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

Leonie G. Mikael,¹ Peter D. Pawelek,² Josée Labrie,¹ Marc Sirois,³ James W. Coulton² and Mario Jacques¹

Author for correspondence: Mario Jacques. Tel: +1 450 773 8521 ext. 8348. Fax: +1 450 778 8108. e-mail: jacquim@medvet.umontreal.ca

¹ Groupe de Recherche sur les Maladies Infectieuses du Porc, Département de Pathologie et Microbiologie, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec, Canada J2S 7C6

² Department of Microbiology and Immunology, McGill University, Montréal, Québec, Canada H3A 2B4

³ Département de Chimie-Biologie, Université du Québec à Trois-Rivières, Trois-Rivières, Québec, Canada G9A 5H7

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).

Abbreviations: DIG, digoxigenin; *fhu*, ferric hydroxamate uptake; OMP, outer-membrane protein; *phoA*, alkaline phosphatase; TbpA, transferrin-binding protein A; TbpB, transferrin-binding protein B.

The GenBank accession number for the sequence of the *fhuCDBA* operon of *Actinobacillus pleuropneumoniae* serotype 1 reference strain 4074 described in this study is AF351135.

Infection by *A. pleuropneumoniae* is a multifactorial 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 & MacInnes, 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; Coulton *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, ϕ 80 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^{3+} -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^{3+} -hydroxamate compounds (Fe^{3+} -aerobactin, Fe^{3+} -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 $15\ \mu\text{g}\ \text{NAD}^+\ \text{ml}^{-1}$. *E. coli* BL21(DE3), *E. coli* CC118 and *E. coli* DH5 α were grown on Luria–Bertani (LB) Miller medium, whereas *E. coli* XL-1 Blue MRF' was grown in NZYCM broth (Gibco-BRL) supplemented with $12.5\ \mu\text{g}\ \text{tetracycline}\ \text{ml}^{-1}$. The phage excision strain of *E. coli*, XL0LR, was grown in LB broth supplemented with $12.5\ \mu\text{g}\ \text{tetracycline}\ \text{ml}^{-1}$. Growth of the phagemid pBK-CMV was carried out for selection on LB agar supplemented with $50\ \mu\text{g}\ \text{kanamycin}\ \text{ml}^{-1}$. Strains harbouring derivatives of the pET30a+ expression vector were grown on LB agar supplemented with $30\ \mu\text{g}\ \text{kanamycin}\ \text{ml}^{-1}$; those harbouring pGEM derivatives required $50\ \mu\text{g}\ \text{ampicillin}\ \text{ml}^{-1}$ to be added to the medium. Blue/white colony selection was achieved by the addition of $40\ \mu\text{g}\ \text{X-Gal}\ \text{ml}^{-1}$ and $1\ \text{mM}\ \text{IPTG}$ to the medium. The addition of $50\ \mu\text{g}\ \text{deferrated ethylenediamine dihydroxyphenyl acetic acid}\ \text{ml}^{-1}$ (Sigma) to the growth medium depleted the iron available to the bacteria. Ferrichrome (Sigma), used as an exogenous source of iron,

Table 1. Bacterial strains and plasmids

Strain/plasmid	Description*	Reference
<i>E. coli</i>		
CC118	<i>phoA recA araD139 Δ(ara-leu)7697 galE galK thi rpsE rpoB argE</i> (Amp)	Mintz & Fives-Taylor (1999)
XL-1 BlueMRF'	<i>Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac F' [proAB lacI^qZAM15 Tn10 (Tet^r)]</i>	Stratagene
XL0LR	<i>Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 thi-1 recA1 gyrA96 relA1 lac F' [proAB lacI^qZAM15 Tn10 (Tet^r)]; Su⁻ λ^r</i>	Stratagene
XL-1 Blue	<i>supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac⁻ F' [proAB⁺ lacI^qlacZAM15 Tn10 (Tet^r)]</i>	Stratagene
DH5α	<i>supE44 ΔlacU169(φ80 lacZAM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Stratagene
BL21(DE3)	<i>F⁻ ompT hsdS_B (r_B m_B) gal dcm</i> (DE3)	Novagen
Plasmid		
pBK-CMV	Km ^r ; cloning vector of pBR322 origin, T3 and T7 promoter	Stratagene
pLM101	Km ^r ; App 7974 bp <i>fhuCDBA</i> operon in pBK-CMV vector	This study
pLM201	Amp ^r ; PCR product of AFor and ARev (mature <i>fhuA</i>) in pGEM	This study
pET30a+	Km ^r ; expression vector with N-terminal histidine tag	Novagen
pLM202	Km ^r ; <i>NotI</i> fragment of pLM201 in pET30a	This study
pHX405	Amp ^r , Tet ^r ; pBR322-based plasmid encoding FhuA-hexahistidine of <i>E. coli</i>	Moeck <i>et al.</i> (1997)

* Su⁻, non-repressing; λ^r, λ-resistant.

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* (Sirois *et al.*, 2001), chromosomal DNA from *A. pleuropneumoniae* serotype 1 strain 4074 was digested with *Sau3AI* 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* *Sau3AI* 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 (XP) ml⁻¹. The translocation of PhoA across the bacterial inner membrane results in the hydrolysis of XP 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 Miniprep 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 5·0 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. Various PCR products were then digoxigenin (DIG)-labelled using the DIG DNA Labelling and Detection Kit (Roche Diagnostics) and used as DNA hybridization probes in Southern blots and plaque-lift assays. Chromosomal DNA was extracted by the method of Pitcher *et al.* (1989). For Southern-blotting experiments, the genomic DNA of the *A. pleuropneumoniae* serotypes was digested with different enzymes, run on a 0·7% agarose gel and then transferred to positively-charged nylon membranes (Ausubel *et al.*, 1998). Conditions of high stringency were applied. Hybridization of the DIG-labelled DNA probes was detected by using phosphatase-labelled anti-DIG antibodies and revealed colorimetrically with the use of NBT-BCIP as substrate.

Construction and screening of an *A. pleuropneumoniae* phage bank. A library of *A. pleuropneumoniae* serotype 1 strain 4074 DNA was constructed (Vézina *et al.*, 1997) in the λZap Express phage vector (Stratagene), following the manufacturer's instructions. Briefly, *A. pleuropneumoniae* genomic DNA was partially digested with *Sau3AI* and the resulting DNA fragments were separated by agarose-gel electrophoresis. The DNA fragments corresponding to approximately 7 to 12 kb in length were excised from the gel and ligated with *Bam*HI-digested λZap 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* XL0LR as the excision plating strain. The library was screened with the probes designed for Southern blotting,

Table 2. Sequences of primers used in this study

Primer	Sequence
foA	5'-CACCCGTTAAACGGCGAGCAC-3'
F7	5'-GCAACCGTCCAATCCA-3'
R7	5'-CATCCTGAAACCAAACGA-3'
FA1	5'-CTCGAGCTCGAGGCAGCAGCAGCCGTCAGGC-3'
5T3W1	5'-CATTGAAAACCTCGAGTCG-3'
5T7W1	5'-GATACCTATCAAGAAGGC-3'
5T3W2	5'-TGGCGCAACAAAGCAAAT-3'
5T7W2	5'-GTTGCCTACGTTATCCAC-3'
5T7W2.5	5'-GTTTGTACGGGCACGATC-3'
5T3W3	5'-ACCGTTGTTCAGCCTTAT-3'
5T7W3	5'-GAGCGTCCACTTTGTATG-3'
5T3W4	5'-AGTTGTTGAGTTTGGCGAT-3'
5T7W4	5'-TCATTGAGTCTGCGCCTT-3'
5T3W5	5'-TGGTAGCAATAGCGGTCG-3'
AFor	5'-GATGAGGTGTCGGTGGTT-3'
ARev	5'-TGTGGCATTGACTTTACG-3'
BFor	5'-CTTATTCAGCGGATTAGCC-3'
BRev	5'-CAACTAATGTGGCGACTAAG-3'
CFor	5'-GCAATTCGAGCAGGGTAAG-3'
CRev	5'-CCGGTCGTTTGGTTTCAGG-3'
DFor	5'-GTGAGCTTCCGTATTTCT-3'
DRev	5'-CGCCTCCGTGCAATAATC-3'
T3	5'-AATTAACCCTCACTAAAGGG-3'
T7	5'-CGGGATATCACTCAGCATAATG-3'

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 *Xba*I and *Sac*I. 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 dideoxy-nucleotide 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, 5T7W4 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 applying 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 *Not*I, and the resulting *Not*I fragment was cloned directionally and in-frame into the *Not*I site of the pET30a+ 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* DH5 α . It was then extracted from DH5 α , 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, 1.0, 1.5 and 3.0 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 anti-swine IgG-horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories). Reactions were revealed by the addition of 4-chloro-1-naphthol and H₂O₂ (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 C α 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 <1.00 Å. The loops and anchor regions modelled by the CODA server are as follows, 158–166, 183–191, 228–236, 291–299, 301–308, 405–412, 538–546, 564–572, 597–604, 610–617 and 630–637. This numbering is based on the amino acid residues of the FhuA sequence of *A. pleuropneumoniae*.

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 region of a heterologous signal sequence plus sequences from a normally exported protein and *'phoA'* may result in the expression of PhoA activity. PhoA-positive colonies are indicative of fusion proteins derived from plasmids containing a foreign DNA insert harbouring a promoter, a translational start site and a functional signal sequence (Mintz & Fives-Taylor, 1999).

When our *A. pleuropneumoniae* signal-sequence library, representing approximately 8250 individual colonies in the *phoA*-negative *E. coli* strain CC118, was screened for the blue colony phenotype on medium containing 5-bromo-4-chloro-3-indolyl phosphate, 95 colonies were found to be PhoA-positive. One plasmid (pI-25) from the PhoA-positive colonies contained a 375 bp *A. pleuropneumoniae* insert that was relevant to our objectives. BLASTX analysis of this fragment showed that it displayed 36 % identity with and 57 % similarity to the

Fig. 1. Map of the *A. pleuropneumoniae* serotype 1 reference strain 4074 *fhu* gene cluster, which is homologous to the *fhuACDB* cluster of *E. coli*. Numbers indicate the base-pairs of the insert within pLM101. ORFs designating the genes *fhuC*, *fhuD*, *fhuB* and *fhuA* are marked by thick solid lines. The direction of transcription is indicated by an arrow.

Rhizobium leguminosarum FhuD 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 pI-25. 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 λ ZAP 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 8.0 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* operons 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 GTG (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

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

Gene	Characteristic of gene sequence								
	Length (bp)	No. amino acids coded	Deduced molecular mass (Da)	Identity with (%)					
				<i>Escherichia coli</i>	<i>Salmonella typhimurium</i>	<i>Rhizobium leguminosarum</i>	<i>Vibrio cholerae</i>	<i>Pantoea agglomerans</i>	<i>Pseudomonas aeruginosa</i>
<i>fhuC</i>	765	255	28 504	53	52	47	41	–	–
<i>fhuD</i>	954	318	35 656	27	26	30	24	–	–
<i>fhuB</i>	1953	651	69 370	27	27	29	25	–	–
<i>fhuA</i>	2088	696	77 120	26	26	–	31	26	27

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 *A. pleuropneumoniae* characterized this protein as having an ATP/GTP-binding-site motif A (GHNGSGKS, amino acids 34–41) and an ABC-transporter-family signature (LSGGERSRIWLAMLL, amino acids 138–152).

Sequence of the *fhuD* region

In *A. pleuropneumoniae*, the last codon (TGA) of *fhuC* overlaps with the initiation codon (ATG) of the following gene *fhuD*, which extends from nucleotide 1656 to nucleotide 2609 (Fig. 1 and Table 3) – this genetic organization matches the overlap of the *fhuC* and *fhuD* genes in *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). FhuD is a periplasmic protein that is responsible for transporting ferrichrome from the FhuA receptor in the outer membrane to the FhuB protein in the cytoplasmic membrane (Köster & Braun, 1989; Moeck *et al.*, 1996). Using software (Nakai & Kanehisa, 1991) for the prediction of signal-sequence-cleavage sites, a possible cleavage site was identified and is proposed to be situated at amino acid 47 for FhuD of *A. pleuropneumoniae*; however, the program failed to characterize this region as an N-terminal signal sequence that is usually displayed by proteins destined for export into the periplasm or outer membrane.

Sequence of the *fhuB* region

The initiation codon (ATG) for *fhuB* is situated 8 bp upstream of the termination codon TAA of the preceding gene *fhuD* in the *fhu* operon of *A. pleuropneumoniae* (Fig. 1). The ORF for FhuB extends from

nucleotide 2603 to nucleotide 4555 (Fig. 1 and Table 3) and terminates with a TAA stop codon. The deduced amino-acid sequence of *fhuB* encodes a protein that displays identity with the FhuB protein of several bacteria (Table 3). Software for the prediction of protein localization (Nakai & Kanehisa, 1991) identified the FhuB homologue in *A. pleuropneumoniae* as being a cytoplasmic-membrane protein with 19 membrane-spanning regions, as compared to the 16 membrane-spanning regions that were predicted for *E. coli*. The FhuB homologue was also characterized as having an ABC-transporter-family signature sequence (IASGDP-RANQLITWT, amino acids 489–503).

Sequence of the *fhuA* region

The last gene in the *A. pleuropneumoniae* *fhu* operon is an ORF that commences 46 bp downstream of *fhuB*, stretching from nucleotide 4602 to nucleotide 6689 (Fig. 1) and terminating with a TAA stop codon. The deduced amino-acid sequence of this ORF encodes a protein which displays identity with the FhuA proteins of several bacteria (Table 3). Software analysis for the prediction of protein localization (Nakai & Kanehisa, 1991) identified the putative *A. pleuropneumoniae* 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 74 750 Da. A multiple-sequence alignment generated using CLUSTAL W allowed a comparison of the amino termini of three known OMPs of *A. pleuropneumoniae*, TbpA (Gonzalez *et al.*, 1995), FhuA (this study) and haemoglobin-binding protein, HgbA (A. Khamessan, 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, EQAVQLNDVYVTG; FhuA, QETAVLDESVVS; HgbA, QEQLDQTVIVKD). In other Gram-negative bacteria, the TonB box serves as a site of physical interaction between some outer-mem-

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-aminoamidazole-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*

Fig. 2. PCR analysis of the *fhu* operon in *A. pleuropneumoniae* serotype reference strains. The amplifications were done by using primers (5T3W1 and 5T7W2) flanking the *fhu* operon and chromosomal DNA from the *A. pleuropneumoniae* reference strains as templates. Lane M, DNA ladder (kb); the numbering of the other lanes relates to the serotypes outlined in Methods. The arrow points to the 6.0 kb mark.

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

Fig. 3. SDS-PAGE (a) and Western blot (b–d) analyses of whole-cell lysates of *E. coli* BL21 cells expressing recombinant FhuA from *A. pleuropneumoniae*. Lanes: 1, *E. coli* BL21(pLM202) with no IPTG added; 2, *E. coli* BL21(pLM202) plus 1 mM IPTG, with 4 h incubation at 25 °C; 3, *E. coli* BL21(pLM202) plus 1 mM IPTG, with 24 h incubation at 25 °C; 4, *E. coli* BL21(pHX405) expressing the FhuA–hexahistidine protein. The gel shown in: (a) was stained with Coomassie blue; (b) was immunoblotted with an mAb against hexahistidine; (c) was immunoblotted with mAb Fhu6.1 directed against *E. coli* FhuA (Moeck *et al.*, 1995); and (d) was immunoblotted with convalescent serum taken from a pig that had been infected experimentally with *A. pleuropneumoniae* serotype 1.

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 (5T3W1 and 5T7W2) 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-

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 or a β 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.

terminal stretch being contained within the adjacent β -strand (Fig. 5a).

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 hydroxymates are the products of the genes *fhuA*, *fhuC*, *fhuD* and *fhuB* where FhuA acts as the receptor for ferri-chrome 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 hydroxymates 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

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 43.2 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

shown by parallel double lines. Extracellular loops are numbered L1–L11, starting from residue 135 of the first β -strand. (b) Proposed tertiary structure (C α trace, stereo view) for the FhuA protein of *A. pleuropneumoniae*. The cork domain is represented by a solid line; the β -barrel domain is represented by a grey line. The positions of loops L3, L4, L6 and L8 are given for reference. The stereo images were generated by using the program MOLSCRIPT (Kraulis, 1991).

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 D352, which align with D358 and D379, respectively, in the *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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