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Expression of Multidrug Resistance Efflux Pump Gene *norA* Is Iron Responsive in *Staphylococcus aureus*

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Staphylococcus aureus **utilizes efflux transporter NorA to pump out a wide range of structurally dissimilar drugs, conferring low-level multidrug resistance. The regulation of *norA* expression has yet to be fully understood although past studies have revealed that this gene is under the control of the global transcriptional regulator MgrA and the two-component system ArlRS. To identify additional regulators of *norA*, we screened a transposon library in strain Newman expressing the transcriptional fusion *norA-lacZ* for altered β-galactosidase activity. We identify a transposon insertion in *fhuB*, a gene that encodes a ferric hydroxamate uptake system permease, and propose that the *norA* transcription is iron responsive. In agreement with this observation, addition of $FeCl_3$ repressed the induction of *norA-lacZ*, suggesting that bacterial iron uptake plays an important role in regulating *norA* transcription. In addition, a *fur* (ferric uptake regulator) deletion exhibited compromised *norA* transcription and reduced resistance to quinolone compared to the wild-type strain, indicating that *fur* functions as a positive regulator of *norA*. A putative Fur box identified in the promoter region of *norA* was confirmed by electrophoretic mobility shift and DNase I footprint assays. Finally, by employing a siderophore secretion assay, we reveal that NorA may contribute to the export of siderophores. Collectively, our experiments uncover some novel interactions between cellular iron level and *norA* regulation in *S. aureus*.**

The Gram-positive bacterium *Staphylococcus aureus* causes serious human infectious diseases worldwide (41). Antibiotic resistance has become a surging problem for the treatment of *S. aureus* infections. Resistance can be gained via drug target modification, drug inactivation, or drug export by efflux pumps (10). *S. aureus* encodes several multidrug resistance (MDR) efflux pumps, among which NorA has been extensively examined. NorA appears to export a variety of structurally unrelated drugs, such as fluoroquinolones, ethidium bromide, cetrimide, benzalkonium chloride, tetraphenylphosphonium bromide, and acriflavine (30, 35, 48, 49).

The transcription of *norA* has been shown to be regulated by the two-component system ArlRS and the global transcription regulator MgrA (25, 36, 62, 63). ArlRS are important for regulating factors involved in adhesion, autolysis, and proteolytic activity of *S. aureus*. Expression of *norA* increased in an *arlS* mutant strain (25). However, the detailed mechanism by which *arlRS* control *norA* expression is still unknown. MgrA tunes the expression of ~350 genes, including *arlRS* and *norA* (42). Overexpression of *mgrA* results in a 2.3-fold reduction of *norA* transcripts, indicating that *mgrA* is a negative regulator of *norA* (36). DNA repeats consisting of the TTAATT sequence have been suggested to be involved in the binding of MgrA to the *norA* promoter (25). Besides *arlRS* and *mgrA*, additional factors could affect *norA* expression; for instance, exposure of *S. aureus* to subinhibitory concentrations of fluoroquinolones induces *norA* transcription. However, the mediator(s) of this effect have not been elucidated (35).

As for almost all other bacteria, iron is an indispensable nutrient for *S. aureus*. Under iron deprivation conditions, *S. aureus* utilizes the iron-responsive transcriptional regulator Fur to activate iron acquisition and tune gene expression (5). *S. aureus* acquires iron through siderophore-dependent and heme-dependent mechanisms. *S. aureus* produces at least three siderophores such as staphyloferrin A, staphyloferrin B, and aureochelin (57). SfaABCD and SbnABCDEFGHI are involved in the biosynthesis of staphyloferrin A and staphyloferrin B, respectively (6, 15). *S. aureus* has also been demonstrated to carry at least four iron-regulated ABC transporter systems reported to import Fe(III)-siderophores composed of the exogenous hydroxamate-type siderophore transporter FhuABG (12, 54), staphyloferrin A transporter HtsABC (6), staphyloferrin B transporter SirABC (15), and SstABCD, whose substrate has yet to be identified (29). In addition, *S. aureus* possesses an iron-regulated surface determinant (Isd) heme uptake system including four surface proteins (IsdABCH) that are anchored in the cell wall, a transporter (IsdDEF), a sortase (SrtB), and two cytoplasmic proteins (IsdGI) (57).

In order to identify additional regulators of *norA*, *S. aureus* Newman transposon mutants were screened on the basis of their altered induction of a *norA-lacZ* reporter gene on agar plate. This study indicates an intimate link between iron concentration and *norA* expression. It reveals that the master iron uptake regulator Fur directly binds to the *norA* promoter and exerts an apo-Fur-dependent activation of *norA* expression. Our results suggest that NorA might be involved in secretion of potential siderophores in *S. aureus*.

MATERIALS AND METHODS

Bacterial growth conditions. The bacterial strains used in this study are listed in Table 1. *S. aureus* strain Newman is a human clinical isolate (22) that stably maintains an agr^+ phenotype, i.e., quorum-controlled toxin

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TABLE 1 Strains and plasmids

Strain or plasmid	Description	Source or reference
S. aureus strains		
RN4220	Restriction deficient, prophage cured	37
Newman	Clinical isolate	22
USA100	Clinical isolate	43
USA300	Clinical isolate	21
USA400	Clinical isolate	47
Newman *norA-lacZ*	*norA-lacZ* in Newman	14
fhuB(pTV1) strain	Tn*917* insertion in fhuB via pTV1	This study
fhuA(*bursa aurealis*) strain	Newman with Tn*917* insertion in *fhuA*	3
fhuB(*bursa aurealis*) strain	Newman with Tn*917* insertion in *fhuB*	3
fhuG(*bursa aurealis*) strain	Newman with Tn*917* insertion in *fhuG*	3
RN4220 *fur*::*tet*	*fur* deletion in RN4220	34
Newman *fur*::*tet*	*fur* deletion in Newman	This study
Newman *norA-lacZ fur*::*tet*	*fur* deletion in Newman *norA-lacZ*	This study
E. coli strains		
DH5		Stratagene
BL21		Invitrogen
Plasmids		
pTV1	For Tn*917* transposon screen	66
pYJ335	*S. aureus-E. coli* shuttle vector, Chl^r	33
pMCSG7	For His-tagged protein expression in *E. coli*	59
pYJ335::*fhuB*	pYJ335 containing *fhuB*	This study
pYJ335::*fur*	pYJ335 containing *fur*	This study
pMCSG7::*fur*	pMCSG7 containing *fur*	This study
pYJ335::*norA*	pYJ335 containing *norA*	This study

TABLE 2 Primers

Primer name	Sequence
Int1	CTATTCCTAAACACTTAAGAGAATTG
Int2	CATTGGTTTAGTGGGAATTTGTACC
Seq1	GGAGAGTATAAATTTGACTTG
Arb1a	GGCCACGCGTCGACTAGTACNNNNNNNNNNGTATA
Arb2	GGCCACGCGTCGACTAGTAC
Int3	CTCACAATAGAGAGATGTCACCGTC
fhuB-F	ATAGAGAGAACCCAACGCCA
fhuB-R	TCATATTGAGCGTCCTCCTT
norA-F	ATGAATAAACAGATTTTTGT
norA-R	CTACATATTTTGTTCTTTCA
norA-GS-F	TCAATCCCCTTTATTTTAATATGTCA
norA-GS-R	TCTTTTCCACGACAGATTGC
norA-FP-6FAM-F	6-FAM-TGGTCATCTGCAAAGGTTGT
norA-FP-R	TCTGTTTATTCATATGCTCACCTCTT
SAV2033F	CCTTTGCTTCAACTGTGTGC
SAV2033R	GTACTTGTAAAGCAATATTTACG
fur-F	TGAGAAAAGCTTGCATTTTATTGA
fur-R	TTGTTTCCATAACCACACCTCT
fur-EX-F	TACTTCCAATCCAATGCCTTGGAAGAACGATTAAATCGCGTTAAGC
fur-EX-R	TTATCCACTTCCAATGTTATCCTTTACCTTTAGCTTGGCACGTTTC
sfaD-F	CAATGCATCGCGAAAACTTA
sfaD-R	CGCCTAAATGGTTTTGAACG
sbnC-F	GAGGGAAGGGTGTCTAAGCA
sbnC-R	TCACCATTGGATTTGGTACG

secretion and the ability to cause animal disease (3, 50). *S. aureus* was cultured in tryptic soy broth ([TSB] Difco) with shaking at 250 rpm or on tryptic soy agar ([TSA] Difco) at 37°C. The recipe for chemically defined medium (CDM) was described previously (31). Residual free iron was chelated from CDM by addition of 1 μM ethylenediamine-*N*,*N*′-bis(2-hydroxyphenylacetic acid) (EDDHA). Ferric(III) chloride is dissolved in water and added into CDM. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth. When necessary, antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; nalidixic acid, 5 μg/ml; erythromycin, 10 μg/ml; chloramphenicol, 10 μg/ml.

pTV1 (Tn*917*-based) transposon screening. Plasmid pTV1 (66) was electroporated into *S. aureus* strain RN4220, purified (QIAprep spin minikit; Qiagen), and then electroporated into *S. aureus* Newman carrying the *norA-lacZ* reporter on the chromosome (14). Transposon screening was performed as previously described (7). The *norA-lacZ* strain containing pTV1 was grown overnight in TSB containing 5 μg/ml nalidixic acid at 30°C, and 10-fold dilutions were plated on TSA plates containing 10 μg/ml erythromycin ($TSA_{erm\text{-}10}$). Plates were incubated overnight at 43°C. A total of 20,000 colonies were screened for dark blue or white colony appearance by patching on $TSA_{erm\text{-}10}$ plates containing 100 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Colonies were picked and cultured in TSB overnight at 37°C. Glycerol was added to a final concentration of 25%, and the mutants were stored at −80°C.

Phage transduction and determination of transposon insertion sites. To avoid unrelated mutations that affect *norA-lacZ* expression, all the mutant *S. aureus* strains used in this study were lysed with phage 85, and transposon insertions were transduced into wild-type strain Newman. Tn*917* insertion sites were mapped by using arbitrary PCR primers (Table 2) and DNA sequencing as previously described (7).

β-Galactosidase measurements. *S. aureus* strains were grown at 37°C overnight in CDM without iron (CDM-Fe), diluted 100-fold in 2 ml of fresh CDM containing a different concentration of ferric(III) chloride, and incubated at 37°C with shaking at 250 rpm for 3 h (optical density at 600 nm [OD_{600}] of ~0.6). After being collected by centrifugation, the cells were suspended in AB buffer (100 mM potassium phosphate, 100 mM NaCl, pH 7.0) and treated with lysostaphin (0.1 μg/ml) at 37°C for 15 min. After the addition of 900 μl of ABT buffer (AB buffer containing 0.1% Triton X-100) to lysostaphin-treated cells, 50 μl of the cell lysate was mixed with 10 μl of MUG (4-methylumbelliferyl-β-D-galactopyranoside, 4 mg/ml; Sigma) and incubated at room temperature for 1 h. Then, 20 μl of the reaction mixture was mixed with 180 μl of ABT buffer, and the emission of fluorescence was measured (355-nm excitation and 445-nm emission). LacZ activity was normalized by cell density at 600 nm, and then the relative activity was calculated. The assay was repeated at least three times with similar results.

MIC measurements. MICs of norfloxacin were determined using a microdilution technique according to NCCLS guidelines in Mueller-Hinton broth. TSB was used as the growth medium.

Plate sensitivity assay. *S. aureus* strains were grown at 37°C overnight in TSB with appropriate antibiotics to ensure plasmid maintenance. Overnight cultures were diluted 100-fold into the same medium without antibiotics and grown at 37°C for ~3 h to reach an OD_{600} of ~0.6, and then the numbers of bacterial cells from all strains were normalized to approximately 5×10^8 CFU/ml with fresh TSB, followed by six 10-fold serial dilutions. Then, 10 μl of each strain of bacterial samples (from 10^{-1} to 10^{-6} dilutions) was spotted onto the TSA plates containing 0.8 μg/ml norfloxacin. All plates were incubated at 37°C for 24 h before being read. The assay was repeated at least for three times with similar results.

RNA isolation and Northern blotting. *S. aureus* strains were grown at 37°C overnight in TSB or CDM, diluted 100-fold in 5 ml of fresh TSB or CDM containing different concentrations of ferric(III) chloride, and incubated at 37°C with shaking at 250 rpm for 3 h (OD_{600} of ~0.6). Two volumes of the RNAprotect bacteria reagent (Qiagen) was added to 1 volume of cell culture and treated at room temperature for 30 min. Cell pellets were collected and lysed by following the enzymatic lysis procedure described in the RNAprotect

Bacteria Reagent Handbook (38) and with mechanical disruption (Fast Prep FP120 instrument; Qbiogene, Heidelberg, Germany). An RNeasy Mini Kit (Qiagen) was used for subsequent RNA purification with DNase I treatment. RNA concentration and purity were determined by reading absorbances at 260 and 280 nm. Northern blotting was performed as previously reported (38). Primers used for amplification of *norA*, *sbnC*, and *sfaD* coding regions are listed in Table 2. Each Northern analysis was repeated at least two times with similar results.

Construction of plasmids for complementation of *fhuB* mutant, *fur* deletion, and *norA* deletion. The shuttle plasmid pYJ335 (33) was used for complementation study. To construct pYJ335::*fhuB*, a 1-kb DNA fragment containing the gene NWMN_0617 was amplified from strain Newman genomic DNA by using the primers fhuB-F and fhuB-R. To construct pYJ335::*fur*, a 0.45-kb DNA fragment containing the gene NWMN_1406 was amplified by using the primers fur-F and fur-R. To construct pYJ335::*norA*, a 1.5-kb DNA fragment containing the *norA* gene was amplified by using the primers norA-F and norA-R. DNA fragments were cloned into pYJ335 as described previously (14). In this study, the primer pairs tetRFor399/fhuB-R and tetRFor399/fur-R were used to select the plasmid clones in which *fhuB* and *fur* are located downstream of the *xyl-tetO* promoter. The selected plasmid clones were sequenced to confirm that no additional mutations were introduced by PCR. The correct plasmids were transformed into RN4220 and then into the corresponding mutant strain by electroporation. The pYJ335 vector alone was transformed into RN4220 and then into the mutant strain and wild-type Newman as the control.

Fur protein purification. For expression of the full-length *S. aureus* Fur protein, we used the ligation-independent cloning (LIC) method (60). The coding region of Fur was PCR amplified from strain Newman chromosomal DNA with primers Fur-EX-F and Fur-EX-R, listed in Table 2. The PCR products were treated with T4 DNA polymerase in the presence of dCTP for 30 min at room temperature. Target vector pMCSG7 (59) was digested with SspI, gel purified, and then treated with T4 DNA polymerase in the presence of dGTP for 15 min at 16°C. The T4 DNA polymerase-treated plasmid vector and PCR product were gel purified, mixed, incubated for 5 min at room temperature, and then transformed into *E. coli* strain DH5α. The resulting plasmid was transformed again into BL21 Star(DE3) (Science Reagents, Inc.), and the transformants were selected on LB agar plates with 100 μg/ml ampicillin. The BL21 Star(DE3) strain carrying the plasmid was grown in LB medium to an optical density at 600 nm (OD_{600}) of 0.6, and then 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added. After overnight induction at 16°C, the cells were harvested and frozen at −80°C. The expressed protein was purified from the frozen cells with a HisTrap column (GE Healthcare, Inc.) by following the column manufacturer's recommendations. The purified protein was supplemented with 20% glycerol and stored at −80°C.

Colorimetric ferrozine assay. Iron concentrations in *S. aureus* were determined by the ferrozine assay developed by Riemer et al. (52). In order to measure staphylococcal intracellular iron concentration in CDM supplemented with different concentrations of $FeCl_3$, bacterial lysate was prepared by mechanical disruption (Fast Prep FP120 instrument; Qbiogene, Heidelberg, Germany) and then processed following the protocol by Riemer et al. (52). The assay was repeated at least three times with similar results.

Electrophoretic mobility shift assay (EMSA). DNA probes were PCR amplified using primers norA-GS-F and norA-GS-R (listed in Table 2) and then radiolabeled with T4 polynucleotide kinase (NEB) and [γ-^{32}P]ATP (Perkin-Elmer). The radioactive probe (2 ng) was mixed with various amounts of the Fur protein in 20 μl of gel shift loading buffer (24% glycerol, 40 mM Tris-Cl, pH 8.0, 150 mM KCl, 2 mM dithiothreitol [DTT], 600 μg/ml bovine serum albumin, and 50 ng of herring sperm DNA) (23). In order to obtain apo-Fur, recombinant Fur protein was treated overnight with 200 mM EDTA, 20 mM HEPES (pH 8.0), 20 mM NaCl, and 5% glycerol at 4°C, followed by buffer exchange (55). Either 200 μM manganese chloride ($MnCl_2$) or 200 μM EDTA was added to reaction mixtures containing Fur or apo-Fur, respectively (55). SAV2033 promoter DNA was used for the negative control. After being incubated at room temperature for 20 min, the samples were analyzed by 8% polyacrylamide gel electrophoresis (100 V for prerun; 85 V for 45 min for sample separation). The gels were dried and subjected to autoradiography on a phosphor screen (BAS-IP; Fuji). The assay was repeated at least three times with similar results.

Dye primer-based DNase I footprint assay. The DNase I footprint procedures were modified according to Zianni et al. (67). A 313-bp fragment that encompasses bases −300 to +13 of the promoter region from *norA* was generated by PCR with the primers norA-FP-6FAM and norA-FR (Table 2). About 50 ng of 6-carboxyfluorescein (FAM)-labeled *norA* promoter was incubated with various amounts of apo-Fur protein ranging from 0 to 8 μM in a binding buffer (24% glycerol, 40 mM Tris-Cl, pH 8.0, 150 mM KCl, 2 mM DTT, 600 μg/ml bovine serum albumin, and 50 ng of herring sperm DNA). After several optimization experiments, the DNase I digestion was found to work best with 0.05 Kunitz units of DNase I (New England BioLabs) per 20-μl reaction mixture for 5 min at room temperature. The reaction was stopped with 0.25 M EDTA and extracted with phenol-chloroform-isoamyl alcohol (25:24:1). The DNA fragments were purified with a QIAquick PCR Purification kit (Qiagen) and eluted in 15 μl of distilled water. About 5 μl of digested DNA was added to 4.9 μl of HiDi formamide (Applied Biosystems) and 0.1 μl of GeneScan-500 LIZ size standards (Applied Biosystems). The samples were analyzed with a 3730 DNA Analyzer, with a G5 dye set, running an altered default genotyping module that increased the injection time to 30 s and the injection voltage to 3 kV, in the sequencing facility at the University of Chicago. Results were analyzed with Peak Scanner software (Applied Biosystems). The assay was repeated at least three times with similar results.

Siderophore secretion assay. Siderophore secretion in the supernatant of cultures grown in iron-limiting CDM for 5 days was tested by classic chrome azurol S (CAS) assay as previously reported (16, 53, 56). Culture supernatants were mixed with equal volumes of CAS solution and allowed to interact for 30 min at room temperature. With CDM serving as the blank and deferoxamine mesylate (Desferal) as the reference standard, the A_{630} was recorded. Siderophore units were calculated by the following equation: (A_{630} of CDM − A_{630} of sample)/A_{630} of CDM × 100% (17). The assay was repeated at least three times with similar results.

RESULTS

Screening of a transposon library identifies that loss of *fhuB* affects expression of *norA*. *S. aureus* Newman carrying the chromosomal *norA-lacZ* reporter was subjected to Tn*917* mutagenesis using plasmid pTV1. In total, about 20,000 mutant clones were screened for altered *norA-lacZ* activity. White or dark blue clones that displayed altered LacZ activity on agar plates containing X-Gal were recovered. Transposon insertion sites in these candidate mutants were determined by two-stage arbitrary PCR (7), and the flanking sequences were searched against the *S. aureus* Newman genomic sequence (2).

Among the mutants we found 25 isolates forming dark blue colonies on the plate that carried the Tn*917* transposon in the same *fhuB* gene. The isolates were not isogenic as insertion occurred throughout the gene. FhuB has been characterized as a permease in a three-gene operon, *fhuABG*, involved in transporting hydroxamate siderophores (12, 54) (Fig. 1A). These results indicated that the loss of *fhuB* could alter *norA* expression. NorA is an efflux pump for quinolone antibiotics. Changed expression of *norA* resulted in altered MICs of quinolones (63). Consistent with our observation on enhanced expression of *norA-lacZ* in the *fhuB* mutant, the MIC of norfloxacin for the *fhuB* mutant was 2-fold higher than that for the wild-type bacteria (0.6 μg/ml) (Fig. 1B). This phenotype was further confirmed by the complementation

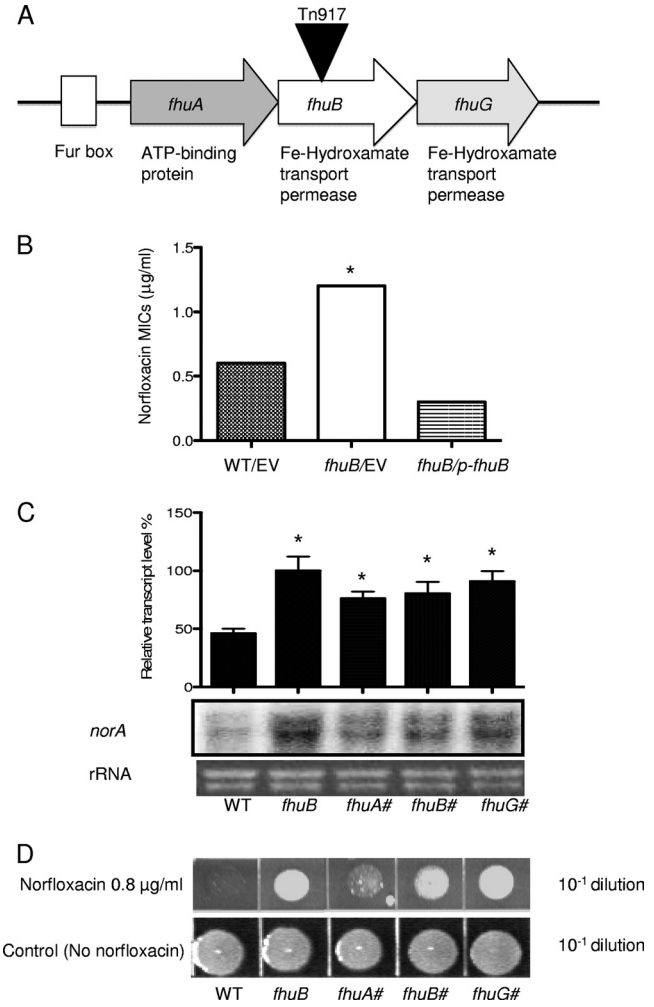

FIG 1 Identification of *fhuB* that is involved in the *norA* regulation. (A) Gene organization and Tn*917* transposon insertion in the *fhuABG* loci of *S. aureus*. (B) MIC of norfloxacin for strains in Mueller-Hinton broth supplemented with 10 μg/ml chloramphenicol. WT/EV, wild-type Newman strain containing pYJ335 empty vector; *fhuB*/EV, *fhuB* mutant containing pYJ335; *fhuB*/p-*fhuB*, *fhuB* complementation by pYJ335::*fhuB*. An asterisk indicates that the value in the *fhuB* mutant is statistically different from that of the other two strains as determined by a Student's *t* test ($P < 0.05$). (C) Mutations in the *fhuABG* operon resulted in enhanced expression of *norA*. Bacteria were grown in TSB until mid-log phase before RNA extraction. Total RNA was analyzed by RNA blotting with radiolabeled *norA* probe. The ethidium bromide-stained RNA gel indicates the loading of RNA samples. The *norA* transcript levels were quantified, and statistical analysis was performed. An asterisk indicates that the value is statistically different from that of the wild type as determined by a Student's *t* test ($P < 0.05$). #, mutants are from the Phoenix library (3). (D) Susceptibilities of strains (10^{-1} dilution) to 0.8 μg/ml norfloxacin on TSA plates. Control plates contained no antibiotics.

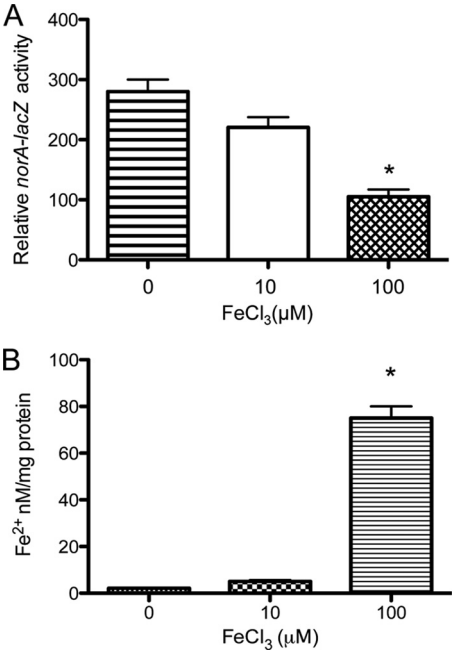

FIG 2 The *norA-lacZ* activity is iron responsive. (A) Transcription of *norA* is affected by iron. β-Galactosidase activity of the *S. aureus* strain containing the *norA-lacZ* reporter fusion was determined by MUG units (1 MUG unit = 1 pmol of MUG cleaved by β-galactosidase per min per OD_{600} unit). An asterisk indicates that the *norA-lacZ* activity in medium containing 100 μM $FeCl_3$ is statistically different from that of the control without iron supplement as determined by a Student's *t* test ($P < 0.05$). (B) Iron uptake by *S. aureus* Newman wild type in CDM supplemented with different concentrations of $FeCl_3$. An asterisk indicates that the value is statistically different from others as determined by a Student's *t* test ($P < 0.05$).

assay, in which overexpression of the wild-type *fhuB* gene in the *fhuB* mutant strain resulted in lower quinolone MICs (Fig. 1B).

Loss of *fhuA* or *fhuG* also affects *norA* expression. Since *fhuB* is the middle gene of the three-gene operon *fhuABG* involved in hydroxamate siderophore uptake, we suspected that the other two genes may also affect *norA* expression. In order to test the potential role of *fhuA* and *fhuG* in *norA* regulation, we obtained the corresponding *bursa aurealis* transposon insertion mutants from the Phoenix Library (3). As expected, all three *bursa aurealis* mutants displayed phenotypes similar to that of the Tn*917* insertion in *fhuB*. We performed Northern hybridization, and the results confirmed that inactivation of the *fhu* operon resulted in enhanced levels of *norA* transcription in TSB compared to levels of the wild-type bacteria (Fig. 1C). There was a doublet of *norA* transcript in the Northern blot, indicating that there are two different transcripts (Fig. 1C), which is echoed by two previous studies (26, 63). Furthermore, all mutants were more resistant to norfloxacin on plate assays than the wild-type parent (Fig. 1D). Taken together, these data indicated that loss of the *fhu* operon affects *norA* expression.

***norA* expression is iron responsive.** FhuABG have been well documented as a major uptake system for hydroxamate-type siderophores in numerous bacteria, including *E. coli* and *S. aureus* (12, 24, 54). Given that *fhuABG* are involved in iron uptake and that their mutants displayed enhanced *norA* expression and higher resistance to quinolones, we hypothesized that iron availability might influence *norA* expression. In order to test the role of iron in regulating *norA* expression, we measured the *norA-lacZ* activity over different concentrations of $FeCl_3$ in CDM. As expected, we observed a clearly decreasing level of *norA-lacZ* when we added increasing concentrations of $FeCl_3$ (0 to 100 μM) to the CDM (Fig. 2A). Meanwhile, the ferrozine assay showed that the intracellular iron concentration of *S. aureus* increased after cells were cultured in CDM with 100 μM $FeCl_3$ for 3 h (Fig. 2B). These results indicate that iron uptake represses *norA* expression in *S. aureus*. Consistently, Northern hybridization confirmed a reduction of *norA* transcripts when there was more iron in the environ-

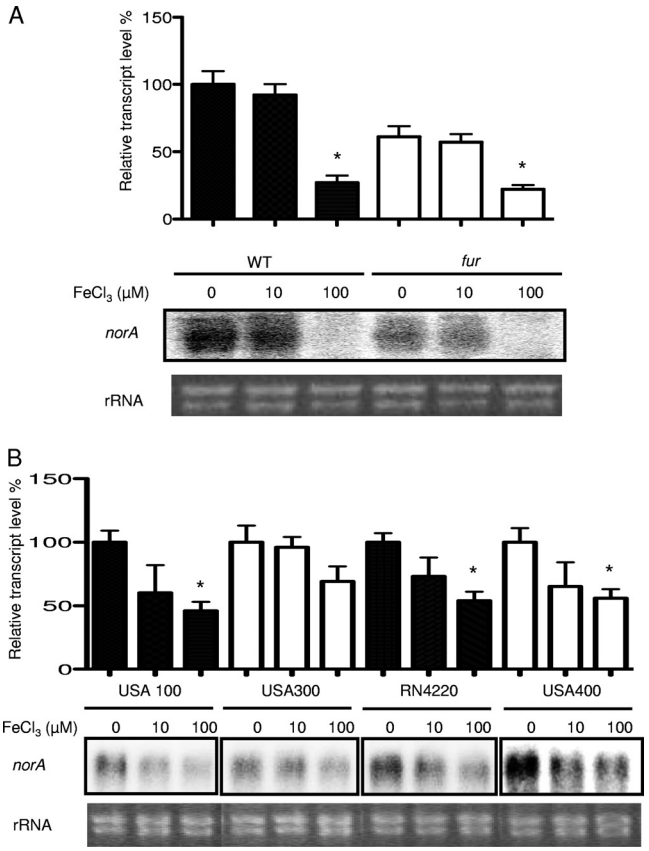

FIG 3 *norA* transcription is iron responsive. (A) Addition of iron reduced *norA* expression. Bacteria were grown in CDM containing different concentrations of Fe(III) until mid-log phase before RNA extraction. Total RNA was analyzed by RNA blotting with α-^{32}P-labeled *norA* probe. The ethidium bromide-stained RNA gel indicates the loading of RNA samples. The *norA* transcript levels were quantified, and statistical analysis was performed. An asterisk indicates that the values for the wild type (WT) and the *fur* mutant are statistically different from the value for the control without iron supplement as determined by a Student's *t* test ($P < 0.05$). (B) Addition of iron reduced *norA* expression in other *S. aureus* strains, such as USA100, USA300, RN4220, and USA400. Bacteria were grown in CDM containing different concentrations of Fe(III) until mid-log phase before RNA extraction. Total RNA was analyzed by RNA blotting with α-^{32}P-labeled *norA* probe. The ethidium bromide-stained RNA gel indicates the loading of RNA samples. The *norA* transcript levels were quantified, and statistical analysis was performed. An asterisk indicates that the value is statistically different from the control without iron supplement, as determined by a Student's *t* test ($P < 0.05$).

ment (Fig. 3A). Interestingly, we noticed a similar decrease of *norA* expression in the *fur* deletion background (that produces about half the amount of *norA* transcript compared to the wild type), suggesting that other factors are also involved in the repression of *norA* expression by iron uptake. In order to test if *norA* repression is also iron responsive in other *S. aureus* strains, we applied Northern hybridization to the USA100, USA300, RN4220, and USA400 strains. All tested strains displayed results similar to those of the Newman strain (Fig. 3B) although the changes of the *norA* expression levels in these new strains were less significant than the change in the Newman strain.

***fur* is a positive regulator of *norA*.** Since we showed that the expression of *norA* is iron responsive, we hypothesized that regulators for iron uptake may affect *norA* expression. The ferric uptake regulator protein Fur is the master regulator and plays a significant role in iron uptake and storage for a diverse number of bacteria (4, 13). In order to test the potential role of Fur on *norA* expression, we obtained a *fur*::*tet* strain in RN4220 (29) and transduced the allele into wild-type Newman *norA-lacZ*. LacZ assays revealed that the *fur* deletion strain displayed less than half of the *norA-lacZ* activity of the wild-type strain, indicating that *fur* is a positive regulator of *norA* expression (Fig. 4A). Consistent with this observation, we also observed a 2-fold reduction of the MIC of norfloxacin to the *fur* deletion strain compared to that for the wild-type Newman (Fig. 4B). These phenotypes of the *fur* deletion strain were successfully complemented by overexpressing *fur* with the *E. coli-S. aureus* shuttle vector pYJ335 in the deletion strain.

Apo-Fur directly binds to *norA* promoter that contains a putative Fur box. Since our genetic data indicated that Fur is a new positive regulator of *norA*, we were curious to test if Fur directly binds to the *norA* promoter. We purified a His_6-tagged full-length *S. aureus* Fur protein (purity of >90%) from *E. coli* grown in rich Luria broth (Fig. 5A). However, this recombinant Fur protein hardly bound to the *norA* promoter (Fig. 5B). It has been documented that iron-free apo-Fur protein has a different DNA-binding affinity and regulatory role from iron-bound Fur (19, 20). We speculated that only apo-Fur protein binds to the *norA* promoter. To test this possibility, apo-Fur was obtained by treating Fur with 200 mM EDTA overnight (55). EDTA was removed by buffer exchange, and the apo-Fur was used in the EMSA. The iron content of apo-Fur was confirmed to be less than 1%, as determined by the ferrozine assay. As expected, the EMSA showed that apo-Fur binds to the *norA* promoter efficiently; the complex can be dissociated in a competition reaction mixture containing 100× cold DNA (Fig. 5B). In addition, only a minor interaction was seen between a control probe (the SAV2033 promoter DNA) and apo-Fur protein at 8 μM, which indicates that the binding between apo-Fur and the *norA* promoter is specific (Fig. 5C).

In order to further confirm the binding site of Fur on the *norA* promoter, we performed a DNase I footprint assay by using dye primer sequencing on the Applied Biosystems 3730 DNA Analyzer (67). PCR was performed with a 6-FAM-labeled primer (Table 2, norA-FP-6FAM-F and norA-FP-R) to amplify a 313-bp *norA* promoter region. This PCR product was then incubated with 4 μM apo-Fur protein and then partially digested with DNase I. After a 5-min incubation, the reaction was terminated, and the product was analyzed on the 3730 DNA Analyzer with the default genotyping module and the G5 dye set. By comparing electropherograms with or without apo-Fur (Fig. 6A and B) using of Peak Scanner software (Applied Biosystems), we were able to uncover a specific apo-Fur-protected region (−163 to −130 away from ATG) on the *norA* promoter (Fig. 6C). Interestingly, a putative apo-Fur box (AATAGTGATAATTACAAAG, from −157 to −139) with seven mismatches (underlined) (Fig. 6D) was found in this protected region. The predicted putative apo-Fur box is located in the middle of the protected region that is upstream of the −35 and −10 consensus sequences (Fig. 6C), which is characteristic for positive regulation by transcription factors (8).

NorA may be involved in pumping out siderophores. We demonstrated that the multidrug efflux pump gene *norA* is under the control of *fur*, a master regulator of iron uptake and storage. However, we were puzzled by the intriguing relationship between iron uptake and *norA* in *S. aureus*. A group of studies has revealed that efflux pumps are involved in the secretion of siderophores in

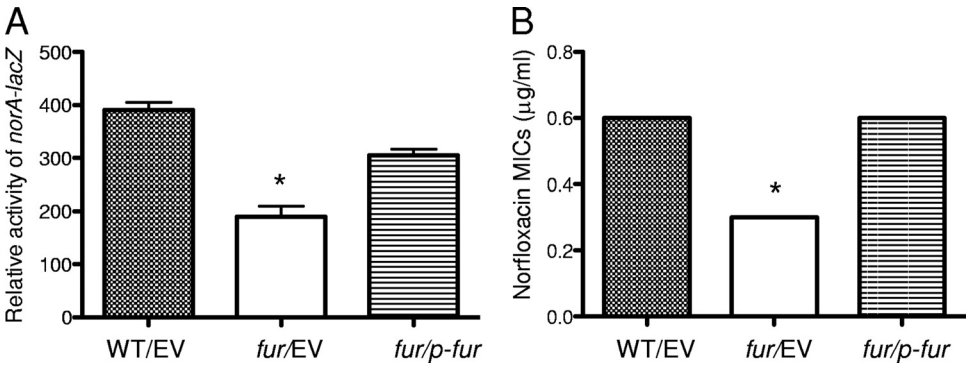

FIG 4 Transcription of *norA* is affected by *fur*. (A) β-Galactosidase activity of the *S. aureus* strain containing the *norA-lacZ* reporter fusion was determined by MUG units (1 MUG unit = 1 pmol of MUG cleaved by β-galactosidase per min per OD_{600} unit). An asterisk indicates that the value is statistically different from other strains as determined by a Student's *t* test ($P < 0.05$). (B) Susceptibilities of strains to 0.8 μg/ml norfloxacin in Mueller-Hinton broth supplemented with 10 μg/ml chloramphenicol. WT/EV, wild-type Newman strain containing pYJ335 empty vector; *fur*/EV, *fur* deletion containing pYJ335; *fur*/p-*fur*, *fur* complementation by pYJ335::*fur*. The experiment was repeated in triplicates, and identical results were obtained. An asterisk indicates that the value is statistically different from other strains as determined by a Student's *t* test ($P < 0.05$).

other microorganisms (27, 46). Inspired by these findings, we compared general siderophore secretion between the wild type and the *norA* deletion strain by using the CAS assay (53). We were able to use the *norA-lacZ* reporter strain as a *norA* deletion strain because the *lacZ* gene replaced the coding region of *norA* in the chromosome (14). In the CDM without iron supplement, the *norA-lacZ* strain showed only about half of the siderophore secretion of the wild-type bacteria after 2 days (Fig. 7A), suggesting that NorA may participate in the secretion of staphylococcal siderophores. However, at day 4, the difference between the *norA-lacZ* strain and the wild type was gone (Fig. 7A), which may suggest that the low siderophore secretion in the *norA-lacZ* strain may be compensated for by other mechanism(s) at a later time. The decreased siderophore secretion of *norA* deletion strain at day 2 could be complemented by pYJ335::*norA* (Fig. 7A). In order to rule out that the deletion of *norA* may downregulate staphylococcal siderophore synthesis pathways, we tested the expression levels of two genes (*sfaD* and *sbnC*) that belong to the staphyloferrin A and B biosynthesis pathways by performing Northern hybridization. At day 2 in CDM without iron, the Newman wild-type, the *norA* deletion mutant, and the complementation strain shared almost the same RNA levels of *sfaD* and *sbnC* (Fig. 7B). Collectively, our results suggest that NorA is involved in siderophore transport.

DISCUSSION

The hydroxamate-type siderophore transporter FhuABG is one of four iron-regulated ABC transporter systems reported to import Fe(III)-siderophore in *S. aureus* (12, 54). HtsABC and SirABC are involved in the uptake of the siderophores staphyloferrin A and staphyloferrin B, respectively (6, 15, 18). The substrate for the fourth transporter, SstABCD, has yet to be identified (29). *S. aureus* has so far been demonstrated to produce at least three siderophores including staphyloferrin A, staphyloferrin B, and aureochelin. None of them belongs to the hydroxamate family of siderophores, which leads to the conclusion that the hydroxamate-type siderophores might be exogenous for *S. aureus*.

Given that there is no hydroxamate-type siderophore when *S. aureus* is grown in TSA, the observation of enhanced *norA* expression in the *fhuB* mutant and under iron depletion suggests two possibilities. First, *S. aureus* may secrete hydroxamate-type sid-

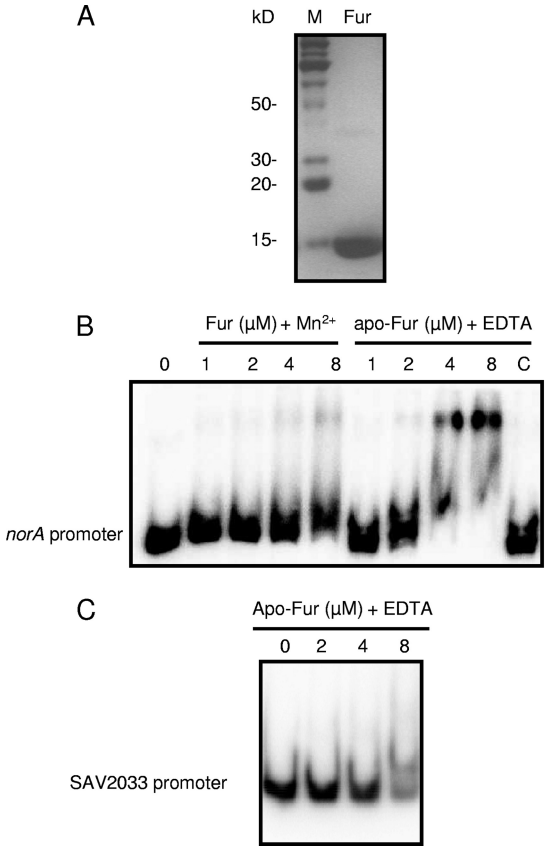

FIG 5 Apo-Fur binds to the *norA* promoter. (A) Purification of His_6-tagged full-length *S. aureus* Fur from *E. coli*. The purified Fur was analyzed by silver-stained nonreducing SDS-PAGE. (B) Apo-Fur was obtained by treating Fur with 200 mM EDTA overnight. EMSA showing direct binding of apo-Fur, but not Fur, to the *norA* promoter. In the experiment shown in the left panel, 2 ng of γ-^{32}P-end-labeled *norA* promoter fragment was incubated with the indicated concentration of Fur in a binding buffer containing 200 μM $MnCl_2$ at room temperature for 20 min. In the experiment shown in the right panel, 200 μM EDTA was incubated with the apo-Fur and *norA* promoter complex. Lane C, a cold competition with 100× unlabeled *norA* promoter DNA. (C) EMSA showing that apo-Fur did not bind to the SAV2033 promoter DNA as a negative control.

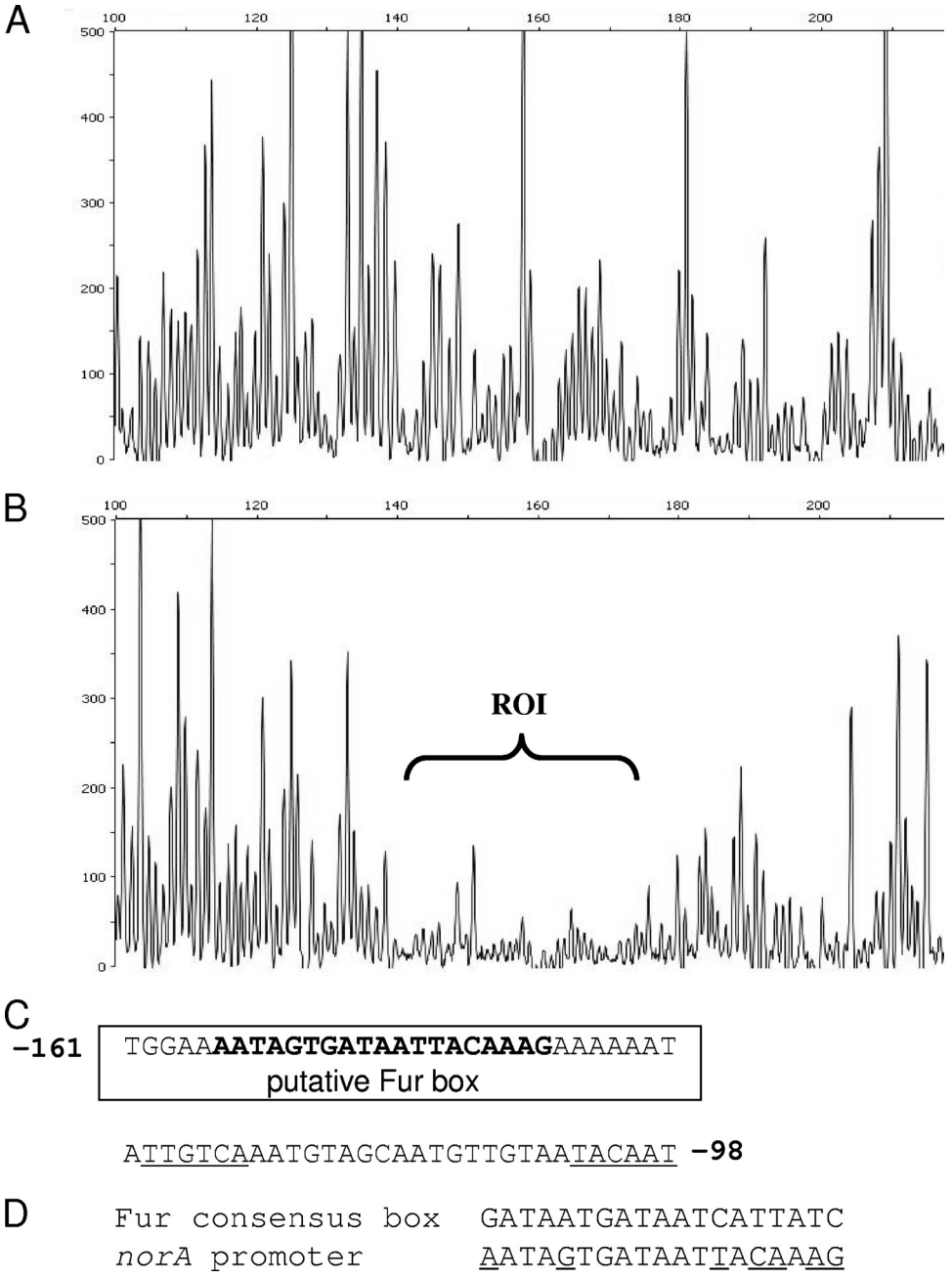

FIG 6 Apo-Fur directly binds to the putative Fur box in the *norA* promoter as determined by dye primer-based DNase I footprint assay. Electropherograms show the protection pattern of the *norA* promoter after digestion with DNase I following incubation in the absence (A) or presence (B) of 4 μM apo-Fur. ROI, region of interest. (C) *norA* promoter sequence (−161 to −98 from ATG) with a summary of the DNase I footprint assay results. The −35 and −10 promoter regions are underlined. The Fur-protected region is in the solid box, and the putative Fur box is in boldface. (D) The *norA* promoter contains a putative Fur-binding box. Letters underlined are mismatches from the consensus Fur box in *E. coli*.

erophores. However, based on a homology search, *S. aureus* does not seem to possess genes that produce hydroxamate-type siderophores. Previous attempts have also failed to detect hydroxamate siderophore production in *S. aureus* by the Csaky test (16). The second explanation is that FhuABG could be involved in the import of other nonhydroxamate siderophores. In concurrence with this notion, FhuA ATPase has been demonstrated to be required for both staphyloferrin A and staphyloferrin B transport (58). It is also possible that *fhuBG* are partially involved in the import of iron through other siderophores or pathways. In order to test this hypothesis, the iron concentrations in the *fhu* mutants were measured by the ferrozine assay after samples were cultured in TSB overnight. Compared with the wild type, all *fhu* mutants showed about a 40% decrease in iron level, which suggests that FhuABG are involved in the import of other siderophores (Fig. 8).

A classical hallmark of Fur is the differential activity of iron-bound Fur and apo-Fur. Iron-bound Fur is a repressor that forms dimers and binds to a specific Fur box DNA sequence element (13,

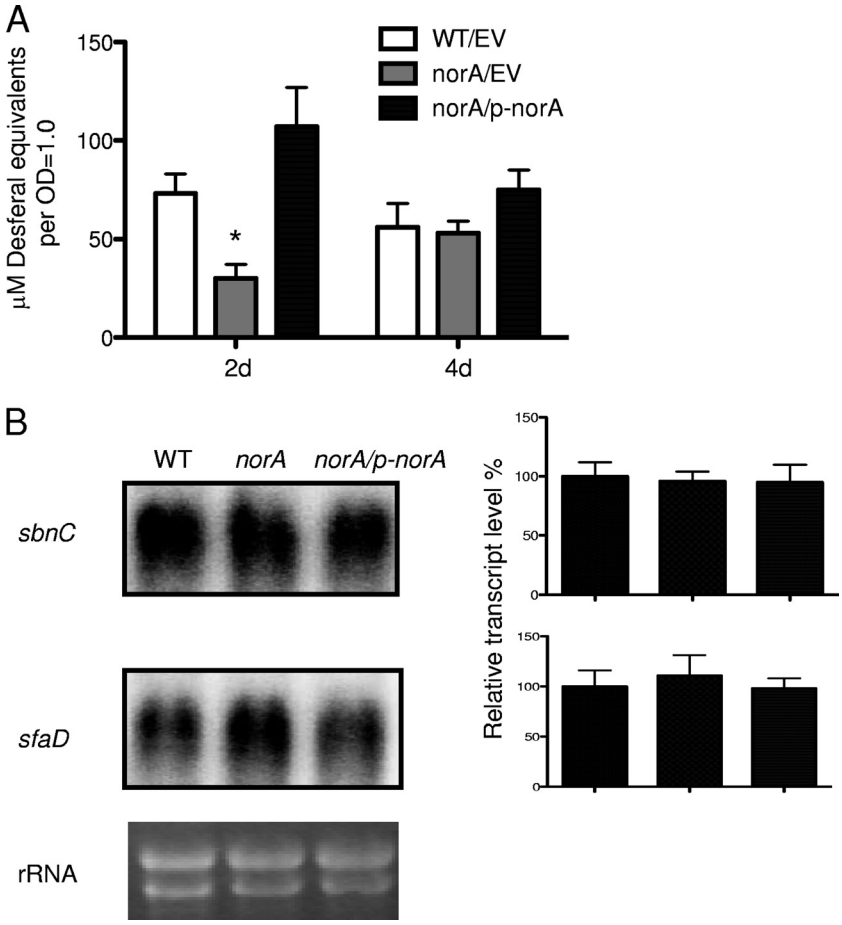

FIG 7 NorA is involved in siderophore secretion in *S. aureus*. (A) Wild-type Newman strain, a *norA* deletion strain, and a *norA* deletion strain complemented by pYJ335-*norA* were cultured in CDM-Fe for 4 days at 37°C. A CAS assay was used to quantify the general siderophore secretion in the supernatant of cultured medium at 2 days and 4 days. Deferoxamine mesylate was used as the reference standard. An asterisk indicates that the siderophore secretion in the *norA* deletion strain is statistically different from that in both the wild type and the complemented (*norA*/p-*norA*) strain at day 2 but not at day 4, as determined by a Student's *t* test ($P < 0.05$). (B) Deletion in *norA* did not change the expression of two genes (*sfaD* and *sbnC*) that belong to two major siderophore synthesis pathways (staphyloferrin A and B, respectively). Bacteria were grown in CDM without iron for 2 days before RNA extraction. Total RNA was analyzed by RNA blotting with α-^{32}P-labeled *sbnC* or *sfaD* probe. The ethidium bromide-stained RNA gel indicates the loading of RNA samples. The transcript levels were quantified, and statistical analysis was performed. No statistical difference was observed among three strains as determined by a Student's *t* test ($P > 0.05$).

28). Fur boxes are usually located inside the −35 region of the promoter; thus, Fur binding blocks the binding of RNA polymerase and prevents transcription of its target genes (13). Fur regulation is best understood in *E. coli*, where a 19-bp Fur box (GATAA TGATAATCATTATC) has been used as the standard to which other Fur binding sites are compared, even in other organisms (4). However, bacterial Fur regulation is extremely complicated inasmuch as it involves apo-Fur repression, apo-Fur activation, and iron-bound Fur activation (13). Our biochemical and genetic evidence strongly suggests that apo-Fur, but not iron-bound Fur, positively regulates *norA* (Fig. 5 and 6). More specifically, apo-Fur has been shown to directly interact with the putative apo-Fur box that is located outside the −35/−10 region (Fig. 6C), a feature characteristic of positive regulation by transcription factors. Similarly, catalase is also subject to positive regulation by Fur in *S. aureus* (29). However, its mechanism has not been demonstrated. Apo-Fur activation has also been reported in conjunction with *fur* autoregulation in *Helicobacter pylori* (19, 20). The authors showed that other than Fur boxes, apo-Fur binds to a distal upstream region that is essential for the antirepression function (20). The complexity of *fur* regulation also includes few poorly conserved Fur boxes in some organisms. For example, based on an alignment of Fur-regulated promoters, the consensus *H. pylori* Fur box is

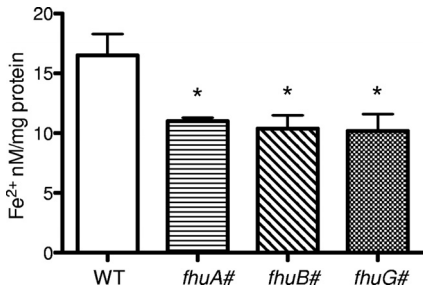

FIG 8 The iron content in the *fhu* mutants was lower than that in the wild-type *S. aureus*. The strains were cultured in TSB overnight before the iron contents were determined by the ferrozine assay. #, mutants are from the Phoenix library (3).

NNNNNAATAATNNTNANN, which is significantly different from the counterpart in *E. coli* (44, 45).

Compared to *fur* regulation other microorganisms, *fur* regulation in *S. aureus* is relatively poorly understood. In *S. aureus*, *fur* has been demonstrated to control directly only several loci that are involved in siderophore biosynthesis and transport systems, which were supported by the identification of highly conserved Fur boxes in the promoters (13). In contrast to the classic iron-bound repressor mechanism on *fhuABG* and *sirA* by *fur* in *S. aureus* (18, 65), we observed positive regulation of *norA* by apo-Fur. As in our study of *S. aureus fur*, positive autoregulation of *fur* in iron-depleted medium has been demonstrated in *Vibrio vulnificus* (39), despite the presence of Fe-Fur repression of other genes in the same organism (40, 64). Through the use of a DNase I footprint assay, the authors identified a novel Fur-binding sequence that consists of two direct repeats of AAATTGT, which is also dramatically different from the canonical Fur box in *E. coli*. Furthermore, the affinity of Fur to this unconventional Fur-binding site (~3 μM) is 10- to 100-fold lower than that of the Fur boxes in *E. coli* and *V. vulnificus*. A plausible explanation is that Fe-Fur or apo-Fur binds to different regions with different binding affinities, thus leading to variable regulatory effects. A recent study showed that *fur* tunes the expression of multiple virulence factors in *S. aureus*, such as cytolysins and immunomodulatory proteins (61). However, the authors of that study were unable to pinpoint a classic Fur box to account for direct regulation and attributed Fur activity to cross talk with other regulatory systems (61). However, based on our results, it seems that Fur could recognize less-conserved Fur boxes.

In contrast to microbial siderophore uptake machineries that have been well documented, very few secretion systems for siderophores have been characterized (46). The majority of the known siderophore exporters are efflux pumps while some are ATP-binding cassette transporters (46). These siderophore-secreting efflux pumps include EntS in *E. coli* (9), LbtB in *Legionella pneumophila* (1), CsbX in *Azotobacter vinelandii* (51), AlcS of *Bordetella pertussis* and *Bordetella bronchiseptica* (11), and PvdRT-OpmQ from *Pseudomonas aeruginosa* (27, 32). Consistent with this, our siderophore secretion assay of the *norA-lacZ* strain suggested that NorA is involved in siderophore secretion in *S. aureus* (Fig. 7A). To the best of our knowledge, this is the first evidence of a siderophore exporter in *S. aureus*.

In summary, our results unveiled a novel relationship between cellular iron level and the multidrug efflux pump gene *norA*, which also expands the knowledge of the complex *fur* regulon and regulation in *S. aureus*. Based on the data presented in this study, we propose that in the absence of iron (or when iron concentration is low inside *S. aureus*), apo-Fur acts as an activator of *norA* transcription by directly binding to the putative apo-Fur box in the promoter. When the iron concentration is higher, iron(II) dissociates Fur from the *norA* promoter, thus limiting the transcription of *norA*. However, besides *fur*, additional factors are also involved in the iron uptake-dependent repression of *norA* expression. Being a part of the *fur* regulon, NorA is involved in the secretion/recycling of siderophores in *S. aureus*.

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