Effect of Iron Restriction on the Outer Membrane Proteins of *Actinobacillus (Haemophilus) pleuropneumoniae*†

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The outer membrane protein profile of *Actinobacillus (Haemophilus) pleuropneumoniae* grown under iron-restricted and iron-replete conditions was studied by polyacrylamide gel electrophoresis and immunoblotting. A virulent serotype 1 isolate synthesized a novel protein with an apparent molecular weight of 105,000 (105K) and increased the synthesis of a 76K protein under iron-restricted conditions. Both proteins were synthesized within 15 min of establishment of iron-restricted conditions. Proteins of equivalent molecular weights could also be induced by iron restriction in serotype 2, 3, 4, 5, and 7 isolates of *A. pleuropneumoniae*. Convalescent-phase sera from serotype 1-infected pigs contained antibodies which recognized both the 105K and 76K proteins from all six serotypes examined, indicating that these proteins were expressed in vivo and were immunologically conserved. Cells expressing the 105K and 76K proteins also displayed an enhanced ability to bind Congo red and hemin, suggesting that one or both of these proteins functioned to acquire complexed iron during in vivo growth.

*Actinobacillus (Haemophilus) pleuropneumoniae* is an economically important swine pathogen that causes an acute pneumonia with fibrous pleuritis or chronic lung lesions in infected animals (31). Control of the disease by vaccination has largely been unsuccessful because whole-cell bacterins do not induce immunity toward heterologous serotypes of *A. pleuropneumoniae* or prevent chronic forms of the disease or development of the subclinical carrier state (13, 24). The failure of current vaccines is reflected in part by the lack of a basic understanding of the virulence mechanisms and pathobiology of *A. pleuropneumoniae*. Although hemolysin (18), capsular polysaccharide (1), lipopolysaccharide (9) and, recently, outer membrane protein (OMP) (27, 30) antigens have all been implicated as virulence factors, little is known about the in vivo characteristics of *A. pleuropneumoniae* and how they differ from those of in vitro-cultured organisms.

It is well known that many factors in the host environment during infection can markedly influence the phenotype of the invading pathogen (11); one common host defense mechanism is the withholding of essential bacterial nutrients such as iron. 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. To obtain this unavailable iron most pathogenic bacteria have developed high-affinity iron uptake systems which usually involve two components: low-molecular-weight siderophores which chelate iron and iron-repressible OMPs (IROMPs) which function as receptors of the iron-siderophore complexes (4, 19, 23). In *Escherichia coli*, IROMPs ranging in apparent molecular weight from 74,000 to 81,000 are expressed in vitro during growth on iron-limited media and in vivo during experimentally established infections (12, 20). Furthermore, IROMPs are frequently immunogenic and in many cases show a significant degree of immunological cross-reactivity between different serogroups of one species (2, 10). In addition, Bolin and Jensen (3) have shown that turkeys passively immunized with antibodies against the *E. coli* IROMPs can be partially protected against *E. coli* septicemia. These results suggest that IROMPs may be useful as protective antigens by inducing antibodies which block siderophore-mediated iron uptake and/or which promote phagocytosis.

In the present study we demonstrate that *A. pleuropneumoniae* is also capable of expressing IROMPs and show immunologically that these IROMPs are expressed in vivo and are present in other serotypes of this pathogen. Furthermore, we present indirect evidence that these IROMPs may be involved in the binding of heme compounds by *A. pleuropneumoniae*.


**MATERIALS AND METHODS**

**Bacterial strains.** *A. pleuropneumoniae* 79-9 was obtained from the Western College of Veterinary Medicine, Saskatoon, Saskatchewan, Canada, and is a highly virulent serotype 1 strain originally isolated from the pulmonary tissues of a pig which died of acute pleuropneumonia (32). Strains of *A. pleuropneumoniae* representing serotypes 2, 3, 4, 5, and 7 were all obtained from clinical cases of porcine pleuropneumonia in western Canada. Strains were routinely stored at −70°C either as samples of infected lung tissue or after passage in chicken egg yolk sacs (32). As previously reported by Rapp et al. (27), repeated subculturing did not alter the OMP profile of any *A. pleuropneumoniae* strain examined here (results not shown). No attempt was made to segregate smooth from adherent forms of the organism; consequently, a mixture of both types was used for all experiments.

**Growth conditions.** Strains were maintained on PPLO agar (Difco Laboratories, Detroit, Mich.) plates supplemented with 50 μg of NAD per ml and 0.1% (wt/vol) glucose. For

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iron-restricted growth, PPLO-NAD-glucose broth was supplemented with the iron chelator 2,2’-dipyridyl (Sigma Chemical Co., St. Louis, Mo.) to a final concentration of 200 μM. This medium is referred to as PPLO-D. For some experiments, PPLO-NAD-glucose broth was supplemented with 200 μM iron-free (29) ethylenediaminedihydroxyphenylacetic acid (EDDA; Sigma) or 1.5 mg of iron-free conalbumin (Sigma) per ml as an iron chelator. Iron-replete bacteria were grown in PPLO-NAD-glucose broth alone or in PPLO-D containing 50 μM Fe(NO₃)₂.

For the preparation of outer membranes, A. pleuropneumoniae was grown at 37°C in PPLO-NAD-glucose broth until an optical density at 660 nm (OD₆60) of 0.07 to 0.08 was reached. At this time, an iron chelator was added at the concentrations indicated above and growth was continued for 10 h. The pellet was removed by centrifugation at 3,000 x g (Braunsonic 1510; Rapp et al. (27)). Harvested cells were removed, and 2,2’-dipyridyl (200 μM) was added to the supernatant. After incubation for 10 h, the pellet was centrifuged for 30 s in an Eppendorf centrifuge (15,500 x g) to pellet the cells, and the supernatant was assayed spectrophotometrically (OD₆60). Aliquots of cells were removed at 15-min intervals, collected by centrifugation (6,000 x g, 10 min), and used for the preparation of outer membranes.

Preparation of OMP-enriched fractions. Sarcosyl-insoluble OMP-enriched fractions were prepared by the method of Rapp et al. (27). Harvested cells were suspended in 10 mM N-2-hydroxyethylpipеразине-N’-2-ethanesulfonic acid (HEPES)(pH 7.4) buffer and sonicated intermittently for 90 s (Braunsonic 1510; intermediate probe; 60 W). Debris was removed by centrifugation at 3,000 x g for 10 min; a 0.5 volume of 2% (wt/vol) sarcosyl (sodium N-lauroylsarcosinate; Sigma) was added to the supernatant. After incubation at room temperature for 10 min, the partially purified outer membrane fraction was pelleted by centrifugation at 100,000 x g for 1 h. The pellet was suspended in 10 mM HEPES buffer and treated with 1 volume of 2% sarcosyl for 20 min at room temperature. This procedure was found to produce an outer membrane fraction of greater purity than could be obtained with only a single sarcosyl extraction. The final sarcosyl-insoluble, OMP-enriched fraction was sedimented by centrifugation as described above, and the pellet was suspended in 10 mM HEPES buffer before storage at −20°C. The protein concentration of each sample was determined as described by Markwell et al. (17).

SDS-PAGE and immunoblotting. Proteins were separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 4% stacking gels and 8 or 12% separating gels (15). OMP preparations (10 to 15 μg of total protein) were mixed with equal volumes of solubilization buffer (2.5% SDS, 5% 2-mercaptoethanol, 25% glycerol, and 0.003% bromophenol blue in 0.05 M Tris hydrochloride [pH 6.8]) and boiled for 7 min prior to electrophoresis. Gels were stained with Coomassie brilliant blue R250 or used for immunoblotting.

Electrophoretic transfer of proteins to nitrocellulose membranes and immunoblotting were performed essentially as described by Towbin et al. (35). Nonspecific binding sites were blocked by incubating the nitrocellulose overnight in TST buffer (100 mM Tris hydrochloride, 150 mM NaCl, 0.05% Tween 20 [pH 7.5]) containing 4% bovine serum albumin and 5% horse serum. Membranes were incubated for 2 h with antiseras diluted in TST buffer and then for 1 h with alkaline phosphatase-conjugated goat anti-swine immunoglobulin G (heavy- and light-chain specific; Kirkegaard and Perry Laboratories, Gaithersburg, Md.). After being washed, membranes were developed with 5-bromo-4-chloro-3-indolyl phosphate-Nitro Blue Tetrazolium substrate (Bio-Rad Laboratories, Richmond, Calif.).

Immune antiseras were obtained from swine which had recovered from natural serotype 1 A. pleuropneumoniae infections and from swine which were found to be chronic carriers of serotype 1 organisms. Serum from 6-week-old gnotobiotic pigs was used as a negative control in immunoblots. Gnotobiotic serum was used undiluted, while immune antiseras were diluted 1:250 prior to use.

Congo red- and hemin-binding assays. The binding of Congo red and hemin to A. pleuropneumoniae cells was measured by modifications of the procedures described by Kay et al. (14) and Daskaleros and Payne (8). A. pleuropneumoniae 79-9 cells grown in PPLO-NAD-glucose or PPLO-D broth were harvested, washed in phosphate-buffered saline, and suspended in phosphate-buffered saline to an OD₆60 of 1.5. Congo red (Sigma) or hemin (Sigma) was added to a final concentration of 30 or 40 μM, respectively. A 1-ml sample was immediately removed and centrifuged for 30 s at 15,500 x g in an Eppendorf centrifuge (15,500 x g) to pellet the cells, and the supernatant was assayed spectrophotometrically for Congo red (OD₆60) or hemin (OD₆40). The remaining cells were shaken at 37°C and assayed at 15-min intervals for residual Congo red or hemin in the supernatant as described above.

For competition assays, Congo red (30 μg/ml) was allowed to prebind to A. pleuropneumoniae 79-9 cells in PPLO-NAD-glucose or PPLO-D broth for 30 min at 37°C, after which the cells were pelleted, suspended in phosphate-buffered saline, and used in the hemin-binding assay as described above.

Porphyrin utilization assay. The utilization of porphyrin by iron-restricted A. pleuropneumoniae was determined by a plate assay as described by Daskaleros and Payne (8). Bacteria (10⁵ CFU/ml) were spread onto the surface of a PPLO-NAD-glucose agar plate containing 300 μM 2,2’-dipyridyl. Wells (6 mm) were cut into the solidified agar and filled with porphyrin solutions at concentrations of 10 mM. Zones of growth around the wells were measured after incubation at 37°C for 24 to 48 h.

RESULTS

Effects of iron restriction on the OMP composition of A. pleuropneumoniae 79-9. The effects of iron-restricted conditions were determined by growing the serotype 1 strain of A. pleuropneumoniae (79-9) in PPLO-NAD-glucose broth supplemented with one of three types of iron chelator: 2,2’-dipyridyl, EDDA, or conalbumin. OMPs were compared by SDS-PAGE to OMPs from cells grown under iron-replete conditions. Iron restriction induced by all three chelators resulted in the appearance of a novel OMP with a relative molecular weight of 105,000 (105K) (Fig. 1). In addition, the use of EDDA or 2,2’-dipyridyl as an iron chelator resulted in a very large increase in the synthesis of a 76K protein, whereas the use of conalbumin brought about a more modest but noticeable increase in this protein. Iron restriction induced by EDDA or conalbumin resulted in increased amounts of a 67K protein, an effect not readily apparent when 2,2’-dipyridyl was used. There were occasional differences seen among smaller, minor membrane proteins, depending on the type of iron chelator used, but they were never consistently observed in all OMP preparations. Inter-
Interestingly however, iron restriction routinely resulted in the disappearance of a 23K or 24K OMP seen in iron-replete cells.

We next wanted to ensure that the changes in the major 105K and 76K IROMPs were not the result of other environmental factors such as generally poor growth conditions. For these experiments, the medium was PPLO-NAD-glucose containing sufficient iron for growth. Firstly, the generation time of the cells was increased by reducing the aeration of the culture (30 rpm versus 180 rpm). Secondly, fully aerated cells were harvested at the mid-log, late-log, or stationary phase of growth. Thirdly, an aerated culture was grown to the mid-log phase, and aeration was stopped for 3 h prior to harvesting of cells and preparation of outer membranes. While there were minor changes evident among the lower-molecular-weight OMPs under some of these conditions, in no case was induction of the 105K and 76K IROMPs observed (data not shown).

**Kinetics of IROMP induction.** Cultures of *A. pleuropneumoniae* 79-9 in PPLO-NAD-glucose were grown in parallel, and 2,2'-dipyridyl was added to one to induce iron restriction. An effect of iron restriction on the growth rate was noted within 30 to 45 min after the addition of the acid chelator, and growth appeared to stop completely after about 120 min (Fig. 2A). Analysis of outer membranes prepared from cells sampled after 2,2'-dipyridyl addition (Fig. 2B) indicated that induction of the 105K and 76K IROMPs occurred within 15 min after a shift to iron-restricted conditions and reached a maximum after about 45 min. Analysis of outer membranes prepared from the parallel *A. pleuropneumoniae* culture grown under iron-replete conditions revealed no major changes throughout this growth period (data not shown).

**Serotype specificity of *A. pleuropneumoniae* IROMPs.** Isolates of *A. pleuropneumoniae* exhibit considerable serological heterogeneity; up to 12 serotypes based on capsule-associated, heat-stable, and heat-labile antigens have been reported (25). We therefore wished to determine firstly whether other *A. pleuropneumoniae* serotypes produced IROMPs and secondly whether these IROMPs were homologous to those of the serotype 1 strain. Clinical field isolates representing serotypes 1, 2, 3, 4, 5, and 7 were grown under iron-replete and iron-restricted (2,2'-dipyridyl) conditions, and their OMPs were compared by SDS-PAGE (Fig. 3). A
A pleuropneumoniae iron-repressible proteins

FIG. 3. IROMPs of other A. pleuropneumoniae serotypes. Strains were grown in PPL0-NAD-glucose or PPL0-D as described in the text, and OMP-enriched preparations were separated on 8% SDS-polyacrylamide gels. Only the relevant portion of the gels is shown. OMPs from iron-restricted cells are shown in the left lane of each pair, and OMPs from iron-repeat cells are shown in the right lane. The serotype of each pair of samples is shown at the top. The two outside lanes contain OMP preparations from iron-restricted serotype 1 cells (strain 79-9). Large arrowheads indicate the positions of the 105K and 76K IROMPs in the serotype 1 sample. Small arrowheads indicate the analogous IROMPs in the serotype 2, 3, 4, 5, and 7 samples.

A novel protein similar in size to the 105K IROMP of A. pleuropneumoniae 79-9 (serotype 1) was seen in all serotypes after iron restriction, although this IROMP appeared to have a slightly higher molecular weight in the serotype 4 and 7 strains. Furthermore, this IROMP was apparently synthesized in lesser amounts in the serotype 2, 4, and 7 strains than in the serotype 1, 3, and 5 strains. A protein equivalent to the serotype 1 76K IROMP was also induced by iron restriction in all serotypes, although again the relative amounts varied considerably from one serotype to another. Also, the 67K OMP, which in the serotype 1 strain was not responsive to iron restriction by 2,2′-dipyridyl, was induced somewhat in the serotype 2 to 7 strains and was especially prominent in the serotype 7 strain. Finally, a ca. 100K protein was present in substantially greater amounts in iron-restricted cells of the serotype 2, 4, 5, and 7 strains than in those of the serotype 1 and 3 strains. At least three different isolates of each serotype were examined in this way, and the results were equivalent in all cases (data not shown).

Immunoblot analysis of A. pleuropneumoniae IROMPs. To assess the immunogenicity of the A. pleuropneumoniae IROMPs, we performed Western blotting (immunoblotting) with OMPs from iron-starved cells representing serotypes 1, 2, 3, 4, 5, and 7. Proteins were electrophoretically transferred to nitrocellulose paper and reacted with convalescent-phase antisera from a number of pigs infected with serotype 1 A. pleuropneumoniae. Antibodies directed against most of the OMPs of the homologous serotype 1 strain could be detected in these sera, including antibodies against both the 105K and 76K IROMPs (Fig. 4, lane 1). In control experiments, serum from 6-week-old gnotobiotic pigs failed to recognize any OMP other than a 40K major OMP (a very weak reaction) (lane 7). The serotype 1-specific immune sera also recognized the equivalent 105K and 76K IROMPs of the serotype 2, 3, 4, 5, and 7 strains (lanes 2 to 6), indicating that there was considerable cross-reactivity between these proteins of different serotypes. Interestingly, both a 100K OMP and 67K OMP which appeared to be iron repressible in some but not all serotypes (see above) reacted strongly with these antisera. Immunoblot analysis analogous to that shown in Fig. 4 were also made with antisera from pigs found to be chronically infected with a serotype 1 A. pleuropneumoniae strain but showing no signs of an acute disease. In these cases, an identical pattern of reactivity was seen (data not shown).

Congo red and hemin binding by A. pleuropneumoniae. We wanted to obtain evidence that the IROMPs of A. pleuropneumoniae did indeed play a role in iron acquisition by this organism. It has been suggested that the ability of Yersinia species to bind the aromatic dye Congo red is related to their ability to sequester iron (26) and that a strong correlation exists between Congo red binding and hemin adsorption (34). To see if a similar relationship was true for A. pleuropneumoniae, we grew serotype 1 strain 79-9 in the presence of 2,2′-dipyridyl to induce the 105K and 76K IROMPs and measured the Congo red-binding ability of these cells. A. pleuropneumoniae strains not expressing IROMPs had only a modest ability to bind Congo red, whereas cells expressing the 105K and 76K IROMPs showed a marked increase in dye adsorption (Fig. 5). The same effect was noted when the hemin-binding ability of A. pleuropneumoniae was measured; cells expressing IROMPs showed a greatly enhanced ability to adsorb this porphyrin compound as compared with cells which had been grown under iron-replete conditions (Fig. 6). To see if Congo red and hemin were bound at the same sites on the cell surface, we performed a competition assay in which Congo red was prebound to iron-starved A. pleuropneumoniae cells prior to measurement of their hemin-binding ability. Prebinding of Congo red essentially eliminated the ability of the cells to bind hemin (Fig. 6). In this case the levels of hemin adsorption were reduced to a level equivalent to that seen with iron-replete cells.

Use of porphyrin compounds as iron sources by A. pleuropneumoniae. To see if A. pleuropneumoniae 79-9 could utilize heme compounds as sole sources of iron, we carried out plate assays in which the ability of various porphyrins to overcome 2,2′-dipyridyl-induced iron restriction was measured. Free heme, hemin, hematin, and hemoglobin were all able to stimulate the growth of iron-restricted A. pleuropneumoniae, while the iron-free precursor to heme biosynthesis, protoporphyrin IX, was unable to do so (Table 1).
Cultures function of Congo gens iron-protei ability environmental grown Congo IROMPs; was uptake of iron-replete conditions and measured complexes. Binding of Congo red by A. pleuropneumoniae grown here as depletion of the dye from solution.

**DISCUSSION**

Pathogenic bacteria have a strict nutritional requirement for iron, but in vivo they must contend with the natural ability of the host to withhold free iron in the form of iron-protein complexes. To obtain bound iron, many pathogens possess high-affinity iron uptake systems which consist in part of novel OMPs inducible under conditions of environmental iron limitations. Evidence to date suggests that the presence of iron uptake systems can be an important bacterial virulence mechanism. For this reason we have been examining the role of iron in the pathobiology of *A. pleuropneumoniae*, an important and highly virulent respiratory pathogen of swine. We show here that *A. pleuropneumoniae* is able to respond to iron-restricted conditions by inducing the synthesis of a specific subset of OMPs. One of these proteins, a 105K OMP, was apparently completely absent in iron-replete cells, whereas a 76K OMP was detected in minor amounts when sufficient iron was present but was increased greatly in response to iron restriction. It should be noted that, as other workers have found (6), the choice of chelator used to create an iron-restricted environment also influenced the OMP profile. Use of the synthetic chelators 2,2’-dipyridyl and EDDA resulted in a substantial increase in the synthesis of the 76K OMP, while the natural iron-binding glycoprotein conalbumin produced a relatively modest increase. Likewise, the use of conalbumin and EDDA produced a slight increase in the amount of a 67K OMP, whereas iron chelation with 2,2’-dipyridyl had little or no effect on this protein. The status of the 67K OMP in terms of its response to iron restriction is therefore unclear at this point. The 105K OMP was consistently observed irrespective of the type of chelator used, so it and probably the 76K OMP can be considered to be true IROMPs.

Induction of the *A. pleuropneumoniae* IROMPs occurred rapidly; they could be detected in outer membrane preparations within 15 min of establishment of iron-restricted conditions and at least 15 min prior to a decline in cellular growth rate caused by iron deprivation. Rapid induction of IROMPs would presumably be necessary to ensure that the remaining intracellular iron stores were not depleted before a bacterial iron acquisition system could become fully operable. A similar time course for IROMP expression has also been observed in the bovine pathogen *Pasteurella haemolytica* (8a).

If the 105K and 76K OMPs of serotype 1 *A. pleuropneumoniae* are indeed part of an iron acquisition system, then proteins analogous to them would be expected to be present in other serotypes as well, since all serotypes of *A. pleuropneumoniae* are capable of causing disease. Our study shows that at least for field isolates representing the predominant serotypes 2, 3, 4, 5, and 7, common IROMPs appear to be expressed. All serotypes induced a novel ca. 105K OMP under iron-restricted conditions, although in the serotype 4 and 7 isolates this protein appeared slightly larger. It is unclear whether this represents a true difference in size or is due to the fact that with some strains, large amounts of lipopolysaccharide comigrate in this region of the gel (28; unpublished results) and can cause anomalous banding patterns. In addition to the novel 105K OMP, an increase in the synthesis of the 76K IROMP as well as variable increases in

<table>
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<tr>
<th>Iron source</th>
<th>Zone size (mm)</th>
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<tr>
<td>Fe(NO₃)₃</td>
<td>9.5</td>
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<tr>
<td>Heme</td>
<td>7.5</td>
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<tr>
<td>Hemin</td>
<td>8.0</td>
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<td>Hematin</td>
<td>7.0</td>
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<tr>
<td>Hemoglobin</td>
<td>6.0</td>
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<td>Protoporphyrin IX</td>
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* Each well contained 10 mM heme compound or 10 µg of iron as Fe(NO₃)₃.
* Zone size was measured from the edge of a well to the outer periphery of bacterial growth.
the synthesis of a 67K OMP were noted in all serotypes. These results suggest that the serotypes of *A. pleuropneumoniae* examined here share a common mechanism for iron acquisition, at least as far as the OMP component of this mechanism is concerned.

Finally, immunological analysis was used to examine some of the in vivo properties of the *A. pleuropneumoniae* IROMPs and to confirm the apparent relatedness of IROMPs between different serotypes. Sera from swine infected with serotype 1 *A. pleuropneumoniae* were found to contain antibodies which reacted with both the 105K and 76K IROMPs from the homologous serotype 1 strain 79-9, suggesting that these proteins are expressed during in vivo growth of the organism and are possibly exposed on the bacterial cell surface. This is to be expected, since any role for these proteins as receptors in a bacterial high-affinity iron acquisition system would necessitate that they be both localized in the outer membrane of the cells and surface exposed. Surface exposure would in turn allow interaction with the host immune system during an active infection. Indeed, IROMPs from a number of gram-negative pathogens, including *Neisseria* species (2, 10), *Pseudomonas aeruginosa* (5), and Klebsiella pneumonias (33), have been shown to elicit an antibody response during naturally occurring infections, and we now extend these observations to include the IROMPs from *A. pleuropneumoniae*. We also showed that the serotype 1-specific antisera contained antibodies which recognized both the 105K and 75K IROMPs from the serotype 2, 3, 4, 5, and 7 isolates. The antigenic cross-reactivity between IROMPs of different serotypes again suggests that in vivo iron acquisition by *A. pleuropneumoniae* is not serotype specific but instead involves a common mechanism that uses conserved OMPs. This finding, combined with our observation that pigs chronically infected with *A. pleuropneumoniae* also carry antibodies against IROMPs (unpublished data), suggests that the IROMPs of *A. pleuropneumoniae* represent effective targets in the development of vaccines which offer serotypic cross-protection as well as the ability to prevent chronic infections.

At present the function of the 105K and 76K IROMPs is unknown, although it is tempting to speculate that one or both of these proteins may serve as a receptor for siderophore-iron complexes or in some way interact directly with host iron-carrying compounds. Siderophorelike molecules have not been described in *A. pleuropneumoniae*, and in preliminary bioassays we have not been able to demonstrate this type of iron-chelating activity (H. Deneer, unpublished data). However, it is possible that *A. pleuropneumoniae* can obtain iron in vivo directly from host sources in a manner similar to that of *Neisseria* species, which also apparently do not produce siderophores (21, 36). In this regard we have shown that *A. pleuropneumoniae* can overcome 2,2'-dipyridyl-induced iron restriction by utilizing various porphyrin compounds such as heme, hemin, hematin, and hemoglobin as sources of iron. Similar results have been reported for *Haemophilus influenza* (7) and *Neisseria* species (22) as well as for *Shigella flexneri*, in which a hemin transport system appears to be independent of siderophore-mediated iron acquisition (16). Furthermore, previous reports have shown that some pathogenic bacteria can adsorb hemin and the structurally similar aromatic dye Congo red and that this ability is strongly correlated with virulence (8, 26). The molecular basis for this adsorption is unclear, although studies with the fish pathogen *Aeromonas salmonicida* have indicated that Congo red, hemin, and protoporphyrin IX are all bound by a constitutive 49,000-molecular-weight protein which comprises the cell surface protein array known as the A layer (14). In our efforts to determine a function for the 105K and 76K IROMPs of *A. pleuropneumoniae*, we showed that Congo red and hemin could also be bound by *A. pleuropneumoniae* cells but that this binding occurred only in a significant extent if the cells were previously grown under iron-restrictive conditions and hence were expressing the 105K and 76K proteins. Cells grown in iron-replete media bound only modest amounts of Congo red and hemin; this may have been the result of nonspecific, hydrophobic interactions (14). Furthermore, we have provided evidence that Congo red and hemin are bound by the same *A. pleuropneumoniae* cell surface receptor, since prebinding of Congo red effectively eliminated the subsequent uptake of hemin. Thus, although not conclusive, these results suggest that there may be an association between the expression of the 105K and 76K IROMPs of *A. pleuropneumoniae* and the scavenging of porphyrin compounds by this organism and represent, to our knowledge, the first report of the involvement of IROMPs as opposed to constitutive OMPs in such a phenomenon. Further experiments, including an analysis of *A. pleuropneumoniae* mutants lacking IROMPs, are necessary to confirm the role of these proteins in Congo red or porphyrin binding and indeed to resolve the question of whether only one protein or both are required. Similarly, it remains unclear whether the very small amounts of free heme or hemoglobin normally found in vivo are sufficient to meet the iron requirements of *A. pleuropneumoniae* or whether in fact the heme bound to serum hemopexin or hemoglobin complexed with haptoglobin can be utilized. *A. pleuropneumoniae* is known to secrete a potent hemolysin, and it is possible that the hemoglobin released from lysed erythrocytes is an adequate source of iron for in vivo growth. The role of *A. pleuropneumoniae* IROMPs may then simply be to serve as receptors for these free heme compounds and to mediate their transport into the cell. Clearly, the relationship between IROMPs and iron uptake and its contribution to the overall virulence of *A. pleuropneumoniae* remain to be fully defined.

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LITERATURE CITED