The *Pasteurella haemolytica* 35 kDa iron-regulated protein is an FbpA homologue

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In a previous investigation, a 35 kDa iron-regulated protein was identified from total cellular proteins of *Pasteurella haemolytica* grown under iron-depleted conditions. This study reports identification of the gene (fbpA) encoding the 35 kDa protein based on complementation of an entA *Escherichia coli* strain transformed with a plasmid derived from a *P. haemolytica* lambda ZAP II library. Cross-reactivity was demonstrated between an anti-35 kDa mAb and a 35 kDa protein expressed in this strain. Furthermore, a translated ORF identified on the recombinant plasmid corresponded with the N-terminal amino acid sequence of the intact and a CNBr-cleaved fragment of the 35 kDa iron-regulated protein. Nucleotide sequence analysis of the gene encoding the 35 kDa protein demonstrated homology with the cluster 1 group of extracellular solute-binding proteins, especially to the iron-binding proteins of this family. Complete sequence analysis of the recombinant plasmid insert identified three other predominant ORFs, two of which appeared to be in an operonic organization with fbpA. These latter components (fbpB and fbpC) showed homology to the transmembrane and ATPase components of ATP-binding cassette (ABC)-type uptake systems, respectively. Based on amino acid/DNA sequencing, citrate competition assay of iron affinity and visible wavelength spectra, it was concluded that the *P. haemolytica* 35 kDa protein functions as an FbpA homologue (referred to as PFbpA) and that the gene encoding this protein is part of an operon comprising a member of the FbpABC family of iron uptake systems. Primary sequence analysis revealed rather surprisingly that PFbpA is more closely related to the intracellular Mn/Fe-binding protein IdiA found in cyanobacteria than to any of the homologous FbpA proteins currently known in commensal or pathogenic members of the *Pasteurellaceae* or *Neisseriaceae*.

**Keywords:** *Pasteurella haemolytica*, iron, FbpA, ABC transporter

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**INTRODUCTION**

Iron is an essential and limiting resource in most environments. In the mammalian host this is largely due to the iron-sequestering effects of the mammalian iron-binding proteins transferrin (in sera) and lactoferrin (on mucosal surfaces). In response to the difficulties associated with acquiring iron, many micro-organisms have developed high-affinity iron acquisition systems to obtain this nutrient (Mietzner & Morse, 1994). The expression of genes encoding proteins involved in the iron acquisition process is often coordinately regulated by the availability of iron. In bacterial pathogens, low levels of iron serve as a signal for expression of a number of bacterial genes involved in adaptation to the host, including genes responsible for the pathogenesis of infection. Thus, proteins expressed under iron-limited conditions may be of essential function for bacterial survival and may ultimately serve as immuno- or chemotherapeutic targets.

*Pasteurella haemolytica* is the causative agent of bovine
pneumonic pasteurellosis (shipping fever), responsible for a significant economic loss to the cattle industry. Initial difficulties encountered in developing vaccines to P. haemolytica prompted investigators to identify and study proteins which were preferentially expressed under iron-limiting conditions. A number of such iron-regulated proteins have been identified, including proteins of molecular mass 100, 77, 71, 42, 35 and 31 kDa (Deneer & Potter, 1989; Murray et al., 1992; Tabatabai & Frank, 1997). The 100 kDa protein has been demonstrated to possess transferrin-binding activity (Ogunnariwo & Schryvers, 1990; Gray-Owen & Schryvers, 1996) and the genes encoding the 100 and 71 kDa proteins in P. haemolytica have since been cloned, sequenced and identified as the TbpA and TbpB transferrin-binding receptors (Ogunnariwo et al., 1997).

TbpA and TbpB form a host-specific outer-membrane receptor complex necessary for the removal of iron from transferrin at the cell surface (Gray-Owen & Schryvers, 1996). The TbpB receptor is a largely hydrophilic molecule, believed to be anchored to the cell surface through a lipitated acyl tail (Gray-Owen & Schryvers, 1996). The TbpA receptor is believed to function as an integral membrane gated porin through which iron is translocated after having been removed from transferrin. Upon removal from transferrin and translocation across the outer membrane, iron becomes associated with a periplasmic ferric-ion-binding protein (FbpA). FbpA has been demonstrated to function in complexing and transporting this iron through the periplasmic space (Chen et al., 1993; Kirby et al., 1997; Khun et al., 1998). FbpA is the periplasmic component of an iron ATP-binding cassette (ABC) transporter system (Tam & Saier, 1993), which also includes an inner-transmembrane protein, FbpB, and a cytoplasmic ATPase, FbpC (believed to be necessary for the transport of FbpA-bound iron across the inner membrane and mobilization of iron from FbpA, respectively). This integrated cascade of proteins results in transferrin-bound iron being mobilized from the cell surface, transported through the periplasmic space and deposited into the cytoplasm without the incorporation of the transferrin molecule into the bacterial cell.

In a previous investigation, the 35 kDa iron-regulated protein was identified by comparison of autoradiographic patterns between iodinated total cellular proteins of P. haemolytica grown in iron-replete and iron-depleted media (Lainson et al., 1991). An anti-35 kDa mAb demonstrated this protein to be localized in the periplasm. It was proposed that the 35 kDa protein, given its size, location and regulation, may function in the uptake or transport of chelated iron. In this investigation, we undertook to identify and characterize the gene encoding the 35 kDa protein using a P. haemolytica phagemid library to complement in trans an iron-utilization-deficient strain of Escherichia coli. Evidence is provided that the P. haemolytica fbpA gene encodes the 35 kDa protein. Biochemical properties of the P. haemolytica FbpA protein are examined and its relationship to other known FbpA proteins is presented.

**METHODS**

**Strains and growth media.** Bacterial strains, plasmids and deoxyxyligonucleotides used in this study are shown in Table 1. P. haemolytica and E. coli strains were both stored in 30% glycerol at −70 °C. Fresh P. haemolytica cultures were grown on BHI (brain-heart-infusion medium; Difco) plates incubated overnight at 37 °C in a 5% CO₂ atmosphere. Fresh E. coli cultures were prepared on LA or LT plates (Luria agar (Gibco-BRL) supplemented with 100 μg ampicillin (Sigma) ml⁻¹ or 12.5 μg tetracycline (Sigma) ml⁻¹, respectively) and incubated overnight at 37 °C. Liquid cultures of P. haemolytica were prepared in BHI broth. Liquid E. coli cultures were prepared in LA or LT broth (Luria broth base (Gibco-BRL) supplemented with 100 μg ampicillin ml⁻¹ or 12.5 μg tetracycline ml⁻¹, respectively). Iron-restricted cultures of P. haemolytica were prepared by subculturing overnight BHI cultures into fresh BHI broth to an OD₆₀₀ of 0.2, at which point the cultures were supplemented to 100 μM with EDDHA (ethylenediamine-di(o-hydroxyphenylacetic acid); Sigma). Whole cells were used for Western blot analysis after an additional 2 h growth at 37 °C. NBDA agar [8% (w/v) nutrient broth (Difco), 5% (w/v) NaCl, 1.5% (w/v) agar (Difco), pH 7.0 supplemented to 0.2 mM with 2,2'-dipyridyl (Sigma) and 100 μg ampicillin ml⁻¹ (Eick-Helmerich et al., 1987) and 2x YT broth [16% (w/v) Bactotryptone, 10% (w/v) Bactoyeast extract, 5% NaCl, pH 7.0] were used in the library complementation experiment.

**N-terminal amino acid sequence analysis of the 35 kDa protein.** The 35 kDa protein was semi-purified by reversed-phase chromatography. It was then further purified by separation on SDS-PAGE gel, blotted onto Immobilon-P membrane (Millipore) and recovered for analysis. For internal sequencing, the 35 kDa protein was affinity purified as previously described (Murray et al., 1992). It was then cleaved with CNBr and a 14 kDa internal fragment was N-terminally sequenced. Sequence analyses were carried out on an Applied Biosystems 470 gas-phase protein sequencer at the Babraham Research Institute, Cambridge, UK.

**Complementation of e573 for growth on NBDA media using a P. haemolytica lambda ZAP II library.** Briefly, the following protocol [a hybrid of protocols described by Zimmermann et al. (1989) and Williamson & Slocum (1994)] was used to complement e573 (entA) for growth on NBDA media using a P. haemolytica h044 lambda ZAP II library (Stratagene). This library had been prepared as previously described for a Haemophilus influenzae library (Adhikari et al., 1995). e136 (SURE strain) and e573 (entA) cultures were grown in 50 ml LT broth supplemented with 0.2% maltose and 10 mM MgSO₄ overnight at 37 °C in a shaking incubator. After overnight incubation the e573 culture was stored until needed at 4 °C. Five microlitres of a 10¹⁰ p.f.u. ml⁻¹ P. haemolytica lambda ZAP II library was diluted in 195 μl 2 x YT broth. Aliquots (200 μl) of the diluted library were mixed with 200 μl of an overnight e136 culture and 10 μl of R408 helper phage (7.5 x 10¹⁰ p.f.u. ml⁻¹) and incubated at 37 °C for 15 min. The mixture was added to 5 ml prewarmed (37 °C) 2 x YT broth in a 15 ml polystyrene tube and incubated for 3 h in a 37 °C shaking incubator. The tube was then heated to 70 °C for 20 min, centrifuged in a horizontal centrifuge at 4000 g for 15 min and the supernatant transferred to a fresh 15 ml polystyrene tube. The resulting infective ss-phage was immediately rescued to phage-lac plasmid by mixing 1 ml phage with 1 ml prewarmed e573 in a stationary 37 °C incubator for 30 min. The 2 ml of culture were then added to 50 ml of 5:1 dilution of BHI/NBDA broth
Table 1. Bacterial strains, plasmids and primers

<table>
<thead>
<tr>
<th>Strain, plasmid or primer</th>
<th>Description</th>
<th>Source</th>
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<tr>
<td>Strains</td>
<td></td>
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<tr>
<td>e136 E. coli SURE strain</td>
<td>E. coli DS strain F' strain</td>
<td>Stratagen</td>
</tr>
<tr>
<td>e157 E. coli BL21(DE3)</td>
<td>PLYSs T7 expression strain</td>
<td>Liss (1987)</td>
</tr>
<tr>
<td>e201 E. coli BL21(DE3)</td>
<td>pLYSs T7 expression strain</td>
<td>Novagen</td>
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<tr>
<td>e573 Enterocin-deficient E. coli strain F', entA, Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>R. Kazmer, Charlotte. USA</td>
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<td>e638 e573 with plasmid pBSPH1 (f6pABC operon)</td>
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<tr>
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<td>e1124 e157 with plasmid pT7-7 PHFA</td>
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<td>e1267 e201 with plasmid pT7-7</td>
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<td></td>
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<tr>
<td>h098 Serotype A2 P. haemolytica strain</td>
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<td>Plasmids</td>
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<tr>
<td>pBluescript SK II(+)</td>
<td>High-copy general cloning vector</td>
<td>Stratagen</td>
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<tr>
<td>pBSPH1</td>
<td>pBluescript SK II(+) vector with recombinant f6psABC insert</td>
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<td>Promega</td>
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<td>504-505 PHFA PCR product cloned into pGEM vector</td>
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<td>pT7-7</td>
<td>T7 expression vector</td>
<td>Tabor (1994)</td>
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<td>This study</td>
</tr>
<tr>
<td>Oligo 505</td>
<td>5' AGGATCCAAAAGTTGTTAGAGCAGCAATAC 3'</td>
<td>This study</td>
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</table>

and incubated overnight at 37 °C in a shaking incubator. The cells were subcultured in broth, rather than directly plating to solid media, to provide an environment selective for growth and subsequent enrichment of the culture of those clones capable of growing under moderately iron-restricted conditions. After overnight incubation, 200 μl of culture was plated in duplicate on LA agar (to determine the efficiency of the phagemid transfection) and NBDA agar (to select for clones capable of complementing the siderophore-deficient host). Pinpoint colonies on NBDA agar could be identified after 48–72 h incubation at 37 °C. Subsequent colonies were replated on NBDA agar. Colonies capable of growing on fresh NBDA agar were screened for siderophore production by plating on siderophore detection media (Schwyn & Neilands, 1987). Clones negative for siderophore production were further investigated.

**PCR and sequence analysis.** PCR reactions were performed using Taq thermostable DNA polymerase (BRL) on a Perkin Elmer model 480 thermocycler. The following profile was used to amplify PCR products: 1 x (94 °C for 2 min), 30 x (94 °C for 1 min, 50 °C for 1 min, 92 °C for 1 min) and 1 x (72 °C for 10 min). All other PCR parameters were performed as described by Innis & Gelfand (1990). Plasmids were prepared for sequencing using a double-stranded nested deletion kit (Pharmacia). Derivatives of plasmids were prepared using Qiagen plasmid miniprep columns and sequencing was performed using Thermosequenase fluorescent dye-primer cycle sequencing with 7-deaza-dGTP (Amersham) on an ABI PRISM 377 DNA sequencer.

**Subcloning, overexpression and purification of P. haemolytica FbpA (PFbpA).** Oligodeoxynucleotide primers were designed to PCR-amplify the f6pA gene, in order to clone and overexpress the gene product in pT7-7 vector (Tabor, 1994). Upstream primer 504 was designed to amplify the (predicted) native Shine–Dalgarno (underlined, Table 1) region of f6pA. It also possesses an EcoRI site for cloning purposes (bold region, Table 1). Primer 505 was designed to amplify the intergenic region between f6pA and f6pB, 144 bases downstream of the stop codon of f6pA, and possesses a BamHI site for subcloning purposes (bold region, Table 1). A 504-505 PCR product amplified from genomic h044 DNA was cloned into pGEM T-vector, constructing pGEM PHFA and strain e1103. The EcoRI/BamHI f6pA cassette was removed from pGEM PHFA and spliced into similarly cut pT7-7 vector, producing pT7-7 PHFA and strain e1124. pT7-7 PHFA was transformed into the lambda lysogen E. coli strain BL21(DE3), which directs expression of T7 RNA polymerase under the control of the IPTG-inducible promoter, lacUV5, constructing strain e1126. PFBP was overexpressed and purified from strain e1126 using IPTG induction and osmotic shock procedures described for the production and isolation of periplasmic maltose-binding fusion proteins from pMal-F2 (Riggs, 1994). Osmotic shock fluid was lyophilized and resuspended in appropriate buffers for further analysis.

**Electrophoresis and Western blotting.** All SDS-PAGE gels were run with the buffer system of Laemmli (1970). Urea denaturing IEF gels were run using Dry-IEF gels reconstituted with 6 M urea/10 mM Tris (pH 7-2) on a Phast-gel electrophoresis system using carbamylyte calibration standards (Pharmacia). TAE (Tris/acetate/EDTA) agarose gels, standard molecular biology techniques and immunological analy-
sis of electroblotted proteins were performed as previously described (Sambrook et al., 1989). Whole cells were applied to SDS-PAGE gels and a previously prepared murine mAb (Lainson et al., 1991) specific for the *P. haemolytica* 35 kDa protein was used to develop the blots using ECL detection reagent (Amersham).

**Buffer exchange, preparation of holo- and apo-PFbpA, wavelength scans and citrate competition assay.** To prepare protein for IEF, protein samples were dialysed overnight with one change of 6 M urea/10 mM Tris (pH 7.2). Holo-PFbpA was prepared by resuspending lyophilized protein in 0.1 M sodium citrate/0.1 M NaHCO₃ buffer (pH 8.6), adding 25-fold molar excess FeCl₃ and incubating at room temperature for 10 min. The solution was then either gel-filtered using Econo-Pac 10 DG columns (Bio-Rad) or dialysed against two overnight changes of 10 mM Tris/200 mM NaCl (pH 8.0).

Apo-PFbpA was prepared by resuspending the protein with 0.1 M sodium acetate/0.1 M NaH₂PO₄ (pH 5.5), dialysing PFbpA against two overnight changes of 0.1 M sodium acetate/0.1 M NaH₂PO₄/25 mM Na₂EDTA/0.4% (w/v) Chelex 100 (pH 5.5) and one overnight change of 10 mM Tris/200 mM NaCl (pH 8.0). Protein samples were quantified using a Bio-Rad DC Protein Assay and a Beckman DU-64 spectrophotometer. Wavelength scans of holo- and apo-PFbpA were performed on a Beckman DU-640 spectrophotometer. Citrate competition assays of PFbpA iron affinity were performed as described by Chen et al. (1993), except that visible absorbance was monitored at the peak Fe absorbance for PFbpA (419 nm).

**RESULTS**

**Isolation of a *P. haemolytica* fbpABC operon**

Our approach to cloning the *fbpA* gene was based on the premise that it would be part of an operon capable of mediating high-affinity iron transport in an *E. coli* mutant deficient in this process (Zimmerman et al., 1989). We used a siderophore-deficient (entA) strain as a host. This strain was incapable of growing on nutrient broth supplemented with the iron-chelating agent diprydyl. The entA strain was transfected with a lambda ZAP II phagemid library generated from genomic DNA of a serotype A1 *P. haemolytica* strain (Ogunnarwoo et al., 1997) and selected on the iron-limited medium. Upon selection, colonies were found which were negative for siderophore production, but positive for growth reploting on fresh iron-limited medium. One clone, designated e638, was randomly selected for further analysis.

Plasmid analysis of strain e638 yielded a recombinant plasmid, pBSPH1, with a 5-5 kb insert. Whole-cell Western blots were performed to determine if this recombinant strain expressed the previously identified 35 kDa protein (Lainson et al., 1991). A mAb reacting with the 35 kDa protein isolated from a serotype A2 strain of *P. haemolytica* was used to screen the electroblots. Extracts from iron-starved cells of both A1 and A2 serotypes were included in the analysis to ensure that the mAb was capable of detecting the 35 kDa protein from a serotype A1 strain (the source of the genomic library). A single protein of 35 kDa was detected in both serotypes of *P. haemolytica*, reaffirming the conserved antigenic nature of this protein between A serotypes of *P. haemolytica* (lanes 3 and 4, Fig. 1; Murray et al., 1992). In e638 two bands were detected, a predominant band at 35 kDa and a minor band at a slightly higher molecular mass (lane 1, Fig. 1).

**Sequence analysis of pBSPH1**

DNA sequence analysis of the 5527 bp insert in pBSPH1 identified four ORFs with homology to other known proteins (Fig. 2). Three of these ORFs appeared in tandem arrangement, while a fourth partial coding sequence was identified in opposite orientation to the former genes. The first ORF (*fbpA*) encoded a protein which bears homology to the iron-binding proteins belonging to the cluster 1 group of periplasmic binding proteins (Tam & Saier, 1993). A Shine–Dalgarno sequence immediately upstream of the *fbpA* coding sequence corresponds to 9/13 nucleotides of the *E. coli* consensus sequence (Shine & Dalgarno, 1974). A potential −10 region (TAAGAA) region was also identified upstream of *fbpA*. The recombinant gene insert ends immediately upstream of this −10 region; however the fusion of the recombinant insert to the pBluescript SK II(+) vector appears to have created an artificial −35 region (TTGATA), generating a functional promoter. Previous work has indicated that FbpA is an iron-regulated protein (Lainson et al., 1991), but unfortunately truncation of the promoter/operator regions in pBSPH1 precludes analysis of this region or the potential search for a *fur* consensus sequence.

An intergenic region of 187 bp with a number of potential regions of secondary structure separates *fbpA* from a potential TTG start codon of the next ORF. The second ORF (*fbpB*) encodes a highly hydrophobic protein with homology to the family of cytoplasmic membrane-spanning proteins believed to interact with the iron-binding cluster 1 periplasmic binding proteins. Based on this TTG start codon, the calculated molecular mass of FbpB would be 58.7 kDa. Two regions of FbpB match the permease consensus sequence EAA--G--------I-LP, common to the transmembrane component of these ABC transporter systems (Saurin et al., 1994; Adhikari et al., 1996). The third gene (*fbpC*), with a

**Fig. 1.** Western blot analysis of 12% SDS-PAGE-separated whole-cell extracts of e638 (pBSPH1) (lane 1); e573 (entA host strain) (lane 2); h044 (serotype A1 *P. haemolytica*) (lane 3); and h098 (serotype A2 *P. haemolytica*) (lane 4). *P. haemolytica* cultures were iron-starved prior to analysis. Blots were probed with a previously prepared anti-35 kDa mAb (Lainson et al., 1991).
calculated molecular mass of 37.7 kDa, has the typical signature sequences of an ATPase (Walker A and B motifs; Higgins, 1992) and begins 9 bp downstream of FbpB. The fourth truncated ORF (54-3) illustrates homology to a hypothetical protein of 54.3 kDa (Burland et al., 1995) identified in the genome of E. coli (51% identity with the available P. haemolytica sequence; ~65% of the gene sequenced, based on comparison with the E. coli gene). Two small potential ORFs (Fig. 2) immediately following FbpC [ORF5 of 389 bp (~15 kDa) and ORF 6 of 284 bp (~10 kDa)] show no significant homology to any known proteins, but are reminiscent of the small potential ORF (524 bp; ~19 kDa) following sfbC of Serratia marcescens (Angerer et al., 1990).

The fbpA gene encodes a 38 kDa protein containing a characteristic signal sequence (Fig. 2) which is cleaved from the mature protein following secretion. The predicted size of the mature PFbpA which would be exported to the periplasm is 35.8 kDa. Thus the higher molecular mass protein detected in Western blots of the strain containing pBSPH1 (Fig. 1) corresponds to the 38 kDa unprocessed precursor protein of PFbpA. As pBSPH1 is a high-copy plasmid, the presence of the higher molecular mass PFbpA in E. coli, but not in the native strains, may indicate a saturation of the E. coli periplasmic secretion apparatus. Two regions of the translated PFbpA protein correspond with the amino acid sequence derived from the 35 kDa protein (Table 2), confirming that the 35 kDa protein is encoded by the fbpA gene. The N-terminal amino acid sequence of the intact 35 kDa protein corresponds exactly to that of the translated DNA sequence. In contrast, there are several differences between the experimentally determined amino acid sequence of the 14 kDa polypeptide and the translated sequence from the fbpA gene. This may represent an artifact of DNA- or amino acid sequencing or perhaps is attributable to differences between PFbpA from the A1 and A2 serotypes.

The predicted amino acid sequence of the mature PFbpA
Table 2. N-terminal amino acid sequences of the 35 kDa PFbpA and CNBr-cleaved 14 kDa subfragment, and the corresponding translated DNA sequences of \( fbpA \)

Bracketed letters represent questionable amino acid identifications.

<table>
<thead>
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<th>Sequence source</th>
<th>Peptide sequence</th>
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<tr>
<td>PFbpA N-terminal sequence</td>
<td>ANEVNYSYRQPYLIEPMLK</td>
</tr>
<tr>
<td>Translated DNA sequence</td>
<td>ANEVNYSYRQPYLIEPMLK</td>
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<tr>
<td>14 kDa fragment N-terminal sequence</td>
<td>MLDEEKQS (C) AEAAlI (N) FPS</td>
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<tr>
<td>Translated DNA sequence</td>
<td>MLDEEKQS W AEAAlI N FPS</td>
</tr>
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</table>

Fig. 3. Primary sequence alignment between the mature proteins of \( P. haemolytica \) FbpA, \( Synechococcus \) sp. PCC6301 IdiA and \( H. influenzae \) FbpA. Boxed are the TGIKV and PADV sequences characteristic of the iron-binding protein subclass of cluster 1 periplasmic binding proteins. Outlined are the iron (I)-binding ligands and in bold are the phosphate (P)-binding ligands known from the \( H. influenzae \) FbpA crystal structure (Bruns et al., 1997). Identical residues with reference to the PFbpA primary sequence are illustrated by dots; dashes represent spaces introduced to maximize alignment.

was aligned with the sequences of homologues found in other bacterial species. Surprisingly, there was greater identity with IdiA (iron-deficiency-induced Protein A) from the cyanobacterium \( Synechococcus \) than with FbpA proteins from other pathogens, such as \( H. influenzae \), belonging to the \( Pasteurellaceae \) (Fig. 3). The analysis also revealed that there are two short regions of identity which appear common to virtually all Fe-binding proteins of this class (TGIKV and PADV) and thus may serve as signatures of this protein family. It is pertinent to note that these do not represent any of the amino acids involved in liganding the metal ion (Bruns et al., 1997). A dendrogram was compiled to illustrate the similarities and relationships of the various iron-ligand-binding proteins relative to one another and to the larger family of cluster 1 extracellular solute-binding proteins (Tam & Saier, 1993), including the carbohydrate-binding proteins MalE, MalX, MsmE and UgpB (Fig. 4). The resulting non-rooted tree was compiled using primary protein sequence comparison and the neighbour-joining method of the CLUSTAL W 1.6 program (Higgins et al., 1996). Confidence in the reliability of the branch points in the dendrogram is illustrated by the boxed bootstrap value assigned at each branch point (out of 10000 bootstrap samples).

Biochemical characterization of PFbpA

The \( P. haemolytica \) \( fbpA \) gene was subcloned into the pT7-7 expression vector in order to provide substantial quantities of protein for further biochemical analysis. Simple overexpression of the recombinant FbpA and isolation of osmotic shock fluid provided a relatively pure preparation of protein. The process of iron loading and dialysis removed some minor contaminants such that the resulting protein was pure enough for bio-
Fig. 4. Relationship of *P. haemolytica* FbpA to other members of the iron-binding protein subfamily and proteins of the cluster 1 group of extracellular solute-binding proteins (Tam & Saier, 1993). Analysis was performed using the Power Macintosh versions of CLUSTAL W 1.6 (Higgins *et al.*, 1996). Illustrated are relative genetic distances between proteins (fractional numbers) and bootstrap branching confidence values (out of 10000 samples, in boxes).
Fig. 5. Coomassie-stained 12% SDS-PAGE gel of holo-PFbpA protein produced by IPTG induction and concentrated osmotic shock fractionation from strain e1126 (pT7-7 PHFA) (lane 1), and an equivalent amount of sample produced by IPTG induction and concentrated osmotic shock fractionation from the negative control strain e1267 (pT7-7) (lane 2). Lane M contains prestained SDS-7B molecular mass markers (Sigma).

Fig. 6. Wavelength spectral scan from 300 to 700 nm of holo- (-) and apo- (-----) forms of PFbpA.

chemical analyses (Fig. 5). Iron-loaded (holo-) and apo-forms of PFbpA were subjected to wavelength scans from 300 to 700 nm. Peak visible absorbance was determined to be 419 nm with an estimated molar absorption coefficient of 5426 M$^{-1}$ cm$^{-1}$ (Fig. 6). Concentrated holo-PFbpA exhibits an orange–yellow coloration which is distinct from that of the reddish-pink coloration previously reported for holo-H. influenzae FbpA, Neisseria gonorrhoeae FbpA and human transferrin (data not shown). Citrate competition assays confirmed a relative iron affinity similar to that reported for other FbpAs (approximate $K_d$ of $10^{18}$–$10^{20}$; data not shown). IEF of PFbpA revealed an isoelectric point slightly less than that of H. influenzae FbpA at pI $\sim$ 7.5 (position $\sim$ 4 on a GADPH carboxylation train; Pharmacia) (data not shown).

DISCUSSION

We initially attempted to isolate the gene encoding the 35 kDa protein using a PCR-based approach, designing oligonucleotides to amino acid sequences generated from the intact and a CNBr-cleaved fragment of the 35 kDa protein. However, we were unsuccessful at amplifying a specific PCR product from P. haemolytica chromosomal DNA during these experiments (data not shown). This may be in part attributable to the differences between the N-terminal amino acid and DNA sequences of the 14 kDa peptide (Table 2). We decided to pursue an alternative approach based on the method previously utilized by Zimmermann et al. (1989) to isolate the sfuABC operon from S. marcescens. We proceeded on the premise (i) that the 35 kDa protein may be an FbpA homologue organized in a three-gene operon similar to that of other fipABC operons, and (ii) these iron uptake systems may all share a similar affinity for iron, making it possible to clone the gene for the 35 kDa protein based on the ability of the associated operon to complement an iron uptake mutant of E. coli. Using a P. haemolytica A1 lambda ZAP II library we were successful in identifying a clone capable of complementing an entA E. coli strain on iron-restricted media. This clone was verified to be negative for siderophore production on the siderophore detection media of Schwyn & Neilands (1987). DNA sequencing using M13F universal primer and Western blotting using an anti-35 kDa mAb verified that the plasmid pBSPH1 possessed a translatable sequence which corresponded with the N-terminal amino acid sequence of the 35 kDa protein, and that the strain e638 produced a 35 kDa protein cross-reactive with the mAb. Overexpression and purification of this protein revealed reversible iron binding with a peak absorbance at 419 nm and a relative iron affinity similar to that reported for FbpAs from H. influenzae and N. gonorrhoeae.

Complete sequence analysis of pBSPH1 identified three genes in tandem arrangement with motifs characteristic of bacterial ABC importer systems (Higgins, 1992) and in particular, the three-component iron uptake operons within this family of transporters. Members of this ‘operon’ transporter family have been identified in a number of eubacterial species, including: N. gonorrhoeae (Berish et al., 1990b), Neisseria meningitidis (Berish et al., 1990a), H. influenzae (Sanders et al., 1994), Actinobacillus actinomycetemcomitans (GenBank accession number 2340838), S. marcescens (Angerer et al., 1990), Yersinia enterocolitica
HFbpA is, in addition to being an iron-binding protein, a phosphate-binding protein. Complexed exogenous phosphate binds irreversibly with iron. A central α-helix implicated in anion binding appears to be the most structurally universal motif conserved between HFbp, sulfate- and phosphate-binding proteins.

Despite low sequence identity, two of the four amino acids involved in iron binding (Y198 and Y199) are conserved among primary sequence alignment can be identified by alignment of PFbpA with HFbpA. In addition, three of the six amino acids implicated in PO₄ binding in HFbp (Q58, N175, N193 and the α-helix S137, G138, K139) are also present in PFbpA, including N196 and what appears to be a homologue of the central α-helix: S137, G138, K139. Currently ongoing crystallographic analysis of this protein should reveal whether it also binds phosphate or instead uses an alternative exogenous anionic ligand. Differences in the anion-iron-binding ligands in PFbpA may be responsible for the blue-shifted absorbance spectra of this protein. Preliminary crystallographic data reveal that PFbpA cannot be exactly modelled on the HFbpA crystal structure, indicating that structural differences do exist between these two proteins (D. McRee, personal communication).

Early in our work we had anticipated a large degree of sequence conservation in the FbpA homologues between the various pathogenic members of the Pasteurellaceae, including the representative species P. haemolytica (bovine pathogen), A. pleuropneumoniae (porcine pathogen) and H. influenzae (human pathogen). This postulate was based on the proposal that FbpA represents a critical component of transferrin-receptor-mediated iron uptake pathways and, due to constraints of protein-protein interactions with other components of the uptake pathway (i.e. FbpB and possibly TbpA), would be restricted in the degree of permissible variation which would continue to allow this system to remain functional. Furthermore, as FbpA is a periplasmic entity it would not be expected to be under immunological pressure for variation and therefore should not vary more than any cellular ‘house keeping’ genes. It must be stated that it has been suggested that FbpA may be transiently surface-exposed (Gomez et al., 1996). However, the majority of the evidence, including that for PFbpA, suggests that this protein is predominantly a periplasmic entity (Lainson et al., 1991; Berish et al., 1991; Lainson et al., 1997; Bruns et al., 1997; Kaneko et al., 1995). IdiA has been demonstrated to be involved in the mobilization of both Fe and Mn necessary for growth and photosynthetic activity under Fe and/or Mn limitation in these species. To further compound our surprise, however, was the fact that IdiA is a periplasmic protein, but rather is associated with thylakoid membranes (photosynthetic pseudoorganelles) within the cytoplasm of the cell. Both in this investigation (on the basis of an osmotic shock purification scheme and biochemical characterization) and in a previous study (Lainson et al., 1991) it has been demonstrated that PFbpA is a periplasmic protein that reversibly and with high affinity associates with iron. As this protein and its cognate inner-membrane receptor complex were cloned functionally on the basis of their ability to rescue a siderophile-deficient strain of E. coli for growth under iron-limited conditions, it can be concluded that the PFbpABC operon is an iron ABC uptake transporter system, despite the similarity of PFbpA to IdiA. It is an interesting example of biological conservation, however, that two highly related iron-binding proteins have been adapted to different roles and subcellular locations within different species of eubacteria.

The recent resolution of the crystal structure of the H. influenzae FbpA (HFbpA) protein (Bruns et al., 1997) has provided some insight into the origins and function of this class of proteins. Structural data reveal that HFbpA is, in addition to being an iron-binding protein, a phosphate-binding protein. Complexed exogenous phosphate provides a necessary O ligand for iron binding and is a structural analogue of the carbonate ion necessary for iron binding by transferrins and lactoferrins. Both transferrin N-lobe and HFbp bind iron with a similar spectrum of ligands (H249, Y95, Y188, D63 and two O ligands from exogenous CO₂ in transferrin; H9, Y195, Y196, E57, one O ligand from exogenous PO₄ and one O ligand from H₂O in HFbp), however, source of these ligands is non-analogous regions of the primary structure of these proteins. There is also extremely low sequence identity between the N-lobe of transferrin and HFbp (~ 10%). This, coupled with the fact that HFbp has more in common structurally with sugar- and anion-binding periplasmic proteins than transferrin, has led to the proposal that high-efficiency iron-binding has evolved independently multiple times over the course of evolution and, in the case of HFbp, has resulted from selective pressure for a phosphate-binding protein to associate avidly, but reversibly, with iron. A central α-helix implicated in anion binding appears to be the most structurally universal motif conserved between HFbp, sulfate- and phosphate-binding proteins.
1992). It has now become apparent from the numerous fbpA gene sequences available that, although highly conserved between different biovariants within species (Murray et al., 1992; Genco et al., 1994), there exists an enormous degree of sequence divergence in this gene between species (Fig. 4). This is surprising given the fact that other proteins, such as a 31 kDa iron-regulated periplasmic protein found in both H. influenzae and P. haemolytica, appear to be strongly conserved with ~95% identity over available sequence (Harkness et al., 1992; Tabatabai & Frank, 1997). This would suggest that it is not lack of phylogenetic overlap which is responsible for the degree of sequence divergence in fbpA between species, but perhaps biological constraints dictating variation. If FbpA directly interacts with surface receptors during iron uptake, one possible explanation for this divergence could be that immunological pressure for antigenic variation in TbpA and TbpB may have resulted in co-variation in FbpA, such that protein–protein interactions between the surface receptors and their cognate periplasmic protein remain viable for iron uptake. Although it remains to be demonstrated whether FbpA directly interacts with transferrin or lactoferrin surface receptor proteins in any species which possess this iron uptake strategy, that FbpA is necessary for transferrin iron uptake has been verified by the fact that mutant strains incapable of producing FbpA are abrogated in high-efficiency iron acquisition from transferrin and free iron salts in H. influenzae (Kirby et al., 1997) and transferrin, lactoferrin and free iron salts in N. meningitidis (Khun et al., 1998).

It is likely that PFbpA functions in a similar capacity with the P. haemolytica Tbp A/B proteins, for which the corresponding genes have recently been cloned and sequenced (Ogunnariwo et al., 1997).

Within the cluster 1 group of periplasmic binding proteins, PFbpA and, to a greater extent, A. pleuropneumoniae FbpA, stand out as highly divergent from other members of this iron-binding protein subfamily (Fig. 4). It could be suggested that this divergence may have been the result of isolation of these pathogens from other members of the Pasteurellaceae as a result of the development of host specificity (at least in part due to specificity of bacterial transferrin receptors for their cognate host transferrin) (Schryvers & Gonzalez, 1990). However, the strong degree of identity between not only the P. haemolytica fbpA gene and that of homologues identified within Synechococcus and Synechocystis spp., but also between P. haemolytica FbpB (~42% identity vs ~17–24% of other FbpBs) and P. haemolytica FbpC (~44% identity vs ~32–38%) and homologues within these cyanobacteria is a curiosity, as obviously the cyanobacteria have diverged from the Pasteurellaceae long before the development of host specificity within the Pasteurellaceae. We cannot, however, give a rational explanation for how such a relationship could have developed, although the limitations of phylogenetic analysis and the complications of horizontal genetic transfer have been well-documented by others (Syvanen, 1994). Although resistance to challenge infection by P. haemolytica serotype A1 in sheep has been demonstrated to be correlated with a rise in titre of anti-35 kDa serum antibodies (possibly anti-PFbpA) (Mosier et al., 1989) and the 35 kDa protein is highly immunogenic in sheep (Lainson et al., 1991), it is unlikely that the 35 kDa protein (PFbpA) alone could make an effective immunoprophylactic target given its lack of surface accessibility. However, it is unknown whether the strong humoral response to PFbpA could augment overall immune response to P. haemolytica infection. It has recently been suggested that FbpA could make a potential chemo-therapeutic target, given the recent understanding of the mechanism of iron binding revealed by the crystal structure of HFbpA (Bruns et al., 1997). This is supported by the facts that iron acquisition is a critical component of bacterial pathogenesis in vivo and FbpA is a focal point for high-affinity iron uptake from both free iron sources and iron complexed by transferrin and lactoferrin within the host. Despite low overall sequence identity, it appeared that the mechanism of iron binding by FbpA was universally conserved between different species, as the core iron-binding ligands could be identified in all representative members of this protein; an attractive feature for a broad-spectrum target. However, the recent identification of ‘outliers’ for which these iron-binding ligands cannot be identified in primary sequence alignments of PFbpA and, to a larger extent, A. pleuropneumoniae FbpA underscores the question of whether alternative mechanisms of iron binding exist in these related pathogens. Structural information on these proteins will answer this question and may reveal whether FbpA should be further pursued as a target for antibacterial therapy.

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