNOWLEDGEMENTS

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC OPG0003428 to MJ), and from a research partnership program involving NSERC, Agriculture Canada, and Fédération des Producteurs de Porcs du Québec. Sonia-Élaine Paradis is the recipient of a studentship from Fonds pour la Formation de Chercheurs et l'Aide à la Recherche. We are grateful to France Dumas (Biotechnology Research Institute, National Research Council, Montréal, Canada) for performing the N-terminal amino acid sequencing, to Serge Dea (Institut Armand-Frappier, Laval, Canada), Ludovic Bourré, and Pierre Hébert for technical support relating to porcine alveolar macrophages. We also want to thank Caroline Bégin for technical

assistance with the Fab fragments preparation, and Maan Abul-Milh for critical review of the manuscript.

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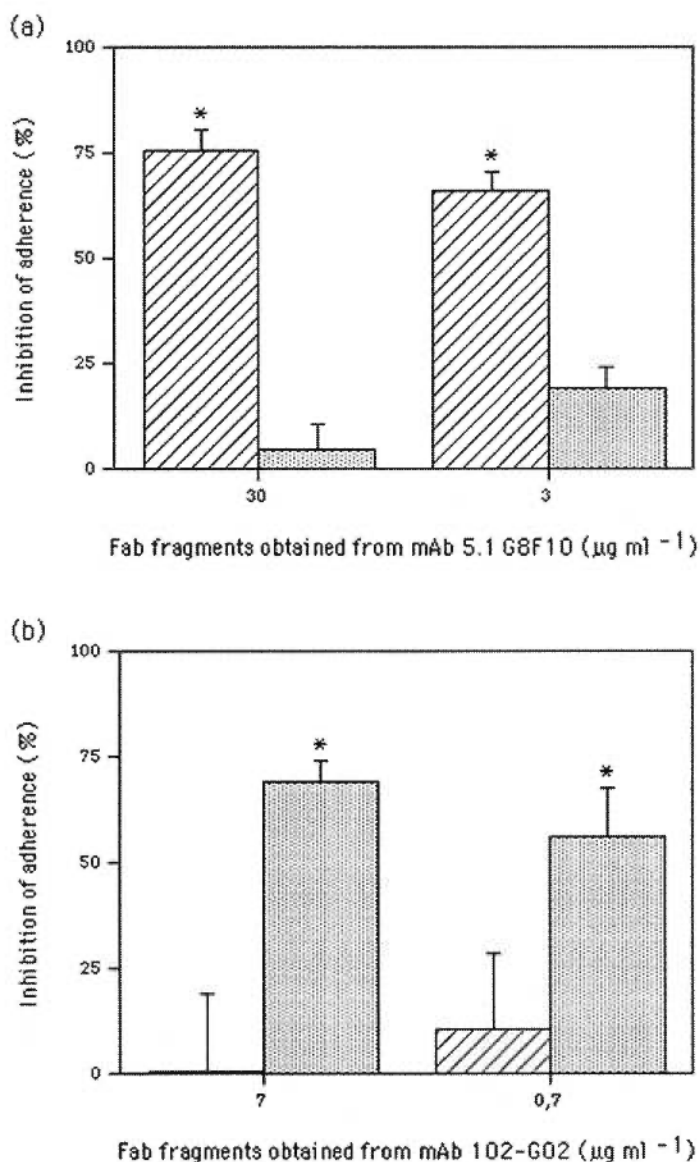

Fig. 1. Inhibition of adherence of *A. pleuropneumoniae* strain 4074 (hatched bars) and strain 4226 (spotted bars) to porcine tracheal frozen sections by different concentrations of Fab fragments obtained from mAb 5·1 G8F10 (specific for serotype 1 O-Ag) (a) or mAb 102-G02 (specific for serotype 2 O-Ag) (b). Results shown are the mean of at least two experiments with SD bars. The columns marqued (*) were significantly different from the controls incubated without Fab fragments ($P = 0{\cdot}0001$).

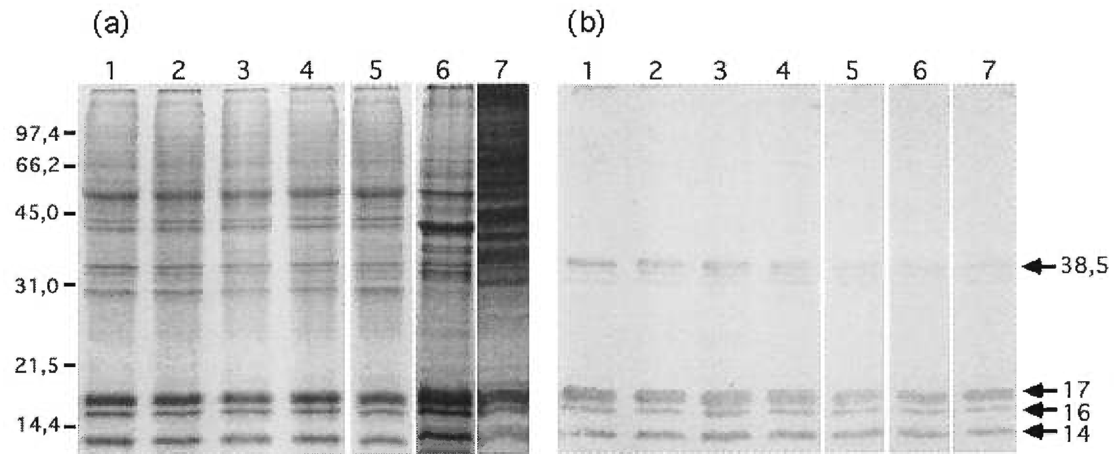

Fig. 2. Electrophoretic and Far-Western analyses of porcine respiratory tract cells preparation. Preparations were Coomassie brilliant blue-stained (a) or transferred to a nitrocellulose membrane, incubated with *A. pleuropneumoniae* serotype 2 reference strain cells and probed with homologous rabbit antiserum (b). Controls were made by incubating the preparations on the nitrocellulose membranes with the antiserum against *A. pleuropneumoniae* serotype 2 (data not shown). Tracheal cells collected from 3-day old Duroc piglets (lanes 1); Hamphsire Duroc piglets (lanes 2); Landrace piglets (lanes 3); or Landrace Duroc piglets (lanes 4). Tracheal cells collected from 7-8-week old piglets (lanes 5), and from adult pigs at slaughter (lanes 6). Alveolar macrophages from 4-week old piglets (lanes 7). The position of molecular mass markers (in kilodaltons) are indicated on the left. Arrows indicate the apparent molecular mass of the putative receptors.

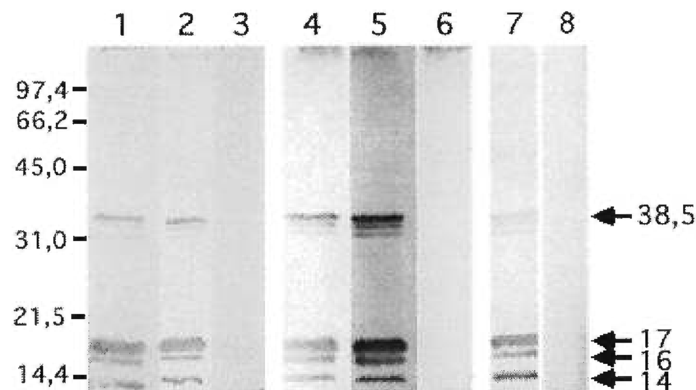

Fig. 3. Far-Western analyses of porcine tracheal cells preparation. The nitrocellulose membranes were incubated with cells of *A. pleuropneumoniae* serotype 1 reference strain (lane 1) and isolate FMV87-682 (lane 2); or cells of *A. pleuropneumoniae* serotype 2 reference strain (lane 4) and isolate 84-4397 (lane 5). They were probed with homologous rabbit antiserum. Nitrocellulose membranes were incubated with extracted LPS from serotype 1 reference strain and probed with mAb 5·1 G8F10 (lane 7). Controls were made by incubating the preparations on the nitrocellulose membranes with the same antibody (lanes 3, 6, and 8). The position of molecular mass markers (in kilodaltons) are indicated on the left. Arrows indicate the apparent molecular mass of the putative receptors.

IV. DISCUSSION

L'établissement d'un microorganisme menant à la colonisation et à l'infection d'un hôte, est possible grâce à une interaction spécifique entre des molécules complémentaires appartenant à chacun des partis et ayant une affinité les unes pour les autres. Le microorganisme doit obligatoirement se fixer, à l'aide de ses adhésines, à des récepteurs présents au niveau des muqueuses, et ce pour éviter d'être expulsé par les différents mécanismes de défense de l'hôte et ainsi parvenir à se multiplier pour envahir l'hôte (Ofek et Doyle, 1994). Les adhésines utilisées par les différents microorganismes sont de nature très diversifiées; elles peuvent être protéiques (e.g. fimbriae, fibrils) ou polysaccharidiques (e.g. LOS, LPS, CPS). De plus, plusieurs types d'adhésines peuvent être retrouvés chez un même microorganisme, augmentant ainsi les possibilités d'interactions de ce dernier avec différents récepteurs situés au niveau des muqueuses de l'hôte.

A. pleuropneumoniae est un pathogène respiratoire de l'espèce porcine qui utilise ses LPS comme adhésine majeure. Plusieurs autres bactéries Gram-négatif impliquées dans diverses infections chez différents mammifères utilisent elles aussi leurs LPS ou LOS comme adhésines (Jacques, 1996). Ce n'est pas si surprenant que par besoin d'économie énergétique, ces bactéries emploient des structures déjà bien en place à bon escient et à d'innombrables tâches. Chez *A. pleuropneumoniae*, Bélanger *et al.* (1990) ont mis en évidence cette propriété en utilisant un modèle *in vitro* composé d'anneaux de trachée maintenus en culture; c'est à l'aide de ce modèle qu'ils ont démontré qu'il était possible d'inhiber l'adhérence d'*A. pleuropneumoniae* par des LPS purifiés. Ils avaient également noté que les souches d'*A. pleuropneumoniae* présentant des LPS de type lisse adhéraient de façon plus importante que les souches présentant des LPS de type semi-rugueux.

Nous avons voulu, par le présent projet, approfondir notre compréhension de l'interaction adhésine-récepteur chez *A. pleuropneumoniae*. Tout d'abord, il nous importait de confirmer l'accessibilité de l'adhésine à la surface bactérienne, ce qui est un prérequis indéniable pour permettre aux LPS d'interagir avec d'éventuels récepteurs présents dans l'environnement. Chez *A. pleuropneumoniae*, la présence de nombreuses vésicules extra-membranaires (ou "blebs") avaient déjà été observée et pouvait expliquer l'accessibilité des LPS à la surface malgré la présence de matériel capsulaire (Jacques *et al.*, 1988). De même, l'adhérence similaire, aux anneaux de trachée maintenus en culture,

d'isolats capsulés et de leurs variants non capsulés, laissait aussi présager que les LPS n'étaient pas entièrement masqués (Bélanger *et al.*, 1990). Nous avons donc marqué des cellules bactériennes d'*A. pleuropneumoniae* grâce à des mAb dirigés spécifiquement contre les Ag-O, que ce soit i) en MET, à l'aide d'anticorps secondaires conjugués à des billes d'or colloïdal, ou ii) en cytofluorométrie, à l'aide d'anticorps secondaires conjugués à de la fluorescéine; un fort marquage a été détecté, nous démontrant bien que la capsule ne réussi pas à masquer les LPS ou du moins ne réussi qu'en partie. Il serait intéressant de comparer quantitativement, par cytofluorométrie, le marquage d'un variant acapsulé ou, mieux encore, d'une souche mutante acapsulée isogénique pour vérifier la proportion de LPS cachés par les polysaccharides capsulaires.

Dans le but de poursuivre la caractérisation de l'adhésine d'*A. pleuropneumoniae*, le premier objectif fut de miniaturiser le modèle d'adhérence *in vitro* afin de réduire les quantités de LPS nécessaires aux tests d'inhibition. À partir d'un autre modèle d'adhérence *in vitro*, utilisant cette fois des coupes congelées de poumon et s'étant aussi avéré efficace pour démontrer l'adhérence d'*A. pleuropneumoniae* (Jacques *et al.*, 1991), nous avons développé un modèle d'adhérence aux coupes congelées de trachée. La trachée, à cause du tissu cartilagineux qui la maintient, nous a permis d'obtenir des coupes plus facilement maniables que celles obtenues à partir de poumons et donc beaucoup plus homogènes. De plus, comme la surface de l'épithélium trachéal correspond à la circonférence de la lumière de la trachée, cela nous a permis de comparer des comptes bactériens sur une région bien définie et bien conservée entre des coupes successives, ce qui n'était pas le cas lorsque l'on essayait de retrouver des régions alvéolaires du poumon de dimensions semblables.

À l'aide d'une coloration immunoenzymatique, utilisant ici des anticorps dirigés contre *A. pleuropneumoniae* ou contre ses Ag-O, nous avons pu démontrer sur les coupes congelées de trachée, incubées en présence de LPS purifiés, que l'adhésine majeure d'*A. pleuropneumoniae* se lie bel et bien à l'épithélium trachéal. La détection du site de liaison des LPS a été effectuée grâce à des anticorps secondaires conjugués à la phosphatase alcaline et qui, en présence du substrat, produisaient un précipité brunâtre à l'endroit même où s'étaient fixés les LPS i.e. au niveau de l'épithélium trachéal. Des tests d'inhibition de l'adhérence d'*A. pleuropneumoniae* à l'aide de LPS purifiés, nous ont convaincus

que le modèle des coupes congelées de trachée reproduisait bien les résultats obtenus avec les anneaux de trachée maintenus en culture, tout en étant nettement moins laborieux. La même corrélation entre l'adhérence des souches d'*A. pleuropneumoniae* et le profil des LPS de ces dernières était encore observée. En effet, la souche de référence du sérotype 2 ainsi que des isolats de sérotype 2, ayant donc un profil lisse de LPS, adhéraient de façon beaucoup plus importante aux coupes congelées de trachée en comparaison à la souche de référence du sérotype 1 ou à des isolats de sérotype 1, ayant un profil semi-rugueux de LPS.

Par hydrolyse acide, il a été possible de scinder les molécules de LPS en leur partie polysaccharidique et leur partie lipidique. Les polysaccharides (soit l'ensemble du noyau OS et de l'Ag-O) ont démontré un pouvoir inhibiteur sur l'adhérence d'*A. pleuropneumoniae* presque aussi fort que les LPS complets. Le lipide A ne semble donc pas être impliqué dans le processus de l'adhérence. Il faut toutefois considérer la nature très hydrophobe du lipide A qui amène celui-ci à se regrouper en micelles et qui, par le fait même, réduit sa dispersion et modifie sa conformation. Ces altérations pourraient effectivement nuire à d'éventuelles interactions. Il ne faut toutefois pas écarter la possibilité, aussi petite soit elle, que les LPS d'*A. pleuropneumonie* à l'état natif soient capables d'interagir de manières variées avec de nombreuses molécules présentes à la surface des muqueuses, et ce à l'aide de ses différentes composantes.

Comme les LPS se présentent à la surface bactérienne en des chaînes de longueurs variables, surtout chez les souches présentant des LPS de type lisse ou semi-rugueux, nous avons voulu vérifier si la longueur des chaînes polysaccharidiques avait une influence sur leur capacité à inhiber l'adhérence. À l'aide de chromatographie sur gel de Sephacryl S-300 SF en présence de désoxycholate, afin d'obtenir une séparation optimale satisfaisante, les LPS ont pu être dissociés en fractions de masse moléculaire décroissantes. Le profil des fractions de LPS sur les gels SDS-PAGE nous a permis de constater qu'*A. pleuropneumoniae* sérotype 1 produit bel et bien de très longues chaînes d'Ag-O, sans toutefois présenter un profil en échelle typique des LPS de type lisse. Chez *A. pleuropneumoniae* sérotype 1, il semble donc y avoir une préférence pour certaines longueurs de chaînes, ce qui crée un profil bimodal i.e. à deux régions principales sans formes intermédiaires, contrairement à ce que l'on observe chez

A. pleuropneumoniae sérotype 2 où toutes les longueurs de chaînes semblent être présentes, ce qui produit le profil typique en échelle. Nous avons observé que seules les chaînes de très haute masse moléculaire, présentes chez les deux sérotypes étudiés, permettaient d'inhiber l'adhérence d'*A. pleuropneumoniae*, en système homologue. Ces fractions de très haute masse moléculaire n'étaient pas visibles sur gel SDS-PAGE coloré au nitrate d'argent. Cela était probablement dû à l'incapacité du matériel à pénétrer dans le gel (e.g. s'il n'y pas suffisamment de lipides) ou à l'absence de groupes réactifs nécessaires pour la détection au nitrate d'argent (e.g. s'il n'y pas au moins une paire de groupements hydroxylés substitués, présents dans le noyau OS, qui sont clivés par l'oxydation au périodate ou si les LPS ne sont pas précipités par la solution aqueuse d'isopropanol et d'acide acétique).

Par coloration négative au phosphotungstate, l'arrangement des LPS contenus dans les différentes fractions a pu être visualisé. L'arrangement des LPS que l'on retrouve dans un extrait de LPS lisses a précédemment été décrit par Kato (1996) chez d'autres espèces bactériennes telles que *E. coli*, *Salmonella minnesota* et *K. pneumoniae* comme s'organisant sous forme de rubans, de boucles et de sphères couverts de fines structures chevelues. Dans notre fraction de faible masse moléculaire, ne contenant pas d'Ag-O, les LPS de composition majoritairement lipidique se combinaient entre eux, comme attendu, pour former des micelles. Dans nos fractions intermédiaires, des formes de rubans ressemblant à ceux retrouvés dans l'extrait total de LPS étaient observables. Tandis que dans la fraction de très haute masse moléculaire, où aucun matériel n'était détecté sur gel coloré au nitrate d'argent, que de fins filaments étaient perceptibles. Ce qui nous permet de supposer que l'incapacité de migrer sur gel SDS-PAGE ainsi que le non regroupement des molécules en rubans ou en micelles seraient dû à l'absence de lipide A dans cette fraction. Ici s'ajoute donc des résultats nous permettant de conclure que la partie polysaccharidique est effectivement impliqué dans l'interaction adhésine-récepteur, ce qui répond au deuxième objectif du projet. Cette implication de la partie polysaccharidique dans l'adhérence n'est pas une particularité d'*A. pleuropneumoniae* (Cf. la section 3.3.2 de la revue de littérature), car plusieurs autres bactéries Gram négatif emploient une telle stratégie.

Si l'adhérence, reconnue comme l'étape initiale de l'infection, est bloquée, est-il possible de prévenir l'infection? Le troisième objectif de ce travail voulait donc vérifier le pouvoir inhibiteur d'anticorps dirigés contre l'adhésine, donc dirigés contre les LPS d'*A. pleuropneumoniae.* Pour ce faire, nous avons produit des fragments Fab à partir des mAb 5.1 G8F10 et 102-G02 dirigés spécifiquement contre les Ag-O d'*A. pleuropneumoniae* sérotype 1 et 2 respectivement. L'adhérence des bactéries, préincubées en présence des Fab dirigés contre leur Ag-O a été comparée, sur des coupes congelées de trachée, à celles de bactéries préincubées en absence de Fab ainsi qu'à celle de bactéries incubées en présence des Fab dirigés contre l'Ag-O du sérotype hétérologue. L'adhérence d'*A. pleuropneumoniae* sérotype 1 et 2 a été inhibée par les Fab dirigés spécifiquement contre leur Ag-O, donc en système homologue seulement. Il n'est pas surprenant d'obtenir des inhibitions qu'en système homologue, car chez *A. pleuropneumoniae* des différences de composition importantes sont présentes entre les Ag-O des différents sérotypes (Perry *et al.*, 1990). Certains sérotypes de LPS possèdent des épitopes communs, donnant lieu à des réactions croisées, mais aucune réaction n'a été reportée entre les Ag-O des sérotypes 1 et 2, ni entre les Ag-O des sérotypes 1 et 5. *In vitro*, cette expérience confirme le potentiel protecteur des anticorps dirigés contre les LPS d'*A. pleuropneumoniae.* Ce qui nous permet d'affirmer avec encore plus de certitude que l'adhésine d'*A. pleuropneumoniae* se situe dans la région polysaccharidique de ses LPS.

Dans notre laboratoire, Rioux *et al.* (1997) ont procédé à des études de protection *in vivo* chez la souris et chez les porcs (1996). Des souris ont été immunisées avec une préparation d'Ag-O du sérotype 1 conjugué à de l'albumine sérique de bovin. Cette préparation avait les avantages de présenter à l'hôte la molécule servant d'adhésine à la bactérie, éliminait du même coup la toxicité du lipide A et permettait d'induire une réponse T-dépendante. Ces souris ont été protégées à 80% contre une infection homologue (souche de sérotype 1) et non contre une infection hétérologue (souche de sérotype 5). Chez les porcs, il y a également été possible d'observer une protection contre une infection homologue en immunisant les animaux à l'aide d'un vaccin contenant des LPS détoxiqués. De plus, il a été rapporté qu'une protection passive à l'aide d'un mAb dirigé contre les LPS, le H-1b, chez des souris, était possible contre une dose léthale d'*A. pleuropneumoniae* (Oishi *et al.*, 1993).

Bien que nos résultats tendent à incriminer les unités répétitives de polysaccharides formant l'Ag-O comme composante de la molécule du LPS responsable de l'adhérence, il est essentiel de garder en tête que le noyau OS pourrait avoir une implication plus importante que prévue. Il serait très plausible que les anticorps ne bloquent l'adhérence que par empêchement stérique. Pour résoudre cette interrogation, il faudrait disposer d'anticorps spécifiquement dirigés contre les deux types de noyaux OS retrouvés chez *A. pleuropneumoniae.*

En effet, chez *A. pleuropneumoniae* deux types de noyaux OS sont identifiables par i) leur mobilité sur gel TSDS-PAGE ainsi que par ii) leur antigénicité, car un antisérum prélevé chez un porc infecté expérimentalement avec une souche d'*A. pleuropneumoniae* possédant un noyau OS de type I, ne reconnaîtra pas en immunobuvardage un noyau OS de type II, et vice-versa. Les types de noyaux OS peuvent aussi être responsables de la non inhibition de l'adhérence d'*A. pleuropneumoniae* par ses LPS ou des composantes de ces derniers, en systèmes hétérologues, car en plus de posséder des Ag-O très différents, les sérotypes 1 et 2 d'*A. pleuropneumoniae* possèdent des noyaux OS de type différent (le sérotype O1 appartient au type de noyau OS I et le sérotype O2 appartient au type de noyau OS II). Si des anticorps dirigés contre les noyaux OS étaient disponibles, l'accessibilité des noyaux OS à la surface bactérienne pourraient être mis en évidence tout comme le marquage avec le mAb dirigé contre le lipide A, nous a permis de confirmer que même cette partie du LPS pouvait être accessible et disponible pour d'éventuelles interactions chez *A. pleuropneumoniae.*

Le noyau OS interagit par une de ces deux extrémités avec le lipide A et par l'autre extrémité avec l'Ag-O. Le noyau OS adopte fort probablement une conformation spatiale très différente s'il reste lié i) au lipide A, comme dans les fractions de faible masse moléculaire trier par la chromatographie sur gel de filtration qui ne réussissent pas à inhiber l'adhérence d'*A. pleuropneumoniae;* ou ii) à l'Ag-O, comme lors de l'hydrolyse acide qui engendre une portion saccharidique en brisant le lien que forme la première molécule de KDO avec lipide A et comme lors de la chromatographie sur gel de filtration où les fractions de haute masse moléculaire semblent n'être composées que de saccharides incluant du KDO et qui inhibent l'adhérence d'*A. pleuropneumoniae* dans notre modèle de coupes congelées.

Des mutants sans Ag-O nous permettraient aussi de confirmer le rôle de ces derniers dans l'adhérence. Tout récemment, des mutants sans Ag-O de *K. pneumoniae* ont permis de démontrer clairement l'importance de l'Ag-O dans l'adhérence de ce microorganisme aux cellules uroépithéliales. Ces mutants adhéraient effectivement beaucoup moins que la souche parentale (Merino *et al.*, 1997).

Parallèlement, nous avons cherché à savoir si des conditions de croissance réduites en fer, telles que retrouvées dans les tissus animaux, influençaient la production des polysaccharides de surface chez *A. pleuropneumoniae* comme elles le peuvent pour l'expression de certaines protéines de la membrane externe d'*A. pleuropneumoniae*. Nous désirions principalement vérifier si les LPS pouvaient être plus exposés soit par une diminution de la production du matériel capsulaire, soit par une augmentation de la production des LPS. Par cytofluorométrie, nous n'avons pas observé de différence dans la production des CPS. Les marquages, des bactéries de sérotype 1 ayant poussé dans le milieu contenant du fer ou dans le milieu réduit en fer par l'ajout de 160 μM de 2,2'-dipyridyl, ont été effectués à l'aide du mAb LA 40#1.M et ont démontrés des quantités de CPS similaires.

Toujours par cytofluorométrie, nous avons observé chez la souche de référence d'*A. pleuropneumoniae* sérotype 1, par marquage de l'Ag-O à l'aide du mAb 5.1 G8F10, deux sous-populations que l'on pouvait aussi distinguer en MET. Dans le milieu riche en fer, la sous-population I est plus importante en nombre de cellules (77%) que la sous-population II (23%), mais les cellules de la sous-populations II sont plus fluorescentes, i.e. d'une différence de 1 logarithme, donc auraient environ 10X plus d'Ag-O marqués. Par contre, lorsque les cellules sont cultivées dans le milieu pauvre en fer, les deux sous-populations sont quasi équivalentes en nombre de cellules. Ce qui pourrait vouloir dire que lorsque cette souche se retrouve chez l'hôte, la faible concentration de fer disponible pourrait inciter la bactérie à allonger ses molécules de LPS de plus d'unités répétitives d'Ag-O.

La possibilité que plus de LPS/cellule soient produits semble moins probable puisque un marquage des cellules cultivées dans chacun des milieux a été effectué avec un mAb dirigé contre le lipide A et aucune différence significative

n'a été observée. Ce phénomène pourrait permettre à la bactérie d'augmenter la quantité de ses adhésines et ainsi ses chances d'adhérer et d'infecter l'hôte. Il serait donc intéressant, à la lueur de ces résultats, de comparer l'adhérence d'*A. pleuropneumoniae* cultivé dans un milieu riche ou pauvre en fer. Ce n'est cependant pas toutes les souches du sérotype 1 d'*A. pleuropneumoniae* qui présentent deux sous-populations. Ainsi chez l'isolat FMV87-682, ne présentant qu'une seule population, la concentration de fer ne change en rien le marquage des Ag-O.

Finalement, comme dernier objectif, nous avons voulu identifier des récepteurs potentiels pour les LPS d'*A. pleuropneumoniae* au niveau des voies respiratoires porcines. Pour ce faire, nous avons utilisé des cellules épithéliales de trachée porcine puisque, précédemment, nous avions étudié l'adhérence d'*A. pleuropneumoniae* au niveau de ce tissu. Pour la recherche de récepteurs, l'utilisation de cellules prélevées directement chez l'animal, peut amener quelques difficultés de reproductibilité car l'expression des récepteurs peut être sujette à des variations entre différents individus d'une même espèce ainsi qu'à différents âges chez un même individu. Nous nous sommes principalement intéressés au contenu protéique des cellules épithéliales, donc après avoir solubilisé les protéines des cellules épithéliales, elles ont été séparées par électrophorèse et transférées sur une membrane de nitrocellulose. Les membranes de nitrocellulose ayant l'empreinte du profil protéique des cellules épithéliales de trachée ont ensuite été traitées de nombreuses manières. Grâce à cette méthodologie, nous avons pu mettre en évidence sept principales bandes dont un triplet à Mr 38 500, un doublet à 17 000 et deux bandes simples à 16 000 et 14 000. Toutes ces bandes réactives étaient de nature protéique; leur activité était totalement inhibée par une digestion à la protéinase K et non par un traitement au périodate de sodium. De plus, elles n'étaient pas détectées comme étant des glycoprotéines.

Pour vérifier la variabilité de l'expression des récepteurs "potentiels" identifiés, nous avons refait le test d'adhérence d'*A. pleuropneumoniae* aux cellules de trachée provenant de quatre races hybrides de porcelets. Nous avons aussi comparé l'adhérence d'*A. pleuropneumoniae* aux cellules de trachée provenant de porcelets non sevrés (ayant trois jours d'âge), de porcelets sevrés (ayant 7-8 semaines d'âge) et de porcs à l'abattage (ayant environ 6 mois d'âge). Les

protéines se liant aux LPS d'*A. pleuropneumoniae* étaient toujours présentes dans les différentes préparations. Ce qui n'est pas surprenant car les porcs sont susceptibles à une infection à *A. pleuropneumoniae* aussitôt qu'ils sont mis dans des conditions d'élevage intensif. Les porcelets ne sont pas moins susceptibles, sauf s'ils recoivent des anticorps maternels lors de l'allaitement.

Par séquençage des acides aminés de l'extrémité N-terminale, les deux protéines de 17 kDa seraient l'histone H3.1/H3.2 et l'histone H2B1. De plus, par homologie du profil des masses moléculaires, il est fort probable que les bandes de plus faibles masses moléculaires, soit celles de 16 et 14 kDa soient les histones H2A et H4, respectivement. Quoique habituellement intracellulaires, ces protéines cationiques ont été détectées à la surface de monocytes (Holers et Kotzin, 1985). Il pourrait donc être intéressant à l'aide d'anticorps dirigés contre ces différentes histones de vérifier la réactivité des bandes avec ces anticorps ainsi que la possibilité de bloquer l'adhérence d'*A. pleuropneumoniae* à ces bandes. De plus, nous pourrions utiliser ces anticorps pour marquer des coupes de tissu et vérifier si des histones se retrouvent à la surface des cellules épithéliales de trachée porcine. Cependant, il est peu probable que les histones représentent des récepteurs pour les LPS.

Pour ce qui est de la bande de 38 500 qui semble être un récepteur potentiel pour les LPS d'*A. pleuropneumoniae* aucune séquence en acides aminés n'a pu être obtenue dû, ici aussi, au blocage de la région N-terminale. Des essais devront être faits avec les bandes sous-jacentes. Nous avons aussi testé la présence de protéines liant les LPS d'*A. pleuropneumoniae* chez les macrophages alvéolaires et avons observé les mêmes bandes. Les récepteurs de LPS ayant été étudiés maintes fois chez les macrophages, nous avons pu retrouver dans la littérature des protéines ayant une masse moléculaire correspondante à la nôtre et ayant la capacité de lier les LPS telles qu'une galectine-3 de 31-32 kDa observée par Gupta *et al.* (1997); une protéine de 38 kDa identifiée par Halling *et al.* (1992); une protéine cationique de 37 kDa étudiée par Pereira *et al.* (1993) ainsi que par Brakett *et al.* (Brackett *et al.*, 1997); et les surfactants pulmonaires SP-A et SP-D ayant comme sous unités des protéines de 28-36 kDa et de 42-44 kDa, respectivement. Des efforts devront être déployés afin de confirmer la présence de cette protéine au niveau membranaire. Enfin, des résultats

préliminaires, nous indiquent qu'il serait intéressant de vérifier l'adhérence des LPS d'*A. pleuropneumoniae* à divers glycolipides.

V. CONCLUSION

Au cours de ce projet, le développement du modèle d'adhérence sur coupes congelées de tissus nous a permis de démontrer que:

i) *A. pleuropneumoniae* adhère à l'épithélium de la trachée ainsi qu'à l'endothélium vasculaire du poumon chez les porcelets;

ii) les LPS d'*A. pleuropneumoniae* inhibent l'adhérence de ce microorganisme;

iii) la partie polysaccharidique des LPS d'*A. pleuropneumoniae* est impliquée dans l'adhérence de ce microorganisme;

iv) des anticorps dirigés contre l'Ag-O des LPS d'*A. pleuropneumoniae* inhibent l'adhérence de ce microorganisme;

De plus, nous avons réussi à mettre en évidence que:

v) l'Ag-O des LPS d'*A. pleuropneumoniae* est bel et bien accessible à la surface de ce microorganisme encapsulé;

vi) deux types de noyau OS-lipide A ayant des propriétés différentes existent parmi les nombreux sérotypes d'Ag-O des LPS d'*A. pleuropneumoniae;*

vii) chez la souche de référence d'*A. pleuropneumoniae* sérotype 1, les conditions de croissance réduites en fer n'influencent pas la production des CPS, mais augmentent la production des Ag-O des LPS;

viii) les LPS d'*A. pleuropneumoniae* se lient aux histones et à des protéines d'environ 38,5 kDa.

Idéalement, la production de mutants isogéniques sans Ag-O et/ou de mutants isogéniques possédant toujours l'Ag-O, mais où le noyau OS serait tronqué offrirait des outils permettant de mieux évaluer le rôle de chaque composante du LPS dans l'interaction adhésine-récepteur. De plus, l'utilisation d'autres systèmes d'adhérence tenant compte des divers types moléculaires et cellulaires présents au niveau des voies respiratoires porcines pourrait permettre la mise en évidence d'autres récepteurs pour les LPS d'*A. pleuropneumoniae.*

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VII. ANNEXES

Acte de colloque 1

Localization of *Actinobacillus pleuropneumoniae* lipopolysaccharides by immunoelectron microscopy

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21st Annual Meeting of the Microscopial Society of Canada.
Montréal, Québec: 12 au 15 juin 1994, pp.130-131.

J'ai participé à l'analyse des résultats ainsi qu'à la rédaction de ce rapport.

LOCALIZATION OF ACTINOBACILLUS PLEUROPNEUMONIAE LIPOPOLYSACCHARIDES BY IMMUNOELECTRON MICROSCOPY.

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1. INTRODUCTION

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a disease causing tremendous economic loss to the swine industry. We previously demonstrated that lipopolysaccharides (LPS) were the major adhesin of A. pleuropneumoniae involved in adherence to porcine respiratory tract cells and mucus (Bélanger et al. 1990, 1992, 1994). LPS are amphiphilic molecules and represent a major component of the outer membrane of gram-negative bacteria. The purpose of the present study was to show accessibility of LPS molecules at the surface of this encapsulated microorganism, an essential prerequisite for a bacterial adhesin.

2. MATERIALS AND METHODS

2.1 Immunostabilization of the capsular material

Capsular material of A. pleuropneumoniae serotype 1 reference strain 4074 was stabilized using a mouse monoclonal antibody against A. pleuropneumoniae serotype 1 capsular antigen (1.5 C5 F4), and stained with ruthenium red before examination by transmission electron microscopy as described by Jacques et al. (1988).

2.2 Immunolabeling of LPS

Bacteria were resuspended in PBS (0.01M, pH 7.4). Single drops of bacterial suspension were placed on 200-mesh Formvar-coated grids and were allowed to partially air dry. The grids were then placed sequentially on drops of PBS containing 1% egg albumin for 5 min and mouse monoclonal against A. pleuropneumoniae serotype 1 O-antigen (5.1 G8F10; kindly supplied by E. Stenbaek, Denmark) for 30 min. They were then washed in distilled water and placed on drops of colloidal gold particles (10 nm) conjugated to goat anti-mouse IgG for 30 min. After a final wash in distilled water, they were stained with 0.2% phosphotungstate (pH 7.1) and then examined with a Philips 201 electron microscope at an accelerating voltage of 60 kV.

3. RESULTS AND DISCUSSION

We first confirmed that A. pleuropneumoniae cells were covered by a thick layer of polysaccharidic capsular material. Since conventional fixation for electron microscopy is insufficient to preserve bacterial capsular material, which comprises a highly hydrated polyanionic structure that collapses during dehydration, immunostabilization had to be used. Cells of A. pleuropneumoniae 4074 incubated with monoclonal antibody 1.5 C5 F4 (against serotype 1 capsular antigen) and stained with ruthenium red were indeed covered by a 190 - 210 nm layer of capsular material (Figure 1A). We then evaluated the accessibility of LPS molecules at the surface of this encapsulated microorganism by incubating cells of A. pleuropneumoniae 4074 with monoclonal

antibody 5.1 G8F10 (against serotype 1 O-antigen) and goat anti-mouse IgG-gold particles (10 nm). As shown in Figure 1B, bacterial cells were heavily labeled with gold particles despite the presence of a thick capsular material layer.

Results of the present study indicate that monoclonal antibodies against the capsular antigen could be used, instead of a rabbit polyclonal antiserum against whole bacterial cells (Jacques et al. 1988), to specifically stabilize the capsular material of A. pleuropneumoniae. Results, using immunoelectron microscopy, also indicate that LPS are indeed exposed at the surface of this encapsulated microorganism and may, therefore, interact with host components.

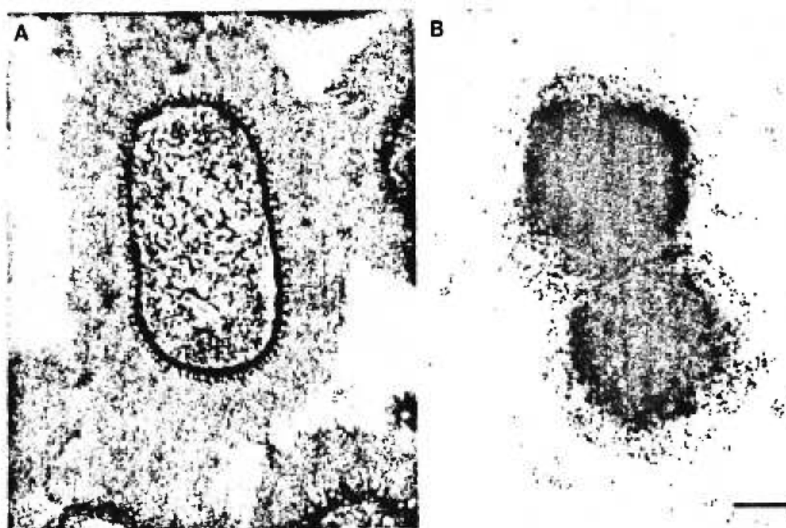

Figure 1. (A) Transmission electron micrograph of thin sections of A. pleuropneumoniae 4074 cells immunostabilized with monoclonal antibody (1.5 C5 F4) against serotype 1 capsular antigen and stained with ruthenium red. (B) Transmission electron micrograph of whole cells of A. pleuropneumoniae 4074 probed with monoclonal antibody (5.1 G8F10) against serotype 1 O-antigen and goat anti-mouse IgG-gold particles (10 nm). Bar, 200 nm.

Bélanger M. et al. (1990) Infect. Immun. **58**:3523-3530.
Bélanger M. et al. (1992) FEMS Microbiol. Lett. **97**:119-126.
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Jacques M. et al. (1988) J. Bacteriol. **170**:3314-3318.

Acte de colloque 2

Electron microscopic examination of *Actinobacillus pleuropneumoniae* lipopolysaccharides (LPS) fractionnated by gel chromatography

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13th International Congress for Electron Microscopy.
Paris, France: 17 au 22 juillet 1994, volume 3B:1325-1326.

J'ai effectué la purification des LPS et leur fractionnement sur colonne ainsi que les différents dosages. De plus, j'ai participé à l'analyse des résultats et à la rédaction de cette présentation.

ICEM 13-PARIS
17-22 July 1994

Electron Microscopic Examination of *Actinobacillus pleuropneumoniae* Lipopolysaccharides (LPS) Fractionated by Gel Chromatography

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1. INTRODUCTION

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a disease causing tremendous economic loss to the swine industry. We previously demonstrated that lipopolysaccharides (LPS) were the major adhesin of A. pleuropneumoniae involved in adherence to porcine respiratory tract cells and mucus (Bélanger et al. 1990, 1992, 1994). LPS are amphiphilic molecules and represent a major component of the outer membrane of gram-negative bacteria. LPS molecules are composed of three well-defined regions: the lipid A; the core, an oligosaccharide containing KDO; and the O-antigen, a chain of polysaccharides consisting of repeating units. The purpose of the present study was to examine, by negative staining, preparations of LPS molecules separated by chromatography according to their molecular mass.

2. MATERIALS AND METHODS

2.1 Extraction and fractionation of LPS

LPS from A. pleuropneumoniae serotypes 1 and 2 were extracted and isolated by the method of Darveau and Hancock (1983). Extracted LPS were separated in the presence of sodium deoxycholate by chromatography on a Sephacryl S-300SF (Pharmacia) column according to the procedure described by Peterson and McGroarty (1985).

2.2 Negative staining

A drop of each LPS preparations, sonicated for 5 minutes, was placed on 200-mesh Formvar-coated grids. A drop of 1% (wt/vol) phosphotungstate (pH 7.1) was then applied to the grids which were then examined with a Philips 201 electron microscope at an accelerating voltage of 60 kV.

3. RESULTS AND DISCUSSION

LPS molecules were separated according to their molecular mass by gel chromatography (Figure 1), and fractions were examined by transmission electron microscopy after negative staining. Molecular aggregates of extracted LPS as well as fractions 188 to 236 appeared as ribbons of approximately 12 nm wide (Figure 2B). High-molecular mass fractions (104 to 176) contained thin filaments of approximately 2 nm wide (Figure 2A) whereas vesicles of 55-75 nm in diameter were observed in the low-molecular-mass fractions 248 to 332 (Figure 2C). Our results suggest that A. pleuropneumoniae LPS aggregates adopt different shapes depending on the O-antigen chain length as well as the lipid/carbohydrate ratio.

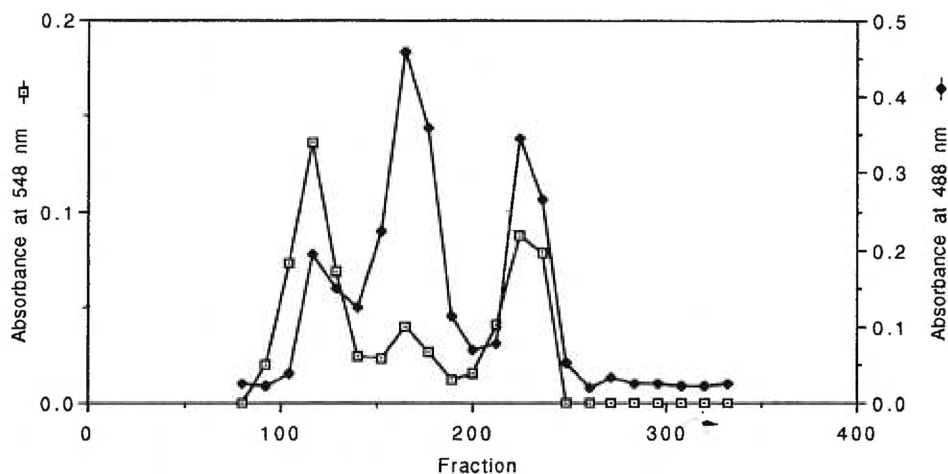

Figure 1: Fractionation of extracted LPS from A. pleuropneumoniae serotype 1 on a Sephacryl S-300SF column in the presence of 0.25% deoxycholate. Fractions (in ml) were analyzed for KDO (squares) and for neutral sugars (diamonds).

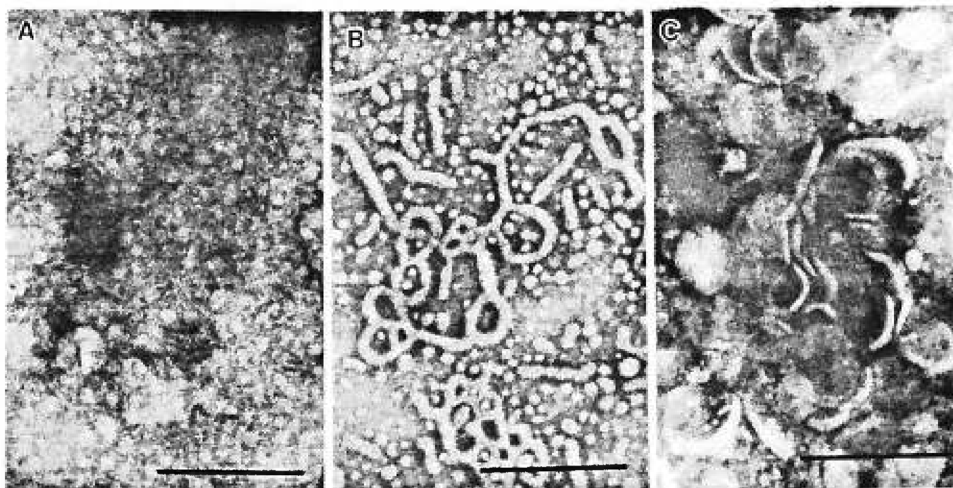

Figure 2: Transmission electron micrographs of negatively stained A. pleuroneumoniae serotype 1 LPS. LPS preparations obtained after chromatography on a Sephacryl S-300SF column: (A) fractions 104 to 128; (B) fractions 224 to 236; (C) fractions 248 to 332. Bars, 200 nm.

Bélanger M. et al. (1990) *Infect. Immun.* **58**:3523-3530.
Bélanger M. et al. (1992) *FEMS Microbiol. Lett.* **97**:119-126.
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Darveau R.P. and Hancock R.E.W. (1983) *J. Bacteriol.* **155**:831-838.
Peterson A.A. and McGroarty E.J. (1985) *J. Bacteriol.* **162**:738-745.

Article 5

Effects of pig hemoglobin binding on some physical and biological properties of *Actinobacillus pleuropneumoniae* lipopolysaccharides

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Journal of Endotoxin Research. **4:** 53-65, 1997

Dans cette étude, j'ai effectué les tests de cytofluorométrie. J'ai aussi contribué à la mise au point de la centrifugation des LPS sur gradient de sucrose, ainsi qu'à celle des conditions d'électrophorèse et d'immunobuvardage des fractions récoltées. De plus, j'ai participé à l'analyse des résultats et à la révision du manuscrit.

Research

Journal of Endotoxin Research (1997) 4(1), 53–65
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Effects of pig hemoglobin binding on some physical and biological properties of *Actinobacillus pleuropneumoniae* lipopolysaccharides

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Summary Binding of pig hemoglobin (Hb) to *Actinobacillus pleuropneumoniae* lipopolysaccharide (LPS), either extracted or present at the surface of whole cells, was studied. After a short incubation period, pig Hb seemed to cover the bacterial cell surface and enhanced the cells' contrast when examined by transmission electron microscopy (TEM). Energy-dispersive X-ray spectroscopy analysis showed that the amount of elemental iron detected was increased when cells of *A. pleuropneumoniae* were incubated with pig Hb. Coating with pig Hb, however, did not interfere with the accessibility of O- and capsular antigens to antibodies on the bacterial cell surface. Binding of pig Hb and polymyxin B to lipid A of *A. pleuropneumoniae* was confirmed with a fluorescent probe (dansylcadaverine) displacement assay. The binding of pig Hb to extracted LPS resulted in a disaggregation of LPS as observed by TEM after negative staining. Additional evidence for a direct physical interaction between pig Hb and *A. pleuropneumoniae* LPS was demonstrated by a shift in the sedimentation velocity of LPS-Hb complexes determined by sucrose gradient centrifugation. Pig Hb binding to extracted LPS or to bacterial cells resulted in a significant decrease of chromogenic *Limulus* amebocyte lysate activation. Finally, the capacity of extracted LPS to induce NO_2^- in the presence of pig Hb was tested by using cell line J774 and determined by the Greiss' reaction. LPS alone induced, as expected, NO_2^- production, whereas the presence of pig Hb significantly reduced NO_2^- production by murine macrophages. Taken together, our results indicate that binding of pig Hb affected some physical and biological properties of *A. pleuropneumoniae* LPS.

INTRODUCTION

Recent studies have clearly demonstrated that in vitro, complex formation occurs between endotoxin (LPS or lipopolysaccharide) and human hemoglobin (Hb), and that the procoagulant activity of these complexes was increased compared with endotoxin alone as determined by LAL (*Limulus* amebocyte lysate) gelation or chromogenic tests.[1–3] In addition, these endotoxin-Hb complexes have increased potency for the production of tissue factor by cultured human umbilical vein endothelial cells[4] and human monocytes.[5] However, the mechanisms of their synergistic interaction are not known. It has been also demonstrated that binding of LPS to the β-chains of Hb was particularly prominent and that a portion of LPS in the presence of Hb had increased electrophoretic mobility, and co-electrophoresed with

Received 21 June 1996
Revised 16 December 1996
Accepted 3 January 1997

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Hb.[2] A reduction in LPS sedimentation velocity was observed when LPS was incubated with Hb and then subjected to a sucrose gradient centrifugation.[2] In addition, synergistic in vivo toxicities of endotoxin and Hb have been reported.[6,7]

Actinobacillus pleuropneumoniae, a member of the *Pasteurellaceae*, is the etiological agent of porcine pleuropneumonia, a severe disease that causes economic loss to the swine industry. Several bacterial components, including hemolysins and/or cytolysins, LPS, and capsular polysaccharides, appear to contribute to the disease process.[8] We previously demonstrated that LPS was the major adhesin of *A. pleuropneumoniae* involved in adherence to porcine respiratory tract cells.[9–11] More recently, we observed the binding of pig Hb to *A. pleuropneumoniae* LPS.[12] We demonstrated that lipid A was involved and that polymyxin B completely inhibited binding of Hb to *A. pleuropneumoniae* lipid A. Interestingly, this hemolytic microorganism is also able to use pig Hb as a sole source of iron for growth.[12,13]

The current study was undertaken to further investigate the binding of pig Hb to *A. pleuropneumoniae* LPS, either extracted or present at the surface of whole cells, in order to determine whether such interactions might affect some physical and biological properties of *A. pleuropneumoniae* LPS.

MATERIALS AND METHODS

Bacterial isolates and growth conditions

A. pleuropneumoniae reference strains of serotype 1 (strain 4074; semi-rough LPS profile) and serotype 2 (strain 4226; smooth LPS profile) were provided by A. Gunnarson, National Veterinary Institute, Uppsala, Sweden. Bacteria were grown on brain heart infusion agar plates (Difco Laboratories, Detroit, MI, USA) supplemented with 15 µg of NAD/ml. Plates were incubated at 37°C for 18 h.

Reagents

Pig and human Hb, and polymyxin B sulfate were obtained from Sigma Chemical Co., St Louis, MO, USA. Dansylcadaverine was obtained from Molecular Probes (Eugene, OR, USA). LPS from *Salmonella minnesota* (smooth and Re 595) and *Escherichia coli* (0111:B4 and Rd F583) were obtained from Sigma. Pig and human Hb stock solutions (1 mg/ml) were prepared in endotoxin free water (Associates of Cape Cod, Woods Hole, MA, USA) and contained less than 0.6 EU/ml of endotoxin as determined by cLAL.

Extraction of LPS

LPS from *A. pleuropneumoniae* was extracted and isolated by the method of Darveau and Hancock[14] with some modifications.[15] Briefly, disrupted cells were treated with DNase, RNase, pronase and sodium dodecyl sulfate (SDS), and then subjected to $MgCl_2$ precipitation and high-speed centrifugation.

Electron microscopy

Negative staining of LPS aggregates

Extracted LPS from *A. pleuropneumoniae* (1 mg/ml) was incubated for 1 h or 18 h at 37°C with either pig Hb (1 mg/ml) or polymyxin B sulfate (0.1 or 1 mg/ml). Samples were recovered after ultracentrifugation at 80 000 *g* for 6 h. A drop of each LPS preparation was placed on 200-mesh Formvar-coated grids that were then stained with 1 % (w/v) phosphotungstate. The grids were examined with a Phillips 201 electron microscope at an accelerating voltage of 60 kV. LPS of *A. pleuropneumoniae* that had not been incubated with Hb or polymyxin B served as control.

Whole cells

Whole cells of *A. pleuropneumoniae* were incubated for 30 min at 37°C with pig Hb (1 mg/ml of PBS, 0.01 M, pH 7.4). A single drop of the sample was placed on Formvar-coated grids and was allowed to partially air dry. The grids were washed in distilled water and examined without any additional staining by transmission electron microscopy as described above. Cells of *A. pleuropneumoniae* that have not been incubated with pig Hb served as controls.

Energy-dispersive X-ray spectroscopy analysis

Grids with whole cells of *A. pleuropneumoniae* prepared as described above were also viewed with a Phillips EM400T transmission electron microscope equipped with a model LZ-5 light element detector and an exL multichannel analyzer (both from LINK Analytical) operating at 100 kV, with a cold trap in place, to obtain elemental analysis. Energy-dispersive X-ray spectroscopy (EDS) spectra were taken by using a beam current of 0.1 µA and a spot size of 200 nm. The counting time was 100 s (live time).[16]

Flow cytometry

Overnight cultures of *A. pleuropneumoniae* were washed and resuspended in PBS to an A_{540} of 0.2, equivalent to approximately 10^8 CFU/ml. Bacterial suspensions (1 ml) were incubated with pig Hb (1 mg/ml) for 60 min at 37°C. Cells were washed twice in PBS and centrifuged at

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10 000 *g* for 2 min, then the pellets were resuspended in 1 ml of a dilution of a monoclonal antibody against serotype 1 O-antigen (5.1G8F10) or serotype 1 capsular-antigen (1.5C5F4), or serotype 2 O-antigen (102-G02) (all kindly supplied by M. Gottschalk, Faculté de médecine vétérinaire, Université de Montréal, Montréal, Canada) and incubated for 60 min at room temperature. Samples were washed twice in PBS, and then 0.5 ml of sheep fluorescein isothiocyanate-conjugated anti-mouse IgG (Boehringer Mannheim, Laval, Québec, Canada) diluted 1:50 in PBS was added. After a further incubation of 60 min at room temperature, samples were washed twice in PBS and fixed with 2% paraformaldehyde. Cells were kept in the dark at 4°C until analyzed by flow cytometry as previously described.[10] Suspensions of cells of *A. pleuropneumoniae* that have not been incubated with pig Hb served as controls.

Sucrose centrifugation

Continuous sucrose density gradients (12 ml; 5–20%) were prepared in 0.05 M Tris and 0.25% deoxycholic acid in pyrogen-free water. LPS from *A. pleuropneumoniae*, final concentration of 1 mg/ml, was added to a pig Hb solution of 10 mg/ml and the mixture was incubated for 1 h at 37°C. Aliquots (100 µl) of LPS-Hb mixtures, LPS alone, or Hb alone were then layered over a sucrose gradient and centrifuged at 52 000 *g* for 4 h in a Beckman L7-65 ultracentrifuge using a SW28 rotor. Following centrifugation, the tubes were punctured and 1.5 ml fractions were collected and analyzed by gel electrophoresis.

Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted by the discontinuous buffer system of Laemmli,[17] with a 4.5% polyacrylamide stacking gel and a 15% polyacrylamide running gel. Samples were boiled for 15 min in solubilization buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.025% bromophenol blue). Prestained and low-molecular mass markers were obtained from Bio-Rad (Mississauga, Ontario, Canada). Gels were run in a Mini-PROTEAN II vertical slab electrophoresis cell (Bio-Rad) and were either stained with silver nitrate as described by Tsai and Frasch[18] or stained with Coomassie brilliant blue R-250 or transferred to nitocellulose for Western blotting.

Western Blot

Western blots were carried out as described by Towbin et al.[19] All incubations were followed by four 3 min washes with a Tris-saline buffer (10 mM Tris, 150 mM NaCl, pH 7.4). The membrane were first incubated at room temperature for 1 h with 2% casein (a blocking solution) and then incubated overnight at 4°C with serum from a pig experimentally infected with *A. pleuropneumoniae* serotype 2 or a rabbit antiserum against pig Hb. The membranes were then incubated at room temperature for 1 h with a goat anti-pig IgG (heavy plus light chains) horseradish peroxidase conjugate (Jackson Immunoresearch Lab) or a goat anti-rabbit IgG (heavy plus light chains) horseradish peroxidase conjugate (Bio-Rad). Reactions were revealed by addition of 4-chloro-1-naphthol and hydrogen peroxide (Sigma) as chromogenic substrate.

Dansylcadaverine displacement assay

The use of dansylcadaverine (DC) as a fluorescent displacement probe to characterize the interaction of lipid A-binding molecules has been described by David et al.[20,21] The binding of the probe to lipid A results in an intensity enhancement in the emission spectrum of DC. Compounds which bind lipid A displace the probe, resulting in quenching of fluorescence. Probe displacement is analyzed as a function of displacer (e.g. polymyxin B or pig Hb) concentration. In this study, we compared the relative binding affinities of polymyxin B and pig Hb to lipid A of *A. pleuropneumoniae* strain 4074. *A. pleuropneumoniae* lipid A was obtained after acid hydrolysis of extracted LPS.[12] The relative binding affinities were measured by DC displacement assays as described by David et al.[20,21] using a spectrofluorometer Fluorolog CM-III (Spex industries, Edison, NJ, USA). The excitation wavelength was 340 nm. Emission wavelength is an interval between two wavelengths (450 and 660 nm) where bandpasses for excitation and emission were 5 nm for both monochromators. Briefly, 50 µM of DC and 20 µg of *A. pleuropneumoniae* lipid A were mixed together in 1 ml of 50 mM Tris-HCl buffer (pH 7.4) which resulted in maximum fluorescence. Different concentrations of polymyxin B or pig Hb were added to the solution and the subsequent decrease in fluorescence owing to the displacement of the DC from lipid A was recorded after each addition.

Chromogenic *Limulus* amebocyte lysate (cLAL) test

Activation of cLAL by endotoxin was used to compare the biological activity of extracted LPS or LPS of whole cells in the presence or absence of pig Hb. Sterile, endotoxin-free water and microplates (Pyroplates) were purchased from Associates of Cape Cod. Glassware was heated at 190°C in a dry oven for 5 h. Some experiments were conducted with two-fold dilutions of extracted LPS (starting concentration 1 µg/ml) or two-fold dilutions of bacterial cells (starting concentration 1 mg/ml), and 50 µl of Hb (1 mg/ml) was added to diluted LPS or diluted bac-

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terial cells. Other experiments were conducted with 10-fold dilutions of Hb (starting concentration 1 mg/ml) or polymyxin B sulfate (starting concentration 1 mg/ml), and 50 µl of LPS (1 µg/ml) was added to diluted Hb or diluted polymyxin B. Dilutions were prepared using pyrogen-free water, in nontreated polystyrene microplates. 50 µl of cLAL (Pyrochrome, Associates of Cape Cod) was then added to each well and the plates were incubated at 37°C for 30 min. Absorbance was determined with an automated plate reader (Dynatech Laboratories Inc, Virginia, USA) at 410 nm. Background absorbance at 410 nm, which included a component of absorbance due to Hb, was subtracted from each reading. Polymyxin B sulfate, a well known inhibitor of LPS biological activity, was used as control. Activation of cLAL by LPS from *S. minnesota* or *E. coli* with or without human Hb was also evaluated. In some experiments, gelation LAL test (gLAL, Associates of Cape Cod) was done following the same protocol as for cLAL but instead of chromogen quantification, spectrophotometric measurement of turbidity was determined with the automated plate reader at 410 nm.

Nitric oxide production

Murine macrophages, cell line J774, were cultured in RPMI-1640 (Gibco BRL, Burlington, Ontario, Canada) without phenol containing 10% heat-inactivated FBS (HyClone Laboratories Inc., Logan, UT, USA), 5% L-glutamine (Gibco BRL) and 5% penicillin-streptomycin (Gibco BRL), stimulated with *A. pleuropneumoniae* serotype 2 LPS preincubated or not with pig Hb, and assayed for production of nitric oxide (NO_2^-) as described.[22] Adherent macrophage monolayers were obtained by plating the cells in 24-well microtiter plates (Falcon 3047, Becton Dickinson Labware, Lincoln Park, NJ, USA) at 5×10^5 cells/well for 4 h at 37°C in 5% CO_2 and monolayers were then washed with warm culture medium. Cells were then exposed to dilutions of LPS (1 µg/ml to 0.001 µg/ml) preincubated (30 min) or not with pig Hb (1 mg/ml or 10 mg/ml) for 24 h at 37°C in 5% CO_2. Following incubation, cell culture supernatants were collected and NO_2^- production determined by the Greiss' reaction. Briefly, aliquots of 300 µl were removed from conditioned medium and incubated with an equal volume of Greiss' reagent (1% (w/v) sulfanilamide and 0.1% (w/v) naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid) at room temperature for 15 min. Absorbance was determined with an automated plate reader at 540 nm. Cell medium background absorbance at 540 nm, which included a component of absorbance due to Hb, was subtracted from each reading. NO_2^- concentration was determined by using sodium nitrite as a standard. The production was expressed as a µM/5×10^5 cells.

Data analysis

All experiments were performed at least twice. The significance was evaluated by the one-tailed paired two group Student's *t*-test.

RESULTS

Electron microscopy and EDS of whole cells

Unstained preparations of *A. pleuropneumoniae* cells were examined by transmission electron microscopy. As expected, untreated cells (not stained and not incubated with pig Hb) offered a very poor contrast (Fig. 1A). However, cells of *A. pleuropneumoniae* incubated with pig Hb were more contrasted (Fig. 1B) which suggests that pig Hb, a relatively large molecule, was binding to

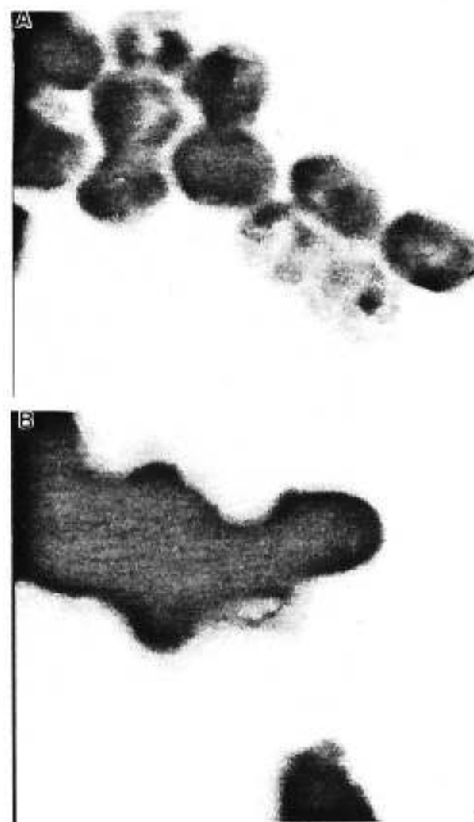

Fig. 1 Electron transmission micrographs of whole cells of *A. pleuropneumoniae* serotype 1. (A) Control cells that were not stained, nor incubated with pig Hb. (B) Unstained cells that were incubated during 30 min with pig Hb (1 mg/ml). Bar, 200 nm.

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the cell surface and coating the cells. The same preparations were also analyzed by EDS (Fig. 2). The amount of elemental iron detected was increased 4–8-fold when cells of *A. pleuropneumoniae* were incubated with pig Hb as compared to control cells not incubated with pig Hb. The amount of Fe, expressed as atom %, increased from 4.47 to 18.64, and from 0.93 to 7.77, when cells of *A. pleuropneumoniae* serotype 1 and serotype 2, respectively, were incubated with pig Hb. EDS does not differentiate, however, the iron present at the cell surface from the intracellular iron.

Flow cytometry

Since cells of *A. pleuropneumoniae* appeared to be coated with pig Hb after a short incubation period, we then determined whether binding of pig Hb to whole cells interfere with the accessibility of antibodies to surface antigens. Cells, incubated with or without pig Hb, were incubated with monoclonal antibodies against O- or capsular-antigens and analyzed by flow cytometry. Figure 3 shows representative flow cytometry profiles of whole cells of *A. pleuropneumoniae* incubated or not with pig Hb. The accessibility of LPS O-antigen (Fig. 3A,B) or capsular antigens (Fig. 3 C, D) to antibodies did not seem to be affected by a pre-incubation with pig Hb, since the fluorescence patterns (Fig. 3B,D) obtained were similar to the fluorescence patterns of control cells not pre-incubated with pig Hb (Fig. 3A,C).

Electron microscopy examination of extracted LPS

This experiment was conducted to evaluate the effect of pig Hb binding on the morphology of LPS aggregates. Molecular aggregates of *A. pleuropneumoniae* extracted LPS appeared as ribbons of approximately 12 nm wide in the absence of pig Hb (Fig. 4A) as previously described.[10] Incubation of pig Hb with extracted LPS for 1 h resulted in the disaggregation of LPS into smaller aggregates (Fig. 4B). After a longer incubation period (18 h) with pig Hb, small spherical particles which seem to be micelles were observed (Fig. 4C). The effect of pig Hb on LPS was different from the hexagonal lattice observed when *A. pleuropneumoniae* LPS was incubated with polymyxin B (Fig. 4D).

Sucrose density centrifugation

Mixtures of *A. pleuropneumoniae* serotype 2 LPS and pig Hb were layered over a sucrose gradient. After ultracentrifugation and SDS-PAGE, Western blot was performed to demonstrate the distribution of LPS in the different fractions. LPS alone sedimented into all fractions (Fig. 5A, lanes 1–8) of the sucrose gradient, whereas most of the LPS in Hb/LPS mixtures (Fig. 5B, lanes 7,8) had a sedimentation rate similar to that of Hb alone (Fig. 5C, lanes 7,8). Most of the Hb from Hb/LPS mixtures (Fig. 5D, lanes 7,8) and Hb alone remained in the top layer of the sucrose gradient. Similar results were obtained with *A. pleuropneumoniae* serotype 1 LPS. Therefore, incubation with pig Hb decreased the density of LPS, resulting in a different migration of LPS in a sucrose gradient.

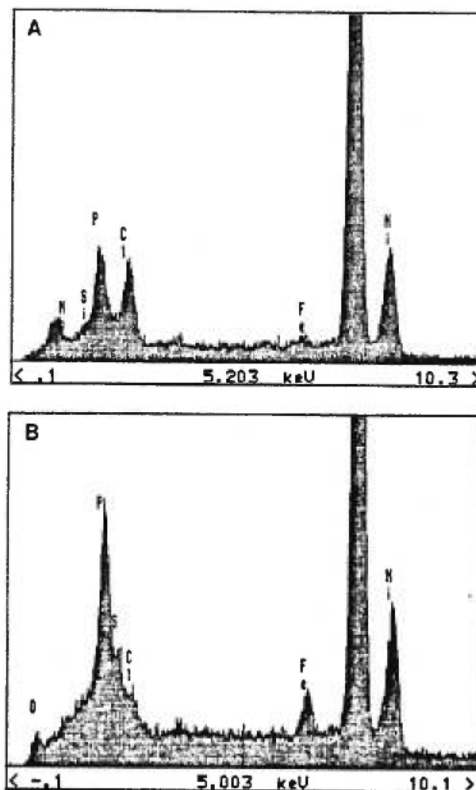

Fig. 2 EDS spectrum of whole cells of *A. pleuropneumoniae* serotype 1 shown in Figure 1. Elemental analysis of control cells (A) or cells incubated with pig Hb (B). An 8-fold increase in iron (Fe) was observed in cells previously incubated with pig Hb.

Dansylcadaverine displacement assay

We tested the ability of polymyxin B or pig Hb to displace DC from *A. pleuropneumoniae* lipid A. The addition of *A. pleuropneumoniae* lipid A to DC resulted in a marked

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enhancement of fluorescence intensity which is indicative of interaction of DC with lipid A. The maximal fluorescence of DC was achieved with 20 μg/ml of *A. pleuropneumoniae* lipid A. The addition of polymyxin B or pig Hb to the mixture of lipid A and DC resulted in an attenuation of fluorescence intensity (Fig. 6) which is indicative of the displacement of bound DC to lipid A by these compounds.

cLAL assay with extracted LPS and whole cells

We used a chromogenic LAL test to study the influence of pig Hb on LPS biological activity. A decrease in cLAL activity was obtained after addition of different dilutions of pig Hb to a constant concentration of extracted LPS or bacterial cells (Fig. 7A). As expected, we observed a reduction of cLAL activation when LPS were incubated with polymyxin B (Fig. 7B). Used at the same concentration (e.g. 0.01 mg/ml), pig Hb (0.15 nM) was a better inhibitor of cLAL activation (1.5–2-fold) than polymyxin B (6.89 nM). LAL gelation tests were used to confirm the results obtained with cLAL. As shown in Figure 8, addition of human Hb to *Enterobacteriaceae* LPS from *S. minnesota* (smooth and rough Re 595) or *E. coli* (O111:B4 and Rd F583) (data not shown) also significantly decreased LAL activation.

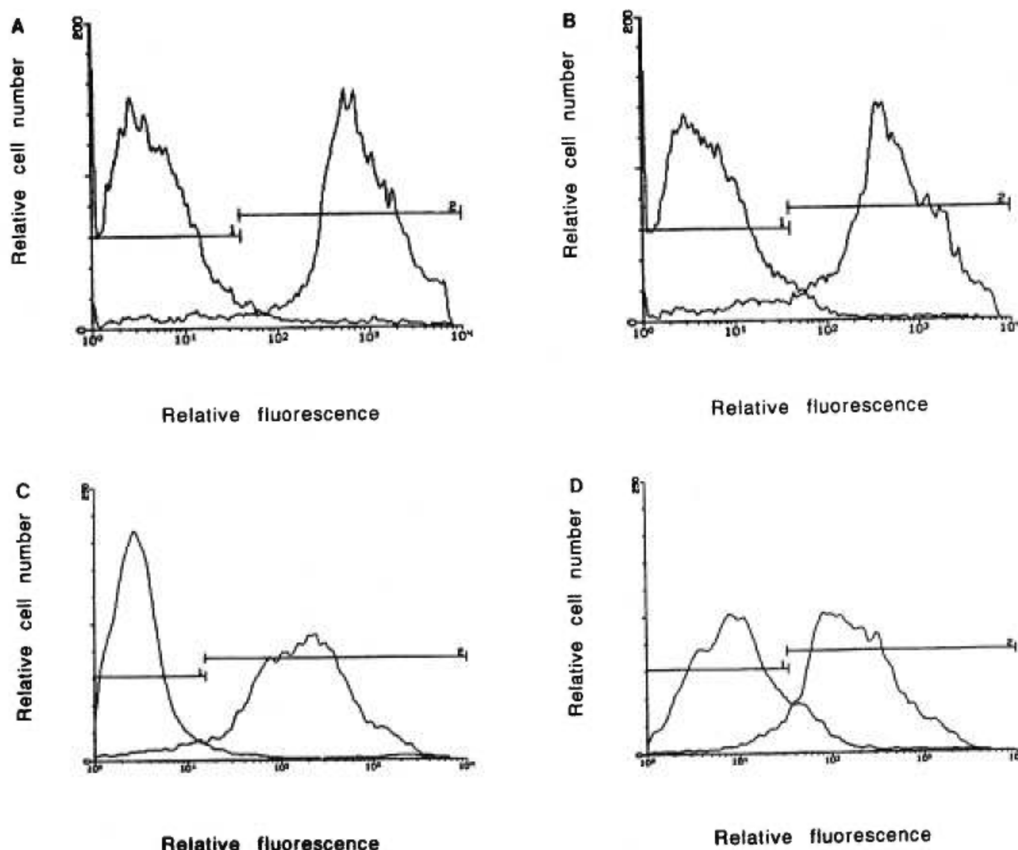

Fig. 3 Flow cytometry analysis of whole cells of *A. pleuropneumoniae* serotype 2 not incubated with pig Hb (A) or incubated with pig Hb (B) were labeled with the anti-mouse IgG fluorescein isothiocyanate (FITC)-conjugated antibody (left peak, control) or with monoclonal antibodies against serotype 2 O-antigen plus the anti-mouse IgG FITC-conjugated antibody (right peak). Whole cells of *A. pleuropneumoniae* serotype 1 not incubated with pig Hb (C) or incubated with pig Hb (D) were labeled with the anti-mouse IgG FITC-conjugated antibody (left peak, control) or with monoclonal antibodies against serotype 1 capsular-antigen plus the anti-mouse IgG FITC-conjugated antibody (right peak). The horizontal bars labelled '1' indicate non-specific fluorescence while bars labelled '2' indicate specific fluorescence.

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Nitric oxide production

We compared LPS alone and LPS-Hb complexes for their ability to decrease murine macrophages NO_2^- releasing capacity. LPS alone (0.1–1 μg/ml) induced substantial NO_2^- release (Fig. 9), whereas incubation of LPS (0.1–1 μg/ml) with pig Hb (1 mg/ml) significantly decreased release of NO_2^-. Incubation of LPS with pig Hb (10 mg/ml) resulted in NO_2^- production similar to the background level (macrophages alone in RPMI-1640) (data not shown).

DISCUSSION

We previously demonstrated that LPS was the major adhesin of *A. pleuropneumoniae* involved in adherence to porcine respiratory tract cells.[9,10] More recently, we observed the binding of pig Hb to the lipid A portion of *A. pleuropneumoniae* LPS.[12] The current study was undertaken to further examine the binding of pig Hb to *A. pleuropneumoniae* LPS from serotypes 1 and 2, either extracted or present at the surface of whole cells. These 2 serotypes were selected because they represent the two LPS profiles found in *A. pleuropneumoniae*, semi-rough (serotype 1) or smooth (serotype 2).

We first used electron microscopy which allowed the visualization of pig Hb at the surface of *A. pleuropneumoniae* whole cells. Pig Hb covered the bacterial cell surface and enhanced the cells' contrast. The same preparations were also analyzed by EDS and the amount of elemental iron detected at the surface or inside the cell was increased when *A. pleuropneumoniae* were incubated

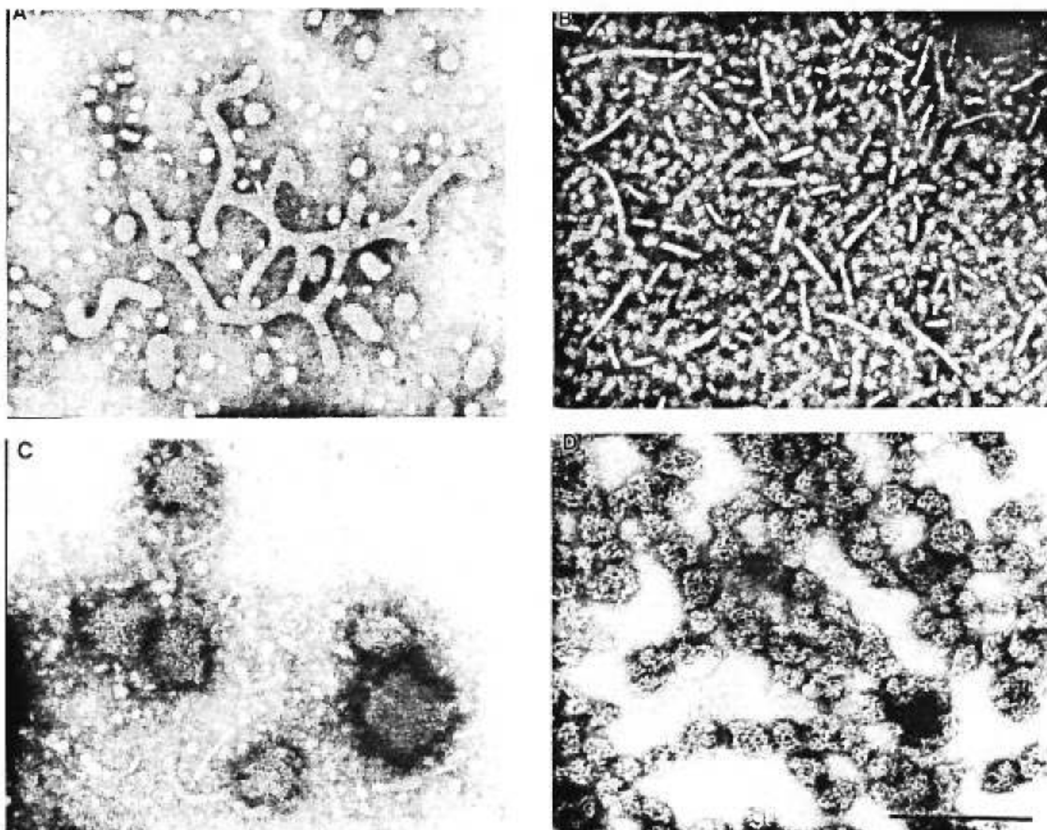

Fig. 4 Transmission electron micrographs of negatively stained *A. pleuropneumoniae* serotype 2 extracted LPS. In the absence of Hb, the LPS appeared as ribbon-like structures with frequent branching (**A**). When LPS was incubated with Hb (1 mg/ml) for 1 h, the typical structure was broken down into shorter molecular aggregates or completely disaggregated in small particles (**B**). After 18 h of incubation with pig Hb, micelles were observed (**C**). A hexagonal lattice was observed when LPS from *A.pleuropneumoniae* was incubated with polymyxin B (1 mg/ml) (**D**). Bar, 200 nm.

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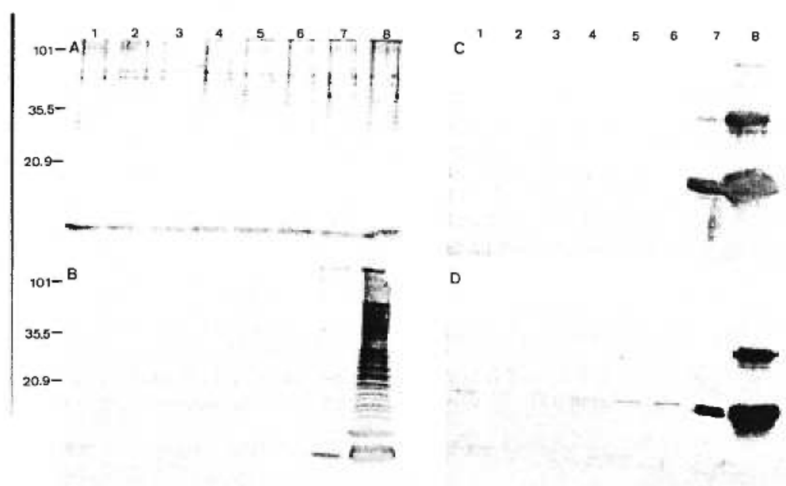

Fig. 5 Western Blot analysis of fractions obtained following sucrose density centrifugation. *A. pleuropneumoniae* serotype 2 extracted LPS (**A**), pig Hb (**C**), or *A.pleuropneumoniae* serotype 2 extracted LPS incubated with pig Hb (**B**,**D**) were layered over a 5–20% continuous sucrose gradient. After centrifugation, 8 fractions (of 1.5 ml) were recovered and analyzed by SDS-PAGE and Western blot; fractions 1 and 8 correspond to the bottom and the top of the tubes, respectively. Nitrocellulose membranes were either incubated with a serum from a pig experimentally infected with *A. pleuropneumoniae* (**A**,**B**) or with a rabbit antiserum against pig Hb (**C**,**D**).

with pig Hb. The ability of *A. pleuropneumoniae* to bind Hb to its surface might be quite advantageous for this microorganism knowing that it can express two hemolysins[23] and can use Hb as a source of iron for growth.[12,23] Our observations suggest that *A. pleuropneumoniae* LPS might represent a non-specific 'receptor' for pig hemoglobin or that it might perform a 'docking' function. It is possible that pig Hb would then be transferred to a specific receptor, such as the hemoglobin-binding proteins that have been reported in *Haemophilus ducreyi*,[24] *Haemophilus influenzae*,[24,25] and *Neisseria meningitidis*.[26] Additional work is needed to identify the proteins that are involved in the subsequent uptake of iron from pig Hb.

It is conceivable that binding of a host iron-containing protein such as Hb by *A. pleuropneumoniae* serves a dual function as a source of iron and as a protective coat. Hb binding to the bacterial cell surface may be operational in the ability of pathogens in evading host responses, in particular, activation of complement. However, addition of Hb had little or no effect on the intrinsic complement fixing abilities of endotoxin or endotoxin partial structures.[27]

Binding of host proteins by bacteria has been shown to change their physicochemical surface properties.[28,29] Flow cytometry experiments were conducted to determine whether Hb binding would also modify the cell surface characteristics of *A. pleuropneumoniae* and by this means interfere with the antibodies accessibility to surface antigens. Our results indicated that binding of pig Hb to the cell surface did not interfere with the accessibility of antibodies to O- and capsular antigens. We and others have shown that Hb was binding to the lipidic region of LPS.[2,12] We previously obtained evidence, using a monoclonal antibody (E5), directed against bacterial lipid A, that some lipid A residues are accessible at the surface of *A. pleuropneumoniae* cells, especially around outer membrane blebs.[12] Preferential binding of pig Hb to outer membrane blebs might explain why the binding of pig Hb to the cell surface did not interfere with the accessibility of antibodies to O- and capsular antigens. We have shown that LPS was the adhesin of *A. pleuropneumoniae* and that the active component of LPS involved in adherence was not the lipid A but the polysaccharides.[10] Further studies will be needed to verify that Hb binding to lipid A residues does not affect *A. pleuropneumoniae* adherence to porcine respiratory tract cells. It would also be quite interesting to determine whether Hb binding to LPS could affect *A. pleuropneumoniae* interaction with host alveolar macrophages where lipid A certainly plays a major role.

To determine whether Hb binding altered some physical properties of *A. pleuropneumoniae* LPS, we visualized the binding of pig Hb to *A. pleuropneumoniae* extracted LPS by electron microscopy after negative staining. In the absence of Hb, the LPS isolated from *A. pleuropneumoniae* appeared as ribbon-like structures with frequent branching. When the LPS was incubated with Hb, the typical structure was broken down in shorter molecular

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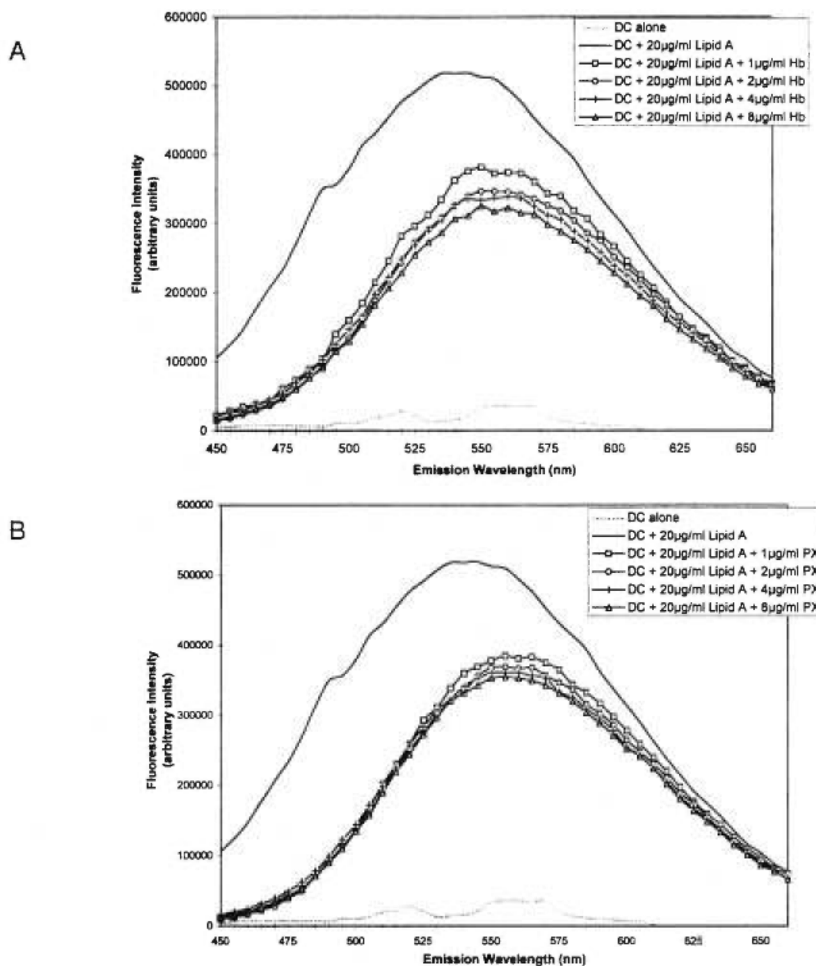

Fig. 6 Dansylcadaverine displacement assay: representative emission spectra. The maximum fluorescence of DC was achieved by using 20 µg/ml of *A. pleuropneumoniae* lipid A. The addition of different concentration of pig Hb (**A**) or polymyxin B (**B**) to the mixture of lipid A and DC resulted in an attenuation of fluorescence intensity which is indicative of the displacement of bound DC to lipid A by these compounds. DC alone (50 µM) was used as control.

aggregates or completely disaggregated into small particles. Similarly, disaggregation of enterobacterial LPS by human Hb visualized by electron microscopy has recently been reported by Roth.[30] We also observed disaggregation of *A. pleuropneumoniae* LPS with polymyxin B which confirmed previous results by Lopes et al.,[31] who demonstrated that incubation of *E. coli* LPS with polymyxin resulted in LPS fragmentation. Additional evidence for a direct physical interaction between pig Hb and *A. pleuropneumoniae* LPS was demonstrated by a shift in the sedimentation velocity of LPS-Hb complexes determined by sucrose density centrifugation. Whereas LPS alone sedimented in all fractions of the sucrose gradient, most of the LPS in Hb-LPS complexes had a sedimentation rate similar to that of Hb. These results are consistent with a process of LPS disaggregation and Hb-LPS complex formation demonstrated by Kaca et al.[1] for enterobacterial endotoxin and human Hb.

We previously observed that polymyxin B completely inhibited binding of pig Hb to *A. pleuropneumoniae* lipid A

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and were possibly competing for binding sites.[12] In the present study polymyxin B and pig Hb were both able to displace dansylcadaverine, a fluorescent probe used for quantitating the interactions of various compounds with endotoxic lipid A.[20,21]

Several studies have demonstrated that proteins such as melitin,[32] lysozyme,[33,34] synthetic peptides,[35] bactericidal/permeability-increasing protein,[36–38] LPS-binding protein,[39,40] glycopeptide antibiotics (mideplanin),[41] granulocyte-derived peptide (rabbit cationic antimicrobial

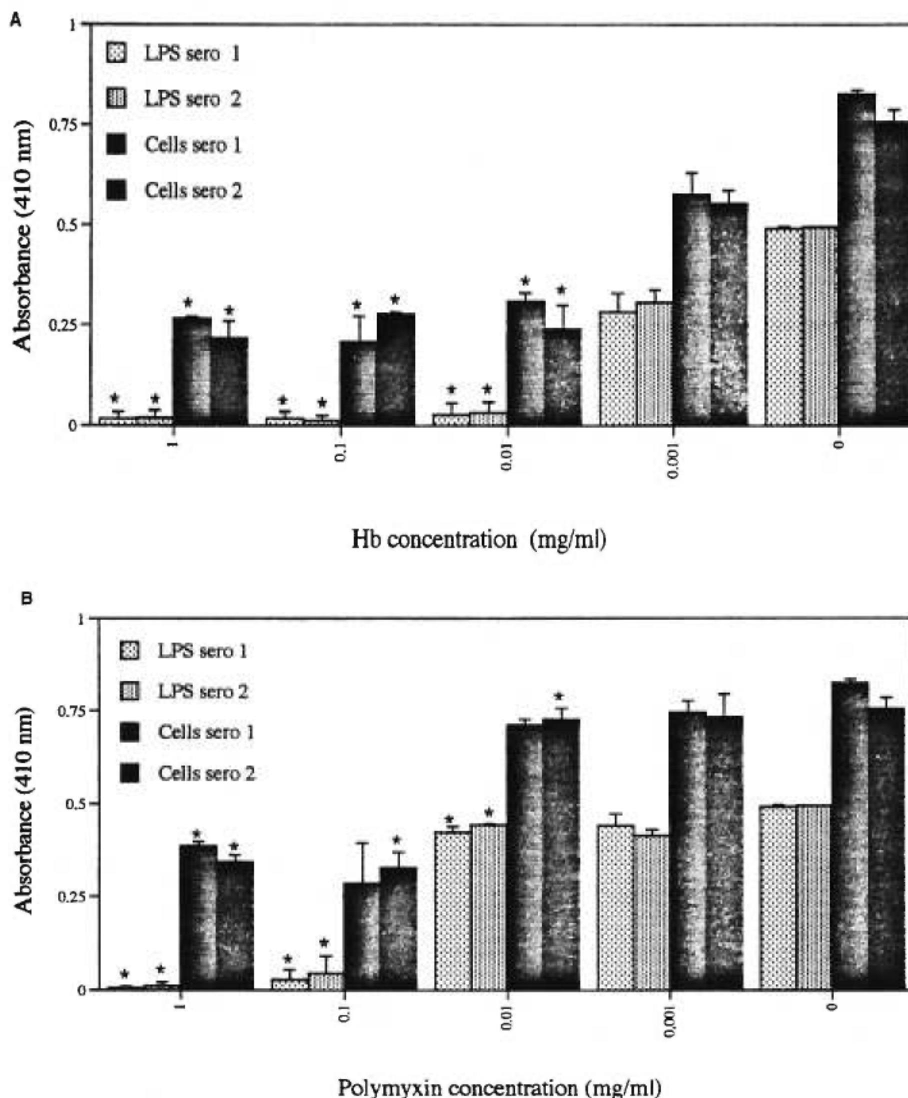

Fig. 7 Inhibition of extracted LPS or bacterial cells activation of cLAL by pig Hb or polymyxin B. A decrease was obtained in cLAL activation from the addition of 10-fold diluted pig Hb (starting concentration 1 mg/ml) (A) or polymyxin B (starting concentration 1 mg/ml) (B) to a constant concentration of extracted LPS from serotype 1 or serotype 2 (1 μg/ml) or bacterial cells of serotype 1 or serotype 2 (1 mg/ml). Two independent experiments were performed, $^{*}P < 0.05$.

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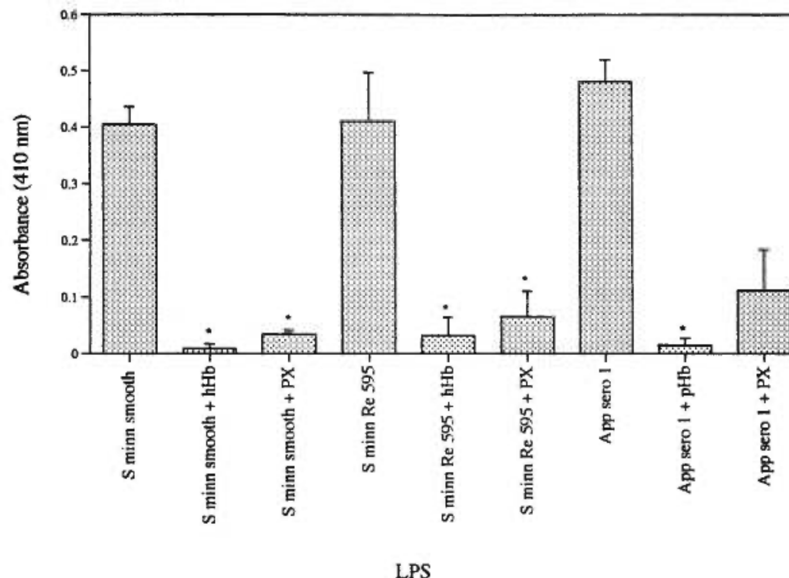

Fig. 8 Effect of pig Hb (pHb), human Hb (hHb), or polymyxin B (PX) on LPS biological activity of *S. minnesota* smooth or Re 595 or *A. pleuropneumoniae* serotype 1 in the cLAL test. The addition of pHb (1 mg/ml) or hHb (1 mg/ml) or PX (1 mg/ml) to extracted LPS (1 μg/ml) resulted in a significant decrease of cLAL activation, $^*P < 0.05$.

protein)[42] and the polypeptide polymyxin B,[43] bind LPS and decrease its biological activity. To determine whether pig Hb binding could alter some biological properties of *A. pleuropneumoniae* LPS, we used cLAL and gLAL tests.

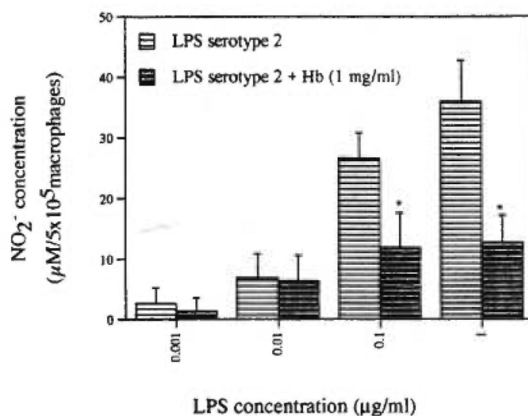

Fig. 9 Effect of pig Hb on LPS biological activity of *A. pleuropneumoniae* serotype 2 in inducing NO_2^- production in J774 cells. The addition of a constant concentration of pig Hb (1 mg/ml) to 10-fold diluted extracted LPS (starting concentration 1 μg/ml) resulted in a significant decrease of NO_2^- production, $^*P < 0.05$.

Interaction of LPS with pig Hb or with polymyxin B was associated with decreased biological activity of LPS in these tests. Considering that both, pig Hb and polymyxin B, bind to lipid A, as confirmed with the fluorescent probe displacement assay, and cause disaggregation of extracted LPS, it is not surprising that both substances have a similar effect on LAL activation. It is known that the spatial conformation of lipid A is an important determinant for its endotoxic activity.[44] A study by Shnyra et al.[45] has shown that the biological and endotoxic properties of LPS are significantly influenced by the physical state of its aggregates in aqueous solutions. These authors suggest that LPS must be in a highly aggregate state to be endotoxic.

Our data contrast however with the observation of Kaca et al.[1,3] who showed that addition of human Hb to *Enterobacteriaceae* LPS enhanced LAL activation, and of Takayama et al.[46] who demonstrated that monomeric Re lipopolysaccharide from *E. coli* is more active than the aggregated form in the *Limulus* amebocyte lysate assay and in inducing Egr-1 mRNA in murine peritoneal macrophages. The reasons for this discrepancy are unclear. One possible explanation might involve the structure of *A. pleuropneumoniae* lipid A. Although lipid A of distinct bacterial origin share certain basic structural elements, they differ in some chemical features.[47]

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Variations in structure result from the type of hexosamine present, the degree of phosphorylation, and most notably, the nature, chain length, number, and location of acyl groups.[48] The composition of lipid A has been shown to be more conserved within a bacterial family.[49] Kaca et al.[1] used *Enterobacteriaceae* LPS and human Hb whereas we used *Pasteurellaceae* LPS and pig Hb. In addition, LAL test, reagents and the LPS extraction method were different. In view of the fact that some of our findings contrast with information existing in the current literature and in order to understand these potentially conflicting results, we performed cLAL using enterobacterial LPS and human Hb. In our hands, addition of human Hb to *Enterobacteriaceae* LPS decreased LAL activation. We believe that LAL tests and reagents used for the assay might explain, at least in part, the difference observed between results of the two groups.

We then selected another in vitro biological assay for LPS and evaluated whether the reactive nitrogen intermediates (RNI) production as measured by NO_2^- is modified by LPS-Hb complexes compared to LPS alone. Among the numerous secretory products of macrophages,[50] RNI is a group of inorganic compounds with a high degree of chemical reactivity.[51] Macrophage-derived RNI are of interest because they play an important role in antimicrobial actions of activated macrophages. It is tempting to speculate that the inability of the LPS-Hb complexes to increase macrophage NO_2^--releasing capacity might be due to the LPS disaggregation by pig Hb or to the fact that Hb is binding to lipid A and is interfering with its recognition by cell receptors.

Our data indicate that binding of pig Hb, as well as polymyxin B, to extracted LPS of *A. pleuropneumoniae* causes fragmentation of LPS molecular aggregates and a marked reduction in their biological activity as determined by LAL activation and NO_2^- production by murine macrophages. Further studies are needed to determine whether Hb binding affects other biological properties of LPS and the virulence of *A. pleuropneumoniae*. Nevertheless, binding of pig Hb to the cell surface of *A. pleuropneumoniae* increased the amount of iron associated with the cells, and this might also influence virulence of *A. pleuropneumoniae*.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from Natural Sciences and Engineering Research Council of Canada (OGP0003428) to MJ. We are grateful to S. Sénéchal for performing flow cytometry and J.Y. Lapointe (Département de physique, Université de Montréal) for his expertise in spectrofluorometry. We thank R. Harris and T.J. Beveridge (Department of Microbiology, University of Guelph) for performing EDS analysis. We also acknowledge Claudine Matte and Jean-François Marquis for their excellent technical assistance and stimulating discussion.

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Article 6

Adhesin-receptor interactions in *Pasteurellaceae*

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FEMS Microbiology Reviews. **22:** 45-59, 1998

Pour la rédaction de cette revue, j'ai réalisé les recherches bibliographiques, effectuer une sélection des articles d'intérêt et créer la banque de références. De plus, j'ai contribué à la révision du manuscrit.

Review

Adhesin-Receptor Interactions in *Pasteurellaceae*

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Key words: Adherence; Adhesin; Receptor; *Actinobacillus*; *Haemophilus*; *Pasteurella*

This paper was presented at the
Haemophilus, Actinobacillus, Pasteurella International Conference (HAP'96) held in
Acapulco, Mexico, October 13-17, 1996

Abstract

The ability of bacteria to adhere to mucosal epithelium is dependent on the expression of adhesive molecules or structures, called adhesins, that allow attachment of the organisms to complementary molecules on mucosal surfaces, the receptors. Important human and animal pathogens are found among the *Pasteurellaceae* family which includes *Haemophilus*, *Actinobacillus*, and *Pasteurella* organisms. The purpose of this paper is to review the adhesin-receptor systems found in *Pasteurellaceae*, with an emphasis on recent developments in this specific area. Most of these organisms can employ multiple molecular mechanisms of adherence (or multiple adhesins) to initiate infection. Indeed, a wide variety of adhesins are expressed by members of the *Pasteurellaceae*, and different proteins (e.g. fimbriae, fibrils, outer membrane proteins) as well as polysaccharides (lipooligosaccharides, lipopolysaccharides, capsular polysaccharides) were clearly shown to play an important role in adherence. In many instances, these adhesins have proved to represent good vaccine candidates. Surprisingly, the receptors on host mucosal surfaces have yet been identified in very few cases.

Contents

1. Introduction xlvi
2. *Haemophilus* xlvi
   2.1 *Haemophilus ducreyi* xlvi
   2.2 *Haemophilus influenzae* xlviii
      2.2.1 Capsule xlviii
      2.2.2 Fimbriae (pili) xlviii
      2.2.3 Surface fibrils l
      2.2.4 High-molecular-weight adhesion proteins li
      2.2.5 Other putative adhesins lii
      2.2.6 Binding to mucus liii
      2.2.7 Binding to extracellular matrix components liv
      2.2.8 Cell invasion liv
3. *Actinobacillus* liv
   3.1 *Actinobacillus actinomycetemcomitans* liv
   3.2 *Actinobacillus pleuropneumoniae* lvi
4. *Pasteurella* lviii

4.1 *Pasteurella haemolytica* lviii
4.2 *Pasteurella multocida* lviii
5. Conclusion lx
Acknowledgments lx
References lxi

1. Introduction

The initial event in bacterial colonization of their host organisms is the adherence of microorganisms to the epithelial cells and/or mucus layer of the mucosal surfaces, which involves specific interactions between bacterial adhesins and host receptors [1]. Bacterial adherence endows the pathogen with the ability to withstand normal host defense cleansing mechanisms on mucosal surfaces. Adherence also confers a number of advantages on the bacterium, including enhanced toxicity to the host and increased resistance to deleterious agents. The fact that bacterial colonization and infection can be prevented by blocking adherence has stimulated research on the identification of adhesins, their molecular biology, and their binding specificities.

Members of the *Pasteurellaceae* are small Gram-negative rods that can colonize the mucosal surface of the respiratory and genital tracts. Important human and animal pathogens are found among this bacterial family (Table 1) [2]. The purpose of this paper is to review the adhesin-receptor systems found in *Pasteurellaceae*, i.e. organisms of the *Haemophilus, Actinobacillus,* and *Pasteurella* (HAP) group, with an emphasis on recent developments in this specific area. The literature review for this article ended in May 1997.

2. *Haemophilus*

2.1 Haemophilus ducreyi

H. ducreyi is the etiological agent of chancroid, a sexually transmitted disease that is common in developing countries and that has characteristic genital mucocutaneous ulcerative lesions (for a recent review see [3]). The disease has received renewed attention after reports that genital ulcers facilitate the transmission of the human immunodeficiency virus in endemic populations. It has been proposed [3, 4] that bacterial adherence to epithelial cells is the first

step in the pathogenesis of *H. ducreyi* infection. Adherence is followed by the growth of bacteria on the epithelial cells and the secretion of cytotoxin which result in cell damage that may be responsible for the development of ulcers.

H. ducreyi adheres strongly to many cultured cell lines including HEp-2 (human laryngeal epidermoid carcinoma), CHO (Chinese hamster ovary), MRC-C (human embryo lung), C16 (clone of MRC-C), HeLa (human cervical carcinoma), HEC-1-B (endometrial adenocarcinoma), and A549 (human lung carcinoma), to human foreskin cells, foreskin fibroblasts, and keratinocytes [4-10]. Cell invasion has been observed with some of these cell lines [3]. *In vitro*, all the strains tested could survive and multiply on cell monolayers, and a small fraction of bacteria could also survive inside eukaryotic cells. However, because many cells, including epithelial cells, can phagocytose foreign particles, cell invasion may reflect the phagocytic activity of the epithelial cells rather than the invasive properties of the bacteria. *H. ducreyi* adheres also to extracellular matrix proteins such as fibrinogen, fibronectin, collagen, gelatin and laminin [11].

This microorganism expresses surface structures that resemble pili or fimbriae [12]. Although their role in adherence to host cells has not been demonstrated they seemed to be involved in binding to laminin [11]. These fine tangled pili are composed predominantly of a protein whose apparent molecular mass is 24 kDa [12, 13]. The gene (*ftp*A) coding for the major subunit has been recently cloned [13]. These pili represent a novel class of pili since the sequence lacks a clivable signal sequence and has no homology to known pilin sequences. The major subunit FtpA does not mediate binding to laminin since an isogenic pilin mutant binds to laminin [13]. Interestingly, vaccination with a *H. ducreyi* pilus preparation confers protection in an animal model of chancroid, through cell-mediated immunity [14].

Lipooligosaccharides (LOS), present in the outer membrane, seem to play a role in adherence of *H. ducreyi* to human keratinocytes which may represent the first cells this microorganism encounters in the host [15, 16]. A *Tn*916 mutant of strain 3500 (mutant 1381) has LOS which lack the Gal-ß-1-4-GlcNAc structure, and is deficient in adherence and invasion of human keratinocytes. The mutation is located in a rfaK-like gene (D-glycero-D-manno-heptosyltransferase). In addition, Alfa and DeGagne [17] recently showed that purified LOS was able to inhibit attachment of *H. ducreyi* to foreskin fibroblasts in a dose dependent manner. In addition, proteinase K treatment of *H. ducreyi* significantly reduced attachment suggesting protein involvement. It appeared

that *H. ducreyi* binds to fibronectin in the extracellular matrix of human foreskin fibroblasts since fibronectin was able to significantly reduce attachment. These authors hypothesized that the attachment of *H. ducreyi* involves both a protein mediator (likely pili) as well as LOS and that one or both of these bacterial surface components interacts with fibronectin to mediate attachment to human foreskin fibroblasts.

2.2 Haemophilus influenzae

H. influenzae is an important human pathogen worldwide. Non-encapsulated isolates, known as non-typable *H. influenzae* (NTHi), are a common cause of otitis media, sinusitis, conjunctivitis and acute lower respiratory tract infections. Capsular type b isolates (Hib) cause invasive, bacteremic infections such as meningitis, septicemia, and epiglottitis, particularly in infants. *H. influenzae* is a typical example of microorganisms expressing multiple adhesins [18, 19].

2.2.1 Capsule

Hib isolates adhere markedly less than isogenic non-encapsulated variants to cultured epithelial cells and nasal turbinates [19] suggesting that the capsule of Hib is not involved in adherence but rather masks the adhesin(s). Antibodies specific for Hib capsular polysaccharide however, were shown to block adherence of Hib to epithelial cells [20]. Swelling of the capsular material caused by these antibodies may have resulted in steric hindrance and interfered with fimbriae-mediated adherence.

2.2.2 Fimbriae (pili)

Fimbriae, on the other hand, seem to play an important role and fimbriated Hib isolates adhere better to organ cultures than do non-fimbriated Hib [21-23]. Hib isolates from the nasopharynx, in contrast to blood and cerebrospinal fluid isolates, usually express fimbriae. It has been therefore postulated that expression of fimbriae is beneficial during the initial stages of colonization and infection, but disadvantageous in establishing systemic disease.

It has been shown that *H. influenzae* fimbriae mediate adherence to oropharyngeal epithelial cells and erythrocytes, and that the adhesive properties reside in the major fimbrial subunit [19]. The complete fimbrial gene cluster of Hib has been characterized and showed to contain 5 transcribed open reading

frames (ORF; *hif*A-*hif*E) [24]. Using mutants that were inactivated in distinct fimbrial genes, van Ham *et al.* [25] have shown that the adhesive domain resides in the major subunit (HifA) and also showed that both the major and minor subunits (HifD and HifE) were required for adherence of *H. influenzae* to oropharyngeal epithelial cells and human erythrocytes carrying the AnWj antigen (see below). However, the role of the various fimbrial proteins in *H. influenzae* adhesion without interference from their function in fimbriae biogenesis was successfully established by constructing *Escherichia coli* recombinants expressing distinct combinations of *H. influenzae* fimbrial genes. A clone expressing only the major subunit HifA without coexpression of both minor subunits exhibited the specific adherence properties of *H. influenzae* fimbriae, implying that the minor subunits are dispensable for adherence and that the adhesive domain resides in the major subunit. The minor subunits probably play a role in adherence by raising the number of fimbriae above the minimal level required to establish adherence since insertion of a kanamycin cassette in *hifD* or *hifE* reduces the level of fimbriation. They may also play a role in adherence to other substrates via a distinct receptor-ligand interaction.

A more recent study revealed that *H. influenzae* fimbriae are composite structures like type 1, and P fimbriae of *E. coli* [26]. The structure is composed of a flexible two-stranded helical rod comprised of HifA and a short, thin, distal tip fibrillum containing HifD. In type 1 and P fimbriae, the pilus tip contains the adhesive subunit responsible for binding to the relevant host cell receptor. Results of this study raise the possibility that a minor subunit localized to the tip of *H. influenzae* fimbriae is the true adhesin.

The receptor for Hib fimbriae on human erythrocytes was identified as the blood group AnWj antigen [27]. Inhibition experiments with purified gangliosides revealed that GM1, GM2, GM3, and GD1a inhibited both adherence of fimbriated *H. influenzae* to epithelial cells and hemagglutination. The asialo derivative of GM1 (without a sialic residue) was a poor inhibitor. The glycolipid GM3 (sialyl-lactosylceramide or NeuAcß2-3Galß1-4Glcß1-1-ceramide) is the minimum structure for the fimbriae-dependent binding of *H. influenzae* to its receptor on oropharyngeal cells and erythrocytes. The fimbriae of *H. influenzae* thus belong to the family of low-affinity lactosyl-ceramide binding fimbriae [28].

The gene coding for the fimbrial subunit protein (fimbrin) of NTHi was also cloned and sequenced [29]. When the fimbrin gene was disrupted, a reduced

adherence to human oropharyngeal cells was observed. Immunization with isolated fimbrial protein conferred partial protection against transbullar challenge in a chinchilla model. Their data suggest that fimbrin could be useful as a component of a vaccine to protect against otitis media. *H. influenzae* fimbriae are antigenically diverse however, and protection might be strain specific, although a degree of serological relatedness among fimbrin proteins was observed.

Sterk *et al.* [30] studied fimbriae dependent binding to human tissue sections, and showed that the binding to epithelial cells and other cells was involved in colonization and infection by *H. influenzae*. Their data also suggest that a shift to the non-fimbriated form is required for bacteria in the bloodstream to escape clearance mechanisms mediated by blood cells. A study by Gilsdorf *et al.* [31] showed that *H. influenzae* isolates adhere to all of the human respiratory tract cell types they examined. Their results indicate that fimbriae of Hib as well as NTHi isolates mediate adherence to some, but not all, cells derived from human respiratory tissues, and suggest that nonpilus adhesins are also involved in adherence.

2.2.3 Surface fibrils

Short, thin surface fibrils distinct from fimbriae were visualized on Hib isolates [32]. The genetic locus encoding these fibrils was characterized recently [33] and shown to be composed of one long ORF, designated *hsf*, which encodes a protein (Hsf) with a molecular mass of 240 kDa. The derived amino acid sequence demonstrated homology with the NTHi adhesin Hia (see below), as well as with other bacterial adherence factors including AIDA-1 (an adhesion protein expressed by some diarrheagenic *E. coli* strains), Tsh (a hemagglutinin produced by an avian-pathogenic *E. coli* strain), and SepA (a *Shigella flexneri* secreted protein that appears to play a role in tissue invasion). Mutagenesis of this locus resulted in the loss of expression of surface fibrils and a marked decrease in attachment to cultured human epithelial cells (Chang, HeLa and HEp-2).

In another recent study, St-Geme III and Cutter [34] evaluated the influence of fimbriae, fibrils, and capsule on adherence of Hib. They found that fimbriae and surface fibrils have distinct cellular binding specificities, suggesting that these adhesive molecules recognize different host-cell receptors. Fimbriae promoted adherence to human buccal epithelial cells, but failed to recognize any

of the cultured cell lines (Chang, KB, HEp-2, and HaCaT). In contrast, fibrils were associated with adherence to these cell lines, but failed to influence attachment to buccal epithelial cells. They also noted that capsular material inhibited fibril recognition of the host-cell surface. A model for Hib colonization has been proposed [18, 34]. Between host, the organism must avoid desiccation in order to survive, and thus encapsulation is increased. Upon entry of the organism into a new host, the presence of a dense polysaccharide capsule necessitates that fimbriae, which extend beyond the capsule, promote the initial binding event. However, because fimbriae appear to mediate a low-affinity interaction, fibril-mediated adherence may be critical for persistent colonization. Thus, over time, encapsulation is decreased and piliation turned off in order to allow for surface fibril interaction with the appropriate host cell receptor structures. In the occasional circumstance that the organism invades the bloodstream, encapsulation again confers a survival advantage and levels are increased accordingly.

2.2.4 High-molecular-weight adhesion proteins

Two non-fimbrial adhesins, present in NTHi, have been reported and designated HMW1 and HMW2 proteins [18, 35, 36]. These adhesins are antigenically related to the filamentous hemagglutinin of *Bordetella pertussis* and mediate binding to distinct cell lines. The HMW1 protein is 125 kDa in size, while the HMW2 protein has an apparent molecular mass of 120 kDa. The first 1259 base pairs of the *hmw1* and *hmw2* coding regions are identical; thereafter, the sequences diverge somewhat but are 80% identical overall. The derived amino acid sequences show 70% identity. The receptors for HMW proteins appear to be negatively charged glycoconjugates; sulfated glycosaminoglycans as well as a glycoprotein containing N-linked oligosaccharide chains with sialic acid in an alpha 2-3 configuration have been identified as putative receptors [37, 38].

Attachment of NTHi to the epithelial surface of the upper respiratory tract is a critical first step in colonization of the human host. In theory, interruption of this colonization process should prevent disease. Interestingly a partial protection was obtained in an experimental model of otitis media when animals were immunized with HMW1 and HMW2 adhesion proteins [39], suggesting that they might represent one component of a multicomponent NTHi vaccine. In addition, the recent identification of shared surface-exposed B-cell epitopes on

HMW adhesion proteins by using monoclonal antibodies suggests the possibility of developing recombinant or synthetic peptide-based vaccines [40]. At least one surface-exposed B-cell epitope, define by monoclonal antibody AD6, was shared by most NTHi strains which express HMW1-HMW2-like proteins. This epitope mapped to the last 75 amino acids at the carboxy termini of the two proteins. However, a second family of HMW adhesion proteins expressed by NTHi HMW1/HMW2-deficient isolates was recently reported [41]. The gene, designated *hia* (for *Haemophilus influenzae* adhesin), encodes for a protein of 114 kDa. An Hia isogenic mutant was constructed and showed a reduced adherence to Chang epithelial cells. The Hsf (encoding surface fibrils) and Hia proteins were found to confer the same binding specificities (using a panel of 8 cultured cell lines) [29], suggesting that *hsf* and *hia* are alleles of the same locus. Approximately 75% of NHTi isolates express HMW1/HMW2-like adhesins, and most of the remaining isolates contain an Hia homolog [41]. It might therefore be possible of developing vaccines based upon a combination of HMW1/HMW2 proteins and Hia protein which would be protective against disease caused by most or all NTHi.

2.2.5 Other putative adhesins

An IgA protease-like protein (Hap) of NTHi has been shown to promote intimate interaction with human epithelial cells [42]. However, the mechanism by which Hap facilitates cellular invasion is not known [18].

H. influenzae undergoes spontaneous phase variation in colony opacity. Weiser *et al.* [43] have identified a locus contributing to opacity variation and containing to genes *oap*A and *oap*B. Mutagenesis of *oap*A resulted in loss of the ability to colonize the nasopharynx of infant rats.

Using two-dimensional thin-layer chromatography (TLC) and ^{3}H-labeled NTHi as well as fimbriated and non-fimbriated Hib isolates, it was recently shown that *H. influenzae* binds to minor gangliosides of HEp-2 cells [44]. The lipid-binding specificity of *H. influenzae* isolates was determined by TLC [45]. The 13 clinical isolates tested recognized different lipids including phosphatidylethanolamine (PE). A PE affinity matrix was used to purify an adhesin of 46 kDa from both Hib and NTHi. This adhesin was a potent inhibitor of PE binding *in vitro*, and polyclonal antibodies specific for this protein prevented the attachment of *H. influenzae* to cultured HEp-2 epithelial cells. The same group observed that after a brief heat shock treatment, NTHi strains show

a long-lasting change in the binding specificity for glycolipids [46]. After exposure of the organisms to brief heat shock, Western blotting of a surface extract of *H. influenzae* with anti-bovine-brain hsp-70 monoclonal antibody showed an increase in two protein bands at 82 and 60 kDa. In addition, this antibody was a potent inhibitor of the binding of heat-shocked *H. influenzae* to sulfoglycolipids. Their data indicate that cell surface hsp-70-related heat shock proteins can mediate *H. influenzae* attachment to sulfoglycolipids following heat shock. The authors suggest this phenomenon may be a response to fever following *H. influenzae* infection in humans, since a 39°C heat shock, often occuring during fever, was enough for the effect. Interestingly, heat shock proteins have recently been implicated in adherence of other microorganisms including *Mycoplasma* [47],*Chlamydia trachomatis* [48], and *Helicobacter pylori* [49].

The effect of mutations in genes required for LPS synthesis on the interaction of Hib with human nasal turbinate tissue maintained in an organ culture was recently examined [50]. Isogenic mutants expressing truncated LPS due to mutations in *lic*1*lic*2 or *gal*EK genes were used. Unlike studies with *H. ducreyi* in which LOS are involved in adherence, their data do not support a role for LPS as an adhesin for Hib.

2.2.6 Binding to mucus

Respiratory tract mucins may function as receptor molecules for NTHi and thus play an important role in colonization. Several studies have demonstrated that most NTHi isolates can bind to mucus from human respiratory tract [51-53]. However, encapsulated and some NTHi isolates failed to interact with mucins [45]. These isolates would, therefore, adhere only after disruption of the mucus layer and exposure of cellular receptors. Thus, differences in tissue toxicity and invasiveness among *H. influenzae* isolates may also be influenced by the mucin interactions of the isolates.

The binding of NTHi to human nasopharyngeal mucin, evaluated using an overlay assay, appeared to be mediated by outer membrane proteins (OMPs) of *H. influenzae* and sialic acid-containing oligosaccharides of mucin [54]. On the basis of electrophoretic mobility and Western blotting, these proteins were identified as OMPs P2 and P5. Unequivocal identification of OMPs P2 and P5 was made by employing OMPs of bacterial strains and their respective mutants lacking the respective OMP in binding assays. Miyamoto and Bakaletz [55] also observed that OMP P5 (which is in fact a fimbrin), but not OMP P2, contributes

to the binding of NTHi to mucus and epithelial cells of chinchilla Eustachian tube.

2.2.7 Binding to extracellular matrix components

Strains of *H. influenzae* adhere to the extracellular matrix (ECM) and to its isolated components, including laminin, fibronectin, and various collagens [56]. In addition, plasmin generated on *H. influenzae* plasminogen receptors degraded laminin and fibronectin as well as ECM from human endothelial cells. Plasmin bound on *H. influenzae* cells also potentiated penetration of bacteria through a basement membrane preparation reconstituted on membrane filters. Their data give evidence for a role of ECM adherence and plasminogen activation in the spread of *H. influenzae* through tissue barriers.

2.2.8 Cell invasion

Hib as well as NTHi are able to penetrate the mucosal surface predominantly between epithelial cells (a process known as paracytosis) [57]. The bacterial cells are mainly found in clusters (or microcolonies) in crevices between the cells. The passage time of *H. influenzae* through cell layers of NCI-H292 lung epithelial cells (originating from a human lung mucoepidermoid carcinoma) was not influenced by the presence of capsule or fimbriae or by the ability of the bacteria to adhere to the epithelial cells [57]. However, highly adherent strains showed greater paracytosis. More recently, it has been observed that Hib occasionally exhibits highly invasive behavior with HEp-2, HeLa, and MDCK cell lines [58]. The phenomenon was not inhibited by colchicine or cytochalasin but was dependent on the presence of physiological levels of CO_2. These authors proposed that the sensing by Hib of an increase of CO_2 concentration may lead to phenotypic changes, such as increased invasiveness, that enhance colonization and persistence.

3. *Actinobacillus*

3.1 Actinobacillus actinomycetemcomitans

A. actinomycetemcomitans is an important periodontopathogen that has been implicated in juvenile and adult periodontitis, diseases characterized by rapid destruction of the tooth-supporting tissues. This organism possesses a large

number of virulence factors which enable it to colonize the oral cavity, invade periodontal tissues, evade host defences, initiate connective tissue destruction and interfere with tissue repair (for recent reviews see [59, 60]).

Most fresh isolates of *A. actinomycetemcomitans* are fimbriated, and binding of this microorganism to solid surfaces (e.g. hydroxyapatite and saliva-coated hydroxyapatite) may involve fimbriae [61]. The parameters of *A. actinomycetemcomitans* adhesion to epithelial cells were assayed by use of different cell lines including the KB cell line, derived from a human oral epidermoid carcinoma [62-64]. Adhesion of *A. actinomycetemcomitans* to epithelial cells involves multiple determinants (fimbriae, OMPs, vesicles, and/or an extracellular amorphous material) and is influenced by both bacterial and host environmental conditions. Optimal adherence was observed after growth of the bacterial cells in broth under anaerobic conditions [56]. The adhesins that mediate adherence are associated with the outer membrane or are released into the surrounding medium in the form of vesicles [59, 60, 62]. As opposed to *A. pleuropneumoniae* (see below), LPS does not appear to be involved in adhesion of *A. actinomycetemcomitans* to epithelial cells [64].

Oligopeptides were synthesized according to the amino acid sequence of the fimbrial protein of *A. actinomycetemcomitans*, conjugated with branched lysine polymer resin beads, and used to immunize rabbits [65]. An antiserum which reacted with a 54 kDa protein of the fimbriae from *A. actinomycetemcomitans* strongly inhibited the attachment of fimbriated *A. actinomycetemcomitans* to saliva-coated hydroxyapatite beads, buccal epithelial cells, and a fibroblast cell line, Gin-1. Such synthetic fimbrial peptide antigen might act as a vaccine for inducing an antibody response that inhibits *A. actinomycetemcomitans* colonization. Recently, the fimbriae associated protein (*fap*) gene was cloned [66]. The 228 bp ORF encoded a 7.9 kDa protein. It is not known at this moment whether the 54 kDa protein previously reported is a complex of 7.9 kDa protein or the 7.9 kDa protein is one of the components of the mature fimbriae.

Mintz and Fives-Taylor [64] have shown that, in the presence of saliva, adherence of *A. actinomycetemcomitans* to human oral epithelial cells is inhibited. Salivary components which interact with *A. actinomycetemcomitans* were investigated recently by Groenink *et al.* [67]. Their results indicate that IgA, the low-molecular mucin MG2, parotid agglutinin, and a 300 kDa sublingual and submandibular glycoprotein, bound to the bacterial strains tested. In addition, sialic acid residues on MG2 appear to be involved in the binding.

Alugupalli and Kalfas [68] have demonstrated that lactoferrin, which is found in increased concentrations at sites of inflammation and in the gingival crevicular fluid of diseased periodontium, inhibited the adhesion of *A. actinomycetemcomitans* to different cell monolayers including KB cells. In *A. actinomycetemcomitans*, lactoferrin binds to a major 34 kDa heat-modifiable outer membrane protein [69]. This bacterium is also able to bind to the basement membrane matrix and its isolated components such as fibronectin, laminin and type IV collagen [70]. Interestingly, the above-mentioned heat-modifiable OMP has also been found to bind the basement membrane protein laminin, and lactoferrin can inhibit and displace the laminin-bacteria interaction [71]. These results indicate that lactoferrin may prevent the establishment of bacteria in periodontal tissues through adhesion-counteracting mechanisms in addition to its bacteriostatic and bactericidal properties.

Once colonization has taken place, there is some evidence that *A. actinomycetemcomitans* can invade the tissues of the periodontium. The invasion process was studied using both KB and MDCK epithelial cells [72]. The frequency of invasion by *A. actinomycetemcomitans* is comparable with the invasion of other cultured cells by known invasive microorganisms [55]. The degree of invasion by *A. actinomycetemcomitans* is greater in KB cells than in cells of non-oral origin or other commonly used cell lines. The work of Meyer *et al.* [72] revealed that invasion of epithelial cells is a multistep process which involved attachment to the epithelial cell and triggering of the movement of actin, entry into the host cell in an endosome, escape from the endosome, rapid multiplication, and intracellular and intercellular spread. Soon after entry of *A. actinomycetemcomitans* bacteria into epithelial cells, they may be found in protrusions which sometimes extend between neighboring epithelial cells. These protrusions are though to mediate the cell-to-cell spread of *A. actinomycetemcomitans*. Meyer *et al.* [72] postulated that the events they described are involved in the ability of *A. actinomycetemcomitans* to spread to the gingival and connective tissue and cause destruction.

3.2 Actinobacillus pleuropneumoniae

A. pleuropneumoniae is the causative agent of porcine pleuropneumonia, a disease found worldwide that causes tremendous economic loss to the swine industry. Several bacterial components, including hemolysins and/or

cytolysins, LPS, and capsular polysaccharides, appear to contribute to the disease process (for a recent review see [73]). Fimbriae have been demonstrated on some isolates of *A. pleuropneumoniae* [74-76] but their role in adherence has not been established.

We have previously showed that *A. pleuropneumoniae* was able to agglutinate erythrocytes from various animal species [77] and adheres *in vitro* to porcine tracheal rings maintained in culture [78] and to porcine frozen tracheal and lung sections [79]. Dom *et al.* [75] evaluated the *in vivo* association of a serotype 2 strain with the respiratory epithelium of pigs. As soon as 30 min postinoculation, bacteria were intimately associated with the epithelium of the alveoli or the cilia of the terminal bronchioli.

Our group demonstrated that LPS were the major adhesin involved in adherence to porcine respiratory tract cells [78] and mucus [80, 81]. We observed that the degree of adherence of *A. pleuropneumoniae* to porcine tracheal rings was related to LPS profiles and that adherence was blocked by purified LPS [70]. Isolates with a smooth LPS profile adhered in larger numbers to tracheal rings than isolates with a semirough LPS profile. We then found, by using extracted LPS from serotypes 1 and 2, that the polysaccharidic part of this complex molecule, but not the lipidic part, was involved in binding to host cells [82]. We also showed in the latter study, using immunoelectron microscopy and flow cytometry, that LPS were well exposed at the surface of this heavily encapsulated bacterium, an essential prerequisite for a bacterial adhesin. Recent results, using mini-*Tn*10-generated isogenic serotype 1 LPS mutants, indicate that the core region of LPS might play a determinant role in adherence of *A. pleuropneumoniae* [83]. It is noteworthy that with *Pseudomonas aeruginosa*, LPS outer core is an important bacterial ligand involved in the binding and ingestion by airway epithelial cells expressing CFTR (cystic fibrosis transmembrane conductance regulator) [84] and by corneal cells [85]. Preliminary experiments identified two proteins of approximately 15 and 39 kDa as putative receptors for *A. pleuropneumoniae* LPS on porcine tracheal cells [86].

Interestingly, immunization with different adhesin (LPS)-based vaccine preparations has been shown to induce a good protection against challenge with a virulent strain of *A. pleuropneumoniae* in mice [87] and in pigs [88]. These preparations, however, did not induce cross-protection against other serotypes.

4. *Pasteurella*

4.1 *Pasteurella haemolytica*

P. haemolytica is the cause of bovine pneumonic pasteurellosis, also known as shipping fever, as well as pneumonic pasteurellosis in sheep [89, 90]. The adhesins involved in colonization of host mucosal surfaces are not known [91], although two types of fimbriae have been described [92]. It has been proposed that capsular polysaccharides (CPS), LPS, and OMPs may function as adhesins in the absence of fimbriae [91].

Interestingly, LPS are present on epithelial surfaces of experimentally infected calves [93] which might suggest a role in adherence as observed in some other *Pasteurellaceae* [94]. Lipopolysaccharides of *P. haemolytica* have also been shown to complex with lung surfactant covering the alveoli [95]. However, this mechanism may help clear certain Gram-negative bacteria from the lungs of sheep as a part of the pulmonary innate defense system. Ovine pulmonary surfactant induces killing of *P. haemolytica* [96].

It is hypothesized that predisposing factors, management and environmental stress factors or viral infection, alter the upper respiratory epithelium allowing *P. haemolytica* to colonize [90, 91]. In a recent study, Mosier *et al.* [97] examined the lectin histochemistry of normal and bovine herpesvirus-1 (BHV1)-infected bovine nasal mucosa. They found a greater reactivity in samples from BHV1-infected than from normal cattle for all lectins tested. The BHV1-induced alteration of nasal mucosal glycoconjugates could enhance adhesion and colonization of *P. haemolytica* to nasal surfaces. Indeed, using an in vitro assay, it has been observed that infection of bovine cell lines by BHV1 resulted in an increased adherence by *P. haemolytica* [98].

4.2 *Pasteurella multocida*

Pasteurella multocida is an important veterinary pathogen causing diseases in a variety of domestic mammals and birds; in pigs, *P. multocida* is associated with atrophic rhinitis (toxigenic, capsular type D strains) and pneumonia (non-toxigenic, capsular type A strains) [99]. *P. multocida* is considered to be a poor colonizer and, as for *P. haemolytica* in bovine, a variety of factors can predispose the pig nasal cavity to colonization by toxigenic strains including treatment with

a mild chemical irritant (e.g. diluted acetic acid) [100] or exposure to ammonia [101], or pre-infection with *Bordetella bronchiseptica* [100, 102]. *B. bronchiseptica* appears to facilitate upper respiratory tract colonization by *P. multocida* by a process which involves a low molecular mass substance, possibly the tracheal cytotoxin [103]. This toxin induces ciliostasis of the tracheal epithelium with a concomitant accumulation of mucus.

Several studies have evaluated the attachment of *P. multocida* to various porcine respiratory tract cells, and to porcine respiratory tract mucus (e.g. [102, 104-107]), but the adhesins have not been clearly identified. Hemagglutinins [108] and fimbriae [109] have been reported, but it was found that these two factors were independent. More recently, two types of fimbriae (curly or rigid) were observed on toxigenic strains of *P. multocida* [110], but piliated cells failed to attach to red blood cells and to immobilized respiratory tract mucus. It is still not known whether these structures function as adhesins *in vivo*. The rigid fimbriae, which are approximately 7 nm in diameter, were identified as type 4 fimbriae based on N-terminal amino acid sequence of the 18-kDa fimbrial subunit [111].

Lipopolysaccharides may be involved in adherence of *P. multocida* to porcine respiratory tract cells [112]. However, the partial inhibition of adherence to porcine tracheal rings maintained in culture by *P. multocida* LPS suggests that there may be non-LPS components that are also important in adherence. Esslinger *et al.* [113] found that capsular type A isolates adhered strongly to HeLa cells and to alveolar macrophages from different animal species. Interestingly, they suggested a possible role of capsular hyaluronic acid in adhesion since it was reduced by hyaluronidase or hyaluronic acid. A similar observation was made by Pruimboom *et al.* [114]. Their data indicate that adhesion to turkey air sac macrophages is mediated by capsular hyaluronic acid and that the receptor is a glycoprotein. The same group reported that a monocytic CD44 isoform is the receptor for capsular hyaluronic acid on cultured turkey peripheral blood monocytes [115].

A surface protein of *P. multocida* has also been implicated in adherence. It has been shown that the capacity of OMP preparations of *P. multocida* to bind to respiratory mucosal surface preparations was inhibited by antibodies against the major OMP (35 kDa, p35) of *P. multocida* [116]. These antibodies also cross-reacted with a 44-kDa major OMP of *P. haemolytica* and, according to these

authors, p35 could therefore represent a potential candidate for a subunit vaccine against pneumonic pasteurellosis.

Finally, avian strains of *P. multocida* were shown to be able to invade porcine epithelial cells (PK15) and feline epithelial cells (CRFK) in culture, but not rabbit epithelial cells (RK13) [117]. Apparently, avian *P. multocida* enters polarized epithelial cells by interacting with host F-actin [118]. Invasion might be a mechanism of pathogenicity for *P. multocida*, contributing to colonization or virulence of avian strains.

5. Conclusion

Most HAP organisms can employ multiple molecular mechanisms of adherence (or multiple adhesins) to initiate infection (Table 2). Indeed, a wide variety of adhesins are expressed by members of the *Pasteurellaceae*. Proteins (e.g. fimbriae, fibrils, OMPs) and/or polysaccharides (LOS, LPS, CPS) were shown to play a role in adherence of these organisms. These multiple adhesins are capable of reacting with different receptors. Furthermore, these multiple adhesins may be utilized in a stepwise fashion during colonization. It seems reasonable that evolutionary pressures have selected organisms that are capable of demonstrating more than one mechanisms of adherence [1]. The multiplicity of HAP organisms adherence interactions accounts, at least in part, for their success in colonizing the mucosal surfaces of their hosts. In many instances, adhesins of HAP organisms have been shown to represent good vaccine candidates.

Many important questions remain to be answered regarding HAP adhesins, regulation of adhesins expression, and prevalence of adhesins or adhesin genes among isolates. Surprisingly, not much is known about the receptors recognized by HAP adhesins (Table 2). New information on HAP adhesins and their receptor will allow for the rational design of novel subunit or recombinant vaccines, and adhesion agonists for the control or prevention of HAP colonization and diseases.

Acknowledgments

Work in M.J. laboratory was supported by grants from the Natural Sciences and Engineering Research Council of Canada (OGP0003428). We also wish to

thank the other graduate students and collaborators who have been involved in the *A. pleuropneumoniae* LPS project: M. Archambault, M. Bélanger, D. Dubreuil, J. Frey, C. Galarneau, M. Gottschalk, J. Harel, M. Kobisch, J. Nicolet, and S. Rioux. We apologize to the authors whose work could not be quoted, because of space limitations.

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Table 1

Members of the *Pasteurellaceae* family described in the present review and the diseases they cause in their respective host

Organism	Host	Disease
Haemophilus		
H. ducreyi	human	chancroid
H. influenzae capsular type b (Hib)	human	meningitis, septicemia, epiglottitis
H. influenzae non-typable (NTHi)	human	otitis media, sinusitis, conjunctivitis, acute lower respiratory tract infection
Actinobacillus		
A. actinomycetemcomitans	human	juvenile and adult periodontitis
A. pleuropneumoniae	swine	pleuropneumonia
Pasteurella		
P. haemolytica	bovine and sheep	pneumonia
P. multocida	swine	pneumonia, atrophic rhinitis

Table 2

Adhesin-receptor systems found in HAP organisms

HAP organism	Adhesin	Receptor	Reference
H. ducreyi	LOS	-----	[15-17]
	fimbriae (?)	-----	[13]
	-----	ECM components	[11, 17]
H. influenzae	fimbriae (Hib)	blood group AnWj Ag sialyl-lactosylceramide (GM1, GM2, GM3)	[19, 24, 25, 27, 28]
	fimbriae (NTHi)	-----	[29]
	fibrils	-----	[32, 33]
	HMW1/HMW2 adhesion proteins	glycoprotein; sulfated glycosaminoglycans	[35-38]
	Hia adhesion protein	-----	[41]
	Hap (IgA protease-like protein)	-----	[38]
	OapA (opacity protein)	-----	[43]
	OMP 46 kDa	lipids, including PE	[45]
	hsp 70-like proteins	sulfoglycolipids	[46]
	-----	minor gangliosides	[44]
	-----	ECM components	[56]
	-----	mucus/mucins	[51-53]
	OMP P2 and P5	sialic acid-containing oligosaccharides of mucin	[54]
	OMP P5	mucus	[55]
A. actinomycetemcomitans	fimbriae	-----	[61, 65]
	protein(s) found in OM vesicles	-----	[63, 64]
	major OMP (29/34 kDa)	laminin (ECM)	[71]

A. pleuropneumoniae	fimbriae (?)	-----	[74-76]
	LPS	-----	[78, 82]
	LPS core region	-----	[83]
	LPS	15 and 39 kDa proteins of porcine tracheal cells	[86]
P. haemolytica	fimbriae (?)	-----	[92]
	CPS (?) LPS (?) OMPs (?)	-----	[91]
P. multocida	fimbriae (?)	-----	[109-111]
	hemagglutinins	-----	[108]
	major OMP (p35)	-----	[116]
	capsular hyaluronic acid	-----	[113, 114]
	capsular hyaluronic acid	CD44	[115]
	LPS	-----	[112]

CPS, capsular polysaccharides; ECM, extracellular matrix; HSP, heat shock protein; LOS, lipooligosaccharides; LPS, lipopolysaccharides; OM, outer membrane; OMP, outer membrane protein; PE, phosphatidylethanolamine; (?) role in adherence not yet established.