FhuD1, a Ferric Hydroxamate-binding Lipoprotein in Staphylococcus aureus

A CASE OF GENE DUPLICATION AND LATERAL TRANSFER*

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Staphylococcus aureus can utilize ferric hydroxamates as a source of iron under iron-restricted growth conditions. Proteins involved in this transport process are: FhuCBG, which encodes a traffic ATPase; FhuD2, a post-translationally modified lipoprotein that acts as a high affinity receptor at the cytoplasmic membrane for the efficient capture of ferric hydroxamates; and FhuD1, a protein with similarity to FhuD2. Gene duplication likely gave rise to fhuD1 and fhuD2. While the genomic locations of fhuCBG and fhuD2 in S. aureus strains are conserved, both the presence and the location of fhuD1 are variable. The apparent redundancy of FhuD1 led us to examine the role of this protein. We demonstrate that FhuD1 is expressed only under conditions of iron limitation through the regulatory activity of Fur. FhuD1 fractions with the cell membrane and binds hydroxamate siderophores but with lower affinity than FhuD2. Using small angle x-ray scattering, the solution structure of FhuD1 resembles that of FhuD2, and only a small conformational change is associated with ferrichrome binding. FhuD1, therefore, appears to be a receptor for ferric hydroxamates, like FhuD2. Our data to date suggest, however, that FhuD1 is redundant to FhuD2 and plays a minor role in hydroxamate transport. However, given the very real possibility that we have not yet identified the proper conditions where FhuD1 does provide an advantage over FhuD2, we anticipate that FhuD1 serves an enhanced role in the transport of untested hydroxamate siderophores and that it may play a prominent role during the growth of S. aureus in its natural environments.

With few exceptions, all bacteria have an absolute requirement for iron (1, 2). The amount of free, biologically relevant iron, however, is negligible at physiological pH (10^{-18} M) (3). This is primarily caused by the rapid formation of iron(III)-hydroxy precipitates that are highly insoluble. In response to the stress generated from a low iron environment, many microorganisms secrete low molecular weight iron chelating compounds termed siderophores. Siderophores commonly bind ferric iron with extremely high affinities (4) and serve to solubilize iron from the biologically inert iron(III)-hydroxy precipitates. Typically, ferric-siderophores are mobilized across the cell envelope of Gram-negative bacteria expressing cognate outer membrane receptors, periplasmic-binding proteins, and associated ABC1-type transporters (5, 6). In Gram-positive bacteria, ferric siderophores are captured by lipoproteins that function as high affinity receptors and subsequently feed ligand to the ABC transporter in the cytoplasmic membrane. The lipid group on these receptors acts as a tether to anchor the receptor protein to the external face of the cell membrane (7).

Whereas Staphylococcus aureus isolates produce endogenous siderophores (8–11), S. aureus utilizes others that are produced by other microorganisms (so-called xenosiderophores) for growth under conditions of iron deprivation. One group of xenosiderophores that S. aureus can utilize is the hydroxamate-class of siderophore, including aerobactin, coprogen, ferrioxamine B (Desferal™), ferrichrome, and rhodotorulic acid (12). In our previous studies, we showed that S. aureus strain RN6390 possessed at least five different iron-regulated genes whose products were involved in the ferric hydroxamate uptake process (12–14). A three gene operon, fhuCBG, encodes a classical traffic ATPase (for a review of traffic ATPases, see Ref. 15) while a fourth gene, fhuD2, codes for a lipoprotein (12) that functions as a high affinity receptor for aerobactin, coprogen, Desferal™, ferrichrome, and rhodotorulic acid (14). The fifth gene involved in this transport system, fhuD1, is predicted to encode a protein with 50% total similarity to FhuD2. We showed that the fhuD1 gene product could partially compensate for the loss of the fhuD2 gene product (12).

In this communication, we show that the presence and location of fhuCBG and fhuD2 are strictly conserved in several S. aureus genomes. In contrast, the fhuD1 gene is mobile: it is absent in some strains and appears on a genomic island in others. The unusual occurrence of the fhuD1 gene warranted a detailed investigation of the function of the FhuD1 protein.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Media—S. aureus RN6390 (16) served as the wild-type staphylococcal strain in this study, and derivatives H430 (RN6390 fhuD1::Km), H364 (RN6390 fhuD2::Tet), and H295 (RN6390 fur::Km) have been previously described (12, 13). Escherichia coli DH5α (Promega) was used for routine cloning and recombiant protein overexpression. For the routine cultivation of S. aureus, tryptic

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1 The abbreviations used are: ABC, ATP-binding cassette; SAXS, small angle x-ray scattering; MBP, maltose-binding protein.
soy broth (Difco) was the chosen growth medium, and Luria-Bertani broth (Difco) was the selected medium for E. coli. Solid media were obtained by the addition of 1.5% (w/v) Bacto agar (Difco). S. aureus was grown in Tris-minimal sucinate (TMS) medium supplemented with either FeCl3 (50 μM) or ethylenediamine-di(o-hydroxyphenylacetic acid) (EDDHA) (1 μM) to create iron-replete and iron-restricted growth conditions, respectively. TMS was made as follows: 40 mL of a 25× Tris minimal salts stock (in 1 liter was dissolved 145 g of NaCl, 92.5 g of KCl, 27.5 g of NH4Cl, 3.35 g of Na2SO4, 6.8 g of KH2PO4, it was added to 12.1 g of Tris base, 16.6 g of succinate, and 50 ml 20% casamino acids in –800 ml of water, and the pH was adjusted to 7.4 prior to autoclaving. After autoclaving, 2 ml of tryptophan (10 mg/ml) and 1-ml volumes of cysteine (22 mg/ml), thiamine (16.9 mg/ml), nicotinic acid (1.23 mg/ml), pantothenic acid (0.5 mg/ml), biotin (0.01 mg/ml), MgCl2 (95.3 mg/ml), and CaCl2 (11.1 mg/ml) were incorporated and the volume adjusted to 1 liter with sterilized MilliQ water. When required, kanamycin (50 μg/ml) and tetracycline (4 μg/ml) were included in S. aureus growth medium. Ampicillin (100 μg/ml), when appropriate, was incorporated into medium for the growth of E. coli.

**Protein Overexpression and Purification**—The fhuD1 gene, without the predicted signal sequence codons, was PCR-amplified as a 1.0-kb DNA fragment, digested with BamHI and EcoRI (sites were incorporated into the oligonucleotides) and then cloned into BamHI- and EcoRI-digested pET-22-TEV (I4). The resulting plasmid, from which a translational fusion between glutathione S-transferase (GST) and FhuD1 is encoded, was introduced into E. coli strain DH5α to generate strain H659. Strain H659 was grown to an approximate OD∞600 of 0.8, and, following the addition of isopropyl-1-thio-β-D-galactopyranoside (0.4 mM), growth was allowed to continue for 3 h before the cells were lysed. The resulting supernatant was centrifuged at 40000 × g to pellet cellular components and then passed across a 5-ml GSTrap column (Amersham Biosciences) for purification. Once purified, the GST-FhuD1 fusion protein was cleaved by the TEV protease (4 h at 23 °C in 50 mM Tris-Cl, pH 8.0) before it was applied to an 8-m1 Mono-S column (Amersham Biosciences) and recovered using a 0–1 mM NaCl gradient.

**Generation of Polyclonal Antibodies to FhuD1**—The purified FhuD1 was used to generate polyclonal antisera in two New Zealand White rabbits (Charles River). Briefly, 100 μg of protein in Titer Max Gold adjuvant (Cedarlane) was injected subcutaneously into each rabbit. The rabbits were boosted with 50 μg of protein at 2–4 weeks, and at 5 weeks, the rabbits were sacrificed, and sera were collected. Sera were adsorbed three times over polyvinylidene difluoride-immobilized DH5α (pGEX-2-TEV) cell lysate, induced to express glutathione S-transferase.

**Detergent Extraction and Phase Partitioning**—S. aureus cells in late log-phase were collected, washed three times with 0.9% NaCl (w/v), and then equilibrated to an OD600 of 1.0. The cell wall was digested using lysostaphin (50 μg/ml), followed by sonication to lyse cells. Triton X-114 detergent pellets were obtained by the addition of 1.5% (w/v) Bacto agar (Difco). Solid media were obtained by the addition of 1.5% (w/v) Bacto agar (Difco). Siderophore plate bioassays were performed essentially as previously described (15). 5-μl volumes of ferric siderophore solutions, at concentrations as indicated in Table II, were placed onto sterile 6-mm paper discs before being placed on agar plates containing impregnated bacteria. Bacteria were incorporated into the plates at a concentration of 1 × 106 cells/ml. Results were scored after incubation at 37 °C for 48 h followed by 24 h at room temperature.

**Small Angle X-ray Scattering (SAXS)**—SAXS measurements for FhuD2 were carried out as previously described (14). Measurements for FhuD1 were made at the Advanced Photon Source, Beamline ID-18, as follows: the sample temperature was 20 °C, and the protein solution was moved continuously through a 1-mm quartz capillary during the course of the measurement to minimize the effects of radiation damage. Data were collected at 1370 mm using a CCD detector, with X-rays at a wavelength of 1.06 Å, to cover the momentum transfer range 0.027 nm−1 < q < 0.60 nm−1, where q is defined as 2sinθ/λ (2θ is the scattering angle, while λ is the wavelength of the radiation). Note that Q, used in the Guinier plots, is defined as 4πsinθ/λ. For each sample, 5–10 s exposures were recorded that consisted of 3 measurements from the protein solution bracketed by 2 measurements of the buffer solution. Data were integrated using Fit2D (19, 20), and exported into a spreadsheet program. The three protein solution curves and two background buffer curves were averaged and all the background buffer curve was subtracted, with no correction, from the protein solution curve to yield scattering from the hydrated protein.

**RESULTS**

In our previous work that described the properties of the FhuD2 protein in detail (14), we showed that expression of FhuD2 was iron-regulated, that FhuD2 was amphilic and present in the cell membrane, and that purified FhuD2 bound ferric hydroxamates with dissociation constants in the nanomolar to micromolar range. The data suggested that the protein acts as a high affinity receptor for hydroxamate siderophores at the external face of the cytoplasmic membrane in S. aureus (12, 14). In our initial genetic studies, however, we also identified a second gene, fhuD1, that was predicted to encode a protein with ~50% similarity to FhuD2 (12). Examination of the phenotype of fhuD1 and fhuD2 knockout strains demonstrated that the fhuD1 gene product could partially compensate for the loss of fhuD2 in terms of the transport of hydroxamate siderophores. The following studies were undertaken to further characterize the function of FhuD1.

**Variable Occurrence of fhuD1 in S. aureus**—The observation that FhuD1 is more similar in sequence to FhuD2 than to other FhuD homologs encoded from the genomes of other Gram-positive bacteria suggests that they arose from a gene duplication event at some point in the evolution of S. aureus. Seven S. aureus genome bacteria suggesting that they arose from a gene duplication event at some point in the evolution of S. aureus. Seven S. aureus genome bacteria suggesting that they arose from a gene duplication event at some point in the evolution of S. aureus. Seven S. aureus genome bacteria suggesting that they arose from a gene duplication event at some point in the evolution of S. aureus.

**FhuD1 in S. aureus**—53153

MW2, NCTC8325, MSSA476, and COL, and are both absent

This was prepared by methods previously described (18).

**Siderophore Plate Bioassays**—Siderophore plate bioassays were performed essentially as previously described (15). 5-μl volumes of ferric siderophore solutions, at concentrations as indicated in Table II, were placed onto sterile 6-mm paper discs before being placed on agar plates containing impregnated bacteria. Bacteria were incorporated into the plates at a concentration of 1 × 106 cells/ml. Results were scored after incubation at 37 °C for 48 h followed by 24 h at room temperature.

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from the equivalent genomic location in N315, Mu50, and MRSA252. Interestingly, mw1944-fhuD1 (between mw1943 and mw1946) is between a pathogenicity island, SaPIn1 (N315)/SaPIm1 (Mu50), and a bacteriophage, /H9021 N315/H9021 Mu50A, in the genomes of N315 and Mu50 (22). The unusual location/occurrence of the fhuD1 gene within the genomes of S. aureus warranted a functional characterization of the fhuD1 gene product.

S. aureus fhuD1 Encodes an Iron-regulated Lipoprotein—Late log phase cells of S. aureus RN6390 and derivatives were harvested by centrifugation after culture in either iron-replete or iron-restricted medium. Cells were subjected to Triton X-114 phase partitioning, a technique that separates cell components into three fractions: insoluble, aqueous, and detergent soluble (membrane-associated proteins are present within the latter fraction). In Fig. 2, we show that FhuD1 partitions with the detergent soluble fraction, indicating that the protein is amphipathic and associated with the membrane. Similar results using Triton X-114 fractionation have been obtained for several other lipoproteins (12, 24, 25). This result is consistent with the presence of a lipobox motif (15LTAC18) in FhuD1 (26). Our results confirm that FhuD1, although primarily hydrophilic, resides within the detergent soluble membrane component of the cell. Expression of FhuD1 in the cell membrane was regulated by exogenous iron concentrations, since FhuD1 is only detected in cells grown in iron-starved medium, and this regulation is mediated by the activity of the Fur protein, because a fur mutant-expressed FhuD1 in the membrane of cells grows under iron-replete conditions (Fig. 2). No band is observed in the lane loaded with protein extracted from H430 (RN6390 fhuD1::Km) indicating that the antibody is specific for FhuD1 and does not cross-react with FhuD2.

FhuD1 Binds Hydroxamate-type Siderophores but with Less Affinity Than FhuD2—Our previous studies identified a role

**FIG. 1. Genomic locations of fhuCBG, fhuD2, and fhuD1.** Where possible, we have used the mw ORF numbering from the MW2 genome as a reference. A, genomic region surrounding fhuCBG operon. B, genomic region surrounding fhuD2. C, genomic regions surrounding fhuD1, where present. For MRSA252 and N315, strains whose genomes lack fhuD1, shown is the equivalent position of where fhuD1 is situated in other S. aureus genomes. SaP1n1 is an island that is present in N315 and Mu50 and contains tst, the gene encoding toxic shock syndrome toxin-1. mw1941 and mw1942 encode lukF and lukS, respectively, and are carried on bacteriophage DNA.

**FIG. 2. FhuD1 is an iron-regulated lipoprotein.** 4 μg of purified FhuD1 protein was loaded in the lane labeled FhuD1; lane i, Triton X-114-insoluble fraction; lane a, Triton X-114 aqueous fraction; lane d, Triton X-114 detergent fraction. Also shown below gel are + and −, indicating the presence or absence of iron in the growth media. Strains are indicated at the top: RN6390 (wild type), H430 (RN6390 fhuD1::Km), H364 (RN6390 fhuD2::Tet), and H295 (RN6390 fur::Km).
for *fhuD1* in ferric hydroxamate uptake since a *fhuD2* knockout strain that expressed *fhuD1* could still utilize ferric hydroxamates, albeit to a lesser extent than wild-type RN6390 (12). Moreover, since FhuD1 shares 50% total similarity with FhuD2 (12, 14), a protein that our group has characterized as a high affinity receptor for ferric hydroxamates (14), we reasoned that FhuD1 is also a receptor for ferric hydroxamates. To investigate this further, and as an initial attempt to demonstrate that ferric hydroxamates physically interact with FhuD1, we examined in SDS-PAGE the reaction products of FhuD1 treated with protease K after incubation with various ferric siderophores (Fig. 3). We showed that the slowest migrating form of FhuD1 was protected from proteolysis when preincubated with either coprogen, Desferal™ or ferrichrome (Fig. 3, lane 6–8), pyoverdine (lane 9). Ferrated siderophores were all used at a concentration of 5 mM. FhuD1 is marked with an arrowhead.

**TABLE I**

| Protein  | Aerobactin | Desferal™ | Coprogen | Ferrichrome |
|----------|------------|-----------|----------|-------------|
| FhuD1    | 40         | 0.9       | 6.7      | 0.05        |
| FhuD2a   | 0.3        | 0.05      | 1.7      | 0.02        |
| FhuD2b   | 0.4        | 35        | 0.3      | 1.0         |

* S. aureus FhuD2.

* E. coli FhuD values have been previously reported (14, 31).

Effect of Ferrichrome on the Solution Structure of FhuD1—

Many binding proteins undergo a large shape change upon interaction with their cognate ligand; in contrast, FhuD2 only undergoes a very small conformational change (14). To assess the effect of ligand binding on the conformation of FhuD1, we used SAXS to directly measure the change in conformation of FhuD1 when it binds ferrichrome, the siderophore for which we used SAXS to directly measure the change in conformation of FhuD1 when it binds ferrichrome, the siderophore for which

![SAXS curves](image)

**FIG. 3.** Binding of hydroxamate siderophores by FhuD1 enhances its resistance to proteolysis. Lane 1, molecular mass markers; lane 2, 2 μg of purified FhuD1; lanes 3–10, 2 μg of FhuD1 protein incubated for 30 min in the presence of protease K at 55 °C after 15 min of preincubation with water (lane 3), 8.0 mM FeCl₃ (lane 4), aerobactin (lane 5), coprogen (lane 6), Desferal™ (lane 7), ferrichrome (lane 8), pyoverdine (lane 9). Ferrated siderophores were all used at a concentration of 5 mM. FhuD1 is marked with an arrowhead.

**FIG. 4.** Effect of ferrichrome on the structure of FhuD1. To measure ligand-induced structural changes in FhuD1, small-angle X-ray scattering data were collected from a solution of FhuD1 in either the presence or absence of ferrichrome. A, Guinier plots are shown for FhuD1 in either the absence (top curve, with filled circles) or presence (bottom curve, with filled triangles) of ferrichrome. In each case, the complete SAXS data are represented by the dashed curve, while the range of data used to calculate a least-squares linear fit (solid thin line) is indicated by the circles or triangles. The slope of the Guinier plot is related to the radius of gyration (*Rg*), which is 2.05 nm for the unliganded protein, and 2.01 nm for the ferrichrome-bound protein. B, complete SAXS curves for FhuD1 in either the absence (solid curve) or presence (dashed curve) of ferrichrome. The upper curve in this panel is the difference between the ferrichrome-bound and free proteins, as a percentage of the signal from the ferrichrome-bound protein. The change in the curve when ferrichrome is bound is consistent with the smaller slope of the Guinier plot, and indicates a small contraction of FhuD1 upon binding ferrichrome.

FhuD1 has the highest affinity. SAXS data can be used to obtain the radius of gyration (*Rg*), which is defined as the root mean square distance of all atoms from their common center of mass; as such, the *Rg* provides a measure of the overall shape of the particle, and ligand-induced changes in the *Rg* indicate changes in conformation. The *Rg* is measured using a Guinier plot (27), and Fig. 4A shows Guinier plots for FhuD1 in either the absence or presence of ferrichrome. Linear Guinier plots, such as those obtained for FhuD1, indicate that the protein is...
monodisperse in solution. From these plots, we obtained an \( R_g \) of 2.05 nm for the unliganded form of the protein, and 2.01 nm for the ferrichrome-bound form. This decrease in \( R_g \) of 0.04 nm is a relatively minor change, and is discernable only because of the very high quality of the SAXS data; nevertheless, it indicates that the protein undergoes a contraction upon binding ferrichrome. For comparison, maltose-binding protein (MBP) undergoes a large conformational change that has been well characterized by x-ray crystallography (28, 29), and the \( R_g \) of MBP shows a decrease of \( \sim 0.1 \) nm when it binds maltose in solution (30).

To further characterize the shape change in FhuD1, in Fig. 4B we have plotted the entire SAXS curve for the ligand-free and ferrichrome-bound forms of FhuD1. In addition, a curve is provided that plots the difference between the ferrichrome-bound protein and the ligand-free protein, as a percentage of the signal obtained from the ferrichrome-bound protein. There are large differences between the two curves in the higher angle region, \( 0.3 < S < 0.5 \) nm\(^{-1}\); however, because of the very weak scattering from the protein at these higher angles, this region of the SAXS curve is not reliable, as can be seen from the noisiness in the data. On the other hand, there is a significant difference in the curves in the range \( 0.1 < S < 0.3 \) nm\(^{-1}\). Consistent with the \( R_g \) values obtained, this region of the curve shows a ligand-induced transition from a relatively extended to a more globular conformation. This change is qualitatively similar, but much less pronounced, to what is seen with MBP.

We have previously characterized FhuD2 using SAXS (14). A comparison of the SAXS data from both FhuD2 and FhuD1 is illustrated in Fig. 5. The solution x-ray scattering from the proteins, in both the liganded and unliganded states, is almost identical; in fact, the only significant differences occur at relatively high scattering angles, \( 0.3 < S < 0.5 \) nm\(^{-1}\). Although these differences may be due to minor structural alterations, the high degree of correspondence between the curves in the region \( 0.03 < S < 0.3 \) nm\(^{-1}\) indicates that the overall fold and shape of the two proteins will be identical, despite a sequence identity of only 41%.

**FhuD1 Provides Only Marginal Benefit to S. aureus Provided with Hydroxamate Siderophores as Sole Sources of Iron—**Our results indicate that FhuD1, like FhuD2, is a receptor for ferric hydroxamate siderophores and is able to promote transport of siderophores into the cell. This led us to ask the question of whether the presence of FhuD1 is biologically advantageous to *S. aureus*. To address this question, we used the siderophore plate bioassay with decreasing concentrations of siderophore and compared the size of growth halos obtained for RN6390 wild-type strain and its *fhuD1::Km* (H430) and *fhuD2::Tet* (H364) derivatives; the results of these experiments are summarized in Table II. Deletion of FhuD1 had no significant effect on the ability of the cells to use any of the siderophores, indicating that it confers no advantage to cells when they are expressing FhuD2. In contrast, deletion of FhuD2 resulted in cells that would not grow on aerobactin or coprogen, and required relatively high concentrations of ferrichrome and Desferal™ for growth. The inability of coprogen, even at high concentrations, to support the growth of a strain not expressing FhuD2 was somewhat surprising given it bound to FhuD1 with moderate affinity (\( K_d \) 6.7 \( \mu \)M). More interesting, however, were the results obtained for ferrichrome, given the similar high affinity of FhuD1 and FhuD2 for this siderophore. These differences in growth are apparently not caused by differences in protein expression levels because by Western blot analysis we detected similar levels of FhuD1 and FhuD2 in wild-type RN6390 (data not shown). These results suggest that not only is affinity for the siderophore playing a role in the growth phenotype, but that interactions between liganded FhuD1 and the FhuBGC2 complex are likely poorer than those of liganded FhuD2 with the membrane transport complex. Taken together, these results support the conclusion that FhuD2 is the more effective receptor for ferric hydroxamate transport in *S. aureus* and demonstrate that the mere presence of a high affinity-binding protein (e.g. FhuD1) does not necessarily result in efficient transport.

**DISCUSSION**

The ability to acquire iron from the surrounding environment is critical to the growth of virtually all bacteria. *S. aureus* imports ferric iron associated with hydroxamate-type siderophores through the Fhu (ferric hydroxamate uptake) system. The Fhu system in many *S. aureus* strains is composed of
five proteins: FhuD1, FhuD2, and FhuCBG. The FhuCBG proteins represent components of a traffic ATPase (FhuB and FhuG are integral membrane proteins and FhuC has signature sequences of an ATPase) that is essential for the utilization of hydroxamate-type siderophores in \textit{S. aureus} (13). The \textit{fhuD2} gene encodes an iron-regulated lipoprotein that possesses high affinity for hydroxamate-type siderophores and the affinity of FhuD2 for some siderophores is much greater than that of \textit{E. coli} FhuD (14, 31). In this communication, we have expanded our understanding of the Fhu system in \textit{S. aureus} by characterizing the FhuD1 protein and establishing its role in the transport process. We have confirmed that FhuD1 is, indeed, a second lipoprotein in \textit{S. aureus} that acts as a receptor for ferric hydroxamate complexes, but that it functions to a lesser extent than FhuD2 in this capacity.

Although we do not have direct experimental evidence, we hypothesize that both FhuD1 and FhuD2 interact with the FhuBGC\textsubscript{2} complex, with contacts in the extracellular membrane loops of FhuB and FhuG. Indeed, a \textit{S. aureus} RN6390 derivative carrying a transposon insertion in \textit{fhuG} is incapable of transporting ferric hydroxamates (13), ruling out the possibility of two transporters that would interact with the individual FhuD1 and FhuD2 proteins. Although the similarity shared between FhuD1 and FhuD2 is limited to \textasciitilde{}50\%, the structures of FhuD1 and FhuD2 are probably extremely similar, which would be expected given their similar functional roles. Indeed, all residues that were mutated in FhuD2 in an earlier study (14), that gave rise to mutants that demonstrated a ferric hydroxamate transport deficiency, are conserved in FhuD1.

FhuD1 binds aerobactin with much lower affinity (\(K_D\) 40 \(\mu\)M) than FhuD2 (\(K_D\) 0.3 \(\mu\)M), providing an explanation for the observed differences in siderophore utilization in bioassays for strains expressing only FhuD1 or FhuD2, and also explains the results of protease protection assays in that aerobactin was able to protect FhuD2 but not FhuD1 from degradation by proteinase K. An interesting observation was that although the affinities of FhuD1 and FhuD2 for ferrichrome are essentially equivalent, the growth-promoting ability of ferrichrome on a strain expressing FhuD2 is much more pronounced than on an isogenic strain expressing only FhuD1. These observations lend support to the hypothesis that FhuD2 interacts with the FhuBGC\textsubscript{2} transport complex more efficiently than does FhuD1. However, an alternative explanation would suggest that perhaps FhuD2 binds ligand in a slightly different orientation that allows for better recognition by, or interaction with, the FhuBGC\textsubscript{2} transport complex. We had previously speculated that the siderophore itself forms part of the surface that is recognized by the membrane transporter (14).

ABC transporters involved in the transport of siderophores, heme, and vitamin B\textsubscript{12} cluster into a distinct family, and only a few bacterial species lack representatives of this transporter family (5). This family of proteins distinguishes itself from the majority of characterized binding proteins because the structure of proteins within this family lack the classic fold where the two domains of the bilobate structure are linked by a flexible hinge region that is comprised of \(\beta\)-strands at the base of the binding pocket. Instead, the two domains of the bilobate FhuD family of proteins are connected by a single polypeptide chain that forms an inflexible \(\alpha\)-helix, spanning the length of the protein. This structure is thought to result in an inability of these proteins to undergo large scale conformational changes upon ligand binding. Indeed, high resolution structures of \textit{E. coli} BtuF and \textit{Treponema pallidum} TroA, in both the liganded and unliganded forms, indicate this to be the case (32–34).

Our results indicate that, in solution, both FhuD1 and FhuD2 do not demonstrate a ligand-induced, large scale conformational change, in agreement with high resolution crystal structures of other members of this binding protein family. The conformational change associated with ferrichrome binding by FhuD1 resembles that associated with the binding of vitamin B\textsubscript{12} by BtuF, determined by x-ray crystallography (32). In contrast, we previously found that the binding of Desferal\textsuperscript{TM} by FhuD2 was accompanied by an extremely small conformational change (14), and our results from this study show a larger conformational change in FhuD1 when it binds ferrichrome. There are two possibilities that would explain these differences. The first is that the two siderophores produce different changes in protein conformation when they bind; in other words, the conformational change induced in both FhuD1 and FhuD2 may be larger when the proteins bind ferrichrome compared with Desferal\textsuperscript{TM}. In support of this possibility, Guinier curves of FhuD2 indicate that the unliganded protein has an \(R_g\) of 2.06 nm, while ferrichrome- and Desferal\textsuperscript{TM}-bound FhuD2 have \(R_g\) values of 2.03 and 2.05 nm, respectively. A second possibility is that the conformational change in FhuD2 when it binds Desferal\textsuperscript{TM} was underestimated: the SAXS data for the FhuD2- Desferal\textsuperscript{TM} interaction were recorded using several different camera lengths, and therefore there were more data processing and merging steps required to produce

| Table II | Growth promoting ability of hydroxamate siderophores on \textit{S. aureus} |
|----------|-------------------------------------------------|
| Siderophore | Concentration (in \(\mu\)M) | FhuD1 | FhuD2 | FhuBGC\textsubscript{2} |
| Aerobactin | \(3^\circ\) | 100 | 0 | 0 | 0 |
| H430 | 50 | 0 | 0 | 0 | 0 |
| H364 | 0 | 0 | 0 | 0 | 0 |
| Coprogen | \(3^\circ\) | 100 | 0 | 0 | 0 |
| H430 | 50 | 0 | 0 | 0 | 0 |
| H364 | 0 | 0 | 0 | 0 | 0 |
| Desferal\textsuperscript{TM} | \(2^\circ\) | 100 | 0 | 0 | 0 |
| H430 | 50 | 0 | 0 | 0 | 0 |
| H364 | 0 | 0 | 0 | 0 | 0 |
| Ferrichrome | \(2^\circ\) | 100 | 0 | 0 | 0 |
| H430 | 50 | 0 | 0 | 0 | 0 |
| H364 | 0 | 0 | 0 | 0 | 0 |

\(^{a}\) Values in gray bars indicate concentration (in \(\mu\)M) of siderophore.

\(^{b}\) Represents mean diameter (in mm) of growth halo; discs are 6 mm.
the final composite SAXS curve. In contrast, the SAXS data for the FhuD1-ferrichrome interaction were recorded at a single camera length, allowing a more direct comparison between unliganded and ferrichrome-bound FhuD1. In principle, these issues could be resolved by additional SAXS experiments but, in practice, since the conformational changes are very small, only high resolution crystal structures will provide a definitive answer to this question. Nevertheless, that such a muted conformational change can result in a protein form that is more resistant to proteolytic digestion is interesting and suggests that the ligand may protect a protease accessible site or loop structure within the binding pocket. This protection has now been demonstrated for three FhuD family members, E. coli FhuD (35) and S. aureus FhuD2 (14) and FhuD1 (this study).

Whereas fhuD2 and fhuCBG genes are present in all S. aureus strains whose genomes have so far been sequenced, the fhuD1 gene is not universally present (Fig. 1). In an attempt to provide an evolutionary rationale for this curious observation, we have utilized the phylogeny of sequenced S. aureus strains. The phylogeny suggests two distinct groupings (Fig. 6; one represented by the genome of MRSA252, and the other by the genomes of COL, Mu50, N315, NCTC8325, MW2, and MSSA476 (36). This phylogeny is consistent with comparative analysis of genome sequence data that indicates MRSA252 is distinct from other S. aureus isolates. The distribution of paralogous fhuD1 and fhuD2 genes in sequenced genomes suggests that these genes arose by a gene duplication event, but that the timing of this event is uncertain in the absence of more information concerning the distribution of fhuD1 genes in S. aureus isolates. One scenario possibly the gene duplication event after the divergence of the two groups, on the lineage leading to the COL group (Fig. 6). Alternatively, the gene duplication may have pre-dated the divergence of known S. aureus isolates. This scenario, however, would require that the fhuD1 gene was subsequently lost in the lineage leading to MRSA252. For either scenario, genomic and phylogenetic data also indicate that the fhuD1 gene was lost from the lineage leading to N315 (Fig. 6). This loss may have been the result of a deletion mediated by mobile genetic elements. Indeed, fhuD1 is in close proximity to mobile genetic elements such as a bacteriophage and a genomic island (Fig. 1C).

Analysis of genome sequence data reveals that the fhuD1 gene has also been subject to lateral transfer within the S. aureus population after the gene duplication event. This is particularly evident from the sequence of the Mu50 genome as an integrase gene exists near the fhuD1 gene, and both genes are present on a genomic island. Furthermore, in all cases where fhuD1 is present, it lies adjacent to the mu1944 homolog, which is also absent from the genomes of strains that lack the fhuD1 coding region. This linkage of fhuD1 and mu1944, and their association with mobile DNA, strongly suggests that two genes are transferred between genomes as a unit.

Comparative genome analysis has lead to the suggestion that ~75% of the S. aureus genome is composed of “core” components that are associated with central metabolism or housekeeping functions (36). The remaining 25% of the genome is composed of “accessory” genes, usually virulence factors or antibiotic resistance genes, which are subject to frequent lateral transfer by various mechanisms. Presumably, these accessory genes provide a selective advantage to S. aureus in certain environments. It is tempting to speculate that fhuD1 is one such gene, as it is present on a genomic island. Evidence obtained previously (12, 14), in addition to the data presented in this communication, demonstrate functional redundancies between the FhuD1 and FhuD2 proteins. A similar observation was made in the utilization of hydroxamate-type siderophores by B. subtilis (37) and oligopeptide transport in Streptococcus thermophilus and Streptococcus pneumoniae (38, 39). The apparent redundancy within these systems may evoke a selective advantage in diverse microbial communities. Although our data suggest that FhuD1 is the more functionally relevant binding protein, our evidence is based solely on data derived from experiments performed in the laboratory with a small subset of hydroxamate siderophores. However, the retention of FhuD1 in various S. aureus genomes suggests that it likely serves an enhanced role in the transport of untested hydroxamate siderophore derivatives. In contrast, the FhuD1-ferrichrome interaction was recorded at a single log, which is also absent from the genomes of strains that lack the FhuD1-ferrichrome interaction were recorded at a single log, which is also absent from the genomes of strains that lack the FhuD1-ferrichrome interaction were recorded at a single log, which is also absent from the genomes of strains that lack the FhuD1-ferrichrome interaction.
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