Crystal structure of FhuD at 1.6 Å resolution: a ferrichrome-binding protein from the animal and human pathogen *Staphylococcus pseudintermedius*

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Staphylococcus pseudintermedius is a leading cause of disease in dogs, and zoonosis causes human infections. Methicillin-resistant *S. pseudintermedius* strains are emerging, resembling the global health threat of *S. aureus*. Therefore, it is increasingly important to characterize potential targets for intervention against *S. pseudintermedius*. Here, FhuD, an *S. pseudintermedius* surface lipoprotein implicated in iron uptake, was characterized. It was found that FhuD bound ferrichrome in an iron-dependent manner, which increased the thermostability of FhuD by >15°C. The crystal structure of ferrichrome-free FhuD was determined *via* molecular replacement at 1.6 Å resolution. FhuD exhibits the class III solute-binding protein (SBP) fold, with a ligand-binding cavity between the N- and C-terminal lobes, which is here occupied by a PEG molecule. The two lobes of FhuD were oriented in a closed conformation. These results provide the first detailed structural characterization of FhuD, a potential therapeutic target of *S. pseudintermedius*.

1. Introduction

Staphylococcus pseudintermedius is a common commensal bacterium colonizing the skin and mucosa of household animals and is emerging as an important opportunistic pathogen causing canine pyoderma (Bannoehr *et al.*, 2007; Fitzgerald, 2009). Moreover, *S. pseudintermedius* is associated with other canine diseases, including wound infections, urinary-tract infections and otitis externa, thus resulting in significant global morbidity in dogs. Treatment of *S. pseudintermedius* in dogs is often ineffective unless strong systemic antibacterials are used, thus risking an increase in antimicrobial-resistant bacteria in pets that can act as reservoirs for transmission to humans (Van Hoovels *et al.*, 2006). Indeed, methicillin-resistant *S. pseudintermedius* (MRSP) infections resemble human methicillin-resistant *S. aureus* (MRSA) infections (Bannoehr *et al.*, 2007; Fitzgerald, 2009; McCarthy *et al.*, 2015). Although zoonosis is rare, life-threatening *S. pseudintermedius* infections can occur in humans following dog-bite wounds (Guardabassi *et al.*, 2004), and several MRSP outbreaks in humans in hospitals have been reported (Grönthal *et al.*, 2014; Savini *et al.*, 2013; Starlander *et al.*, 2014). Consequently, the development of improved therapeutics against *S. pseudintermedius* could alleviate pathogen-induced disease in animals and humans.

Solute-binding proteins (SBPs) have captured much attention in studies of staphylococci and other pathogens (Chu & Vogel, 2011; Berntsson *et al.*, 2010; Couñago *et al.*, 2012). SBPs typically act *via* an integral membrane protein that mediates transmembrane solute transport. Transport requires

Table 1

Macromolecule-production information.

Source organism	<i>S. pseudintermedius</i> (strain IV369-1041)
DNA source	Clinical sample obtained from Quotient Bioresearch Ltd (GenBank code CP002478)
Expression vector	pET-15
Expression host	<i>E. coli</i> strain BL21(DE3)
Complete amino-acid sequence†	MGSDKIHHHHHENLYFQSEKETKAFNLKTAKGEEKID- IPKDPKRIVVMAPTYAGGLKYLDANIVGVSDQVDQS- PVLAKQKFDVVKVGAEDVEKVASLKPDLIITYNTDK- NTDKLKKIAPTIAFDYAKYNLYEQQEAMGDIVGKSD- EVKKWKADWEKQTAQDSKDIKAHLGDDTSVTFEDF- DKKIYAYGNWGRGSEVLYQAFGLQMPKALDDATKK- EGWTEVPKEEVGKYAGDVIITAKAKDAAQPEFQKTA- MWQNLEAVQNKYAFNVDSVYVYNDPYTLDVIRKDL- KKQLLALPTN

† Complete sequence of the recombinant FhuD protein construct produced. Underlined residues are the hexahistidine tag and the TEV cleavage site derived from cloning procedures and were removed by TEV protease prior to crystallization screening and other experiments.

a cytoplasmic ATP-binding protein, thus comprising the canonical bacterial ABC (ATP-binding cassette) transporter. The SBP architecture features a ligand-binding groove located between two globular domains. The SBP family exhibits three classes, depending largely on the linker(s) connecting the two globular domains (Quioco & Ledvina, 1996). The class I SBPs have three connecting segments between the two domains. In contrast, the class II SBPs have two interdomain crossovers, whereas the class III proteins only have one polypeptide linker connecting the domains (Chu & Vogel, 2011). Different SBPs function by mediating the transport of different solutes, including siderophores, carbohydrates, peptides, amino acids and ions, thus providing nutrients essential for survival, growth and virulence, underlying their biomedical significance. Structurally characterized surface lipoprotein SBPs from *S. aureus* that have also shown promise as protective vaccine antigens in mouse models include FhuD2 (ferric hydroxamate uptake protein D2; Mishra *et al.*, 2012; Mariotti *et al.*, 2013; Podkowa *et al.*, 2014) and MntC (Anderson *et al.*, 2012; Gribenko *et al.*, 2013). To date, the only structurally characterized protein from *S. pseudintermedius* is the Mn²⁺-binding protein SitA (Abate *et al.*, 2014), an orthologue of MntC. Since such lipoproteins are immunogenic and may be important for bacterial virulence, we investigated the structure and ligand-binding ability of FhuD, an orthologue of FhuD2 implicated in the role of iron uptake and a potential therapeutic target of *S. pseudintermedius*.

2. Materials and methods

2.1. Macromolecule production

The *fhuD* gene fragment from *S. pseudintermedius* encoding Ser26–Asn307 was PCR-cloned into a pET-15 vector (Novagen), enabling the expression of recombinant FhuD (UniProt E8SEW8) with an N-terminal hexahistidine tag followed by a *Tobacco etch virus* (TEV) protease cleavage site (Table 1). FhuD was produced in *Escherichia coli* cells grown in Luria–Bertani broth. Protein production was induced at 30°C using 0.15% (w/v) arabinose. The cells were harvested by

Table 2

Crystallization.

Method	Sitting-drop vapour diffusion
Plate type	Low-profile 96-well (Greiner)
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	60
Buffer composition of protein solution	20 mM Tris–HCl pH 8.0, 150 mM NaCl
Composition of reservoir solution	0.1 M CdCl ₂ , 30% (v/v) PEG 400, 0.1 M sodium acetate pH 4.6
Volume and ratio of drop	200 nl protein solution with 200 nl reservoir solution

centrifugation. Protein purification was performed *via* Ni–NTA affinity chromatography essentially as described previously for the *S. pseudintermedius* SitA protein (Abate *et al.*, 2014). Fractions containing FhuD were identified by a band migrating at ~38 kDa in SDS–PAGE analysis (the theoretical size of the tagged construct is 34 171 Da). The N-terminal hexahistidine tag was removed using a solubility-enhanced TEV protease (van den Berg *et al.*, 2006), with cleavage proceeding at room temperature (18–26°C) overnight in buffer consisting of 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 0.5 mM EDTA, 1 mM DTT. Subsequently, the sample was reloaded onto the Ni–NTA resin to recapture the TEV protease and the free hexahistidine tag, thus allowing elution of FhuD in the tagless form in the flowthrough. Finally, FhuD was loaded onto a HiLoad Superdex 75 (16/60) size-exclusion chromatography (SEC) column (GE Healthcare) equilibrated in 20 mM Tris–HCl pH 8.0, 150 mM NaCl. The final yield of purified tagless FhuD (theoretical size 31 784 Da) was >50 mg per litre of growth medium. The final sample quality was checked using 4–12% SDS–PAGE gradient gels in MES buffer; the sample was estimated to be >90% pure.

2.2. Crystallization

Purified FhuD was concentrated using centrifugal concentration devices with 10 000 molecular-weight cutoff membranes (Amicon Ultrafree, Millipore). The protein concentration was determined using the Bradford assay. FhuD was prepared in the absence or presence of ferrichrome (1:1 or 1:2 molar ratios of protein:ligand) for crystallization screening. Drops were dispensed using a Crystal Gryphon robot (Art Robbins Instruments). Over 1500 crystallization conditions were screened, with automatic drop imaging performed using a RockImager 182 (Formulatrix). FhuD crystals were obtained using a reservoir consisting of condition H2 of Structure Screen (Molecular Dimensions), which contains cadmium chloride, PEG 400 and sodium acetate buffered at pH 4.6 (Table 2).

2.3. Data collection and processing

Crystals were cryoprotected by the addition of 20% (w/v) ethylene glycol prior to flash-cooling in liquid nitrogen. Useful data sets were collected on beamlines ID14-1 and ID14-4 at the European Synchrotron Radiation Facility (ESRF), Grenoble and at the Swiss Light Source (SLS), Villigen. The final diffraction data were collected on beamline PXIII of the

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Beamline X06DA, SLS
Wavelength (Å)	1.0
Temperature (K)	100
Detector	Pilatus 2M-F
Space group	$P2_1$
a, b, c (Å)	43.58, 53.82, 120.25
α, β, γ (°)	90, 99.17, 90
Resolution range (Å)	49.02–1.59 (1.65–1.59)
Total No. of reflections	241352 (21049)
No. of unique reflections	72420 (6893)
Completeness (%)	99.2 (94.8)
Multiplicity	3.3 (3.1)
$\langle I/\sigma(I) \rangle^\dagger$	10.66 (1.9)
$R_{\text{r.i.m.}}^\ddagger$	0.108 (0.753)
Overall B factor from Wilson plot (Å ²)	12.28§

[†] The mean $I/\sigma(I)$ in the outer shell is <2.0 when the resolution reaches 1.59 Å. [‡] Estimated $R_{\text{r.i.m.}} = R_{\text{merge}}[N/(N-1)]^{1/2}$, where N is the data multiplicity. [§] No anomalies were observed in the Wilson plot.

SLS. Diffraction data were processed using *iMosflm* (Battye *et al.*, 2011) and scaled using *AIMLESS* (Evans & Murshudov, 2013) from the *CCP4* software suite (Winn *et al.*, 2011). Data-collection and processing statistics are summarized in Table 3.

2.4. Structure solution and refinement

The structure of FhuD was solved by molecular replacement in *Phaser* (McCoy, 2007) using the coordinates of ferrichrome-bound FhuD2 from *S. aureus* (PDB entry 4b8y; Mariotti *et al.*, 2013), which shares 56% sequence identity with FhuD. The structure was refined using *phenix.refine* (Afonine *et al.*, 2012) and *BUSTER* (Bricogne *et al.*, 2011), and model building was performed with *Coot* (Emsley & Cowtan, 2004). The $F_o - F_c$ difference maps revealed several strong peaks of difference density ($>6\sigma$) on the protein surface, which were modelled as Cd^{2+} ions, consistent with previous reports of Cd^{2+} ligation schemes, and were validated using the *Check-MyMetal* metal-binding site validation server (Zheng *et al.*, 2014). The quality of the final structure was assessed using

Table 4

Structure refinement.

Values in parentheses are for the outer shell.

Resolution range (Å)	49.02–1.60 (1.6193–1.5982)
Completeness (%)	99.1
σ Cutoff	$F > 1.99\sigma(F)$
No. of reflections, working set	72391 (2337)
No. of reflections, test set	3650 (122)
Final $R_{\text{cryst}}^\dagger$	0.184 (0.281)
Final $R_{\text{free}}^\ddagger$	0.217 (0.312)
No. of non-H atoms	
Protein	4422
Ion	25
Ligand	45
Water	755
Total	5247
R.m.s. deviations	
Bonds (Å)	0.008
Angles (°)	1.085
Average B factors (Å ²)	
Protein	13.9
Ion	24.1
Ligand	38.9
Water	25.4
Ramachandran plot	
Favoured regions (%)	99.1
Additionally allowed (%)	0.7

[†] $R_{\text{cryst}} = \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$. [‡] R_{free} is calculated as R_{cryst} but for 5.0% of the total reflections that were chosen at random and omitted from refinement.

MolProbity (Chen *et al.*, 2010). Refinement statistics are summarized in Table 4.

3. Results and discussion

3.1. FhuD is strongly stabilized upon binding to ferrichrome

The ectodomain of the surface lipoprotein FhuD (residues Ser26–Asn307) was prepared from the *fhuD* gene of a clinical isolate of *S. pseudintermedius*. The first 25 residues of the wild-type sequence were omitted in order to exclude (i) the signal peptide and lipidation site and (ii) the N-terminal stretch that sequence analyses indicated was likely to be disordered. The soluble recombinant FhuD protein was produced in *E. coli* and was readily purified using standard chromatographic

Figure 1

Biophysical analyses of FhuD and its iron-dependent interaction with ferrichrome. (a) An analytical SEC profile of FhuD injected at 1.5 mg ml⁻¹ showing the absorbance (at 280 nm wavelength) plotted against elution time (min). The peak maximum occurred at 11.1 min. (b) DSC profiles of FhuD recorded in the absence (grey line) or presence (black line) of a 20-fold molar excess of ferrichrome. (c) SPR sensorgrams collected upon the injection of 1 mM ferrichrome (black line) or deferrichrome (iron-free; grey line) revealed that ligand binding to FhuD is dependent on the presence of iron.

techniques. Analytical size-exclusion chromatography (SEC) of the purified protein indicated that it was a monodisperse species migrating with an apparent size of 31 kDa, corresponding closely to the expected size of the FhuD monomer (Fig. 1*a*). The FhuD sample was further analyzed by differential scanning calorimetry (DSC), which revealed a single unfolding transition with a melting temperature (T_m) of 52°C. Upon the addition of a 20-fold molar excess of ferrichrome, FhuD was stabilized by >15°C (Fig. 1*b*). This result suggests that FhuD binds ferrichrome, as observed previously for the *S. aureus* orthologue FhuD2, which showed a 14°C increase in T_m upon the addition of ferrichrome (Mariotti *et al.*, 2013). Using surface plasmon resonance (SPR) binding studies, it was observed that the binding of ferrichrome to FhuD was dependent on the presence of iron, since deferrichrome (which lacks iron) did not interact with FhuD (Fig. 1*c*).

3.2. The crystal structure of FhuD reveals a class III SBP fold

We attempted to crystallize FhuD alone or with ferrichrome. Hundreds of crystallization trials were attempted and crystals were only yielded in the absence of ferrichrome. The crystals contained two FhuD monomers per asymmetric unit and had a solvent content of 41%. The final refined coordinates of FhuD spanned residues Thr30–Thr306, with relatively small fluctuations in B factors. Four Cd^{2+} ions, which originated from the crystallization reservoir solution (Table 2), were modelled bound to the surface of each of the two chains, coordinated by polar or negatively charged side-chain groups, with a ninth Cd^{2+} ion sandwiched between the two chains. In addition to contacting the protein, the Cd^{2+} ions frequently bound one or two chloride ions or water molecules.

The overall fold of FhuD resembles a bilobate bean-like structure typical of the class III SBP proteins, composed of

two globular domains (or lobes) connected by a long α -helix (Ser152–Leu177), with dimensions of $\sim 60 \times 40 \times 35$ Å. The N-terminal lobe contains six β -strands and six α -helices, while the C-terminal lobe contains five β -strands, three well defined α -helices and five short helical turns (Fig. 2). The two chains of FhuD in the asymmetric unit present their ligand-binding grooves facing in opposite directions and are arranged such that the long helix of each molecule lies in the same plane but is rotated by 90° with respect to the other (Fig. 2). This apparent association is likely to merely be a consequence of packing interactions occurring at the very high protein concentration used for crystallization. Indeed, analysis of the crystallographic dimer interface *via* PISA (Krissinel & Henrick, 2007) did not detect any interfaces that were likely to result in the formation of stable quaternary complexes, and the protein is monomeric in solution, as determined by analytical SEC (Fig. 1*a*). Structurally, the two chains of FhuD are essentially identical and can be superimposed with very low root-mean-square deviation (r.m.s.d.) values (<0.2 Å for 277 aligned C^α atoms).

Multiple contacts were observed in the FhuD structure between the N- and C-terminal domains of the protein, which may contribute to the relatively high thermostability of ligand-free FhuD ($T_m = 52^\circ\text{C}$), which was further increased by the addition of ferrichrome. This ligand is a stabilizing factor which, by analogy with *S. aureus* FhuD2, is very likely to bind between the two domains. In the FhuD structure determined here, electron density was clearly observed for a single PEG molecule per protein monomer, which was found in the large pocket of ~ 16 Å in depth located between the two lobes. Notably, the PEG molecule makes contact with many aromatic side chains (including Tyr113, Tyr133, Phe189, Tyr194, Tyr196, Trp200, Trp228, Trp283 and Tyr284) and a few polar or charged residues (Thr59, Gln77, Glu187, Arg202 and

Figure 2
Overall structure of FhuD. (*a*) Cartoon representation of FhuD showing the typical class III SBP fold: the N-terminal lobe (pink) and C-terminal lobe (green) are connected by a single long α -helix (blue). The molecular surface is shown in transparent white to highlight the ligand-binding groove (top centre). The N- and C-termini are labelled. The figure was generated using *PyMOL* (<http://www.pymol.org>). (*b*) Cartoon representation depicting the relative orientation of the two FhuD molecules present in the asymmetric unit.

Asn285) that line the cavity (Fig. 3). Several of the residues (underlined) contacting PEG superimpose very closely with the side chains involved in binding to ferrichrome in the ligand-bound structure of FhuD2 (Mariotti *et al.*, 2013), suggesting that FhuD possesses an active ligand-binding site that is capable of accommodating ferrichrome and possibly also other related siderophores. The similarity of their binding mechanisms is also indicated by the similarly large thermal stabilizations of FhuD and FhuD2 induced by ferrichrome as determined by DSC studies (Fig. 1*b*; Mariotti *et al.*, 2013).

As described previously, the distance separating the C α atoms of the conserved FhuD2 residues Glu97 and Glu231 used to engage the ABC transporter during solute uptake can be used to characterize the conformational state of the SBP (Podkova *et al.*, 2014). Here, for FhuD, the distance between the corresponding residues Glu100 and Glu234 was 44.8 Å (Fig. 3), which is very similar to the distances of 45.5 Å observed in ferrioxamine-bound FhuD2 (Podkova *et al.*, 2014) and 45.4 Å in ferrichrome-bound FhuD2 (Mariotti *et al.*, 2013). These conformations represent the 'closed' state of the protein. In contrast, the structures of unliganded FhuD2 (Podkova *et al.*, 2014) demonstrated more 'open' conformations with an increased Glu97-to-Glu231 distance ranging from 51.1 to 53.2 Å.

3.3. Comparison of FhuD structure with those of FhuD2 and other similar proteins

A search of the Protein Data Bank (PDB) revealed several structures of *S. aureus* FhuD2 that were highly similar to FhuD. Ferrichrome-bound and ferrioxamine-bound forms of FhuD2 could be closely aligned with FhuD, displaying r.m.s.d. values as low as 0.8 and 0.9–1.0 Å, respectively. In contrast, the FhuD structure is more different when compared with the unliganded 'open' forms of FhuD2 (r.m.s.d. of 1.9–2.4 Å). As expected, greater differences are observed when comparing FhuD with SBPs that bind a different class of solute, such as

the divalent metal cation-binding SBP SitA from *S. pseudintermedius* (r.m.s.d. of 3.1 Å; Abate *et al.*, 2014).

4. Closing remarks

Structural and biochemical studies of SBPs have provided many insights into the molecular mechanisms of solute uptake used by bacteria (Chu & Vogel, 2011; Quirocho & Ledvina, 1996; Berntsson *et al.*, 2010; Couñago *et al.*, 2012). Here, we have structurally characterized FhuD from a clinical isolate of *S. pseudintermedius*. Based on sequence analyses alone, FhuD was previously annotated in the UniProt Knowledgebase as a putative ferrichrome-binding protein. To explore this hypothesis experimentally, we performed SPR and DSC experiments, which revealed iron-dependent binding and a large stabilization of FhuD in the presence of ferrichrome, an iron(III) hydroxamate. Together, these data support the hypothesis that FhuD is involved in the capture of iron(III) hydroxamates, which is likely to be essential during the bacterial life cycle. The amino-acid sequence of the FhuD protein studied here is >99% conserved in known *S. pseudintermedius* genomes (Ben Zakour *et al.*, 2011; Tse *et al.*, 2011), suggesting that siderophore binding by FhuD is important in these pathogenic strains. Our results provide the first evidence of siderophore binding and the experimentally determined structure of this surface-exposed lipoprotein from *S. pseudintermedius*, a bacterial pathogen of animals and humans of growing veterinary and medical importance.

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Figure 3

FhuD accommodates one PEG molecule in the central cleft. Cartoon representation looking down into the ligand-binding groove of FhuD (coloured as in Fig. 2). The C α atoms are shown as white spheres for the conserved SBP residues Glu100 and Glu234 (labelled), which are separated by 44.8 Å (indicated by the grey dashed line). The ligand present in the central binding site is a PEG 400 molecule, with C and O atoms shown as grey and red spheres, respectively. Side chains (yellow sticks) contacting the ligand are shown for several aromatic, polar and charged residues.

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