Surface polysaccharides and iron-uptake systems of *Actinobacillus pleuropneumoniae*

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Abstract

Actinobacillus pleuropneumoniae is the etiologic agent of porcine pleuropneumonia. Infection by *A. pleuropneumoniae* is a multifactorial process governed by many virulence factors acting alone or, more often, in concert to establish the pathogen in the porcine host. The aim of this short review is to present recent data concerning important surface molecules of *A. pleuropneumoniae*; namely, lipopolysaccharides, capsular polysaccharides, and a subset of outer membrane proteins involved in iron uptake.

Résumé

Actinobacillus pleuropneumoniae *est l'agent étiologique de la pleuropneumonie porcine. L'infection par* A. pleuropneumoniae *est un processus multifactoriel qui implique plusieurs facteurs de virulence agissant seuls ou, plus souvent, conjointement pour permettre l'établissement de la bactérie pathogène chez son hôte. Le but de cette courte revue est de présenter les données récentes concernant d'importantes molécules de surface telles que les polysaccharides capsulaires, les lipopolysaccharides, et un groupe de protéines de la membrane externe impliquées dans l'acquisition du fer.*

(Traduit par l'auteur)

Introduction

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a disease that contributes important economic losses to the swine industry worldwide. Twelve serotypes (or serovars) of *A. pleuropneumoniae* have been described among the biotype 1 strains, which are dependent on nicotinamide adenine dinucleotide (NAD) (1). Two other serotypes (serotypes 13 and 14) have been described among the biotype 2 strains, which are NAD-independent. Recently, an additional serotype (serotype 15) has been proposed for biotype 1 (2). Serotypes 1, 5, and 7 are the serotypes most commonly found in North America, whereas serotype 2 is predominant in many European countries (1).

Infection by *A. pleuropneumoniae* is a multifactorial process governed by many virulence factors acting alone or in concert to establish the pathogen in the porcine host. Indeed, several bacterial components, including toxins that belong to the RTX (repeats-in-toxin) group of proteins (ApxI–ApxIV), lipopolysaccharides (LPS), capsular polysaccharides (CPS), and some outer membrane proteins (OMPs) appear to contribute to the disease process (1,3–6).

Advanced molecular-based strategies have recently been used to monitor additional *A. pleuropneumoniae* genes required for causing and maintaining infection and for survival. One such new strategy is in vivo expression technology, which allows the identification of gene promoters that are specifically induced in vivo during infections. With *A. pleuropneumoniae* serotype 1, a promoter trap vector was used to complement an attenuated riboflavin-requiring auxotroph (Rib^{-}) (7). If the fragment of *A. pleuropneumoniae* genomic DNA ligated into the vector contains a promoter specifically induced in vivo, the *rib* genes are expressed, allowing the auxotroph to survive and cause disease in experimentally infected pigs. In one set of experiments, Fuller and colleagues (7) tested 2400 transformant clones in pigs. Pools of 300 to 600 clones were inoculated into the upper lungs of animals, and surviving bacteria were isolated from the lungs 12 to 16 h later. From the surviving bacteria, the researchers identified 10 clones containing promoters that were induced in vivo with minimal in vitro expression.

Another strategy to identify genes required for survival and growth in vivo is signature-tagged mutagenesis (STM), in which multiple transposon mutants that are tagged with a unique DNA sequence are screened for their inability to survive in vivo. Fuller and associates (8) used this strategy to screen more than 800 mutants of an *A. pleuropneumoniae* serotype 1 strain in swine. They identified 20 insertion mutations that attenuated virulence. Interestingly, the same group had identified 3 of the genes required for virulence of *A. pleuropneumoniae* in a previous STM study of *Pasteurella multocida*. One of these genes, *exbB*, codes for a component of the ExbB–ExbD–TonB system implicated in iron acquisition. More recently, Sheehan and coworkers (9) also used an STM approach, inoculating pigs with 2064 mutants assembled into pools. They identified 55 separate genes among the 105 mutants that were attenuated. Their results highlight the importance of genes involved in energy metabolism, nutrient

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uptake, stress responses, gene regulation, and the production of cell surface components.

With these and other new technologies, our knowledge of the genes required for virulence and for in vivo survival is likely to increase dramatically and, in turn, should enable us to design novel vaccine candidates or novel targets for antimicrobials. Genomic sequencing is another powerful approach and would also enable researchers to use DNA microarrays to monitor changes in gene expression during infection. Genome sequencing projects with *A. pleuropneumoniae* (serotypes 1, 5, and 7) are under way at the National Research Council of Canada and at the US Advanced Center for Genome Technology.

For many years, we have been investigating the role of *A. pleuropneumoniae* surface polysaccharides (LPS and CPS) in virulence. Recently, we began investigating other important surface molecules of *A. pleuropneumoniae*, notably OMPs involved in iron uptake.

Surface polysaccharides

Lipopolysaccharides

Bacterial surface polysaccharides, LPS and CPS, usually play an important role in virulence. Lipopolysaccharides are complex molecules composed of 3 well-defined regions: (i) lipid A; (ii) the core oligosaccharide, containing 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo); and (iii) the O-antigen, a polysaccharide consisting of repeating units. Studies in our laboratory have shown that LPS of *A. pleuropneumoniae* are involved in the adherence of the bacteria to porcine respiratory tract cells (10,11). By using transposon (mini-Tn*10*) mutagenesis, we have generated different LPS mutants of *A. pleuropneumoniae* serotype 1 (12,13). Mutations in the O-antigen region have apparently no effect on adherence to porcine respiratory tract cells, whereas an intact core oligosaccharide region seems to be required for optimal adherence. The structure of *A. pleuropneumoniae* serotype 1 O-antigen was previously described by Altman, Brisson, and Perry (14) as branched tetrasaccharide repeating units composed of 2 α-L-rhamnopyranosyls, 1 α-D-glycopyranosyl and 1 2-acetamido-2-deoxy-β-D-glucose residues. The structural similarities among the LPS O-antigens of *A. pleuropneumoniae* serotypes 1, 9, and 11 are responsible for serologic cross-reactions between these serotypes (1). A.D. Cox and M. Monteiro and their collaborators at the National Research Council of Canada have recently resolved the structure of the LPS core of *A. pleuropneumoniae* serotype 1 (unpublished data). Work to determine the core structure of LPS in the *A. pleuropneumoniae* LPS core mutants with decreased adherence to host cells is under way. These experiments will allow us to identify with precision the particular LPS residues implicated in host cell recognition.

By analyzing the genes disrupted by mini-Tn*10* insertion in the *A. pleuropneumoniae* LPS mutants generated, we identified a gene cluster responsible for the biosynthesis of the O-antigen in *A. pleuropneumoniae* serotype 1 (15). This cluster displays striking similarity to a gene cluster involved in the biosynthesis of the serotype b O-antigen in *A. actinomycetemcomitans* (a human periodontal pathogen) (16). To determine whether the genes involved in O-antigen biosynthesis of *A. pleuropneumoniae* serotype 1 that we identified by transposon mutagenesis are conserved among serotypes 1 to 12, we performed polymerase chain reaction (PCR) amplification and Southern blot analysis with digoxigenin (DIG)-labelled probes. Not surprisingly, these genes were also present in *A. pleuropneumoniae* serotypes 9 and 11, which express an O-antigen that cross-reacts with serotype 1. We also performed PCRs and Southern blot analyses with 3 genes involved in core LPS biosynthesis of *A. pleuropneumoniae* serotype 1. Our findings showing that these genes were present in serotypes 1 to 12 confirmed that the LPS core is usually more conserved between serotypes than O-antigen, which is the more variable region of LPS.

Evaluation of some biologic properties of the rough LPS mutants we generated indicated that the absence of O-chains does not appear to affect the overall surface hydrophobicity and charge (zeta potential) (15). However, the rough LPS mutants of *A. pleuropneumoniae* serotype 1 were sensitive to normal pig serum, whereas the parent wild-type strain was resistant (13). We recently evaluated the binding of the rough LPS mutants and the LPS core mutants to putative receptors in the porcine respiratory tract, in particular glycolipids and phospholipids extracted from swine lungs (17,18). Our data and recent findings by other groups (19,20) all point toward the presence of multiple adhesin-receptor systems in this microorganism: *A. pleuropneumoniae* would first use low-affinity binding between the O-antigen and phospholipids (phosphatidylethanolamine) or short glycolipids (LacCer and GlcCer) and then rely on the core oligosaccharide of LPS, surface proteins (fimbriae or a 55-kDa OMP), or both to interact more avidly with larger lipidic receptors (GgO_3 and GgO_4) or proteinic receptors.

The virulence of some of the *A. pleuropneumoniae* serotype 1 LPS mutants was evaluated in specific-pathogen-free (SPF) piglets. Although a rough LPS mutant (not expressing the O-antigen) was as virulent as the parent wild-type strain (15), an LPS core mutant was found to be completely avirulent (13). This confirms the important role played by core oligosaccharides of LPS in *A. pleuropneumoniae* serotype 1 during the interaction of the bacteria with their natural host. M. Ramjeet in our laboratory is currently evaluating the susceptibility of these LPS mutants to cationic antimicrobial peptides. In order to generate *A. pleuropneumoniae* deep-rough LPS mutants, we tried to identify and clone genes involved in LPS inner-core biosynthesis by complementing well-characterized *Salmonella enterica* serovar Typhimurium LPS mutants. We were able to complement *Salmonella* SL1102 mutant with an *A. pleuropneumoniae* 4074 genomic library and restore its wild-type smooth LPS phenotype (21). This *Salmonella* deep-rough mutant is defective for *hld*E (formerly *rfa*E), which is a phosphoheptose kinase-ADP-heptose synthase. The generation of an *A. pleuropneumoniae hld*E null mutant using an allelic-exchange strategy was unsuccessful, suggesting that a mutation in the biosynthesis of heptose precursors is probably lethal for this microorganism.

Capsular polysaccharides

We have also generated and characterized an acapsular mutant of *A. pleuropneumoniae* serotype 1 (22) by inactivating a gene (*cpx*C) involved in the transport of CPS across the inner membrane (23). This CPS mutant did not react with a monoclonal antibody against *A. pleuropneumoniae* serotype 1 capsular antigen, as determined by flow cytometry. Absence of the capsule was confirmed by transmission

electron microscopy after polycationic ferritin labelling. The loss of CPS has a marked effect on surface properties, as it decreases the surface zeta potential and increases the surface hydrophobicity of this mutant (15). Furthermore, use of piglet tracheal frozen sections showed that the CPS mutant adhered significantly more than the parent strain, indicating that CPS of serotype 1 are not involved in adherence but, rather, mask the adhesins, at least in part (22). This CPS mutant was resistant to normal pig serum, as was the parent wild-type strain. Finally, when the virulence of this acapsular mutant was evaluated in SPF piglets, the mutant was found to be totally avirulent. These results are in agreement with a recent study of Bandara and colleagues (24), who found an association between the type or quantity of CPS produced and the virulence of *A. pleuropneumoniae*.

The LPS and CPS isogenic mutants of *A. pleuropneumoniae* serotype 1 that we have generated will serve as extremely valuable tools in determining the precise role of these surface polysaccharides in infection.

Iron-uptake systems

Of the many complex interactions between a pathogen and its host, a common essential component of the infectious process is multiplication of the invading microorganism within host tissues. Such growth is critical to the establishment of an infection and depends, in part, on the ability of the pathogen to scavenge certain essential nutrients. Iron is essential for bacterial growth and also acts as an environmental signal that regulates the expression of many virulence factors. In the host, extracellular iron is bound to the iron-binding glycoproteins lactoferrin and transferrin in exocrine secretions, while most of the intracellular iron is sequestered as heme-containing proteins such as hemoglobin (Hb). This sequestration limits the availability of free iron (~10^{-18} M) to levels below that required to support microbial growth (~10^{-6} to 10^{-8} M) (25).

To counteract the scarcity of iron and survive in the host, bacterial pathogens have developed different high-affinity iron-acquisition mechanisms designed to obtain iron (25). One such system comprises the elaboration of siderophores that chelate external iron and then bind to their cognate receptors and subsequent internalization of the ligand. Another system utilizes a receptor-mediated mechanism to acquire iron from lactoferrin, transferrin, or heme-containing proteins. All these high-affinity iron-acquisition systems are TonB-dependent. The TonB system acts as the energy source for promoting the uptake of iron from the outer-membrane-localized proteins that bind the iron chelates at the cell surface. Transport of the iron (from transferrin or hemoglobin) or ferrisiderophore across the outer membrane depends on the ExbB–ExbD–TonB system (26). This cytoplasmic-membrane-localized complex transduces energy from the proton motive force in the cytoplasmic membrane to high-affinity receptors in the outer membrane. Upon ligand binding to iron-chelate receptors, conformational changes are induced, some of which are detected in the periplasm. These structural alterations signal the ligand-loaded status of the receptor and the subsequent requirement for TonB-dependent energy transduction (27).

It is known that *A. pleuropneumoniae* can use host transferrin (28–30) and hemoglobin (31–33), as well as various exogenous microbial siderophores (34), as sole sources of iron for growth. These observations strongly suggest the presence of specific surface receptors for these iron sources. Recent studies by our group, in collaboration with J.W. Coulton's group at McGill University (35–37 and unpublished data), indicate that *A. pleuropneumoniae* strains indeed express receptors for porcine hemoglobin and for ferrichrome, a hydroxamate siderophore produced by fungi, in addition to the receptors for transferrin.

Transferrin-binding proteins

Receptors for transferrin called transferrin-binding proteins (Tbps) are found in *Pasteurellaceae* and *Neisseriaceae* (38) and have been reported in *A. pleuropneumoniae* (28–30) as well. These receptors usually exhibit strict host specificity: for instance, Tbps of *A. pleuropneumoniae* will bind only porcine transferrin and not bovine, ovine, avian, or human transferrin. This iron-uptake system is composed of 2 iron-repressible surface components: TbpB (or TfbA), a lipoprotein of about 60 kDa (28,29), and TbpA (or TfbB), a protein of about 100 kDa (29,30). The 60-kDa component has been shown to be highly divergent among serotypes and to induce a serotype-specific protective immune response (30).

Hemoglobin-binding proteins

Using affinity purification with hemoglobin-agarose (39), we isolated hemoglobin-binding proteins of approximately 75 and 105 kDa from an OMP preparation of *A. pleuropneumoniae* serotype 1 strain 4074 grown under iron restriction (35). Labelling of cells with [^{3}H]palmitate indicated that neither protein was a lipoprotein. The 75-kDa protein seemed able to bind hemin as well. Matrix-assisted laser desorption ionization/time-of-flight analysis was performed on the 75-kDa protein since the amino terminus was blocked. A database search revealed identities with proteins from various gram-negative bacteria, including iron-regulated OMPs and transporter proteins, as well as TonB-dependent receptors. We used the *N*-terminal amino acid sequence of the 105-kDa protein to clone the gene from a genomic library of *A. pleuropneumoniae* serotype 1 strain 4074. The gene product showed high homology with the hemoglobin-binding protein HgbA of other *Pasteurellaceae* (unpublished data). A survey revealed that the *hgb*A gene was present in all reference strains representing serotypes 1 to 12. Interestingly, a deletion mutant of *A. pleuropneumoniae hgb*A generated by allelic replacement showed abolished hemoglobin uptake (unpublished data).

Ferric hydroxamate receptor

The genes involved in the uptake of the hydroxamate siderophore ferrichrome were cloned and sequenced. We screened for exported proteins in *A. pleuropneumoniae*, using a truncated gene for alkaline phosphatase (‘*phoA*) that lacks a functional signal sequence and a signal sequence library of *A. pleuropneumoniae* serotype 1 strain 4074 constructed by M. Sirois (Université du Québec à Trois-Rivières). When the *A. pleuropneumoniae* signal sequence library, representing approximately 8250 individual colonies in the *pho*A^{-} *Escherichia coli* strain CC118, was screened for the blue colony phenotype on media containing 5-bromo-4-chloro-3-indolyl phosphate (XP), 95 colonies were found to be PhoA^{+}. One plasmid (pI-25) from the PhoA^{+} colonies contained a 375-bp *A. pleuropneumoniae* insert that was

relevant to our study. A BLASTX analysis of the sequence carried on the fragment displayed homology with bacterial FhuD proteins. To isolate larger genomic fragments containing the complete gene sequences of interest to this study along with their neighbouring sequences, we screened a genomic library made in Lambda-ZAP Express vector that contained fragments of *A. pleuropneumoniae* strain 4074 with DIG-labelled probes. The plasmid of a positive clone (pLM101) was excised, and restriction analysis revealed an insert of 8.0 kb. In a single operon, we identified 4 genes — *fhuC*, *fhuD*, *fhuB*, and *fhu*A — encoding proteins homologous to proteins of the ferric hydroxamate uptake systems of several bacteria, including *E. coli* (36). The *fhuA* gene encodes the 77-kDa OMP FhuA, the receptor for ferrichrome; FhuD is the 35.6-kDa periplasmic protein responsible for the translocation of ferric hydroxamate from the outer to the inner membrane; FhuC (28.5 kDa) and FhuB (69.4 kDa) are cytoplasmic-membrane-associated proteins, components of an ABC transporter that internalizes the ferric hydroxamate. Reference strains representing serotypes 1 to 12 of *A. pleuropneumoniae* all tested positive by PCR and Southern blot for the 4 *fhu* genes.

The *fhuA* gene of *A. pleuropneumoniae* strain 4074 was cloned into the expression vector pET30a$^+$. The recombinant His6-tagged protein reacted with an anti-His6 monoclonal antibody and with monoclonal antibody Fhu6.1 against *E. coli* FhuA (40). The recombinant His6-tagged protein also reacted with a polyclonal serum from a pig with an *A. pleuropneumoniae* infection. We concluded that *fhuA* is expressed in vivo by *A. pleuropneumoniae*.

Three-dimensional modelling of the FhuA OMP was achieved by threading the protein to the X-ray crystallographic structure of the homologous protein of *E. coli* (41). The model for the *A. pleuropneumoniae* FhuA generated by the JIGSAW 3D Protein Homology Modeling Server (www.bmm.icnet.uk/servers/3djigsaw) showed 11 extracellular loops and 10 periplasmic turns (36). These numbers were consistent with the *E. coli* FhuA structure, as was the presence of 22 β-strands. FhuA from *A. pleuropneumoniae* has the same overall fold as the protein in *E. coli*: both possess 2 domains, an *N*-terminal cork domain, followed by a C-terminal β-barrel domain.

A polyclonal serum to *A. pleuropneumoniae* FhuA reacted with OMP preparations from 12 serotypes of *A. pleuropneumoniae* under iron repletion and iron restriction alike, which suggests that expression of FhuA is not regulated by iron. Reverse-transcription PCR confirmed that, unlike other iron-uptake systems, a low level of iron in the culture medium did not upregulate the expression of *A. pleuropneumoniae fhuA* (37). A deletion mutant of *A. pleuropneumoniae fhuA* generated by a single-step transconjugation system showed that ferrichrome uptake was abolished in the mutant (37). Uptake and use of iron from other sources, such as hemoglobin, hemin, and porcine transferrin, were still possible in the mutant, indicating that these uptake systems were unaffected. This proves unequivocally that the gene *fhuA* codes for the receptor for ferrichrome in *A. pleuropneumoniae* and that the OMP FhuA is specific for this iron source. We found the virulence of *fhu*A-deletion mutants of serotype 1 (L. Mikael, L. Shakarji, M. Kobisch, and M. Jacques: unpublished data) and of serotype 7 (42) not to be attenuated in pigs.

We conclude that hemoglobin-binding proteins and a ferric hydroxamate receptor are present in *A. pleuropneumoniae* in addition to transferrin-binding proteins. Therefore, *A. pleuropneumoniae* seems well-equipped to overcome iron shortages during infection.

Conclusion

Taken together, the results of these recent experiments clearly confirm that infection by *A. pleuropneumoniae* is indeed a multifactorial process and that multiple surface molecules are playing a role in pathogenesis. In addition, *A. pleuropneumoniae* represents a nice model to investigate host–pathogen interactions in the natural host.

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