Molecular cloning and characterization of the ferric hydroxamate uptake (fhu) operon in Actinobacillus pleuropneumoniae

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The bacterium Actinobacillus pleuropneumoniae, a swine pathogen, utilizes ferrichrome as an iron source. This study details the molecular cloning and sequencing of the genes involved in the uptake of this hydroxamate siderophore. Four ferric hydroxamate uptake (fhu) genes, fhuC, fhuD, fhuB and fhuA, were identified in a single operon, and these were found to encode proteins homologous to proteins of the fhu systems of several bacteria, including Escherichia coli. The fhuA gene encodes the 77 kDa outer-membrane protein (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 that are components of an ABC transporter which internalizes the ferric hydroxamate. Reference strains of A. pleuropneumoniae that represented serotypes 1 to 12 of this organism all tested positive for the four fhu genes. When A. pleuropneumoniae FhuA was affinity-tagged with hexahistidine at its amino terminus and expressed in an E. coli host, the recombinant protein reacted with an mAb against E. coli FhuA, as well as with a polyclonal pig serum raised against an A. pleuropneumoniae infection. Hence, the authors conclude that fhuA is expressed in vivo by A. pleuropneumoniae. Three-dimensional modelling of the OMP FhuA was achieved by threading it to the X-ray crystallographic structure of the homologous protein in E. coli. FhuA from A. pleuropneumoniae was found to have the same overall fold as its E. coli homologue, i.e. it possesses an N-terminal cork domain followed by a C-terminal β-barrel domain and displays 11 extracellular loops and 10 periplasmic turns.

Keywords: outer-membrane protein, siderophore transport

INTRODUCTION

Actinobacillus pleuropneumoniae is the aetiological agent of porcine pleuropneumonia, a highly contagious respiratory disease with major economic implications for the swine industry worldwide (Tascón et al., 1996).

The bacterial infection process that is governed by many virulence factors which act alone or in concert to establish the pathogen in the porcine host. Iron has long been associated with bacterial virulence, either as a requirement for bacterial growth or by acting as an environmental signal that regulates the expression of other virulence factors (Braun, 2001). The scarce bioavailability of iron ($10^{-18}$ M) at concentrations lower than are required for most bacteria to grow ($10^{-6}$ to $10^{-8}$ M) necessitates that bacteria utilize mechanisms for high-affinity iron acquisition.

A. pleuropneumoniae is capable of using haemoglobin,
haemin-containing compounds and porcine transferrin as sources of iron for its growth (Bélanger et al., 1995; Deneer & Potter, 1989). In addition, it can produce haemolysins, toxins that belong to the RTX (repeats-in-toxin) group of proteins (Schaller et al., 1999). All of these factors may contribute to the virulence of this bacterium. A. pleuropneumoniae also responds to iron-restricted conditions by inducing the synthesis of a specific subset of outer-membrane proteins (OMPs) (Deneer & Potter, 1989; Niven et al., 1989; Soltes & Maclnnnes, 1994; M. Archambault, personal communication), including two membrane-bound transferrin-specific receptors called TbpA and TbpB (Gerlach et al., 1992a, b; Gonzalez et al., 1995). Although it was tempting to speculate that one of these proteins might serve as a receptor for a siderophore, preliminary bioassays by Deneer & Potter (1989) did not demonstrate any siderophore production in A. pleuropneumoniae. However, it was suggested that A. pleuropneumoniae might obtain iron in vivo directly from host sources in a manner similar to that of Neisseria species, which apparently also do not produce siderophores (Mickelson et al., 1982; West & Sparling, 1985). Niven et al. (1989) were unable to detect hydroxamate and catecholate siderophores in culture supernatants of A. pleuropneumoniae grown under iron-restricted conditions. By contrast, when Diarra et al. (1996) tested the ability of all serotypes of A. pleuropneumoniae to use different exogenous sources of iron (specifically catecholates and hydroxamates), growth promotion assays showed that all of the A. pleuropneumoniae strains tested (with the exception of one field strain of serotype 5) were capable of using ferrichrome as a growth-promoting substance under iron-limited conditions. They also demonstrated that A. pleuropneumoniae strain 87-682 of serotype 1 and strain 2245 of serotype 5 secreted an iron chelator into the culture medium in response to iron stress. However, this potential A. pleuropneumoniae siderophore had a structure that did not conform to that defined by the well-characterized assay for catechols established by Arnow (1937) or the assay of Csaky (1948) for hydroxamates. It is worth noting that some bacteria are known to use siderophores that are produced by other microorganisms; hence, these bacteria must have the necessary receptors for the assimilation of different siderophores.

Several fungi, including Ustilago sphaerogena, synthesize ferrichrome, a hydroxamate siderophore. The ferric hydroxamate uptake (fhu) system in Escherichia coli is well recognized as one of the paradigms for siderophore transport (Braun, 1995; Coutil et al., 1983; Locher et al., 1998). The E. coli fhu system consists of four genes, designated fhuA, fhuC, fhuD and fhuB, which are arranged in one operon at minute 3 of the linkage map (Fecker & Braun, 1984) and transcribed clockwise in the same order. fhuA encodes the multifunctional OMP FhuA (79 kDa) that acts in E. coli as the ferrichrome–iron receptor as well as the receptor for phages T1, T5, φ80 and UC-1, for the bacterial toxin colicin M and for some antibiotics, such as albomycin (a structural analogue of ferrichrome) and rifamycin CGP 4832 (Ferguson et al., 2001a). FhuA is a key player in the fhu system, as it is specific for Fe⁺⁻ ferrichrome and functions as a ligand-specific gated channel (Ferguson et al., 1998a). The elucidation of the high-resolution X-ray crystallographic structure of FhuA from E. coli (Ferguson et al., 1998a; Locher et al., 1998) provided a major advance in the understanding of some of the structure–function relationships of this protein.

The other proteins of the fhu system, namely FhuD, FhuC and FhuB, are also essential to its function. Periplasmic FhuD (31 kDa) and cytoplasmic-membrane-associated FhuC (29 kDa) and FhuB (41 kDa) are proteins necessary for the transport of ferrichrome and other Fe⁺⁻ hydroxamate compounds (Fe³⁺-aerobactin, Fe⁺⁺-coprogen) from the periplasm, across the cytoplasmic membrane into the cytoplasm (Braun et al., 1991; Coulton et al., 1987; Mademidis et al., 1997). The protein complex TonB–ExbB–ExbD (Günter & Braun, 1990; Postle, 1993) provides energy for this process.

Here, we report that the genome of A. pleuropneumoniae contains an operon with genes homologous to those of the E. coli fhu system, albeit in a different gene order. We also studied the distribution of these fhu genes among the different serotypes of A. pleuropneumoniae and the expression of the gene encoding the OMP receptor FhuA. The structural similarities between FhuA of E. coli and FhuA of A. pleuropneumoniae were deduced by three-dimensional modelling.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Strains of E. coli and the plasmids used in this study are listed in Table 1. The A. pleuropneumoniae reference strains (laboratory stock) used in this study were: serotype 1, strain 4074; serotype 2, strain 4226; serotype 3, strain 1421; serotype 4, strain 1462; serotype 5, strain K-17; serotype 6, strain FEMO; serotype 7, strain WF83; serotype 8, strain 405; serotype 9, strain 13261; serotype 10, strain 13039; serotype 11, strain 56153; serotype 12, strain 8329/85. These reference strains were grown on brain–heart infusion agar (Difco Laboratories) supplemented with 15 µg NAD⁺ ml⁻¹. E. coli BL21(DE3), E. coli CC118 and E. coli DH5α were grown on Luria–Bertani (LB) Miller medium, whereas E. coli XL-1 Blue MRF⁺ was grown in NZYCM broth (Gibco-BRL) supplemented with 12.5 µg tetracycline ml⁻¹. The plasmid excision strain of E. coli, XL0LR, was grown in LB broth supplemented with 12.5 µg tetracycline ml⁻¹. Growth of the phagemid pBK-CMV was carried out for selection on LB agar supplemented with 50 µg kanamycin ml⁻¹. Strains harbouring derivatives of the pET30a+ expression vector were grown on LB agar supplemented with 30 µg kanamycin ml⁻¹; those harbouring pGEM derivatives required 50 µg ampicillin ml⁻¹ to be added to the medium. Blue/white colony selection was achieved by the addition of 40 µg X-Gal ml⁻¹ and 1 mM IPTG to the medium. The addition of 50 µg deferrated ethylenediamine dihydroxyphenyl acetic acid ml⁻¹ (Sigma) to the growth medium depleted the iron available to the bacteria. Ferrichrome (Sigma), used as an exogenous source of iron,
was either spotted onto the deferrated medium or incorporated into the top agar.

**Construction of an A. pleuropneumoniae DNA-signal-sequence library.** To screen for exported proteins in *A. pleuropneumoniae* serotype 1 strain 4074 was digested with *Sau*3A1 and then separated by agarose-gel electrophoresis. DNA fragments of 500–1500 bp in size were excised from the gel, and the DNA was extracted using a QIAquick Gel Extraction Kit (Qiagen). The vector (pHRM104; Pearce *et al.*, 1993) containing the truncated alkaline phosphatase gene (*phoA*) was digested with *Bam*HI, dephosphorylated with shrimp alkaline phosphatase and ligated with the *A. pleuropneumoniae Sau*3A1 fragments. Cells from *E. coli phoA* mutant strain CC118 were transformed with the ligation mixture by electroporation and then incubated in LB Miller broth for 1 h. The cells were then plated onto LB agar plates containing 500 μg erythromycin ml⁻¹ and 50 μg 5-bromo-4-chloro-3-indolyl phosphate (XGal) ml⁻¹. The translocation of PhoA across the bacterial inner membrane results in the hydrolysis of XGal and the development of the blue colony phenotype. This indicates that the fusion proteins were derived from plasmids containing an *A. pleuropneumoniae* DNA sequence harbouring a promoter, a translational start site and a functional signal sequence (Mintz & Fives-Taylor, 1999). Restriction and modification enzymes were purchased from Amersham Pharmacia Biotech and Roche Diagnostics, and were used according to the manufacturers’ instructions.

**Genetic techniques.** Plasmids from the PhoA-positive colonies were isolated using the Plasmid QIAprep Spin Minipreps Kit (Qiagen). The *A. pleuropneumoniae* DNA insert was sequenced using the oligonucleotide primer foA (Table 2), which hybridizes to the negative strand of *phoA*. Subsequent primers (F7 and R7; Table 2) were designed from within the plasmid sequence of the positive clone of relevance to this study. An oligonucleotide primer (FA1; Table 2) based on a region of promoter sequences for *fhuA* from *E. coli* was also designed. PCR was carried out with standard conditions and varying annealing temperatures, depending on the sequence of the primers used. When the expected PCR product was larger than 50 kb, the Expand Long Template PCR System 1 (Roche Diagnostics) was used in the reaction, rather than Taq polymerase. DNA sequencing of the PCR product was performed at the DNA Sequencing Core Facility of the University of Maine, by using an ABI model 373A stretch DNA sequencer (Applied Biosystems). Single-stranded synthetic oligonucleotides (Table 2) were synthesized by BioCorp.

## Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description*</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
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<tr>
<td>CC118</td>
<td>*phoA recA araD139 (ara-leu)7697 galE galK thi rpsE rpoB argE (Amp) relA1 lac F’ [proAB lacFZΔAM15 Tn10 (Tet)]</td>
<td>Mintz &amp; Fives-Taylor (1999)</td>
</tr>
<tr>
<td>XL-1 BlueMRF²</td>
<td><em>Δ(mcrA)183 Δ(mcrCB-bsdSMR-mrrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac F’ [proAB lacFZΔAM15 Tn10 (Tet)]; Su ’</em></td>
<td>Stratagene</td>
</tr>
<tr>
<td>XLOLR</td>
<td><em>Δ(mcrA)183 Δ(mcrCB-bsdSMR-mrrr)173 endA1 thi-1 recA1 gyrA96 relA1 lac F’ [proAB lacFZΔAM15 Tn10 (Tet)]; Su ’</em></td>
<td>Stratagene</td>
</tr>
<tr>
<td>XL-1 Blue</td>
<td><em>supE44 bsdR17 recA1 endA1 gyrA46 thi relA1 lac F’ [proAB lacFZΔAM15 Tn10 (Tet)]</em></td>
<td>Stratagene</td>
</tr>
<tr>
<td>DH5α</td>
<td><em>supE44 lacU169 (800 lacZΔAM15) bsdR17 recA1 endA1 gyrA96 thi-1 relA1</em></td>
<td>Stratagene</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F’ompT bsdS6 (r6 m6) gal dcm (DE3)</td>
<td>Novagen</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBK-CMV</td>
<td>Km’; cloning vector of pBR322 origin, T3 and T7 promoter</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pLM101</td>
<td>Km’; App 7974 bp <em>huCDBA</em> operon in pBK-CMV vector</td>
<td>This study</td>
</tr>
<tr>
<td>pLM201</td>
<td>Amp’; PCR product of AFor and ARev (mature <em>fhuA</em>) in pGEM</td>
<td>This study</td>
</tr>
<tr>
<td>pET30a +</td>
<td>Km’; expression vector with N-terminal histidine tag</td>
<td>Novagen</td>
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<tr>
<td>pLM202</td>
<td>NotI fragment of pLM201 in pET30a</td>
<td>This study</td>
</tr>
<tr>
<td>pHX405</td>
<td>Amp’, Tet’; pBR322-based plasmid encoding FhuA–hexahistidine of <em>E. coli</em></td>
<td>Moeck <em>et al.</em> (1997)</td>
</tr>
</tbody>
</table>

*Su’, non-repressing; *λ’, λ-resistant.

### Discussion

The construction and screening of a *A. pleuropneumoniae* phage bank. A library of *A. pleuropneumoniae* serotype 1 strain 4074 DNA was constructed (Vezina *et al.*, 1997) in the *λ*Zap Express phage vector (Stratagene), following the manufacturer’s instructions. Briefly, *A. pleuropneumoniae* genomic DNA was partially digested with *Sau*3A1 and the resulting DNA fragments were separated by agarose-gel electrophoresis. The DNA fragments corresponding to approximately 6 to 12 kb in length were excised from the gel and ligated with *Bam*HI-digested *λ*Zap Express arms and packaged *in vitro* with Stratagene Gold II packaging extracts. The *E. coli* strains used were XL-1 Blue MRF⁺ as the host and *E. coli* XLOLR as the excision plating strain. The library was screened with the probes designed for Southern blotting, and was either spotted onto the deferrated medium or incorporated into the top agar.

**Construction of an A. pleuropneumoniae DNA-signal-sequence library.** To screen for exported proteins in *A. pleuropneumoniae* (Strois *et al.*, 2001), chromosomal DNA from *A. pleuropneumoniae* serotype 1 strain 4074 was digested with *Sau*3A1 and then separated by agarose-gel electrophoresis. DNA fragments of 500–1500 bp in size were excised from the gel, and the DNA was extracted using a QIAquick Gel Extraction Kit (Qiagen). The vector (pHRM104; Pearce *et al.*, 1993) containing the truncated alkaline phosphatase gene (*phoA*) was digested with *Bam*HI, dephosphorylated with shrimp alkaline phosphatase and ligated with the *A. pleuropneumoniae Sau*3A1 fragments. Cells from *E. coli phoA* mutant strain CC118 were transformed with the ligation mixture by electroporation and then incubated in LB Miller broth for 1 h. The cells were then plated onto LB agar plates containing 500 μg erythromycin ml⁻¹ and 50 μg 5-bromo-4-chloro-3-indolyl phosphate (XGal) ml⁻¹. The translocation of PhoA across the bacterial inner membrane results in the hydrolysis of XGal and the development of the blue colony phenotype. This indicates that the fusion proteins were derived from plasmids containing an *A. pleuropneumoniae* DNA sequence harbouring a promoter, a translational start site and a functional signal sequence (Mintz & Fives-Taylor, 1999). Restriction and modification enzymes were purchased from Amersham Pharmacia Biotech and Roche Diagnostics, and were used according to the manufacturers’ instructions.

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**Construction and screening of an A. pleuropneumoniae phage bank.** A library of *A. pleuropneumoniae* serotype 1 strain 4074 DNA was constructed (Vezina *et al.*, 1997) in the *λ*Zap Express phage vector (Stratagene), following the manufacturer’s instructions. Briefly, *A. pleuropneumoniae* genomic DNA was partially digested with *Sau*3A1 and the resulting DNA fragments were separated by agarose-gel electrophoresis. The DNA fragments corresponding to approximately 6 to 12 kb in length were excised from the gel and ligated with *Bam*HI-digested *λ*Zap Express arms and packaged *in vitro* with Stratagene Gold II packaging extracts. The *E. coli* strains used were XL-1 Blue MRF⁺ as the host and *E. coli* XLOLR as the excision plating strain. The library was screened with the probes designed for Southern blotting,
which were DIG-labelled, and the positive plaques were again detected with phosphatase-labelled anti-DIG antibodies with NBT/BCIP as the substrate. The plaques showing a strong reaction were purified by successive rounds of screening. After three rounds of screening, the pBK-CMV phagemid of the positive clones was excised using ExAssist Helper Phage (Stratagene). Finally, the plasmids were digested with restriction enzymes XbaI and SalI. These enzymes are known to cut once each within the multiple-cloning site of the vector, and thereby evaluate the size(s) of the insert(s). The dideoxynucleotide sequencing reactions were carried out with universal primers T3 and T7 (Table 2). To complete walking the entire sequence of the plasmid insert(s), internal primers 5T3W1, 5T7W1, 5T3W2, 5T7W2, 5T7W2.5, 5T3W3, 5T7W3, 5T3W4 and 5T3W5; Table 2) were designed that were based on the sequence information obtained.

Analysis of sequence homology and protein localization.
DNA sequence analysis was carried out with the aid of programs from University of Wisconsin Genetics Computer Group Software (Devereux et al., 1984) using the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov). The multiple-sequence alignment of proteins employed the CLUSTAL alignment algorithm, provided by the Baylor College of Medicine Search Launcher (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.htm). Prediction of protein localization and cleavage sites for signal sequences was carried out by the method of Nakai & Kanehisa (1991).

Expression of recombinant histidine-tagged FhuA. We elected to amplify the coding sequences (amino acids 1–668) of mature FhuA using primers AFor and ARrev (Table 2); these primers were designed to maintain the reading frame of the fhuA gene. The PCR product was purified using a PCR Purification Kit (Qiagen), ligated into the pGEM-T Easy Vector (Promega) and then transformed into E. coli XL-1 Blue cells. The recombinant plasmid, pLM201, was digested with NotI, and the resulting NotI fragment was cloned directionally and in-frame into the NotI site of the pET30α+ expression vector (Novagen); this was done to append a hexahistidine tag to the amino terminus of FhuA. Plasmid pLM202, carrying the fhuA sequence for mature FhuA with a hexahistidine tag at its amino terminus, was transformed into E. coli DH5α. It was then extracted from DH5α, purified and the orientation and reading frame of the fhuA gene were verified by DNA sequencing. pLM202 was then transformed into E. coli BL21 (DE3) (Novagen), the recommended host background for the expression of recombinant proteins in pET vectors. To express the histidine-tagged fusion protein, and following the manufacturer’s suggested procedure, cells containing the recombinant plasmid were grown in broth culture containing kanamycin and induced with IPTG (0–4, 1 mM). Various times (2, 4, 6, 8 and 24 h) and temperatures (37 and 25 °C) were tested for the optimal expression of the fusion protein. Whole-cell protein samples were separated by SDS-PAGE following standard procedures; the proteins within the gel were then either stained with Coomassie blue or transferred to a nitrocellulose membrane for immunoblotting (Harlow & Lane, 1999).

Western blotting. After the proteins had been transferred, the nitrocellulose membranes were blocked for 1 h with a solution of 1% BSA and then incubated overnight at 4 °C with a mouse-anti-hexahistidine mAb (Roche), a mouse mAb against E. coli FhuA (mAb Fhu6.1) (Moeck et al., 1995) or a
convalescent serum obtained from a pig that had been experimentally infected with *A. pleuropneumoniae* serotype 1 (Jacques et al., 1996). The secondary antibodies used were either a goat-anti-mouse IgG + IgM (heavy + light) or an antiswine IgG–horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories). Reactions were revealed by the addition of 4-chloro-1-naphthol and H$_2$O$_2$ (Sigma) to the membranes (Harlow & Lane, 1999).

**Homology model for FhuA of *A. pleuropneumoniae*.** Using the sequence data for the gene encoding *A. pleuropneumoniae* FhuA, the predicted amino-acid sequence (residues 1–673 of the mature protein) of this protein was submitted to the JIGSAW 3D Protein Homology Modelling Server (http://www.bmm.icnet.uk/servers/3djigsaw) (Bates & Sternberg, 1999). The JIGSAW server returned the *E. coli* FhuA structure, PDB code 1qfg (Ferguson et al., 2000), as the only successful structural template within its sample space. Visual inspection of this homology model revealed a number of discontinuities within the extracellular- and periplasmic-loop regions, namely Cz pairs in which the distances were too great to form a covalent bond. To better model the loops of *A. pleuropneumoniae* FhuA, anchor regions flanking these discontinuities were submitted to the CODA server (http://www-cryst.bioc.cam.ac.uk/coda/coda.html) (Deane & Blundell, 2001). CODA models amino-acid sequences of proposed loops by searching against known structures of loop regions within other proteins. The CODA server was able to model all loop regions within the *A. pleuropneumoniae* FhuA sequence that the JIGSAW server could not. The acceptable r.m.s. (root mean square) deviation was <1 Å. The loops and anchor regions modelled by the CODA server are as follows, 158–166, 203–204, 283–284, 321–322, 597–598, 610–611, 630–631.

RESULTS

**Cloning of the fhu operon in *A. pleuropneumoniae***

By using a truncated gene for alkaline phosphatase (*phoA*) that lacks a functional signal sequence, a system was developed that identifies genes encoding exported proteins (Manoil & Beckwith, 1985). This system was modified by Sirois et al. (2001) for use with *A. pleuropneumoniae*. Gene fusions between the coding FhuA protein (amino acids 23–80, out of a total of 301). The 375 bp fragment also showed 35% identity with and 48% similarity to the *E. coli* FhuD protein (amino acids 28–80, out of a total of 296). To obtain the full FhuD sequence from the chromosome of *A. pleuropneumoniae* serotype 1, oligonucleotide primers (F7 and R7; Table 2) were designed from the 5’ and 3’ ends of the sequence in pl25. These were paired with a primer (FA1; Table 2) that was based on a region of promoter sequences for *fhuA* from *E. coli*. A PCR product of 1063 bp was obtained with the primer pair FA1/R7. The deduced amino acids of this amplicon displayed an uninterrupted ORF for a sequence encoding FhuD; this ORF had 27% identity with the protein and 44% similarity to the gene in *E. coli*.

To isolate larger genomic fragments from *A. pleuropneumoniae* that contained *fhuD* and its neighbouring sequences, we used a genomic library of *A. pleuropneumoniae* serotype 1 strain 4074 DNA made in the λZAP Express phage; the fragments in this library were approximately 7–12 kb in size. As a screening tool, we labelled the 1063 bp PCR product (i.e. the *fhuD* sequence) with DIG. From 10 plaques that gave a positive signal with the *fhuD* probe, one was subjected to three rounds of purification; the plasmid (pLM101) of this positive clone was then excised. Restriction analysis of pLM101 (Fig. 1) revealed an insert of 80 kb. The entire insert was then sequenced with universal primers T3 and T7, which annealed with the vector, and then with internal primers designed from the sequence walking. The total sequence information displayed four different ORFs corresponding to the genes *fhuC, fhuD, fhuB* and *fhuA* which appear in the *fhu* operon of several bacteria, including that of *E. coli* (Table 3).

**Sequence of the fhuC region**

The nucleotide sequence encoding *fhuC* contains a single ORF that extends from nucleotide 892 to nucleotide 1656 (Fig. 1 and Table 3). The proposed translation start site is GGT (valine), which is known to act as a start codon in rare instances. The termination codon is TGA. BLASTX analysis of this ORF showed that it had identity with the ATP-binding protein FhuC of several bacteria (Table 3). FhuC is a cytoplasmic protein that belongs to the family of transporters that require binding proteins.
located within the periplasmic space (Linton & Higgins, 1998). It is a hydrophilic but membrane-associated protein that contains two domains which are typical for nucleotide-binding proteins (Burkhardt & Braun, 1987; Coulton et al., 1987; Köster & Braun, 1986, 1989). Protein prediction analysis (Nakai & Kanehisa, 1991) of the FhuC homologue in \textit{A. pleuro pneumoniae} characterized this protein as having an ATP/GTP-binding-site motif A (GHNGSGKS, amino acids 34–41) and an ABC-transporter-family signature (LSGERSRIWLAMLL, amino acids 138–152).

**Sequence of the \textit{fhuD} region**

In \textit{A. pleuro pneumoniae}, the last codon (TGA) of \textit{fhuC} overlaps with the initiation codon (ATG) of the following gene \textit{fhuD}, which extends from nucleotide 1656 to nucleotide 2609 (Fig. 1 and Table 3) – this genetic organization matches the overlap of the \textit{fhuC} and \textit{fhuD} genes in \textit{E. coli} (Coulton et al., 1987). This stretch of nucleotide sequence displays an uninterrupted ORF that terminates with TAA and which encodes a protein whose deduced amino-acid sequence reveals a protein with identity to the FhuD proteins of several bacteria (Table 3). Software analysis for the prediction of protein localization (Nakai & Kanehisa, 1991) identified the FhuD homologue in \textit{A. pleuro pneumoniae} as being a cytoplasmic-membrane protein with 19 membrane-spanning regions, as compared to the 16 membrane-spanning regions that were predicted for \textit{E. coli}. The FhuD homologue was also characterized as having an ABC-transporter-family signature sequence (IASGDPRANQGLWTT, amino acids 489–503).

**Sequence of the \textit{fhuA} region**

The last gene in the \textit{A. pleuro pneumoniae} \textit{fhu} operon is an ORF that commences 46 bp downstream of \textit{fhuB}, stretching from nucleotide 4602 to nucleotide 6689 (Fig. 1) and terminating with a TAA stop codon. The deduced amino-acid sequence of \textit{fhuA} encodes a protein that displays identity with the FhuA protein of several bacteria (Table 3). Software analysis for the prediction of protein localization (Nakai & Kanehisa, 1991) identified the putative \textit{A. pleuro pneumoniae} FhuA homologue as an OMP with 11 extracellular loops and 10 periplasmic turns, as well as a cleavable N-terminal signal sequence characteristic of proteins destined for export to the outer membrane. A potential cleavage site for the signal sequence was detected between amino-acid residues 23 and 24. The predicted molecular mass of the mature protein is 74750 Da. A multiple-sequence alignment generated using \textit{clustal w} allowed a comparison of the amino termini of three known OMPs of \textit{A. pleuro pneumoniae}, TbpA (Gonzalez et al., 1995), FhuA (this study) and haemoglobin-binding protein, HgbA (A. Khamesm, personal communication). A stretch of 6 aa residues (shown in bold) that may act as a TonB box is located within the first 13 aa of these three target sequences (TbpA, EQAVQLNDDYVT; FhuA, QETAFLDEVSVVS; HgbA, VEQMQLDVTVIVKD). In other Gram-negative bacteria, the TonB box serves as a site of physical interaction between some outer-mem-

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**Table 3. Description of the genes in the \textit{fhu} operon of \textit{A. pleuro pneumoniae} serotype 1 reference strain 4074, with the proteins they encode and their identities with homologous proteins from other bacteria**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Length (bp)</th>
<th>No. amino acids coded</th>
<th>Deduced molecular mass (Da)</th>
<th>Identity with (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{fhuC}</td>
<td>765</td>
<td>255</td>
<td>28504</td>
<td>Escherichia coli 53, Salmonella typhimurium 52, Rhizobium leguminosarum 47, Vibrio cholerae 41, Pantoea agglomerans –, Pseudomonas aeruginosa –</td>
</tr>
<tr>
<td>\textit{fhuD}</td>
<td>954</td>
<td>318</td>
<td>35636</td>
<td>Escherichia coli 27, Salmonella typhimurium 26, Rhizobium leguminosarum 29, Vibrio cholerae 25, Pantoea agglomerans –, Pseudomonas aeruginosa –</td>
</tr>
<tr>
<td>\textit{fhuB}</td>
<td>1953</td>
<td>651</td>
<td>69370</td>
<td>Escherichia coli 27, Salmonella typhimurium 27, Rhizobium leguminosarum 29, Vibrio cholerae 25, Pantoea agglomerans –, Pseudomonas aeruginosa –</td>
</tr>
<tr>
<td>\textit{fhuA}</td>
<td>2088</td>
<td>696</td>
<td>77120</td>
<td>Escherichia coli 26, Salmonella typhimurium 26, Rhizobium leguminosarum –, Vibrio cholerae 31, Pantoea agglomerans 26, Pseudomonas aeruginosa 27</td>
</tr>
</tbody>
</table>
brane receptors and TonB, a protein that delivers the proton motive force of the cytoplasmic membrane to the outer membrane (Moeck & Coulton, 1998; Postle, 1993).

**Characterization of the fhu chromosomal locus in A. pleuropneumoniae serotype 1**

In contrast to the fhu operon in E. coli in which fhuA is at the 5' end, fhuA in A. pleuropneumoniae is preceded by the genes fhuC, fhuD and fhuB. Upstream of fhuC in A. pleuropneumoniae is an incomplete ORF in the opposite direction, separated from the 5' start of fhuC by a 244 bp intergenic region and encoding a protein homologous to E. coli YaaH. This region contains promoter sites for the fhu genes, as well as a potential Fur-binding site for their regulation. A putative TAATTA box at –10 (nucleotides 854–859, Fig. 1) and a Shine–Dalgarno sequence (GGAG; nucleotides 883–886, Fig. 1) were also found in this stretch, along with a consensus sequence (TTTAA) for the –35 region (nucleotides 835–839, Fig. 1). Both the –35 and Shine–Dalgarno sequences match the consensus sequence proposed for promoter regions in A. pleuropneumoniae (Doree & Mulks, 2001). The spacing between the –10 and –35 regions was 14 bp, a value which falls within the range of 13 and 16 bp proposed for this region in A. pleuropneumoniae (Doree & Mulks, 2001). In E. coli, the fur gene product is a negative regulator of iron-dependent genes and the consensus sequence for the Fur box is GATAATGATAATC-ATTATC. In A. pleuropneumoniae the region upstream of fhuC (nucleotides 833–851) and the stretch of 46 non-coding base-pairs that falls between fhuB and fhuA (nucleotides 4598–4616) both demonstrated putative Fur boxes – nine out of 19 of the nucleotides were conserved upstream of fhuC, whereas 10 out of 19 of the nucleotides were conserved upstream of fhuA.

Analysis of the DNA sequence immediately downstream of the A. pleuropneumoniae fhuA gene shows that this region displays similarity with a hypothetical protein from Neisseria meningitidis Z2491 (NMA0986; Parkhill et al., 2000), which is transcribed in the same direction as the fhu genes. Four-hundred base-pairs downstream of fhuA a homologue to a Haemophilus influenzae protein is encoded (phospho-ribosyl-aminoimidazole-succinocarboxamide synthase), which is divergently transcribed.

**Distribution of the fhu genes among A. pleuropneumoniae serotype reference strains**

To determine if the fhu operon was unique to the A. pleuropneumoniae serotype 1 reference strain or if it was widely distributed among the different serotypes of this organism, samples of DNA from reference strains representing serotypes 2–12 of A. pleuropneumoniae were investigated by PCR. PCR amplification was carried out with primers 5T3W1 and 5T7W2 (Table 2), which flank the fhu region in A. pleuropneumoniae serotype 1, and the Expand Long Template PCR System 1. Reference strains from serotypes 2, 6, 8, 9, 10, 11 and 12 all showed (Fig. 2) the expected 6.0 kb PCR product, identical to the one observed for serotype 1. Using primers 5T3W1 and 5T7W2 for PCR, some serotype strains yielded a negative PCR result for the entire fhu operon. To investigate these serotype strains further, amplification of the fhu operon was attempted with primers internal to this operon (CFor and ARev; Table 2). Using primers CFor and ARev, the expected PCR product of 5.7 kb in size was obtained for the serotype 3 strain, confirming that the fhu operon is present in this serotype and with an arrangement of fhu genes similar to that seen in the serotype 1 strain. As the reference strains from serotypes 4, 5 and 7 produced a negative PCR result when CFor and ARev were used as primers, these serotypes were investigated with respect to the individual genes within the fhu operon both by PCR and Southern blotting. Pairs of primers internal to each gene of the fhu operon were used for PCR (AFor/ARev for fhuA, BFor/BRev for fhuB, CFor/CRev for fhuC and DFor/DRev for fhuD; Table 2). To elucidate the arrangement of the fhu genes in serotypes 4, 5 and 7, we performed PCR amplifications using various combinations of primers. For the Southern-blot analyses, EcoRI-digested genomic DNA from these serotypes was tested for hybridization to a DIG-labelled PCR product for each gene. Reference strains from serotypes 4, 5 and 7 tested positive for the presence of the fhuA, fhuB, fhuC and fhuD genes both in PCR and Southern-blot analyses, although the conditions of stringency had to be lowered to obtain a PCR amplicon for the fhuA and fhuD genes and a positive hybridization signal for fhuD – this may be due to some dissimilarity in the fhuD and fhuA gene sequences between serotype 1 and serotypes 4, 5 and 7.

Our results show that all of the genes of the fhu operon are present in all of the reference strains that represent serotypes 1–12 of A. pleuropneumoniae, and that they all seem to be organized in a similar arrangement. However, the regions flanking the fhu operon are...
dissimilar in serotypes 3, 4, 5 and 7 compared to the regions flanking the fhu operon in serotypes 1, 2, 6, 8, 9, 10, 11 and 12, as shown by the negative PCR result obtained for these four serotypes when using primers (ST3W1 and ST7W2) that flanked this region.

**Protein expression and Western blotting**

The fhuA gene of the *A. pleuropneumoniae* serotype 1 strain was cloned into the expression vector pET30a+ to yield pLM202 (Table 1). pLM202 was then transformed into *E. coli* BL21 cells. The *E. coli* BL21(pLM202) cells were grown under various conditions to optimize the expression of recombinant FhuA with the amino-terminal histidine tag, i.e. we tested different concentrations of IPTG and varied the parameters of time and temperature. Whole-cell samples of *E. coli* BL21(pLM202) were run on 8 or 12.5 % SDS-polyacrylamide gels and the gels were subsequently stained with Coomassie blue. A protein of approximately 79 kDa in size showed the highest level of expression at 25 °C (Fig. 3a). Induction with IPTG concentrations higher than 1-0 mM and induction periods longer than 6 h did not further enhance the expression of recombinant FhuA. The proteins from the gels were then transferred to a nitrocellulose membrane and blotted with an anti-hexahistidine mAb which reacted specifically with the 79 kDa protein, namely *A. pleuropneumoniae* FhuA with an N-terminal histidine tag (Fig. 3b). We then tested mAb Fhu6.1 (Moeck et al., 1995) (Fig. 3c), which recognizes a linear epitope between amino acids 241 and 281 of *E. coli* FhuA, against the 79 kDa protein. For both Western blots, a purified FhuA–hexahistidine of *E. coli* (Moeck et al., 1996) served as a positive control and uninduced *E. coli* BL21(pLM202) cells served as a negative control. We also tested a polyclonal antiserum taken from a pig infected experimentally with *A. pleuropneumoniae* serotype 1 and were able to show that the 79 kDa protein also reacted with this immune serum (Fig. 3d).

**Homology model for FhuA of *A. pleuropneumoniae* and comparison with the structure of FhuA of *E. coli***

The JIGSAW and CODA servers were used to generate a composite homology model for FhuA of *A. pleuropneumoniae*. Following alignment of the amino-acid sequences of the FhuA proteins of *A. pleuropneumoniae* and *E. coli* (Fig. 4), the model (Fig. 5a) for the FhuA of *A. pleuropneumoniae* generated by the JIGSAW server showed 11 extracellular loops (L1–L11) and 10 periplasmic turns within this protein. These numbers of extracellular loops and periplasmic turns are consistent with the *E. coli* FhuA structure, as is the presence of 22 β-strands. FhuA of *A. pleuropneumoniae* has the same overall fold as FhuA of *E. coli*. Both proteins possess two domains (Fig. 5b). In the *A. pleuropneumoniae* FhuA sequence, these two domains are represented by an N-terminal cork domain (residues 1–134) followed by a C-terminal β-barrel domain (residues 135–673). There were two gaps in the model: the first occurred between residues 74 and 79 in the cork domain and the second occurred between residues 375 and 391 in the β-barrel domain. The second gap overlapped the hexahistidine tag and flanking linker regions (a total of 11 aa) that were inserted into the sequence to facilitate affinity purification of the *E. coli* recombinant FhuA (Ferguson et al., 1998b). It is reasonable to assume that the *A. pleuropneumoniae* model structure should not be threaded against this region, since it is not present in the wild-type *A. pleuropneumoniae* fhuA gene. We hypothesize that residues 375–391 in the *A. pleuropneumoniae* FhuA structure would be predominantly located within extracellular loop L5, with a short C-
Actinobacillus pleuropneumoniae ferrichrome transport

Fig. 4. Structure-based alignment of the A. pleuropneumoniae FhuA sequence relative to that of its template for homology modelling, E. coli FhuA. The numbering of both proteins is based on their mature, processed forms. The N termini (prior to the first gap) of the A. pleuropneumoniae and E. coli sequences are aligned relative to the putative TonB box (LDEVSV, enclosed text) of the A. pleuropneumoniae sequence, which is homologous to the E. coli TonB box. The structural alignment used by the JIGSAW server to produce the homology model begins at position 13 of the A. pleuropneumoniae FhuA protein. Residues corresponding to the recombinant hexahistidine tag and linker regions in the E. coli FhuA primary sequence are indicated in bold; these residues are not included in the 714 aa of the mature, processed FhuA protein from E. coli. Underlined residues in the A. pleuropneumoniae sequence are not present in the homology model for FhuA of this organism. Secondary-structure elements denoted by letters prefixed by an α correspond to those found within the cork domain of FhuA from E. coli; numbered secondary-structure elements prefixed by a β correspond to those found in the β-barrel domain of FhuA from E. coli; the boundaries for each of the 22 β-strands of the β-barrel domain are shown as solid lines below the alignments.

DISCUSSION

Bacterial virulence is determined by the ability of an organism to compete for essential nutrients (Martinez et al., 1990). Because the mammalian host restricts bacterial growth by withholding iron, most bacteria have evolved a diverse series of high-affinity iron-acquisition systems to satisfy their iron requirements. One such system is involved in the synthesis and/or uptake of low-molecular-mass iron chelators, termed siderophores (Braun et al., 1998; Neilands, 1995). Transporters bind iron chelates with high affinity and mediate their uptake across the outer membrane of Gram-negative bacteria. The proteins required for the uptake of ferric hydroxamates are the products of the genes fhuA, fhuC, fhuD and fhuB where FhuA acts as the receptor for ferrichrome in the outer membrane, and its crystal structure has been determined (Ferguson et al., 1998a; Locher et al., 1998). The energy required to translocate ferric hydroxamates across the outer membrane is derived from the proton motive force of the cytoplasmic membrane, as transduced by the TonB–ExbB–ExbD complex (Braun, 1995; Moeck & Coulton, 1998).

A. pleuropneumoniae, a Gram-negative bacterium that is an important swine pathogen, is capable of using transferrin, haemoglobin, haemin and exogenous siderophores, including ferric hydroxamates, as sources of terminal stretch being contained within the adjacent β-strand (Fig. 5a).
Fig. 5. (a) Proposed secondary structure for the β-barrel domain of the FhuA protein of *A. pleuropneumoniae*. Squares containing one-letter amino-acid codes represent residues contained within β-strands; circles containing one-letter amino-acid codes represent residues contained within loop regions. The boundaries between strand and loop regions correspond to those observed by visualization of the three-dimensional homology-based model for FhuA. Residues 375–391 are not present within the homology model, instead their secondary-structure status is predicted from the positions of homologous residues within the *E. coli* FhuA structure. The approximate position of the outer membrane is
iron for growth. The transferrin receptor complex includes TbpA and TbpB in the outer membrane of *A. pleuropneumoniae* (Deneer & Potter, 1989) as well as the *exbB* and *exbD* genes upstream of *tbpA* and *tbpB* in the same operon (Tonpitak et al., 2000). The objectives of the present study were to elucidate the genes and products involved in the uptake of ferric hydroxamates in *A. pleuropneumoniae*. We successfully cloned the homologues of the *E. coli* *fhuACDB* operon from *A. pleuropneumoniae*. In *A. pleuropneumoniae* the proteins encoded by this operon are: FhuA, a 77 kDa OMP that acts as the receptor for ferric hydroxamate; FhuD, the periplasmic protein responsible for the translocation of ferric hydroxamate from the outer to the inner membrane; and FhuC and FhuB, cytoplasmic-membrane-associated proteins that are components of an ABC transporter which internalizes ferric hydroxamate. An ABC-transporter-family signature sequence was identified for the FhuC and FhuB proteins of *A. pleuropneumoniae*. In other Gram-negative bacteria FhuB is a hydrophobic protein that is embedded in the cytoplasmic membrane. It is twice the size of hydrophobic proteins usually found in periplasmic binding transport systems (Linton & Higgins, 1998) and displays an internal amino-acid-sequence homology between its N-terminal and C-terminal halves. This internal homology led Köster & Braun (1989) to suggest that *fhuB* originated from the duplication of an ancestral gene, with the two DNA fragments fusing to form a single gene. The *A. pleuropneumoniae* FhuB homologue is the same size as its *E. coli* counterpart, and comparison of the two halves of the *fhuB* sequence of *A. pleuropneumoniae* revealed 64% sequence identity, hence suggesting similar ancestry. Southern blot and PCR analyses showed that all of the genes of the *fhu* operon are present in all of the reference strains tested here, which represented serotypes 1–12 of *A. pleuropneumoniae*. However, testing for the individual *fhuC, fhuD, fhuB* and *fhuA* genes revealed that although they are present individually in the reference strains of serotypes 4, 5 and 7, the sequences of *fhuA* and *fhuD* are somewhat different in these serotypes compared to the sequences of these genes in the other nine serotypes studied. Nevertheless, it appears that in all of the serotypes of *A. pleuropneumoniae* studied, the *fhuC, fhuD, fhuB* and *fhuA* genes are arranged in the same order as seen in the serotype 1 reference strain 4074.

The genes involved in the uptake of ferrichrome in *A. pleuropneumoniae* are arranged differently than their corresponding genes in *E. coli*. In the latter, the *fhu* receptor gene *fhuA* is located upstream of *fhuCDB*, whereas in *A. pleuropneumoniae* it is the last gene transcribed in the *fhu* operon. This difference in gene organization between the two species may reflect differences in the regulation of their iron-transport systems. To explore this possibility, further analyses are needed on the sequences upstream of *fhuC* and *fhuA* in *A. pleuropneumoniae*. The G+C content of the *fhu* operon in *A. pleuropneumoniae* is 44 mol%, a value that correlates well with the estimated 49.2 mol% reported (Pohl et al., 1983) for the genome of *A. pleuropneumoniae*. Interestingly, Galindo et al. (2001) have reported that the *fhuA* gene in the *fhuABD* operon of *Campylobacter jejuni* is strikingly GC-rich (65 mol%) compared to the *C. jejuni* genome (35 mol%).

The predicted primary sequence of FhuA from *A. pleuropneumoniae* has allowed us to propose a homology-based three-dimensional model for this protein (Fig. 5b). The overall fold of the *A. pleuropneumoniae* FhuA model resembles that of *E. coli* FhuA, for which the crystal structure is known (Ferguson et al., 1998a; Locher et al., 1998), with the most significant deviations from the known structure occurring in the extracellular- and periplasmic-loop regions. Overall, the sizes of the extracellular loops of the FhuA model are similar to the corresponding loops in the *E. coli* structure. However, significant differences between the lengths of two key extracellular-loop regions were observed. Loop L3 in the *E. coli* structure is 31 residues long compared to 35 residues for the same loop in the *A. pleuropneumoniae* FhuA model, a difference due to an extension of the loop at its N-terminal end. Furthermore, L4 in the *A. pleuropneumoniae* FhuA model is considerably longer than the *E. coli* L4 region (28 residues compared to 20 residues) and does not contain the short β-strands that are seen in the *E. coli* FhuA structure. Given that loops L3 and L4 are involved in ligand recognition and uptake (Ferguson et al., 1998a), it remains to be determined whether these structural variations correspond to differences in function of FhuA from *A. pleuropneumoniae* relative to the *E. coli* protein. These differences could also prove to be responsible for the lack of susceptibility of *A. pleuropneumoniae* to the antibiotics albomycin and rifamycin CGP 4832 and to the bacterial toxin colicin M, all of which use FhuA as a docking site for entry into *E. coli* bacterial cells.

The X-ray crystallographic structure of *E. coli* FhuA complexed with ferricrocin indicates that 10 residues of this protein are within 4 Å of the bound ligand atoms (Ferguson et al., 2001b). Inspection of the structure-based alignment of *A. pleuropneumoniae* FhuA with the primary sequence of the *E. coli* protein (Fig. 4) shows that six out of 10 positions are highly homologous. Absolute conservation is observed for residues Y292 and Y294 of the *A. pleuropneumoniae* protein, which align with *E. coli* ligand-binding residues Y313 and Y315. Residues F92 and F224 share the aromatic character of the aligned *E. coli* residues Y116 and W246. A position
of hydrophobicity is maintained at position L364, which aligns with F391 in the *E. coli* sequence. Finally, S222, which has the potential to hydrogen bond to the siderophore ligand, is aligned with *E. coli* residue Y244. Interestingly, two of these six residues retain an aromatic character relative to their homologous *E. coli* ligand-binding residues, yet they do not have the ability to form hydrogen bonds with a siderophore ligand. This suggests either that the mode of ligand binding for the *A. pleuropneumoniae* protein may have hydrophobic-stacking interactions as a more predominant component or that the hydrophobic component of these residues in the *E. coli* protein may be more critical to ligand binding than to their ability to form hydrogen bonds. Functional analysis of the ligand-binding ability of a mutant *E. coli* FhuA protein in which the two aromatic residues have been modified to resemble those in the *A. pleuropneumoniae* sequence (Y116F, W246F) may resolve this issue.

The structure of *E. coli* FhuA also possesses a stretch of highly conserved residues along the longitudinal axis of the inner wall of the β-barrel that is thought to form a ‘staircase’, potentially to facilitate siderophore transfer from the ligand-binding site to the periplasm (Ferguson *et al.*, 1998a, 2001a). Of these eight staircase residues, four homologous positions are conserved in the *A. pleuropneumoniae* primary sequence, as indicated by the structure-based alignment in Fig. 4. With a two-residue shift towards the C terminus, R274 and N276 of the *A. pleuropneumoniae* protein align with *E. coli* residues R297 and N299, respectively. Absolute conservation is seen with two charged residues, D331 and D332, which align with D358 and D379, respectively. In *E. coli* sequence. Position Q397 in the *A. pleuropneumoniae* protein aligns perfectly with *E. coli* staircase residue Q431. Position R333 aligns with *E. coli* staircase residue Q360. Although at this position there is a charge difference between the two proteins, both side-chains are of approximately the same length, suggesting that the role of this staircase residue may be based more on the steric character of the side-chain than on its charge. It is interesting to note that with the exception of the Q397/Q431 pair, the highest degree of homology in the staircase is clustered at its N-terminal end, proximal to the periplasmic end of the β-barrel domain.

This is, to the best of our knowledge, the first description of a siderophore receptor in *A. pleuropneumoniae*. In view of the fact that we now have genetic evidence as well as a three-dimensional model for the OMP FhuA of *A. pleuropneumoniae*, we can now distinguish different molecular mechanisms of siderophore receptors from different bacterial species and compare structural information between these species.

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