Molecular Characterization of the Iron-Hydroxamate Uptake System in *Staphylococcus aureus*

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Received 23 October 2000/Accepted 19 November 2000

To investigate iron uptake, a chromosomal locus containing three consecutive open reading frames, designated *fhuC*, *fhuB*, and *fhuD*, was identified in *Staphylococcus aureus*. Whereas the *fhuC* gene encodes an ATP-binding protein, *fhuB* and *fhuD* code for ferrichrome permeases and thus resemble an ATP-binding cassette transporter. A *fhuB* knockout mutant showed impaired uptake of iron bound to the siderophores but not of ferric chloride, suggesting that this operon is specific for siderophore-mediated iron uptake.

Iron is an essential element for the growth of living cells, and although abundant in nature, under aerobic conditions and at neutral or alkaline pH it is insoluble and thus is not readily available (12, 13). Moreover, in host tissues, iron remains tightly bound to high-affinity iron-binding proteins, such as ferritin, transferrin, lactoferrin, hemoglobin, and iron-sulfur proteins. Because only 10^{-18} M iron is in the free form (8, 12), a concentration not sufficient to sustain bacterial growth, bacteria have evolved various scavenging systems to acquire iron from the environment (6, 11). These systems utilize either cell surface molecules that make direct contact with the host iron-binding proteins (17, 19) or low-molecular-weight molecules, called siderophores, that show a higher affinity for iron than the host iron chelators. The iron-siderophore transport mechanisms are well studied in gram-negative bacteria (6), particularly in *Escherichia coli*, but are understudied in gram-positive bacteria, including staphylococci. Here we report the cloning and characterization of a ferrichrome uptake operon, *fhu*, with three consecutive open reading frames (ORFs), designated *fhuC*, *fhuB*, and *fhuD*. We also show that the *fhuB* null mutation results in reduced ferrichrome uptake.

Staphylococcus aureus strains were grown in tryptic soy broth or defined medium (21), whereas *E. coli* was grown in Luria-Bertani broth with appropriate antibiotics. The iron-limited and iron-rich defined media were prepared by adding 10 μ M dipyrrolyl (Sigma, St. Louis, Mo.) and 10 μ M Fe(III), respectively (4). DNA isolation, cloning, and transformations were performed by standard methods (14, 16). DNA probes were labeled with the Prime-a-Gene labeling system (Promega Corp., Madison, Wis.). The DNA restriction and modification enzymes were obtained from Promega. DNA sequences were determined with an ABI Prism 310 automated sequencer (Perkin-Elmer, Foster City, Calif.), and the sequence data were analyzed by BLAST (1).

The *S. aureus* genome database at the University of Oklahoma (<http://www.genome.ou.edu/staph.html>) revealed the presence of a DNA fragment with strong homology to the *fhuC* gene of *Bacillus subtilis*. The partial *fhuC* sequence was used as a probe to identify an operon in *S. aureus* consisting of three consecutive ORFs (Fig. 1) with an array of GATAAT upstream sequences analogous to the so-called Fur box. The first ORF of this operon, *fhuC*, is homologous to the *fhuA* gene of *B. subtilis* (58% identity; 76% similarity) and the genes encoding ATP-binding proteins of other bacterial iron transport systems. Based on a hydropathy analysis that suggests a cytoplasmic location for this protein and its similarity to the ATP-binding proteins, the protein seems to belong to the family of the ATP-binding cassette transporters. The second and third genes of this operon are homologous to the *fhuB* (43% identity; 67% similarity) and *fhuG* (40% identity; 64% similarity) genes of *B. subtilis*, respectively. They are similar to ferrichrome permeases of other bacteria and thus belong to the FecCD family of membrane transport proteins (17).

To determine the role of the *fhu* operon, we constructed a mutation in the *fhuB* gene as described earlier (22). A 6.7-kb *Hind*III fragment containing the three reading frames was cloned in the vector pTZ18R (10). A 3.2-kb *Eco*RV fragment was deleted from this construct, and then a 1.5-kb kanamycin cassette was inserted at the unique *Kpn*I site of the *fhuB* gene. The 4.8-kb *Hind*III fragment with the disrupted *fhuB* gene was subcloned into the *Hind*III site of the shuttle vector pBT2 (3), which cannot replicate in gram-positive bacteria above the nonpermissive temperature. Approximately 10 μ g of this construct was electroporated into *S. aureus* RN4220 cells, and the transformants were selected on tryptic soy agar plates containing 100 μ g of kanamycin/ml and 10 μ g of chloramphenicol/ml. The transformants were then grown at 43°C, and the chloramphenicol-sensitive clones were checked by Southern blotting and PCR techniques for the replacement of the native *fhuB* gene with the disrupted gene. The mutation in the *fhuB* gene was subsequently transduced into *S. aureus* 8325-4 with a phage 80 α lysate using the method described by Novick et al. (15). To complement mutation in *trans*, the 6.7-kb *Hind*III fragment containing the entire *fhu* operon was cloned into the pCU1 shuttle vector (2) and transferred into the *fhuB* mutant strain.

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FIG. 1. Organization of the *S. aureus* *fhu* operon. The directions of three ORFs, the location of the Fur box, and restriction endonuclease sites are shown. *fhuB* was disrupted by insertion of a kanamycin gene (Kan). The abbreviations for restriction sites are as follows: E, *EcoRV*; H, *HindIII*; K, *KpnI*.

Iron uptake by *S. aureus* strain 8325-4, its *fhuB* mutant, and the *fhuB* mutant complemented with the native gene, were performed as described earlier with slight modifications (9, 17). Cells were grown overnight at 37°C in iron-free defined medium (iron in the medium was chelated by adding 100 μ M dipyrindyl), diluted in 50 ml of fresh iron-free defined medium, and grown to an optical density at 580 nm of 0.6 to 0.7. The cells were subsequently harvested and washed twice with transport buffer (1 g of KH_2PO_4 , 2 g of $(\text{NH}_4)_2\text{SO}_4$, 4 g of NaCl, 17.9 g of tricine, 5 mg of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3 mg of CaCl_2 , 5.7 mg of nitrilotriacetate, and 10 g of glucose in a total volume of 1 liter, pH 8.0). The cells were resuspended in transport buffer to an optical density of 1.0 at 580 nm. Radiolabeled iron purchased as $^{59}\text{FeCl}_3$ in 0.1 N HCl from Amersham Pharmacia Biotech, Inc. (Piscataway, N.J.) [specific activity, 697 MBq/mg of Fe(III)] was used to prepare a 500-fold stock of siderophore (0.2 mM) to give a metal/ligand ratio of 1:10. A 5.0-ml cell suspension was placed in a 50-ml disposable tube to which labeled siderophore was added to a final concentration of 0.4 μ M. Samples (0.5 ml) were taken out at specific time points (0, 5, 10, and 15 min) and filtered through presoaked 0.45- μ m-pore-size filters. The filters were washed with 20 ml of ice-cold transport buffer containing 20 nM FeCl_3 and air dried, and the radioactivity was counted with a Beckman scintillation counter. These experiments were performed in triplicate. The iron uptake studies were also carried out by adding $^{59}\text{FeCl}_3$ to a final concentration of 0.04 μ M to the same density of cells in the transport buffer as described above.

In these assays, the *fhuB* mutant strain showed an impaired ferrichrome-iron uptake compared to the wild-type bacterium (Fig. 2A). However, the *fhuB* mutant complemented with the wild-type gene was able to import iron-ferrichrome to the normal levels (Fig. 2A). In the studies involving radiolabeled FeCl_3 directly, the wild type, the *fhuB* mutant, and the complemented *fhuB* mutant strains acquired iron with roughly similar efficiencies (Fig. 2B), suggesting that the *fhu* operon is specific for ferrichrome-utilizing iron uptake. However, the overall growth kinetics of the mutant did not show any appreciable change compared to those of the wild-type bacterium whether grown in defined medium, defined medium lacking iron, or complex medium (data not shown). This might be supportive of the multiple reports of a very low requirement for iron in *S. aureus* growth (5, 7, 20).

FIG. 2. Ferric ion uptake by *S. aureus* 8325-4 (Δ), *S. aureus* *fhuB* mutant ($\square$), and *S. aureus* *fhuB* mutant complemented ($\blacklozenge$) strains. The uptake studies were carried out as described in the text. The values are averages of three independent experiments. (A) Iron (^{59}Fe)-ferrichrome complex was used as a substrate in the transport study. (B) Ferric chloride (^{59}Fe) was used as a substrate in the uptake study.

Recently, Sebulsky et al. (18) reported a transposon Tn917 insertional mutant in *S. aureus* with impaired uptake of the ferric hydroxamate complexes. Further characterization of this mutant suggested that the transposon had inserted into the same chromosomal locus reported earlier (23) and characterized in this manuscript. Their transposon insertion mutant is in the *fhuG* gene (18), which is *fhuD* (the third ORF of the *fhu* operon) in our nomenclature. It is therefore important to note that mutations in both the permease homologues *fhuB* (present study) and *fhuD* (18) result in similar phenotypes of impaired iron hydroxamate uptake.

In conclusion, we have identified an *S. aureus* operon with three genes that constitutes a transport system which specifically utilizes siderophores to trap iron from the environment. Further characterization of the genes of this locus might help devise strategies to control iron metabolic pathways and in turn to design effective control mechanisms against this important human pathogen.

Nucleotide sequence accession number. The nucleotide sequence reported here has been submitted to GenBank under accession number AF132117.

We are thankful to Anthony Otsuka and Stephanie Coates for critical reading of the manuscript.

This work was supported by a grant from the National Institutes of Health-AREA to R.K.J.

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