Lipopolysaccharides of *Actinobacillus pleuropneumoniae* Bind Pig Hemoglobin

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A previous study indicated that lipopolysaccharides (LPS) extracted from *Actinobacillus pleuropneumoniae* bind two low-molecular-mass proteins, of approximately 10 and 11 kDa, present in porcine respiratory tract secretions (M. Bélanger, D. Dubreuil, and M. Jacques, *Infect. Immun.* 62:868–873, 1994). In the present study, we determined the N-terminal amino acid sequences of these two proteins, which revealed high homology with the α and β chains of pig hemoglobin. Some isolates of *A. pleuropneumoniae* were able to use hemoglobin from various animal species as well as other heme compounds as sole sources of iron for growth, while other isolates were unable to use them. Immunoelectron microscopy showed binding of pig hemoglobin at the surface of all *A. pleuropneumoniae* isolates as well as labeling of outer membrane blebs. We observed, using Western blotting (immunoblotting), that the lipid A-core region of LPS of all isolates was binding pig hemoglobin. Furthermore, lipid A obtained after acid hydrolysis of LPS extracted from *A. pleuropneumoniae* was able to bind pig hemoglobin and this binding was completely abolished by preincubation of lipid A with polymyxin B but was not inhibited by preincubation with glucosamines. Fatty acids constituting the lipid A of *A. pleuropneumoniae*, namely, dodecanoic acid, tetradecanoic acid, 3-hydroxytetradecanoic acid, hexadecanoic acid, and octadecanoic acid, were also binding pig hemoglobin. Our results indicate that LPS of all *A. pleuropneumoniae* isolates tested bind pig hemoglobin and that lipid A is involved in this binding. Our results also indicate that some *A. pleuropneumoniae* isolates are, in addition, able to use hemoglobin for growth. Binding of hemoglobin to LPS might represent an important means by which *A. pleuropneumoniae* acquires iron in vivo from hemoglobin released from erythrocytes lysed by the action of its hemolysins.

Actinobacillus pleuropneumoniae is the causative agent of porcine fibrinohemorrhagic necrotizing pleuropneumonia, a severe disease causing large economic losses in industrialized swine production (23). Of the 12 recognized capsular serotypes (24), serotypes 1 and 5 are predominant in Québec while serotype 2 is important in most European countries (20). Several bacterial structures or products have been suggested as important factors in the virulence of *A. pleuropneumoniae*. The polysaccharidic capsule (14) and lipopolysaccharides (LPS) (8, 39) seem to be involved in virulence. We previously demonstrated that LPS were the major adhesin of *A. pleuropneumoniae* involved in adherence to porcine respiratory tract cells (3, 27). In addition, three pore-forming RTX toxins have been reported (for a review, see reference 9). Two of these toxins (ApxI and ApxII) possess both hemolytic and cytotoxic activities, while the third (ApxIII) is cytotoxic but has no hemolytic activity (9). Outer membrane proteins have also been implicated as virulence factors, as *A. pleuropneumoniae* produces novel proteins under iron-restricted conditions (6, 25). *A. pleuropneumoniae* does not produce siderophores but can bind porcine transferrin to obtain iron from its host (10, 32).

In a recent study, porcine respiratory tract secretions were chromatographed on an affinity column where *A. pleuropneumoniae* LPS were immobilized (4). Two major proteins, of approximately 10 and 11 kDa, binding to *A. pleuropneumoniae* LPS were observed. In the present study, we identified these two low-molecular-mass proteins as α and β chains of pig

hemoglobin and determined which region of the LPS molecules was involved in binding.

MATERIALS AND METHODS

Bacterial isolates. *A. pleuropneumoniae* serotype 1 reference strain 4074 and field isolate FMV87-682, as well as serotype 2 reference strain 4226 and field isolate Q87-981, have been described previously (3, 4). *Escherichia coli* O111 obtained from J. M. Fairbrother (Université de Montréal, St-Hyacinthe, Québec, Canada) was also used in this study.

Extraction and hydrolysis of LPS. LPS from *A. pleuropneumoniae* were extracted by the method of Darveau and Hancock (5). In brief, disrupted cells were treated with DNase, RNase, pronase, and sodium dodecyl sulfate (SDS) and subjected to MgCl₂ 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. To obtain lipid A fraction, LPS from *A. pleuropneumoniae* serotype 1 dissolved in 1% (vol/vol) acetic acid, previously saturated with nitrogen, was heated in a boiling water bath for 2 h. Precipitated lipid A was removed from the cooled solution by centrifugation after neutralization with 5 N NaOH (2). The lipid A fraction was washed five times in distilled water, lyophilized, and resuspended in 20 mM EDTA (pH 8.0).

Affinity chromatography and N-terminal amino acid sequencing. The two proteins, of approximately 10 and 11 kDa, present in porcine respiratory tract secretions which bind *A. pleuropneumoniae* LPS were purified by affinity chromatography as described previously (4). Proteins were electroblotted on Immobilon-P transfer membrane (Millipore Corp., Bedford, Mass.) according to the method of LeGendre and Matsudaira (17). Automated Edman degradation was performed on a Model 470A Applied Biosystems gas phase sequencer equipped with a model 120A on-line phenylthiohydantoin analyzer (Applied Biosystems) according to the general protocol of Hewick et al. (13). 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 (PTH analyzer standards; Applied Biosystems) that were analyzed on-line prior to the sequence analysis.

Heme compound utilization assay. The utilization of heme compounds by iron-restricted *A. pleuropneumoniae* was determined by a plate assay as described by Deneer and Potter (6). Bacterial cells were resuspended to an optical density

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at 540 nm (path length of 1 cm) of 1.0 in saline. Cells were then spread onto the surface of a brain heart infusion-nicotinamide adenine dinucleotide (BHI-NAD) (15 µg/ml) agar plate containing the concentration (200 to 250 µM) of 2,2'-dipyridyl (Sigma Chemical Co., St. Louis, Mo.) appropriate to inhibit growth of each of the isolates. Wells were cut into the solidified agar and filled with 20 µl of a 10 mM solution of either hemin, hematin, or hemoglobin from one of the following: pigs, sheep, humans, goats, bovines, rabbits or horses. Zones of growth around the wells were evaluated after incubation at 37°C for 48 h. A solution of 10 mM FeCl₃ was used as a positive control.

Hemoglobin-binding assay. The binding of hemoglobin to extracted LPS of *A. pleuropneumoniae* was evaluated by incubation of 1 mg of extracted LPS in 1 ml of phosphate-buffered saline (PBS, pH 7.4; Boehringer Mannheim Canada Ltd., Laval, Québec, Canada) containing 2 mg of pig hemoglobin. After a 1-h incubation at 37°C on a rocker platform, the mixture was centrifuged for 6 h at 200,000 × g, at 15°C. The pellet was washed twice with PBS and analyzed by gel electrophoresis. Hemoglobin incubated without extracted LPS was used as a control.

Antiserum production. A rabbit antiserum against pig hemoglobin was prepared by immunization of a male New Zealand White rabbit with a 1-mg/ml solution of pig hemoglobin in incomplete Freund's adjuvant. Intramuscular injections were given every other day for 3 weeks. The antiserum had a titer of 1:1,000 by enzyme-linked immunosorbent assay and reacted with the α and β chains of pig hemoglobin seen by SDS-polyacrylamide gel electrophoresis (SDS-PAGE); control, preimmune serum did not react with pig hemoglobin.

Immunoelectron microscopy. A drop of a dense bacterial suspension was placed on Formvar-coated grids. The grids were incubated for 30 min with 1 mg of pig hemoglobin per ml and blocked with 1% bovine serum albumin. The grids were then incubated for 30 min with a suitable dilution of an antiserum raised against pig hemoglobin followed by an incubation with an anti-rabbit immunoglobulin G (IgG) conjugated with gold particles (10 nm; Sigma). Grids were rinsed, negatively stained with 1% phosphotungstate, and examined with an electron microscope (Philips 201) at an accelerating voltage of 60 kV. Cells of *A. pleuropneumoniae* were also labeled with monoclonal antibody E5 directed against bacterial lipid A (42) and an anti-mouse IgM conjugated with gold particles (10 nm; Sigma). The monoclonal E5 was kindly supplied by P. Dessain, Pfizer Canada Inc., Pointe-Claire, Québec, Canada.

Electrophoresis and Western blot analysis. SDS-PAGE was conducted by the discontinuous buffer system of Laemmli (16), 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. Gels were run on a Mini-PROTEAN II vertical slab electrophoresis cell (Bio-Rad) and were either stained with silver nitrate as described by Tsai and Frasch (38) or transferred to nitrocellulose for Western blotting (immunoblotting). Western blots were carried out as described by Towbin et al. (37). The membranes were subsequently incubated as described below for the dot blot binding assays.

Dot blot binding assays. To evaluate the affinities of lipid A and of polysaccharides of hydrolyzed LPS of *A. pleuropneumoniae* for pig hemoglobin, dot blots were performed. In addition, the fatty acids constituting the lipid A of *A. pleuropneumoniae* serotype 1 (1) were solubilized in methanol (10 mg/ml), and different concentrations (10, 5, 2, 1, 0.5, 0.1, and 0.01 mg/ml) of each (dodecanoic acid, tetradecanoic acid, 3-hydroxytetradecanoic acid, hexadecanoic acid, and octadecanoic acid) were dotted on a membrane. Samples (10 µl) of the solutions were placed on a nitrocellulose membrane (0.22 µm; Bio-Rad). The membranes were first blocked with 2% casein in Tris-saline buffer (TSB; 10 mM Tris, 150 mM NaCl, pH 7.4) and then incubated for 1 h with pig hemoglobin (1 mg/ml in TSB). A 2-h incubation with an antiserum against pig hemoglobin was followed by a 1-h incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad). The reaction was revealed by addition of H₂O₂ and 4-chloro-1-naphthol as the chromogenic substrate.

Inhibition assays. (i) **Amino sugars.** To evaluate inhibition of hemoglobin binding to lipid A, pig hemoglobin was preincubated with different amino sugars. A 1-h incubation of pig hemoglobin (1 mg/ml) with 5, 50, or 100 mM (either) D-glucosamine, D-glucosamine 6-phosphate, N-acetyl-D-glucosamine, N-acetyl-D-glucosamine 1-phosphate, or diglucosamine (β-D-GlcNAc-[1→6]-D-GlcNAc) in TSB was performed at room temperature with agitation before performance of dot binding assays.

(ii) **Polymyxin B sulfate.** Lipid A from *A. pleuropneumoniae* was either dotted on a membrane or transferred on a membrane after SDS-PAGE, which was then blocked with casein and incubated 1 h in polymyxin B sulfate (1 or 10 mg/ml; Sigma) before performance of binding assays.

Outer membrane bleb extraction. Outer membrane blebs were recovered from an overnight BHI-NAD (5 µg/ml) broth culture as described by Pettit and Judd (29) with some modifications. Cells were pelleted by centrifugation at 4,100 × g for 10 min at 4°C, and the supernatant was centrifuged at 12,000 × g for 10 min to assure removal of cells and cell debris. The supernatant was concentrated by ultrafiltration (Amicon, Beverly, Mass.) with a membrane with a molecular weight cutoff of 300,000 and finally was centrifuged at 206,000 × g for 90 min. The pellet was resuspended in PBS.

TABLE 1. Iron acquisition from heme compounds by *A. pleuropneumoniae* isolates

Iron source	Iron acquisition by indicated isolate (serotype)			
	4074 (1)	FMV87-682 (1)	4226 (2)	Q87-981 (2)
Hemin	+	—	+	+
Hematin	+	—	+	—
Hemoglobin ^a	+	—	+	—
FeCl ₃	+	+	+	+

^a Hemoglobin from either pigs, sheep, humans, goats, bovines, rabbits, or horses.

RESULTS

Affinity chromatography of porcine respiratory tract secretions and N-terminal sequence analysis. In a recent study, porcine respiratory tract secretions were chromatographed on a Detoxi-Gel column where *A. pleuropneumoniae* LPS were immobilized (4). Two major proteins, of approximately 10 and 11 kDa, binding to *A. pleuropneumoniae* LPS were observed. N-terminal amino acid sequencing of these two proteins was performed, and sequences were compared by use of the Swiss-Prot database. The N-terminal sequence of the 11-kDa protein was VHLSAEEKEAV, and it had 100% identity with the β chain of pig hemoglobin. The N-terminal sequence of the 10-kDa protein was VLSAADKANVKAAXG, and it had 93.3% identity with the α chain of pig hemoglobin.

Utilization of heme compounds by *A. pleuropneumoniae*. To determine whether *A. pleuropneumoniae* isolates could utilize heme compounds as the sole source of iron, we carried out plate assays in which the abilities of various heme compounds to overcome 2,2'-dipyridyl-induced iron restriction were evaluated. Isolates 4074 and 4226 were able to use all iron sources tested, whereas isolate FMV87-682 used only FeCl₃ and isolate Q87-981 used hemin and FeCl₃ (Table 1). Strains 4074 and 4226 obtained iron from pig hemoglobin as well as from hemoglobin of six other species tested.

Binding of hemoglobin to *A. pleuropneumoniae* whole cells and extracted LPS. Immunoelectron microscopy allowed visualization of binding of pig hemoglobin to *A. pleuropneumoniae* whole cells (Fig. 1A and B). Although only two isolates utilized hemoglobin as a source of iron, all four isolates were binding pig hemoglobin at their surface. Outer membrane blebs shed by bacterial cells were heavily labeled by gold particles (Fig. 1A and B, arrows). Interestingly, bacterial cells incubated with a monoclonal antibody (E5) against lipid A showed a similar pattern of labeling (Fig. 1D). Bacterial cells of the negative controls were not labeled. These negative controls consisted of *A. pleuropneumoniae* cells incubated without pig hemoglobin (Fig. 1C) and *E. coli* O111 cells incubated with pig hemoglobin, a rabbit antiserum against pig hemoglobin, and an anti-rabbit IgG conjugated to gold particles (Fig. 1E).

LPS extracted from *A. pleuropneumoniae* isolates and *E. coli* O111 were tested for their abilities to bind pig hemoglobin. After incubation of extracted LPS with pig hemoglobin for 1 h, LPS were recovered by ultracentrifugation and analyzed by SDS-PAGE (Fig. 2). Two major bands, of approximately 10 and 11 kDa, corresponding to α and β chains of hemoglobin, were seen in the LPS electrophoretic profile of *A. pleuropneumoniae* isolates (Fig. 2, lanes 2 and 4) as well as a minor band of about 28 kDa corresponding probably to a dimer. In contrast, the bands observed with LPS extracted from *E. coli* O111 (Fig. 2, lane 6) were quite similar to the control (Fig. 2, lane 7), in which hemoglobin was incubated without extracted LPS.

Western blot analysis. To determine which region of the

FIG. 1. Transmission electron micrographs of cells of *A. pleuropneumoniae* reference strain 4226. (A and B) Cells incubated with pig hemoglobin, a rabbit antiserum against pig hemoglobin, and an anti-rabbit IgG conjugated to gold particles (10 nm); (C) control cells, without pig hemoglobin; (D) cells labeled with the monoclonal antibody E5 against bacterial lipid A and an anti-mouse IgM conjugated to gold particles (10 nm). (E) Cells of *E. coli* O111 incubated with pig hemoglobin, a rabbit antiserum against pig hemoglobin, and an anti-rabbit IgG conjugated to gold particles (10 nm). Note the gold-labeled outer membrane blebs (arrows in panels A, B, and D). Bars = 200 nm.

FIG. 2. Results of the hemoglobin-binding assay. Silver-stained (A) or Coomassie blue-stained (B) SDS-PAGE profiles of extracted LPS-pig hemoglobin complexes recovered after ultracentrifugation. Extracted LPS of *A. pleuropneumoniae* Q87-981 (lanes 1), *A. pleuropneumoniae* 4226 (lanes 3), and *E. coli* O111 (lanes 5) and complexes of pig hemoglobin and LPS of *A. pleuropneumoniae* Q87-981 (lanes 2), *A. pleuropneumoniae* 4226 (lanes 4), and *E. coli* O111 (lanes 6) are shown. Pig hemoglobin incubated without extracted LPS was used as a control (lanes 7). Molecular mass markers (in kilodaltons) are indicated on the left.

LPS molecules was binding pig hemoglobin, we used Western blot analysis. Proteinase K-digested whole-cell lysates of *A. pleuropneumoniae* and *E. coli* were separated by electrophoresis and transferred onto a nitrocellulose membrane, which was then incubated with pig hemoglobin and probed with an antiserum against pig hemoglobin. Binding of pig hemoglobin was observed for all isolates of *A. pleuropneumoniae*, while only a weak binding was observed for *E. coli* O111 (data not shown). The core-lipid A region of *A. pleuropneumoniae* LPS was binding pig hemoglobin, while the O chains did not show any binding. Furthermore, lipid A obtained after acid hydrolysis of LPS extracted from *A. pleuropneumoniae* also bound pig hemoglobin (Fig. 3, lane 1), and this binding was blocked by a preincubation with polymyxin B (Fig. 3, lane 2).

Dot binding and inhibition assays. Lipid A and polysaccharides obtained after acid hydrolysis of LPS extracted from *A. pleuropneumoniae* serotype 1 were then used in a dot binding

FIG. 3. Western blot incubated with pig hemoglobin and probed with an antiserum against pig hemoglobin. Lane 1, *A. pleuropneumoniae* lipid A. Lane 2, *A. pleuropneumoniae* lipid A incubated in polymyxin B prior to incubation with hemoglobin. Molecular mass markers (in kilodaltons) are indicated on the left.

assay. As observed by Western blot, the lipid A moiety bound pig hemoglobin (Fig. 4A to E) but the polysaccharides did not. Fatty acids constituting the lipid A of *A. pleuropneumoniae* serotype 1, namely, dodecanoic acid, tetradecanoic acid, 3-hydroxytetradecanoic acid, hexadecanoic acid, and octadecanoic acid, were able to bind pig hemoglobin in a dot binding assay. Different concentrations (0.1 to 100 μ g) of lipid A and a fatty acid, octadecanoic acid, were dotted on a membrane. Binding of hemoglobin to lipid A was always more important than binding to the free fatty acid at all concentrations tested (Fig. 4B and G). Incubation of *A. pleuropneumoniae* lipid A with polymyxin B completely inhibited pig hemoglobin binding, as observed previously by Western blot. On the other hand, different glucosamines as well as a diglucosamine did not inhibit binding of pig hemoglobin to *A. pleuropneumoniae* lipid A.

Outer membrane blebs. Since we observed that outer membrane blebs were well labeled when *A. pleuropneumoniae* cells were incubated with pig hemoglobin and probed with an antiserum against pig hemoglobin (Fig. 1), blebs were recovered from a culture supernatant and analyzed by SDS-PAGE. As seen in Fig. 5 (arrowhead), molecules of low molecular mass in

FIG. 4. Dot blot assay used to show binding of pig hemoglobin to lipid A of *A. pleuropneumoniae* serotype 1 or octadecanoic acid. Samples of 20 μ g (A), 10 μ g (B), 5 μ g (C), 1 μ g (D), and 0.1 μ g (E) of lipid A of *A. pleuropneumoniae* and a 10- μ g sample of octadecanoic acid (G) were immobilized on a membrane and sequentially incubated with pig hemoglobin and an antiserum against pig hemoglobin. Controls (10 μ g) of lipid A (F) and octadecanoic acid (H) were incubated without hemoglobin.

FIG. 5. Silver-stained SDS-PAGE profiles of serotype 2 reference strain *A. pleuropneumoniae* 4226. Lane 1, outer membrane bleb proteinase K-treated preparation; lane 2, whole-cell proteinase K-treated preparation. Molecular mass markers (in kilodaltons) are indicated on the left. The arrowhead indicates molecules of low molecular mass in the lipid A-core region.

the lipid A-core region were more important in the LPS profile of outer membrane blebs than in the LPS profile of whole cells when identical amounts of sugars (1.4 μ g) were loaded on the gel.

DISCUSSION

In a recent study, porcine respiratory tract secretions were chromatographed on a Detoxi-Gel column where *A. pleuropneumoniae* LPS were immobilized (4). Two major proteins, of approximately 10 and 11 kDa, binding to *A. pleuropneumoniae* LPS were observed. In the present study, we determined the N-terminal amino acid sequences of these two proteins, which identified them as α and β chains of pig hemoglobin. The fact that the crude mucus preparation we used had been inadvertently contaminated with blood allowed us to detect binding of pig hemoglobin to *A. pleuropneumoniae* LPS, which was unsuspected until then.

We then verified whether *A. pleuropneumoniae* could utilize hemoglobin as well as other heme compounds as the sole source of iron. By carrying out plate assays in which the abilities of various heme compounds to overcome induced iron restriction conditions were evaluated, we showed that some *A. pleuropneumoniae* isolates were able to utilize all iron sources tested whereas other isolates could not. We do not know, at this time, whether the ability of *A. pleuropneumoniae* to use hemoglobin as a source of iron has an effect on virulence in pigs. Utilization of hemoglobin as a source of iron for growth has been reported for one serotype 1 *A. pleuropneumoniae* isolate by Deneer and Potter (6) as well as for other bacterial species such as *Campylobacter jejuni* (30), *Haemophilus influenzae* (31), *Neisseria* spp. (7), and *Vibrio cholerae* (36). Immunoelectron microscopy allowed us to visualize binding of pig hemoglobin to *A. pleuropneumoniae* whole cells. Although only two isolates utilized hemoglobin as a source of iron, all four isolates were binding pig hemoglobin at their surface, which suggested that after binding of hemoglobin to LPS, other molecules, possibly outer membrane proteins and periplasmic proteins, are involved in iron uptake.

In order to find out which region of the LPS molecules binds pig hemoglobin, we used Western blotting with proteinase K-digested whole-cell lysates of *A. pleuropneumoniae*. The fast-migrating core-lipid A region of *A. pleuropneumoniae* LPS bound pig hemoglobin, while the O chains did not show any binding. Lipid A and polysaccharides obtained after acid hydrolysis of LPS extracted from *A. pleuropneumoniae* serotype 1 were then dotted on a membrane. As observed by Western blot, the lipid A moiety bound pig hemoglobin. As the lipid A moiety is composed of a phosphorylated diglucosamine backbone and fatty acids, we therefore looked for the residues of the lipid A moiety that were implicated in binding of hemoglobin. Incubation of *A. pleuropneumoniae* lipid A with polymyxin B, which is known to interact with the lipid A-KDO (3-deoxy-D-manno-octulosonic acid) region of LPS (21), completely inhibited pig hemoglobin binding. Fatty acids constituting the lipid A of *A. pleuropneumoniae* serotype 1, namely, dodecanoic acid, tetradecanoic acid, 3-hydroxytetradecanoic acid, hexadecanoic acid, and octadecanoic acid, were able to bind pig hemoglobin in a dot binding assay. On the other hand, different glucosamines as well as a diglucosamine did not inhibit binding of pig hemoglobin to *A. pleuropneumoniae* lipid A. Taken together, our results suggest that lipid A fatty acids may be involved in hemoglobin binding.

Interestingly, LPS from *E. coli* O111 seemed to bind pig hemoglobin only weakly since proteinase K-digested whole-cell lysate of *E. coli* showed a weak binding of hemoglobin in Western blot. In addition, cells of *E. coli* O111 were not labeled when incubated with pig hemoglobin, as observed by immunoelectron microscopy. The composition of lipid A has been shown to be more conserved within a bacterial family (15). Despite a common architecture, lipids A of different bacterial origins may vary in their fine structure. Variations in structure result from the type of hexosamine present, the degree of phosphorylation, and the nature, chain length, number, and location of acyl groups (33). *E. coli* lipid A is composed of fatty acids with chain lengths of C₁₂ to C₁₄, while fatty acids with longer chain lengths (C₁₄ to C₁₈) are present in *A. pleuropneumoniae* lipid A (1, 15). This might explain, at least in part, the difference between the two species in hemoglobin binding that we observed.

To the best of our knowledge, this study represents the first report of hemoglobin binding to LPS at the bacterial cell surface. Interestingly, endotoxin has been shown by Roth et al. (35) to bind to cross-linked stroma-free hemoglobin, an oxygen-carrying protein being developed as an erythrocyte substitute. More recently, the same group observed that human hemoglobin and LPS form bimolecular complexes and that some biological activities of LPS may be enhanced by the presence of hemoglobin (34). Grenier (11) found that the hemin-binding property of *Porphyromonas gingivalis* appeared to be mediated by LPS, particularly the lipid A region. We do not know, at this time, how lipid A, which should be anchored in the outer membrane, interacts with hemoglobin at the cell surface. Immunoelectron microscopy allowed the visualization of binding of pig hemoglobin to *A. pleuropneumoniae* whole cells. Outer membrane blebs shed by the bacterial cells were also heavily labeled by gold particles. As seen by SDS-PAGE, low-molecular-mass molecules in the lipid A-core region were more important in the LPS profile of outer membrane blebs than in the LPS profile of whole cells. Thus, it is tempting to speculate that during the process of outer membrane bleb formation, short LPS molecules (lipid A-core or lipid A-core substituted with a small number of O-repeating units) are preferentially incorporated into the vesicles, which might, in turn, better expose the hydrophobic region of the LPS mole-

cule at the cell surface. With monoclonal antibody directed against bacterial lipid A, cells as well as outer membrane blebs of *A. pleuropneumoniae* were labeled, as observed by immunoelectron microscopy. This represents additional evidence that at least some lipid A residues are accessible at the surface of *A. pleuropneumoniae* cells.

We observed that the N-terminal amino sequence of the β chain of pig hemoglobin had similarity (5 identical amino acids plus 3 conserved substitutions) with a 10-amino-acid sequence of the extracellular domain of rabbit low-density lipoprotein (LDL) receptor. Interestingly, all lipoprotein classes bind LPS, and it has been suggested that LDL receptor could provide a means for uptake of LPS by cells possessing such a receptor (40). It is not known, however, whether LPS can bind directly to LDL receptors, as they do to acetyl-LDL receptors (or scavenger receptors) (19, 43).

The level of free iron in serum is approximately 10^{-18} M, a concentration well below that required to maintain bacterial growth (12). During infection, intracellular stores increase, a process which further restricts the amounts of iron available to an invading organism (41). Most iron in body fluids is located intracellularly as ferritin or as heme compounds, while extracellular iron is tightly complexed with iron-binding and transport proteins such as transferrin and lactoferrin. Bacteria have several mechanisms to overcome the ability of the host to withhold iron. Many gram-negative bacteria induce production of low-molecular-mass iron chelators (siderophores) in response to iron limitation, but *A. pleuropneumoniae* isolates do not produce siderophores (22, 25). Non-siderophore-producing bacteria may acquire iron by direct binding with transferrin, lactoferrin, or hemoglobin molecules (26, 28). *A. pleuropneumoniae* cells have been shown to express receptors specific for porcine transferrin (10, 32). During infection, *A. pleuropneumoniae* cells produce RTX toxins, two of which are known to have a hemolytic activity, which results in a release of hemoglobin from erythrocytes present in the alveolar exudates (18). *A. pleuropneumoniae* may, therefore, be able to acquire iron in vivo by binding free hemoglobin to its LPS. The present study does not address, however, whether hemoglobin complexed to haptoglobin still binds to LPS or promotes bacterial growth. Additional work is needed to answer these questions as well as to identify the proteins that are involved in the subsequent uptake of iron.

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