Molecular characterization of the ferric-uptake regulator, Fur, from *Staphylococcus aureus*

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Iron is an essential nutrient for the survival and pathogenesis of bacteria, but relatively little is known regarding its transport and regulation in staphylococci. Based on the known sequences of ferric-uptake regulatory (*fur*) genes from several Gram-positive and Gram-negative bacteria, a fragment containing the *fur* homologue was cloned from a genomic library of *Staphylococcus aureus* RN450. Nucleotide sequence analysis of this fragment revealed the presence of a 447 bp ORF that encodes a putative 149 aa polypeptide with an apparent molecular mass of 17 kDa. A putative ferrichrome-uptake (*fhu*) operon, containing the conserved Fur-binding sequences (Fur box) in the promoter region, was also cloned from the same *S. aureus* library. To characterize the impact of Fur on the *fhu* operon, *fur* was cloned, overexpressed as a His-tagged protein and purified by Ni²⁺-affinity column chromatography. The recombinant protein was digested with enterokinase to remove the His tag. Electrophoretic mobility-shift assays indicated that Fur binds to the promoter region of the *fhu* operon in the presence of divalent cations. Fur also interacted with the promoter region of the recently reported *sir* operon that has been proposed to constitute a siderophore-transport system in *S. aureus*. The DNase I-protection assay revealed that Fur specifically binds to the Fur box located in the promoter region of the *fhu* operon. The primer-extension reaction indicated that the transcription-start site of the *fhu* operon was located inside the Fur box. *S. aureus fur* partially complemented a *fur*[−] mutation in *Bacillus subtilis*. The data suggest that Fur regulates iron-transport processes in *S. aureus*.

Keywords: Fur, iron, metal resistance, *Staphylococcus aureus*

INTRODUCTION

Iron plays an essential role in cellular metabolism, toxicity and pathogenesis of bacteria. When colonizing a host, pathogenic bacteria must compete with the host for the available nutrients. Iron in the host tissue normally remains bound to the host proteins such as ferritin, transferrin, lactoferrin, haemoglobin and iron-sulfur proteins, and only a minor fraction ($\sim 10^{-18}$ M) is available in the free form (Guerinot, 1994; Neilands, 1995; Trivier & Courcol, 1996). This concentration is too low to support bacterial growth within the host. To

overcome such an iron deficiency during infection, bacteria have developed various iron-scavenging systems to acquire iron from the host (Guerinot, 1994; Courcol *et al.*, 1997; Modun *et al.*, 1998). In addition to physiological functions in bacteria, iron levels also regulate different virulence determinants such as toxins, enzymes and adhesins. Several iron-regulated virulence factors have been identified in a number of pathogenic bacteria that include iron-repressible outer-membrane proteins in *Escherichia coli*, *Enterobacter cloacae*, *Erwinia* spp., *Klebsiella mobilis*, *Neisseria gonorrhoeae*, *Pseudomonas* spp., *Salmonella typhimurium*, *Shigella flexneri*, *Vibrio* spp. and *Yersinia* spp. (Litwin & Calderwood, 1993a; Dorman, 1994; Crosa, 1997). The haemolysin of *Vibrio cholerae*, diphtheria toxin of *Corynebacterium diphtheriae*, Shiga toxin of *Shigella dysenteriae* and Shiga-like toxin of *E. coli* are also

Abbreviations: EMSA, electrophoretic mobility-shift assay; HTH, helix-turn-helix.

The GenBank accession numbers for the *S. aureus fur* gene and *fhu* operon reported in this paper are AF118839 and AF132117, respectively.

iron-regulated toxins (Lindsay & Riley, 1994). However, iron is toxic for bacterial growth at greater than physiological levels. In particular, iron is notorious in its ability to catalyse the formation of hydroxyl radicals that can cause cellular death (Halliwell & Gutteridge, 1984). Therefore, iron uptake must be tightly regulated to avoid the toxic effects of iron overaccumulation (Braun, 1997).

Intracellular iron concentrations in many bacteria have been reported to be under the control of a ferric-uptake regulator, Fur (Guerinot, 1994; Crosa, 1997). Fur also regulates a variety of iron-dependent cellular processes, such as the acid-shock response (Foster, 1991) and the oxidative-stress response (Tardat & Touati, 1993) and it also regulates the genes involved in the biosynthesis and uptake of several siderophores (Guerinot, 1994). However, the acid-shock response has been reported to be normal in a *fur*⁻ mutant of *Salmonella typhimurium* that was no longer able to sense iron in the environment (Hall & Foster, 1996). Fur has also been reported to regulate metabolic pathways. In *E. coli*, it regulates the genes involved in purine, pyrimidine and methionine biosynthesis (Stojiljkovic *et al.*, 1994).

In Gram-positive bacteria, several iron-dependent repressors have been reported. The DtxR of *C. diphtheriae* and IdeR of *Mycobacterium tuberculosis* show limited homology to the Fur protein of Gram-negative bacteria (Tao *et al.*, 1994; Schmitt *et al.*, 1995). In *Bacillus subtilis*, three distinct Fur homologues designated Fur, Zur and PerR, regulating iron uptake, zinc uptake and the peroxide-stress response, respectively, have been reported (Bsat *et al.*, 1998; Gaballa & Helmann, 1998). The presence of a DtxR homologue, SirR, has been detected in several staphylococcal species (Hill *et al.*, 1998) and a Fur-like protein has been reported in *Staphylococcus epidermidis* (Heidrich *et al.*, 1996).

One of the mechanisms facilitating iron uptake involves several small molecules called siderophores that chelate iron from the host and deliver it into the bacterial cells through specific uptake systems (Guerinot, 1994; Neilands, 1995). Three different siderophores, aureochelin (Courcol *et al.*, 1997), staphyloferrin A (Meiwes *et al.*, 1990) and staphyloferrin B (Haag *et al.*, 1994), have previously been identified in staphylococci. However, very little is known about the mechanism by which these siderophores transport iron into the cells. The cloning of a novel iron-regulated operon consisting of three genes, *sitA*, *-B* and *-C*, encoding an ATPase, a cytoplasmic membrane protein and a lipoprotein, respectively, has been reported in *S. epidermidis* (Cockayne *et al.*, 1998). Whether this transport system is involved in either siderophore- or transferrin-mediated iron uptake in *S. epidermidis* remains to be determined. Recently, a locus from *S. aureus* containing three ORFs with a high sequence homology to the siderophore-acquisition genes of *Erwinia chrysanthemi* was identified (Heinrich *et al.*, 1999). The first ORF, which encodes a membrane-associated siderophore-binding protein SirA, is preceded by a sequence of 19 bp with dyad symmetry,

similar to the Fur boxes of Gram-negative bacteria. It is thus believed that the biosynthesis and uptake of the siderophores among staphylococci are regulated by Fur (Trivier & Courcol, 1996; Cockayne *et al.*, 1998; Heinrich *et al.*, 1999) but there is no direct evidence to support this hypothesis.

Here we report the cloning of a ferric-uptake regulatory gene (*fur*) and a putative high-affinity ferrichrome-uptake operon (*fhu*) from a genomic library of *S. aureus*. For functional analysis, *fur* was overexpressed in *E. coli* and purified to near homogeneity. Electrophoretic mobility-shift and DNase I footprinting assays revealed that Fur specifically interacted with the *fhu* promoter region and therefore possibly regulates the expression of the *fhu* operon in *S. aureus*.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* strains were grown on tryptic soy agar or broth, whereas *E. coli* and *B. subtilis* strains were grown on Luria-Bertani agar or broth at 37 °C. When necessary, ampicillin (50 µg ml⁻¹), kanamycin (30 µg ml⁻¹) or chloramphenicol (10 µg ml⁻¹) were added to the media. The iron-limited minimal media were prepared by incubating the minimal media with Chelex 100 (Sigma) as described by Hill *et al.* (1998).

DNA manipulations. DNA isolation, cloning and transformations were performed using standard methods (Sambrook *et al.*, 1989; Novick, 1990). For DNA amplification, PCRs were performed under the following conditions: initial denaturation of the DNA templates at 94 °C for 3 min (denaturation was for 30 s in subsequent cycles), annealing at 52 °C for 30 s and extension at 72 °C for 1 min in a DNA thermocycler for 30 cycles. DNA probes were randomly labelled by using the Rediprime DNA labelling system (Amersham Life Science). Plasmids were purified using the QIAprep spin miniprep kit (Qiagen) and DNA fragments from agarose gels were purified using the QIAquick gel extraction kit. The DNA restriction and modification enzymes were obtained from Promega. DNA sequences were determined with an ABI Prism 310 genetic analyser system (Perkin Elmer). DNA sequence data were analysed using BLAST and multiple alignments were performed using CLUSTAL W (Thompson *et al.*, 1994).

Cloning of the ferric-uptake regulatory gene, *fur*, and a putative *fhu* operon of *S. aureus*. Based on the DNA sequences of the *fur* gene, specifically of *B. subtilis* and *E. coli*, two oligonucleotide primers were designed using the program CODHOP (Rose *et al.*, 1998). These primers were later modified to reflect the codon usage bias in *S. aureus*. The forward primer used from the highly conserved helix-turn-helix (HTH) domain was 5'-GGCTTGGCGACAGTATACAGC-3' and the reverse primer from the metal-binding region was 5'-ACATTCCATACATACTAAATGATG-3'. PCR was performed using *S. aureus* RN450 chromosomal DNA as the template. The amplified PCR product was sequenced to determine its potential homology with *fur* genes of other bacteria and was later used as a probe to screen the genomic library of *S. aureus* RN450 to clone *fur*.

For functional characterization of the Fur protein, we searched for *fhu*-operon-like sequences in the *S. aureus* genome. The

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Description	Reference
Bacterial strains		
<i>S. aureus</i>		
8325-4	Cured of prophage	Novick (1990)
<i>E. coli</i>		
Jm109	<i>recA1 supE44 endA1 hsdR17</i> <i>gyrA96 relA1 thi Δ(lac-proAB)</i> <i>F'(traD36 proAB⁺ lacI^qZΔM15)</i>	Yanish-Perron <i>et al.</i> (1985)
BL21(DE3)(pLysS)	<i>F⁻ ompT hsdS_B(r_B⁻ m_B⁻) gal dcm</i> (DE3), Tet ^r Cam ^r	Novagen
BL21(DE3)(pLysS)/pSA- <i>fur</i>	BL21(DE3)(pLysS) containing pSA- <i>fur</i> , Tet ^r Cam ^r Amp ^r	This study
<i>B. subtilis</i>		
HB1000	ZB307A attSPβ	Bsat <i>et al.</i> (1998)
HB6543	HB1000 <i>fur::kan^r</i>	Bsat <i>et al.</i> (1998)
HB6543/pCUI	HB6543 containing pCUI1, Kan ^r Cam ^r	This study
HB6543/pCUI- <i>fur</i>	HB6543 containing pCUI1- <i>fur</i> , Kan ^r Cam ^r	This study
Plasmids		
pRSETa	Overexpression vector, Amp ^r	Invitrogen
pSA- <i>fur</i>	pRSETa with <i>fur</i> gene cloned at <i>Bam</i> HI and <i>Hind</i> III sites, Amp ^r	This study
pCR2.1	PCR cloning vector, Amp ^r and Kan ^r	Invitrogen
pCR2.1- <i>fur</i>	pCR2.1 containing 1.9 kb fragment containing <i>fur</i>	This study
PCU1	Shuttle vector, Amp ^r Cam ^r	Augustin <i>et al.</i> (1992)
pCU1- <i>fur</i>	Carries a 1.9 kb fragment containing <i>fur</i> , Amp ^r Cam ^r	This study
PCU1-pfhu	Carries the promoter region of the putative operon <i>fhu</i> , Amp ^r	This study

fhu operon in *B. subtilis* is regulated by Fur (Bsat *et al.*, 1998; Mademedis & Koster, 1998). A fragment with strong homology to the *fhuC* gene of the *fhu* operon of *B. subtilis* was found in the *S. aureus* genome database of the University of Oklahoma's Advanced Center for Genome Technology (www.genome.ou.edu/staph.html). This fragment was PCR amplified and used as a probe to screen the *S. aureus* genomic library. A representative cosmid clone was subsequently sequenced by gene walking to locate the entire *fhu* operon.

Cloning, overexpression and purification of Fur. Two oligonucleotide primers (upstream 5'-GGATCCTTGAAGAAGATTA-3') and (downstream 5'-AAGCTTTTCTATCCTTTACCTT-3') were designed to incorporate a *Bam*HI restriction site at the 5' end and a *Hind*III site at the 3' end of the gene during the amplification of *fur* (restriction enzyme sites underlined). The PCR product was cloned into pCR2.1 (Invitrogen), and then digested with *Bam*HI and *Hind*III. The fragment corresponding to the *fur* coding region was gel purified and subcloned into the *Bam*HI and *Hind*III sites of pRSETa (Invitrogen) to generate plasmid pSA-*fur*. pSA-*fur* was used to transform *E. coli* BL21(DE3)(pLysS). The overnight culture of these transformants was used to inoculate 500 ml fresh LB. At an OD₆₀₀ of 0.4, the cells were induced for the expression of Fur by the addition of 2.5 mM IPTG for 2.5 h. All subsequent procedures were performed at 4 °C

unless stated otherwise. The cells from the culture were harvested and resuspended in 5 ml 50 mM Tris/HCl buffer (pH 7.0). Cell suspensions were subjected to sonication (seven pulses of 30 s spaced 30 s apart, with settings at an output control of 5 and 50 % duty cycle) with a Sonifier Cell Disrupter (Branson Ultrasonic). Cell debris and unbroken cells were removed by centrifugation (30000 g for 15 min at 4 °C). The supernatant was applied to a nickel-charged agarose affinity column and eluted with 200–400 mM imidazole using the Xpress Purification System (Invitrogen). Eluted fractions were subjected to a 15 % SDS-PAGE analysis. Fractions containing the overexpressed His-tagged Fur were pooled and dialysed in a dialysis cassette with molecular-mass cut off of 7 kDa (Pierce) against the dialysis buffer (10 mM MgCl₂, 0.1 mM DTT, 5 %, v/v, glycerol in 20 mM Tris/HCl, pH 7.9). The enterokinase Cleavage Capture Kit (Novagen) was used to cleave the histidine tag from this preparation. A second round of chromatography on a Ni²⁺-affinity column retained the histidine tag peptide and any undigested recombinant protein and the His-tag-free Fur was recovered from the flow-through fraction. The final Fur protein was stored in 20 mM Tris/HCl, pH 7.0, containing 50 mM NaCl, 2 mM CaCl₂ and 33 %, v/v, glycerol, at -20 °C for future use.

Electrophoretic mobility-shift assays (EMSAs). These were performed as described by de Lorenzo *et al.* (1988). Two

oligonucleotide primers were synthesized to amplify the *fhu* promoter from the genomic DNA of *S. aureus*. In the forward primer, an *Xba*I site was incorporated (5'-CGCG-TCTAGAAATTTCCGTACT-3') and a *Kpn*I site was incorporated in the reverse primer (5'-CGTCAGGTACCATG-GTATGAAG-3') (restriction enzyme sites are underlined). The *sir* promoter was also amplified from the genomic DNA of *S. aureus* using two primers (5'-TGGACGGCATACTA-AATCGTGA-3' and 5'-GCTAGTCTAGAGTACCCATTG-CATGTT-3'). The *Xba*I restriction enzyme site in the reverse primer is underlined. The amplified *fhu* and *sir* promoter DNAs were digested with *Xba*I and then end labelled with [α -³²P]dCTP by the fill-in reaction using the Klenow fragment of DNA polymerase I. The standard binding assays contained (in a total volume of 20 μ l) 1 μ g BSA, 1 μ g sheared calf thymus DNA and various concentrations of Fur protein in 1 $\times$ binding buffer (100 μ M MnCl₂, 1 mM MgCl₂ and 25 mM KCl in 10 mM Tris-borate, pH 7.5). The reaction mixture was incubated at 25 °C for 15 min. The diluted end-labelled DNA was added to the reaction mixture and incubated for an additional 20 min at 25 °C. Three microlitres of loading buffer (0.1% cyanol blue and 40% sucrose in DNA-binding buffer) was added and 10 μ l of the mixture was loaded onto a 5% nondenaturing PAGE. Electrophoresis was carried out at 90 V for 2.5 h using 10 mM Tris-borate, pH 7.5, containing 100 μ M MnCl₂ as electrode buffer. The gels were dried and the DNA-protein complexes were visualized by autoradiography.

DNase I-protection assay. A DNA fragment containing the *fhu* promoter region was amplified as above and the amplified DNA fragment was cloned into pCR2.1, resulting in the plasmid pCR2.1-p*fhu*. A 500 ml culture of *E. coli* JM109 containing the above construct was grown and plasmid DNA was isolated using Wizard Plus Maxi Prep Columns (Promega). The isolated DNA (200 μ g) was digested with *Eco*RI and subsequently with *Xba*I. The fragment representing the *fhu* promoter was gel purified and the *Xba*I overhang was end-labelled and used for the DNase I-protection assays essentially as described by Leblanc & Moss (1994) with a slight modification described by Singh *et al.* (1999). First, the binding reaction was performed in a total volume of 50 μ l of the 1 $\times$ binding buffer. The binding assay contained 1 μ g BSA, 20 ng end-labelled DNA and varying concentrations of Fur. After 20 min incubation, 50 μ l cofactor solution (10 mM MgCl₂, 5 mM CaCl₂) and 0.4 units of DNase I (Sigma) were added and incubated for an additional 2 min at room temperature. DNase I activity was terminated by the addition of 100 μ l stop solution (1% SDS, 200 mM NaCl, 20 mM EDTA, pH 8.0). The reaction products were extracted with phenol/chloroform, precipitated with 2.5 vols 100% ethanol and air dried. The DNase I-digested fragments were then analysed on a 5.0% polyacrylamide sequencing gel. To determine the exact area protected, manual nucleotide sequencing reactions of the *fhu* promoter fragment were carried out in parallel to the DNase I-footprint fragments, using the primer 5'-CTAGAAATTTCCCTACTTTC-3'.

Primer extension. Total RNA from *S. aureus* RN450 grown in iron-limited minimal media was isolated with the TRI reagent kit (Molecular Research Center). The primer-extension assay was performed as described previously (Xiong & Jayaswal, 1998), using an oligonucleotide primer (5'-TTCATAATTTCCCTACTTTC-3') specific to the *fhuC* coding region. A sequencing ladder generated by using the same primer on pCR2.1-p*fhu* was coelectrophoresed to determine the position of the transcription-start site.

Complementation of the *fur*⁻ mutation in *B. subtilis*. A ~1.9 kb fragment containing the entire *fur* gene was amplified

using *S. aureus* RN450 genomic DNA as the template. The primers used were 5'-GGTGCAGTTGCTGTTTGTGC-3' and 5'-AGTGGCGTAACGTATGTGGC-3'. The *fur* gene was cloned into pCR2.1 and then subcloned into the *Eco*RI site of the shuttle vector pCU1. To test whether the *S. aureus* Fur complemented the *fur*⁻ mutants, the resulting plasmid pCU1-*fur* was transferred by electroporation into the *fur*⁻ strain of *B. subtilis* HB6543. The complementation test in *B. subtilis* HB6543 was performed by measuring the siderophore production (OD₅₁₀) against the culture density (OD₆₀₀), as described by Chen *et al.* (1993) and Bsat *et al.* (1998).

RESULTS AND DISCUSSION

Cloning and nucleotide sequence analysis of the *fur* gene of *S. aureus*

As described in Methods, two primers were designed from the highly conserved HTH and metal-binding domains of the *fur* genes of many bacteria. PCR with these primers using *S. aureus* RN450 chromosomal DNA as the template resulted in the amplification of a fragment of about 170 bp. The amplified fragment showed significant sequence homology to the *fur* gene of *B. subtilis* (Bsat *et al.*, 1998). A cosmid clone showing strong hybridization with this PCR fragment was identified from the *S. aureus* library. Nucleotide sequencing of a 1900 bp region of the DNA isolated from this cosmid clone revealed the presence of an ORF of 447 bp, designated *fur*. The ribosome-binding sequence (AGTAGG) was found 13 nt upstream of the presumed translation-start codon TTG. Possible -35 (TTAACT) and -10 (TGTAAT) sequences were also identified. Downstream of the *fur* stop codon, there is an inverted repeat (GCGTAGGTAA and TTAACCTT-CGC), followed by a T-rich region which may function as a transcription-termination structure (Fig. 1a). The presence of an ORF (a *xerD* homologue) immediately downstream of the *fur* gene indicates that this gene may have an independent transcription mechanism. In Gram-negative bacteria, the *fur* gene is preceded by a Fur box and is auto-regulated. However, the presence of such a box has not been reported in the upstream regions of the *fur* gene in Gram-positive bacteria. Incidentally, *S. aureus fur* also lacked the Fur box in the upstream region.

The predicted *M_r* and the pI of Fur were 17 224 and 5.78, respectively. As shown in Fig. 1(b), *S. aureus* Fur appeared to be closely related to the Fur of *B. subtilis* (73% identity) and more distantly related to the Fur of Gram-negative bacteria [~30% identity with Fur of *Vibrio vulnificus* (Litwin & Calderwood, 1993b) and of *Pseudomonas aeruginosa* (Prince *et al.*, 1993)]. An additional Fur homologue has been described in the literature in *S. epidermidis* (Heidrich *et al.*, 1996). However, this protein is distantly related to *S. aureus* Fur and is more closely related to *B. subtilis* Zur, which is implicated in regulation of zinc transport (Gaballa & Helmann, 1998).

The hydropathy profile revealed *S. aureus* Fur to be a hydrophilic protein, suggesting a cytoplasmic location. The analysis of the C terminus of Fur indicated the

Fig. 1. (a) The downstream sequence of *S. aureus fur* followed by the *xerD* gene (indicated by white arrows). The presumed -35 and -10 promoter sequences for the *xerD* gene are underlined, while the potential ribosome-binding site (RBS) is indicated in bold type. The inverted repeat, followed by a T-rich region, probably forms a *fur* transcription-termination structure. (b) Alignment of the deduced amino acid sequences of the Fur polypeptide of *S. aureus* (S.a) with that of *E. coli* (E.c), *B. subtilis* (B.s), *S. epidermidis* (S.e), *Vibrio vulnificus* (V.v) and *Pseudomonas aeruginosa* (P.a) by the CLUSTAL W program described by Thompson *et al.* (1994). The total number of residues is shown on the right. White letters in black boxes indicate positions conserved in five or more of six aligned sequences. Black letters in grey boxes indicate position conserved in four out of six aligned sequences. The arrows indicate the location of primers for PCR cloning of *fur*. The consensus sequences of HTH are underlined. The conserved metal-binding motif (HXHHH) is marked by asterisks. The two motifs (CXXC) that coordinate metal binding are also indicated.

Fig. 2. (a) The promoter sequence followed by schematic organization of the *S. aureus fhu* operon containing three ORFs, *fhuC*, *fhuB* and *fhuD*. The potential ribosome-binding site (RBS), -35 and -10 regions of the promoter sequence are underlined. The translation-initiation codon is indicated in bold type. The box indicates the conserved Fur-binding sequences (Fur box). (b) Alignment of the consensus 'Fur box' sequences of *E. coli* (de Lorenzo *et al.*, 1988) with the *dhh* (Rowland *et al.*, 1996) and *fhu* (Bsat *et al.*, 1998) operons of *B. subtilis*, and the *fhu* and *sir* (Heinrich *et al.*, 1999) operons of *S. aureus*. The differences are highlighted in black.

presence of a conserved metal binding domain (HXHHH) and motifs (CXXCG and CXXC) that coordinate the binding of metal ions. The N terminus contains an HTH DNA-binding motif common among

most of the metalloregulatory proteins (Lam *et al.*, 1994; Cook *et al.*, 1998; Pohl *et al.*, 1999). These secondary-structure-associated features suggest that *S. aureus* Fur is a metal-responsive regulatory protein.

Fig. 3. Overexpression and purification of His-tagged Fur. Crude extracts and purified recombinant proteins were analysed on a 12.5% SDS-polyacrylamide gel. Lane 1, standard protein markers; lane 2, uninduced lysate of *E. coli* cells carrying pSA-*fur*; lane 3, induced lysate of *E. coli* cells carrying pSA-*fur*; lane 4, proteins unbound on the nickel column; lane 5, the fraction containing the purified His-tagged Fur.

Cloning of a putative ferrichrome uptake (*fhu*) operon from *S. aureus*

The ferrichrome-uptake system (*fhu*) in *B. subtilis* has been reported to be regulated by Fur (Bsai *et al.*, 1998; Mademidis & Koster, 1998). To determine whether Fur

regulated such a ferrichrome-uptake system in *S. aureus* as well, we cloned a 4 kb fragment containing the *fhu* operon from the cosmid library into pTZ18R. Nucleotide sequence analysis of this fragment revealed three ORFs designated *fhuC*, *fhuB* and *fhuD* (Fig. 2a). *fhuC* showed significant sequence homology with *fhuC* of *B. subtilis*, encoding an ATP-binding protein. *fhuB* and *fhuD* showed significant homologies with many bacterial ferrichrome-transport permeases. In *fhuC*, the ribosome-binding sequence (AGTAGG), was found 6 nt upstream of the proposed translation-initiation codon ATG (Fig. 2a). Possible -35 (TAGTCA) and -10 (TATAAT) sequences were also identified. Three imperfect repetitions of the sequence GATAAT (analogous to the so-called Fur box) were found upstream of *fhuC*. This sequence is identical to the consensus *E. coli* Fur box sequence and to the Fur box of the *dhb* operon of *B. subtilis* except for 2 bp (Fig. 2b) (Rowland *et al.*, 1996; Escolar *et al.*, 1998). However, it differed at five nucleotide positions from the recently identified Fur box upstream of *sirA* in *S. aureus* (Fig. 2b) (Heinrich *et al.*, 1999).

Overproduction and purification of Fur

As described in the Methods, the *fur* gene was cloned in the pRSETa vector and transferred to an *E. coli* overexpression strain. A His-tagged Fur was overproduced (Ramadurai *et al.*, 1999; Singh *et al.*, 1999) from a *tac* promoter by induction with IPTG. The His-tagged Fur was purified by a Ni²⁺-affinity column and it migrated as a protein of approximately 21 kDa (Fig. 3). Fractions containing the overproduced protein were pooled, dialysed and digested with enterokinase. A second round of nickel column chromatography yielded a protein that migrated as 18 kDa protein, unlike the

Fig. 4. (a) EMSAs of the *fhu* promoter in the presence of Fur. All the lanes contained 2 ng end-labelled *fhu* promoter DNA. Lanes 2–6 contained 0.1, 0.2, 0.4, 0.6 and 0.8 µg Fur, respectively. Lane 7 contained 0.4 µg Fur and 10 ng non-labelled *fhu* promoter. Lanes 8 and 9, same as lane 6 with 1 and 2 mM EDTA, respectively. F and C indicate the free and Fur bound *fhu* promoter DNA bands, respectively. (b) EMSAs of the *sir* promoter in the presence of Fur. All the lanes contained 2 ng end-labelled *sir* promoter DNA. Lanes 4 and 5 contained 0.4 and 0.6 µg Fur, respectively. Lanes 1 and 2 contained 0.4 µg Fur and 10 and 5 ng of non-labelled *sir* promoter DNA, respectively. F and C indicate free and Fur bound *sir* promoter DNA bands, respectively.

Fig. 5. Effect of various metal ions on the binding of Fur to the *fhu* promoter. All lanes contained 2 ng end-labelled *fhu* promoter. Lanes 2–10 contained 0.4 μ g Fur. Various metal ions [Mn^{2+} (lane 2), Fe^{2+} (lane 3), Zn^{2+} (lane 4), Co^{2+} (lane 5), Ni^{2+} (lane 6), Cu^{2+} (lane 7), Cd^{2+} (lane 8), Hg^{2+} (lane 9) and Fe^{3+} (lane 10)] were added to a final concentration of 100 μ M in the reaction mixture. F and C indicate free and Fur bound *fhu* promoter DNA bands, respectively.

His-tagged Fur that migrated as a 21 kDa protein. No residual undigested protein was detected in an SDS-PAGE gel, which suggested our Fur preparation to be free of the histidine tag (data not shown).

Fur binds to the promoter regions of the *fhu* and *sir* operons

EMSAs were used to examine the specific interaction of Fur to the Fur boxes upstream of *fhu* and *sir* promoters. Binding of Fur to the *fhu* (Fig. 4a) and *sir* promoter region (Fig. 4b) resulted in decreased migration of both the promoter fragments relative to the free probe on a 5% non-denaturing polyacrylamide gel. To confirm the specificity of binding of Fur with the *fhu* promoter, we challenged radiolabelled DNA with a fivefold excess of non-labelled promoter DNA. Prebound Fur exchanged with non-radiolabelled promoter DNA in the presence of large excess of non-specific calf thymus DNA (Fig. 4a, lane 7; Fig. 4b, lanes 1 and 2), demonstrating that Fur binds specifically to the *fhu* and *sir* promoter regions. While optimizing the conditions for the EMSAs, we found the presence of 100 μ M Mn^{2+} to be critical for the formation of promoter–repressor complexes. When 1–2 mM EDTA was added in the reaction mixture, the promoter–Fur complexes were not seen, as EDTA chelated Mn^{2+} present in the binding assay (Fig. 4a, lanes 8 and 9).

To test if other metal ions could replace Mn^{2+} in the formation of the Fur–*fhu* promoter complex, the same molar concentration (100 μ M) of Cd^{2+} , Co^{2+} , Cu^{2+} , Fe^{2+} , Fe^{3+} , Hg^{2+} , Ni^{2+} and Zn^{2+} was used in place of Mn^{2+} . While Co^{2+} , Cu^{2+} and Ni^{2+} could replace Mn^{2+} , other ions such as Cd^{2+} , Fe^{3+} , Hg^{2+} and Zn^{2+} did not interact with Fur. Fe^{2+} was not as effective in the binding reaction, probably because of its oxidation to Fe^{3+} (Fig. 5). These results are similar to those reported for *E. coli* where Fur interacted with several other divalent cations (Bagg & Neilands, 1987). In *S. epidermidis*, a DtxR like protein, SirR, is also reported to

Fig. 6. Mapping of the Fur-binding site on the *fhu* promoter. Lane 1, 20 ng end-labelled *fhu* promoter DNA digested with 0.4 units DNase I for 2 min. Lanes 2–5 same as lane 1 with 1.0, 2.0, 4.0 and 6.0 μ g Fur, respectively. Lanes C, G, T and A represent dideoxy sequencing ladder of the *fhu* promoter used for DNase I protection (only relevant area is shown). The nucleotide sequence of the protected region is marked on the right.

be regulated by Fe^{2+} and Mn^{2+} , but Co^{2+} , Cu^{2+} , Mg^{2+} , Ni^{2+} and Zn^{2+} did not interact with this repressor (Hill *et al.*, 1998).

DNase I-protection assay

The binding of Fur to the *fhu* promoter protected an area encompassing 31 nt from the DNase I action (Fig. 6). Interestingly, this protected area contained the nucleotide sequences conserved for the Fur box in several bacteria (Crosa, 1997). Thus, Fur specifically interacts with the Fur box located upstream of the *fhu* promoter.

Mapping of the *fhuC* transcription-start site

To define the transcription-start site precisely, the 5' end of the *fhuC* transcript was mapped. A 20 base oligonucleotide primer specific to the coding region was

Fig. 7. Mapping of the transcription-start site of the *S. aureus fhuC* gene. Total RNA isolated from *S. aureus* RN450 grown in iron-limited minimal media was hybridized with an oligonucleotide complementary to the mRNA of *fhuC* locus and extended by avian myeloblastosis virus (AMV) reverse transcriptase (lane P). Lanes A, T, G and C correspond to a dideoxy sequencing reaction performed with the same primer. The sequences encompassing the initiation start (marked by asterisks) are shown on the left.

Fig. 8. Complementation analysis. The production of siderophores by *B. subtilis* HB1000, HB6543, HB6543/pCU1-*fur* and HB6543/pCU1 strains were assayed as described by Chen *et al.* (1993). The white bars represent the ratios of OD₅₁₀/OD₆₀₀ in the iron-limiting minimal media and the black bars indicate the ratios in iron-rich minimal media. The data represent the mean of three independent experiments.

annealed with total RNA and extended in a primer-extension assay. The transcription-start site was located in the Fur box upstream of the *fhuC* gene (Fig. 7). The presence of two bands in Fig. 7 (lane P) may be due to an

alternative transcription-start site in *fhuC*, presence of the degraded mRNA product, or to secondary RNA structures.

Complementation of a *fur*⁻ mutant of *B. subtilis* with *S. aureus fur*

It has been reported that among Gram-negative bacteria, Fur not only shares sequence homology but also has similar structure and function (Stojiljkovic *et al.*, 1994). The *S. aureus fur* gene product showed significant sequence homology with the *fur* genes of *B. subtilis* and to some extent with that of *E. coli*. When *S. aureus fur* was introduced into a *fur*⁻ mutant of *B. subtilis* HB6543, it partially complemented the Fur function in that mutant (Fig. 8). The partial complementation data suggest that *S. aureus* Fur is able to identify Fur-box-like sequences and regulate the iron-dependent cellular processes in *B. subtilis*.

The results of this study indicate that Fur in *S. aureus* is likely to function as an iron-responsive repressor and modulates the expression of the *fhu* and *sir* operons. It seems that distinct metal-responsive regulators modulate the metal-ion transport and dependent metabolic processes in staphylococci. An iron-dependent DtxR-like repressor SirR, regulating the transcription of *S. epidermidis sit* operon, was recently identified, and the presence of this protein is widely distributed among staphylococci (Hill *et al.*, 1998). Besides, *S. aureus* also contains two additional Fur homologues, Zur and PerR, but these proteins are more closely related to Zur and PerR of *B. subtilis* (Bsat *et al.*, 1998). A Fur-like repressor in *S. epidermidis* has also been reported, however the biological function of that protein is yet to be determined (Heidrich *et al.*, 1996). In *S. aureus*, various operons such as the *cadC* system (Endo & Silver, 1995), the *znt* operon (Xiong & Jayaswal, 1998) and the *fur* operon, co-exist, which regulate the levels of various metal ions and their effects are overlapping. It is of utmost significance to determine why bacteria possess multiple metalloregulatory systems and how these different repressors coordinate their function *in vivo* to regulate metal-ion concentration.

In this study, we observed that Co²⁺, Cu²⁺, Mn²⁺ and Ni²⁺ metal ions also interacted with Fur *in vitro*, similar to other systems (Bagg & Neilands, 1987). This suggests that Fur may be regulating not only iron but the intracellular levels of other metal ions as well. However, the interaction of Fur with these cognate metal ions remains controversial. Bsat & Helmann (1999) have recently reported that *B. subtilis* Fur interacted very tightly both *in vitro* and *in vivo*, with the operator region of the *dhb* operon. Interestingly, the affinity of the Fur protein with the *dhb* operon was iron independent. Recently, Althaus *et al.* (1999) have reported that Fur purified from *E. coli* contained two molecules of zinc per Fur molecule. While the first zinc molecule could be easily detached from native Fur, the other could be detached only under denaturing conditions. While the two metalated forms of Fur were able to interact

with the operator–promoter regions, the apoprotein did not bind specifically to these regions.

In conclusion, *S. aureus* Fur is likely to function as an iron-responsive metalloregulatory protein that interacts with at least two operons, *fhu* and *sir*, in *S. aureus*. Currently we are constructing mutations in the *fur* gene, and also in the individual ORFs of the *fhu* operon to determine their biological functions. An understanding of these systems may provide potential targets to control the proliferation of this pathogenic bacterium.

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