

Role of Siderophore Biosynthesis in Virulence of *Staphylococcus aureus*: Identification and Characterization of Genes Involved in Production of a Siderophore

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Molecular determinants underlying the production of siderophores in the human and animal pathogen *Staphylococcus aureus* and the contribution of siderophore production to the virulence of this bacterium have, until now, remained undefined. Here, we show that *S. aureus* strains RN6390 and Newman produce siderophore when the cells are starved for iron. We further identified and characterized a nine-gene, iron-regulated operon, designated *sbn* and situated between *sirABC* and *galE* on the *S. aureus* chromosome, that is involved in the production of a siderophore. Mutation of the *sbnE* gene, in both RN6390 and Newman, eliminates the ability of these strains to produce a siderophore under iron-limited growth conditions, while introduction of multicopy *sbnE* into *sbnE* mutants complemented the inability of the mutants to produce the siderophore. *sbnE* mutants, in both the RN6390 and Newman backgrounds, displayed a drastic growth deficiency, compared to the wild type, in iron-restricted growth medium, whereas no such deficiency was observed during growth in iron-replete medium. Complemented mutants showed a restored ability to grow under iron restriction. We further showed that an *sbnE* mutant was compromised in a murine kidney abscess model of *S. aureus* infection, illustrating the importance of siderophore production to the pathogenicity of *S. aureus*. *sbn* genes were present in all *S. aureus* strains tested (and all *S. aureus* genome sequences) but were undetectable in any of the 13 coagulase-negative staphylococci tested, including *Staphylococcus epidermidis*.

Iron is an absolute requirement for the growth of most microorganisms, with the possible exceptions of lactobacilli (2) and *Borrelia burgdorferi* (24). Despite being the fourth most abundant element in the Earth's crust, iron is frequently a growth-limiting nutrient. In aerobic environments and at physiological pH, iron is present in the ferric (Fe³⁺) state and forms insoluble hydroxide and oxyhydroxide precipitates. Mammals overcome iron restriction by possessing high-affinity iron-binding glycoproteins such as transferrin and lactoferrin that serve to solubilize and deliver iron to host cells (33). This results in a further restriction of free extracellular iron, and, accordingly, the concentration of free iron in the human body is estimated to be 10⁻¹⁸ M, a concentration that is several orders lower than that required to support a productive bacterial infection (3).

To overcome iron restriction, bacteria have evolved several different mechanisms to acquire this essential nutrient. For example, members of the family *Pasteurellaceae* may express receptors for the recognition of iron-loaded forms of transferrin and lactoferrin (9). One of the most common iron acquisition mechanisms, though, is the use of low-molecular-weight, high-affinity iron chelators, termed siderophores, and cognate cell envelope receptors that serve to actively internalize ferric-siderophore complexes. Many siderophores are able to successfully compete with transferrin and lactoferrin for host iron. Indeed, the ferric-siderophore uptake systems are

critical virulence factors in bacteria such as septicemic *E. coli* (34), *Vibrio anguillarum* (6), *Erwinia chrysanthemi* (8), and *Pseudomonas aeruginosa* (19).

Staphylococcus aureus may cause numerous syndromes in humans, ranging from minor skin and wound infections to more serious sequelae such as endocarditis, osteomyelitis, and septicemia (1). The ability of *S. aureus* to invade and colonize many tissues may be ascribed to its capacity to express several virulence factors such as fibronectin-, elastin-, and collagen-binding proteins that aid in tissue adherence and multiple exotoxins and proteases that result in tissue destruction and bacterial dissemination. The ability of this bacterium to acquire iron during in vivo growth is also likely important to its pathogenesis, and several research groups have characterized several different genes whose products are involved in the binding and/or transport of host iron compounds (17, 20, 30).

S. aureus possesses several different iron-regulated ABC transporters, including those encoded by the *sstABCD* (21), *sirABC* (12), and *fhuCBG* (28) operons. While the transported substrates are unknown for the *sst* and *sir* systems, the *fhuCBG* genes, in concert with *fhuD1* and *fhuD2* (27), are involved in the acquisition of iron(III)-hydroxamate complexes. Several members of the staphylococci, including numerous coagulase-negative staphylococci (CoNS) and strains of *S. aureus*, produce siderophores. Two of these siderophores, staphyloferrin A (14, 18) and staphyloferrin B (7, 11), are of the polycarboxylate class, while the third, aureochelin (5), is chemically uncharacterized. Leading into this study, no molecular-genetic information was known about the synthesis of any of the staphylococcal siderophores.

The objectives of this study were to identify genetic loci

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involved in siderophore production in *S. aureus*, to generate siderophore-deficient strains of *S. aureus*, and to utilize the mutants so as to investigate the importance of siderophore production to the iron-limited growth of this bacterium. Finally, we sought to investigate the importance of siderophore production to the virulence of *S. aureus*. We report here the identification and characterization of an iron-regulated, nine-gene operon (designated *sbn*) whose products are involved in the biosynthesis of a siderophore in *S. aureus*. We show that the operon is present in both laboratory strains and clinical isolates of *S. aureus* but is not present in CoNS. Finally, we demonstrate that the expression of this operon not only is important for iron-restricted growth of *S. aureus* in laboratory culture but also prolongs the ability of *S. aureus* to survive in vivo.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. Bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* and *S. aureus* strains were routinely cultured in Luria-Bertani broth (Difco) and tryptic soy broth (Difco), respectively. Iron-restricted bacterial growth was performed in Tris-minimal succinate medium (TMS), the composition of which has been described previously (28). Residual free iron was chelated from TMS medium by the addition of ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDHA) (1 μ M unless otherwise stated), or TMS was made iron replete by the addition of 50 μ M FeCl₃. The following antibiotics were used at the indicated concentrations: erythromycin (5 μ g/ml), lincomycin (20 μ g/ml), neomycin (50 μ g/ml), kanamycin (50 μ g/ml), and tetracycline (4 μ g/ml) for *S. aureus* selection and ampicillin (100 μ g/ml), tetracycline (10 μ g/ml), and erythromycin (300 μ g/ml) for *E. coli* selection. All reagents were made with water purified through a Milli-Q water purification system (Millipore, Mississauga, Ontario, Canada).

Recombinant DNA methodology. Plasmid DNA was isolated from *E. coli* using Qiaprep mini-spin kits (Qiagen). DNA manipulations, including restriction enzyme digestion and DNA ligation, were performed according to standard procedures (25). Restriction enzymes were purchased from Life Technologies, MBI Fermentas, New England Biolabs, or Roche Diagnostics, and DNA ligations were performed using the Roche Rapid DNA Ligation kit. *Pwo*I (Roche) was used for all PCRs. Oligonucleotides were obtained from Life Technologies and are described in Table 1.

Chromosomal DNA isolation and Southern blotting. Chromosomal DNA was isolated from various staphylococcal strains using procedures as previously described (28). Briefly, cells were lysed at 37°C using 10 μ g of lysostaphin (Sigma) in STE (0.1 M NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]), or for CoNS, lysozyme (1 μ g) was added to STE. Sodium dodecyl sulfate (0.1%) and proteinase K (0.5 mg) were added to the preparations and incubated for 2 h at 55°C. Southern blotting techniques were performed essentially as previously described (25), and hybridization was performed with digoxigenin (DIG) (Roche Diagnostics)-labeled probes, prepared and used according to the manufacturer's instructions. Light emission was detected by exposing blots to Hyperfilm ECL (Amersham Biosciences).

Construction of an *sbnE* mutant. A 3,037-bp DNA fragment carrying *sbnE* was PCR amplified from the chromosome of *S. aureus* RN6390 and cloned into pBCSK⁺ (*Bam*HI), generating pSED12. The *sbnE* coding region was interrupted at a unique *Nco*I site (end polished with Klenow enzyme) by the insertion of a kanamycin resistance cassette, derived from plasmid pDG782, to create pSED17. A *Bam*HI fragment containing the disrupted *sbnE* gene was removed from pSED17 and cloned into the temperature-sensitive *S. aureus* suicide plasmid, pAUL-A, to generate pSED18. Plasmid pSED18 was introduced into *S. aureus* RN4220 before being transduced into *S. aureus* RN6390 using bacteriophage 80 α , using methods previously described (28). *S. aureus* RN6390 carrying pSED18 was grown to mid-log phase at 30°C before the growth temperature was shifted to 42°C. After 4 h of incubation at 42°C, the culture was plated onto medium containing kanamycin and neomycin and incubated at 42°C overnight. The *sbnE* mutant, resistant to kanamycin and neomycin and sensitive to erythromycin and lincomycin, was isolated as a result of allelic exchange between chromosomal *sbnE* and the insertionally inactivated copy. The chromosomal insertion of the Km^r cassette into *sbnE* was confirmed by PCR.

Creation of transcriptional *lacZ* fusions and β -galactosidase assays. Internal fragments of individual genes were cloned into the multiple-cloning site of

pMUTIN4 (31), a vector that does not replicate in gram-positive bacteria. *S. aureus* RN4220 was then transformed with recombinant pMUTIN4 plasmids, and homologous recombination between the cloned DNA sequences and those present on the chromosome resulted in the integration of recombinant plasmids into the chromosome. Chromosomal integrations were confirmed by PCR amplification of pMUTIN4-specific DNA sequences.

S. aureus strains bearing transcriptional fusions to *lacZ* were assayed for β -galactosidase activity using previously described methods (30). Briefly, cultures were grown in TMS supplemented with 1 μ M EDDHA or FeCl₃ to an optical density at 600 nm of 0.8. Cells (5×10^8) were lysed in a solution containing 10 mM potassium phosphate buffer (pH 7.8), 15 mM EDTA, 1% Triton X-100, and 10 μ g of lysostaphin at 37°C. After centrifugation of cell debris, 5 μ l of supernatant was assayed for β -galactosidase activity using the Galacto-Light Plus Chemluminescent reporter gene kit (Tropix) in a Berthold luminometer. The background was set at 50 relative light units/s and the data presented are mean relative light units per second for three independent samples \pm standard error.

Siderophore production assays and isolation of siderophore. Siderophore activity in spent culture supernatants was assayed using chrome azurol S (CAS) by procedures previously described (26). Dilutions of culture supernatants were mixed with equal volumes of CAS shuttle solution and allowed to interact for 30 min at room temperature. With TMS medium serving as the blank, and Desferal serving as the reference standard, the absorbance at 630 nm was determined. Siderophore units were calculated as follows: (A_{630} of TMS - A_{630} of sample)/ A_{630} of TMS \times 100%.

For siderophore isolations, *S. aureus* strains were vigorously shaken in TMS for 48 h at 37°C. Culture supernatants were recovered by centrifugation and lyophilized. The concentrated supernatant was resuspended in 100% methanol to 1/10 of the volume of the original culture supernatant and passed through Whatman no. 1 filter paper to remove particulate material. Rotary evaporation was used to reduce the volume before application to an LH-20 column (Amersham Biosciences). Fractions were collected, and those testing positive with CAS shuttle solution and for biological activity in siderophore plate bioassays were dried, resuspended in water, and examined by high-performance liquid chromatography (HPLC). Analytical reversed-phase HPLC was used for final purification of siderophore. The column utilized was a Waters ODS2 Spherisorb column (4.6 by 150 mm). Trifluoroacetic acid (0.1%) in water represented solvent A, whereas 0.1% trifluoroacetic acid in acetonitrile was used as solvent B. The chromatographic method used was as follows: at a flow rate of 0.75 ml/min, 6% B for 3.5 min, followed by a gradient of 6 to 60% B over 20 min. Staphylobactin was detected at 210 nm and had a retention time of approximately 17 min. Staphylobactin was collected, dried, and rechromatographed to check for purity and activity before being analyzed by electrospray ionization-mass spectrometry (ESI-MS).

ESI-MS. ESI-MS and MS-mass spectrography analyses were performed on a quadrupole-time-of-flight (Q-TOF2) mass spectrometer fitted with a Z-spray source (Micromass, Manchester, United Kingdom). The detector was calibrated using an MS-mass spectrography spectrum of [Glu]-fibrinopeptide B. The molecular mass of the siderophore sample was determined by flow injection analysis using a Waters CapLC system with a carrier solvent of 1:1 HPLC-grade methanol-HPLC-grade water at a flow rate of 30 μ l/min. Spectra were acquired in positive-ion mode with an *m/z* range of 50 to 1,800 using the following parameters: capillary voltage, 3.2 kV; cone voltage, 30 to 40 V; desolvation temperature, 200°C; source temperature, 80°C. Tandem mass spectra were acquired for the parent ion of interest using argon as the collision gas and collision energies ranging from 10 to 30 eV. All spectra were acquired and processed using Mass-Lynx 3.5 (Micromass).

Siderophore plate bioassays. The ability of siderophores to promote the iron-restricted growth of *S. aureus* was assessed using siderophore plate bioassays, performed as previously described (28). Briefly, *S. aureus* RN6390 was incorporated into solid TMS medium (1.4×10^4 cells/ml) containing 20 μ M EDDHA. The ability of purified siderophores to promote growth of *S. aureus* was assessed after incubation of plates for 36 h at 37°C.

Mouse kidney abscess experiments. Female Swiss-Webster mice, weighing 25 g, were purchased from Charles River Laboratories Canada, Inc., and housed in microisolator cages. Bacteria were grown overnight in tryptic soy broth (TSB), harvested, and washed three times in sterile saline. Pilot experiments demonstrated that *S. aureus* Newman colonized mice better in this model than did RN6390 and that the optimal amount of *S. aureus* Newman to inject into the tail vein to obtain an acute but nonlethal kidney infection was 10^7 CFU. Bacteria, suspended in sterile saline, were administered intravenously via the tail vein. The number of viable bacteria injected was confirmed by plating serial dilutions of the inoculum on TSB-agar. On days 5 and 6 postinjection, mice were sacrificed and kidneys were aseptically removed. Using a PowerGen 700 Homogenizer, kidneys

TABLE 1. Bacterial strains, plasmids, and oligonucleotides used in this study^a

Bacterial strain, plasmid, or oligonucleotide application	Description or sequences	Source or reference
Bacteria		
<i>E. coli</i> DH5 α	ϕ 80 <i>dlacZ</i> Δ M15 <i>recA1 endA1 gyrA96 thi-1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 relA1 deoR</i> Δ (<i>lacZYA-argF</i>)U169	Promega
<i>S. aureus</i>		
RN4220	r _K ⁻ m _K ⁺ ; accepts foreign DNA	15
RN6390	Prophage-cured wild-type strain	23
Newman	Wild-type strain	O. Schneewind
SA113		T. Foster
ATCC 25923		ATCC
MJH010	8325-4 <i>fur</i> ::Tet; Tet ^r	S. Foster
H295	RN6390 <i>fur</i> ::Km; Km ^r	28
H706	Newman <i>fur</i> ::Km (<i>fur</i> ::Km marker from H295 was transduced into Newman); Km ^r	This study
H438	RN4220 <i>sbnF</i> ::pMUTIN4; Em ^r	This study
H479	H295 <i>sbnF</i> ::pMUTIN4; Em ^r Km ^r	This study
H520	RN4220 SA0121::pMUTIN4; Em ^r	This study
H521	RN4220 <i>galE</i> ::pMUTIN4; Em ^r	This study
H551	RN4220 <i>sbnI</i> ::pMUTIN4; Em ^r	This study
H557	RN4220 <i>sbnH</i> ::pMUTIN4; Em ^r	This study
H572	RN4220 <i>sbnA</i> ::pMUTIN4; Em ^r	This study
H672	RN6390 <i>sbnE</i> ::Km; Km ^r	This study
H675	RN6390 <i>sbnE</i> ::Km <i>fur</i> ::Tet; Km ^r Tet ^r	This study
H686	Newman <i>sbnE</i> ::Km; Km ^r	This study
H16	Clinical isolate	LHSC
H50	Clinical isolate	LHSC
H51	Clinical isolate	LHSC
Coagulase-negative staphylococci (CoNS)		
<i>S. auricularis</i> ATCC 33753		ATCC
<i>S. capitis</i> ATCC 35661		ATCC
<i>S. caprae</i> ATCC 35538		ATCC
<i>S. chromogenes</i> ATCC 43764		ATCC
<i>S. cohnii</i> ATCC 29973		ATCC
<i>S. epidermidis</i> LK819		M. Valvano
<i>S. haemolyticus</i> ATCC 29970		ATCC
<i>S. intermedius</i> ATCC 29663		ATCC
<i>S. hominis</i> ATCC 27846		ATCC
<i>S. sciuri</i> ATCC 29062		ATCC
<i>S. simulans</i> ATCC 27851		ATCC
<i>S. warneri</i> ATCC 27836		ATCC
<i>S. xylosus</i> ATCC 35663		ATCC
<i>Burkholderia cepacia</i> CEP024	Genomovar III isolate from cystic fibrosis patient	M. Valvano
Plasmids		
pAUL-A	Temperature-sensitive <i>S. aureus</i> suicide vector; Em ^r Lc ^r	4
pAW8	<i>E. coli-S. aureus</i> shuttle vector; Tet ^r	32
pBC SK(+)	<i>E. coli</i> cloning vector; Cm ^r	Stratagene
pDG782	pMLT22 derivative that carries a kanamycin resistance cassette; Ap ^r Km ^r	10
pMUTIN4	<i>lacZ</i> fusion vector; Ap ^r (<i>E. coli</i>) Em ^r (<i>S. aureus</i>)	31
pSED12	pBC SK+ derivative carrying <i>sbnE</i> ; Cm ^r	This study
pSED17	pSED12 derivative containing <i>sbnE</i> ::Km; Cm ^r Km ^r	This study
pSED18	pAUL-A derivative containing <i>sbnE</i> ::Km; Km ^r Em ^r	This study
pSED32	pAW8 derivative carrying <i>sbnE</i> ; Tet ^r	This study
Oligonucleotides^b		
Generation of <i>sbnA-lacZ</i> fusion	<u>TTGGATCCAGTATATGAATCCTGGAGGC</u> (forward) <u>TTGGATCCAAAAATGACTGACCCITTCGCATC</u> (reverse)	
Generation of <i>sbnF-lacZ</i> fusion	<u>TGGATCCCATCACCAATTGAGCGTGTCTGATAGGAGAT</u> (forward) <u>TGGATCCTTTCAATTGTATGAGGCGCCAACACTCGT</u> (reverse)	
Generation of <i>sbnH-lacZ</i> fusion	<u>TTCGCGCCGCGATAGATAGAGATATCATT</u> (forward) <u>TTGGATCCTAGTAAACGCCTATGCCACC</u> (reverse)	
Generation of <i>sbnI-lacZ</i> fusion	<u>TTGCGGCCGCCCAACACAATTTGGTATTTCTGAA</u> (forward) <u>TTGGATCCTACTTGAAAATGTGCTTCGC</u> (reverse)	
Generation of SA0121- <i>lacZ</i> fusion	<u>TTGCGGCCGCAAGTTCCATTTGGTGTGTGG</u> (forward) <u>TTGGATCCGGTAAAACAGTAAAAGAGC</u> (reverse)	
Generation of <i>galE-lacZ</i> fusion	<u>TTGCGGCCGCTATTATCGCTTTAGTATTAT</u> (forward) <u>TTGGATCCTCAACGCCTGCTTGAGATGTT</u> (reverse)	
Cloning of <i>sbnE</i> gene	<u>TTGGATCCATTAGCAGACATAGATATAT</u> (forward) <u>TTGGATCCTAGTGCTCATCATTAATCG</u> (reverse)	

^a Abbreviations: Ap^r, Cm^r, Km^r, Lc^r, and Tet^r, resistance to ampicillin, chloramphenicol, kanamycin, lincomycin, and tetracycline, respectively; LHSC, London Health Sciences Centre; ATCC, American Type Culture Collection.^b Data under the heading "Oligonucleotides" are descriptions. Restriction sites for subsequent cloning of the PCR products are underlined.

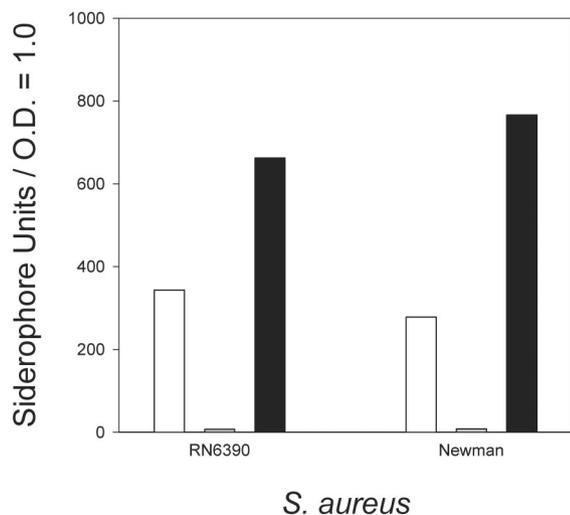


FIG. 1. Siderophore levels in spent culture supernatants of RN6390 and Newman and their respective *fur* derivatives, H295 and H706. Bacteria were grown in an iron-deficient (open bars) or an iron-replete (iron-deficient medium supplemented with 50 μ M iron chloride) (gray bars) medium, while the *fur*::Km derivatives of both RN6390 and Newman (solid bars) were grown in an iron-replete medium. Siderophore units were calculated as described in Materials and Methods. O.D., optical density.

were homogenized for 45 s in sterile phosphate-buffered saline containing 0.1% Triton X-100, and homogenate dilutions were plated on TSB-agar to enumerate recovered bacteria. Data presented are the log CFU recovered per mouse.

Computer analyses. DNA sequence analysis, oligonucleotide primer design and nucleotide sequence alignments were performed using the Vector NTI Suite software package (Informax Inc., Bethesda, Md.).

Nucleotide sequence accession number. The nucleotide sequence for the *sbn* operon has been deposited in GenBank and has been assigned accession no. AY251022.

RESULTS

***S. aureus* RN6390 and Newman produce siderophore.** The objectives of this study were to characterize the role that siderophore production plays in the iron-restricted growth of *S. aureus* in culture and also to examine its importance to in vivo growth and pathogenicity of this bacterium. To accomplish this, our goal was to generate genetically defined siderophore-deficient mutants from siderophore-producing strains of *S. aureus*.

Previous studies have shown that various different isolates of *S. aureus* have the potential to produce multiple siderophores, including staphyloferrin A and staphyloferrin B (18), and that the genetically characterized strain 8325-4 produced siderophore(s), but of undetermined identity (12, 13). We have demonstrated that two additional *S. aureus* strains that are used in our laboratory, strain RN6390 and strain Newman, produce readily detectable quantities of siderophore activity when the cells are grown under conditions of iron starvation but produce very little siderophore during growth in iron-replete medium (Fig. 1). Noting that high-affinity iron acquisition systems, including siderophore production and iron(III)-siderophore uptake, are typically regulated by Fur in many different bacteria, we further showed that, indeed, in strains RN6390 and Newman, siderophore production was regulated

by exogenous iron concentrations via the Fur protein, since *fur* derivatives of both RN6390 (H295) and Newman (H706) produced high levels of siderophore activity even when grown in iron-replete medium (Fig. 1). These findings are consistent with published results of Horsburgh et al., who used *S. aureus* 8325-4 (13).

Isolation of siderophore from *S. aureus*. We wanted to identify which siderophore(s) was produced by *S. aureus* RN6390 and related strains. Given that siderophore production was derepressed in *fur* backgrounds, we isolated siderophore from culture supernatants of strain H295 (RN6390 *fur*::Km). Our initial experiments focused on the isolation of staphyloferrin A and staphyloferrin B using published procedures (11, 18). However, these purifications yielded extremely little CAS-positive material, suggesting that strain RN6390 produces no, or extremely little, staphyloferrin A or staphyloferrin B. Extraction of culture supernatants using a procedure that has previously been used to isolate ornibactins (29) did, however, result in the isolation of significant quantities of CAS-positive material. Chromatography of methanol-extracted culture supernatant through an LH-20 column yielded discrete fractions that both were CAS positive and promoted the iron-restricted growth of *S. aureus* in siderophore plate bioassays. Further purification by reversed-phase HPLC yielded an isolated peak of material that retained biological activity. ESI-MS analysis of the isolated material showed that it contained an abundance of a molecule with an $m/z = 822$, which is significantly greater than that of previously characterized staphylococcal siderophores (staphyloferrin A $m/z = 480$; staphyloferrin B $m/z = 448$). We were unable to detect the presence of compounds in the active LH-20 fractions that matched the masses of either staphyloferrin A or staphyloferrin B. Taken together, these results strongly suggest that we have isolated a siderophore that has not previously been identified in the staphylococci. Efforts are ongoing to elucidate the structure of the molecule. Until such time as we determine that this is not a siderophore previously identified from another organism, we have tentatively named the molecule staphylobactin.

Identification and analysis of a siderophore biosynthetic gene cluster in *S. aureus*. At the time we initiated these studies, genetic information underlying siderophore biosynthesis in the staphylococci had yet to be resolved. We searched *S. aureus* genome sequences from several strains and identified several open reading frames (ORFs) whose products shared significant similarity with enzymes with demonstrated roles in siderophore biosynthesis. In particular, we identified an 11.5-kb gene cluster, situated between the *sirABC* operon and *galE* on the staphylococcal chromosome (Fig. 2), whose products share significant similarity with known or predicted siderophore biosynthetic enzymes in other bacteria (Table 2). While the SirABC proteins share a high degree of similarity to iron(III)-siderophore transport proteins (12), *galE* (encoding UDP-galactose-4-epimerase) is involved in nucleotide-sugar precursor formation. Hypothesizing that the 11.5-kb gene cluster was involved in siderophore biosynthesis, we designated the coding regions *sbn*, for siderophore biosynthesis.

To confirm that the *sbn* gene cluster was involved in siderophore biosynthesis in *S. aureus*, we insertionally inactivated the fifth ORF (*sbnE*) with a kanamycin resistance cassette in *S. aureus* RN6390, thus creating strain H672. Methanol extracts

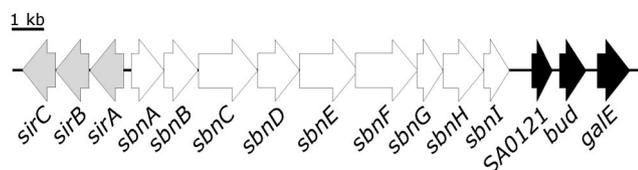


FIG. 2. Schematic representation of the *sir-galE* region of the *S. aureus* chromosome. Arrows are representative of individual coding regions. The coding regions within the *sbn* operon are shown with open arrows, the *sir* coding regions are shown with gray arrows, and coding regions likely not involved in iron uptake are shown with black arrows. SA0121, hypothetical ORF with nomenclature that is derived from N315 genome sequence; *bud*, butanediol dehydrogenase.

of spent culture supernatant from iron-restricted H672 contained no trace of material that promoted *S. aureus* growth in siderophore plate bioassays. Biologically active siderophore was, however, consistently isolated from methanol extracts of iron-restricted supernatants of both the wild-type strain (RN6390) and strain H672 complemented with pSED32, a plasmid carrying *sbnE*, where expression of *sbnE* was driven by the *plac* promoter present on the vector. The staphylobactin molecule isolated from iron-restricted wild-type cultures was completely absent in iron-restricted supernatants of H672 and H675 (RN6390 *fur sbnE*). These results implicated *sbnE* as a key gene involved in the production of a siderophore and, more specifically, staphylobactin. The *sbnE::Km* mutation was also transduced into *S. aureus* Newman, to create strain H686. Whereas staphylobactin was undetectable in supernatants of iron-starved H686, it was readily detectable in culture super-

natants of iron-starved Newman. These results were confirmed by ESI-MS.

The *sbnABCDEFGHI* genes comprise an operon, and iron, via Fur, regulates its transcription. Predicted coding regions of the first nine ORFs of the *sbn* locus either overlap or have very short noncoding segments separating them from one another, whereas approximately 600 bp exists between the 3' end of the 9th coding region and the 5' end of the 10th coding region. This suggested that the operon may be comprised of nine ORFs. The 10th coding region encodes a predicted protein of unknown function, the product of the 11th coding region displays significant similarity to butanediol dehydrogenases (acetoin reductases), and the 12th coding region is *galE*, encoding UDP-galactose-4-epimerase, which is involved in sugar-nucleotide precursor formation in polysaccharide biosynthesis. In an effort to characterize the transcriptional regulation of the *sbn* operon, and to delineate the limits of the operon, chromosomal *lacZ* reporter gene fusions targeted to several coding regions, both within and beyond the putative *sbn* operon, were created. β -Galactosidase expression was then monitored in strains bearing *lacZ* fusions when the cells were grown in either iron-replete or iron-deficient growth medium. When grown in the presence of 50 μ M FeCl₃, expression of β -galactosidase in strains bearing fusions to *sbnA*, *sbnF*, *sbnH*, and *sbnI* was at low, background levels, whereas expression was well above background in strains bearing fusions to SA0121 and *galE* (Table 3). When grown in iron-deficient medium, however, all strains showed high levels of β -galactosidase expression. These results indicate that transcription of the *sbn* operon is iron regulated through the 9th coding region (*sbnI*)

TABLE 2. Amino acid identity and similarity to proteins expressed from the *sbn* operon

Protein	Closest match or function	Bacterium(a)	Identity (%)	Similarity (%)
SbnA	<i>O</i> -Acetyl serine sulfhydrylase	<i>Streptomyces avermitilis</i>	42	62
	<i>O</i> -Acetyl serine sulfhydrylase	<i>E. coli</i>	29	45
SbnB	Ornithine cyclodeaminase	<i>Archaeoglobus fulgidis</i>	32	53
SbnC	AcsA (achromobactin biosynthesis)	<i>Pectobacterium chrysanthemi</i>	32	50
	PvsB (vibrioferrin biosynthesis)	<i>Vibrio parahaemolyticus</i>	23	42
	IucC (aerobactin biosynthesis)	<i>E. coli</i>	24	40
SbnD	Multidrug efflux	<i>Listeria</i> spp.	26	47
SbnE	RhbC (rhizobactin 1021 biosynthesis)	<i>Sinorhizobium meliloti</i>	26	45
	PvsD (vibrioferrin biosynthesis)	<i>Vibrio parahaemolyticus</i>	25	45
	AcsD (achromobactin biosynthesis)	<i>Pectobacterium chrysanthemi</i>	25	43
	IucA (aerobactin biosynthesis)	<i>E. coli</i>	24	42
SbnF	AcsC (achromobactin biosynthesis)	<i>Pectobacterium chrysanthemi</i>	45	63
	RhbF (rhizobactin 1021 biosynthesis)	<i>Sinorhizobium meliloti</i>	28	48
	AlcC (alcaligin biosynthesis)	<i>Bordetella bronchiseptica</i>	25	47
	IucC (aerobactin biosynthesis)	<i>E. coli</i>	25	44
SbnG	AcsB (achromobactin biosynthesis)	<i>Pectobacterium chrysanthemi</i>	47	67
	4-Hydroxy-2-oxovalerate aldolase	<i>Xanthomonas campestris</i>	35	51
	2-Dehydro-3-deoxyglucarate aldolase	<i>E. coli</i>	29	51
SbnH	PvsE (vibrioferrin biosynthesis)	<i>Vibrio parahaemolyticus</i>	42	59
	Diaminopimelate decarboxylase	<i>Xanthomonas campestris</i>	39	57
SbnI	Unknown		ND ^a	ND

^a ND, not determined.

TABLE 3. β -Galactosidase expression from *sbn-lacZ* fusions

Bacterial strain	Presence of Fe	Mean β -galactosidase activity \pm SD (RLU/s)
RN4220	+	0 \pm 0
RN4220	-	0 \pm 0
RN4220 <i>sbnA</i> ::pMUTIN4	+	0 \pm 0
RN4220 <i>sbnA</i> ::pMUTIN4	-	144,763 \pm 6,080
RN4220 <i>sbnF</i> ::pMUTIN4	+	0 \pm 0
RN4220 <i>sbnF</i> ::pMUTIN4	-	193,944 \pm 3,398
RN4220 <i>sbnH</i> ::pMUTIN4	+	0 \pm 0
RN4220 <i>sbnH</i> ::pMUTIN4	-	4,660 \pm 209
RN4220 <i>sbnI</i> ::pMUTIN4	+	0 \pm 0
RN4220 <i>sbnI</i> ::pMUTIN4	-	3,330 \pm 188
RN4220 SA0121::pMUTIN4	+	106 \pm 3
RN4220 SA0121::pMUTIN4	-	89 \pm 10
RN4220 <i>galE</i> ::pMUTIN4	+	3,046 \pm 525
RN4220 <i>galE</i> ::pMUTIN4	-	2,146 \pm 76
RN4220 <i>fur sbnF</i> ::pMUTIN4	+	264,425 \pm 6,581
RN4220 <i>fur sbnF</i> ::pMUTIN4	-	231,425 \pm 5,720

and that expression of the 10th coding region and *galE* is not iron regulated and likely plays no role in the production of siderophore. The observation that *sbnA* and *sbnF* were transcribed to the highest levels under iron-deficient growth conditions, while *sbn* genes further downstream appeared to be transcribed to lesser amounts under similar growth conditions, suggests that expression of the operon is controlled by one iron-regulated promoter element present upstream of the *sbnA* coding region.

The putative *sbnA* start codon is preceded by a sequence which resembles a staphylococcal Shine-Dalgarno sequence (AGGAAGA) (Fig. 3) (22). Approximately 50 bp further upstream, a 19-bp sequence (TGAGAATCATTATCAATTA) that bears a striking resemblance to consensus Fur boxes was found, suggesting that expression of the *sbn* operon is regulated by exogenous iron concentrations via the *S. aureus* Fur homolog. This would be consistent with our earlier observations (see above) that siderophore production was derepressed

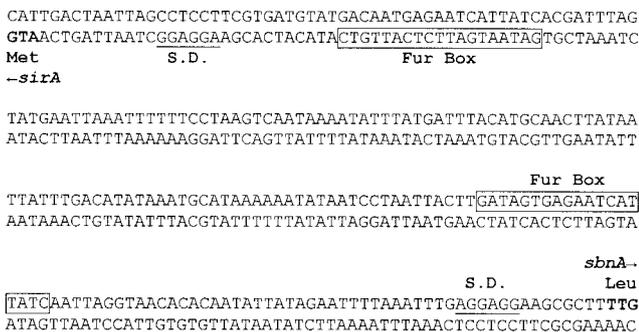


FIG. 3. Promoter region for the *sirABC* and *sbn* operons. Putative Fur box sequences are boxed. Also shown are the predicted start codons for the *sirA* and *sbnA* genes, along with predicted Shine-Dalgarno sequences.

in a *fur* background. Indeed, in a *fur*-deficient background, β -galactosidase expression from the strain bearing an *sbnF-lacZ* fusion was extremely high when the cells were grown in iron-replete medium, indicating that the Fur protein represses transcription of the *sbn* operon under iron-rich growth conditions.

An *sbnE* mutant demonstrates a growth defect in iron-deficient medium. To assess the contribution of siderophore production to in vitro growth of *S. aureus*, RN6390 and Newman, their isogenic *sbnE*::Km mutants (H672 and H686, respectively) and the complemented mutants were grown in defined minimal medium. When grown in TMS medium supplemented with 10 μ M EDDHA and 50 μ M FeCl₃ (iron-replete medium), the growth yields of all of the strains were not appreciably different from one another (Fig. 4A). However, the growth of both H672 and H686 (*sbnE* mutants) was severely impaired, relative to that of their isogenic parents and the *sbnE* mutants carrying plasmid pSED32 (carrying multicopy *sbnE* gene), in the identical medium but lacking FeCl₃ (Fig. 4B). Given that the iron-sufficient versus the iron-deficient medium differed only by the presence or absence of FeCl₃, the suggestion that the poor growth phenotype of the *sbnE* mutants was due to the possible chelation of other essential elements by EDDHA can be ruled out. Thus, the *sbnE* mutants are impaired solely in iron acquisition.

Siderophore production enhances the virulence of *S. aureus*. *S. aureus* can survive and replicate in blood to cause infection despite the fact that this environment is iron restrictive. Moreover, recent reports have demonstrated that *S. aureus* can express proteins with the ability to bind to host iron sources such as heme and hemoglobin (17). Thus, in an effort to determine whether siderophore production in *S. aureus* is involved in the pathogenesis of this bacterium, the ability of the *sbnE* mutant to colonize mice was compared to that of its isogenic parent. Swiss-Webster mice were used in a murine kidney abscess model of *S. aureus* infection. The kidneys of individual mice injected with *S. aureus* Newman contained an average of more than 10⁸ bacteria at both 5 and 6 days postinjection (Fig. 5). Kidneys from these mice possessed multiple cortical and medullary abscesses. In contrast, the kidneys from mice injected with H686 (Newman *sbnE*::Km) lacked observable abscesses, average numbers of bacteria recovered from the kidneys were below 10⁷ at day 5 postinjection, and no bacteria were recoverable at day 6 postinjection (Fig. 5), illustrating that the *sbnE* mutant bacteria were significantly attenuated in this model. These data implicate siderophore production as an important factor in the ability of *S. aureus* to survive in vivo.

The *sbn* operon is present in *S. aureus* but not in the CoNS. Given the demonstrated importance of siderophore production to the pathogenicity of *S. aureus*, we wished to determine whether the *sbn* genes were specific to *S. aureus* or whether they were also present in other staphylococci. Dot blotting experiments, performed under low-stringency hybridization conditions, were performed in efforts to detect *sbnA*, *sbnC*, *sbnE*, and *sbnH* homologs in several other members of the staphylococci. Whereas *sbn* genes were readily detected in all laboratory and clinical strains of *S. aureus* tested (see Table 1 for a complete list of strains used), we were unable to detect the presence of these genes in any of 13 different species of

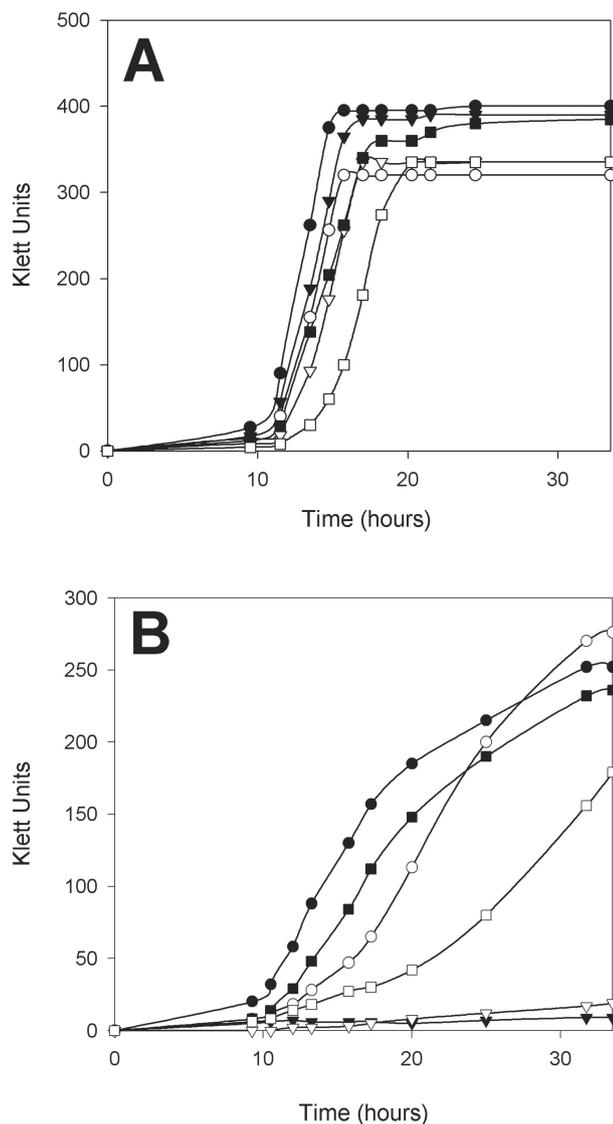


FIG. 4. Effect of an *sbnE* mutation on the growth of *S. aureus*. Shown are growth curves of *S. aureus* RN6390 (●), Newman (○), H672 (RN6390 *sbnE*::Km) (▼), H686 (Newman *sbnE*::Km) (▽), H672 plus pSED32 (■), and H686 plus pSED32 (□) grown in TMS medium supplemented with 10 μM EDDHA in the presence (A) or absence (B) of 50 μM FeCl₃. Bacteria were grown in sidearm flasks with vigorous shaking, and growth was monitored using a Klett meter. Growth experiments were performed in duplicate in three separate experiments. The results of a typical experiment are shown.

CoNS (Table 1). Homologs of these genes are also not present in the genome sequences of *Staphylococcus epidermidis* ATCC 12228 or RP62A. Thus, the *sbn* operon appears to be specific to *S. aureus* among the staphylococci.

The *sbn* operon is found in *Ralstonia solanacearum*. Interestingly, searches of the databases did reveal a similarly sized operon, present on a megaplasmid in the completed genome sequence of the phytopathogen *R.* (formerly *Pseudomonas*) *solanacearum*, whose products bear striking similarity to Sbn proteins (Table 4). Indeed, it is highly likely that the two operons evolved from the same ancestor since the *Ralstonia*

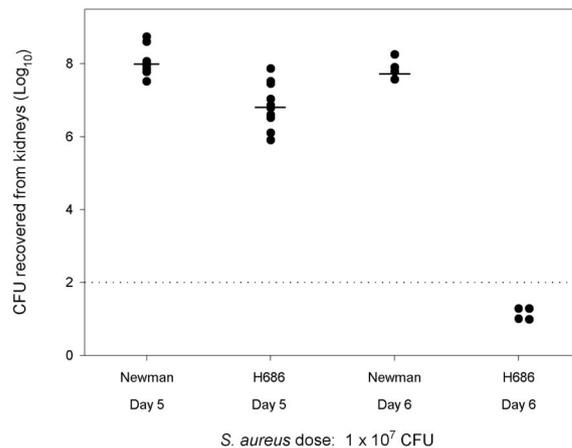


FIG. 5. An *sbnE* mutant is compromised in a murine kidney abscess model. Two groups of 12 mice were injected in the tail vein with 10⁷ bacteria. One group received *S. aureus* Newman, while the second group was infected with H686 (Newman *sbnE*::Km). CFU recovered from the kidneys of mice at both 5 (eight mice) and 6 (four mice) days postinfection are plotted. Each symbol represents the staphylococcal count in the kidneys of one animal, and the dotted line represents the limit of detection for staphylococci in this assay system. Data are representative of three independent experiments. Statistical significance was determined using the Student unpaired *t* test, and differences were found to be highly significant (*P* < 0.003).

homologs are present in the same order as the *sbn* genes in *S. aureus*. The *sbnE* homolog in *Ralstonia*, however, is present on the complementary strand compared with the rest of the coding regions in the *Ralstonia* operon. Another minor difference between the regions in *S. aureus* and *R. solanacearum* is that the *R. solanacearum* *sbnC* and *sbnD* homologs appear to be fused into one coding region. A striking dissimilarity between the *sbn* operon in *S. aureus* and the homologous region of DNA in *R. solanacearum* is the G+C content (in moles percent) of the respective operons. Whereas the operon in *R. solanacearum* has a G+C content of 72 mol%, the *S. aureus* *sbn* operon has a G+C content of 37 mol%. The G+C content of the *S. aureus* genome is approximately 32 mol%.

TABLE 4. Homolog of the *sbn* operon in *R. solanacearum*

Sbn protein	<i>R. solanacearum</i> homolog ^a	
	Identity (%)	Similarity (%)
SbnA	56	75
SbnB	58	75
SbnC	29	44
SbnD	28	42
SbnE	32	52
SbnF	36	54
SbnG	42	59
SbnH	47	63
SbnI	ND ^b	ND

^a Identities and similarities are between the predicted protein products.
^b ND, not determined.

DISCUSSION

S. aureus possesses both siderophore-mediated and nonsiderophore iron uptake systems, and the relative role that each system plays during pathogenesis needs to be resolved. In this study, we characterized a genetic locus (*sbn*) that is involved in the biosynthesis of a siderophore in *S. aureus*. We showed that an *sbnE* mutation correlates with impaired growth in an iron-deficient medium and decreased virulence of *S. aureus* in a murine kidney abscess model.

Mutation of the *sbnE* coding region resulted in the inability of *S. aureus* strains to synthesize an as yet structurally uncharacterized molecule that we have named staphylobactin. Introduction of multicopy *sbnE* into *sbnE* mutants resulted in the restored ability of *S. aureus* to produce staphylobactin, a molecule which when purified by HPLC promoted the iron-restricted growth of *S. aureus*. It would appear, then, that the staphylobactin molecule is a product synthesized from expression of the *sbn* operon; however, in order to validate this hypothesis, an elucidation of the structure of this molecule as well as a detailed functional characterization of the remainder of the *sbn* genes will be required.

The *sbnE* mutant derivatives of RN6390 and Newman, H672 and H686, respectively, grew equivalently to their isogenic wild-type parents in iron-rich medium. In contrast, the *sbnE* mutants were severely compromised in their ability to grow, relative to the wild type, under conditions of severe iron starvation (i.e., TMS supplemented with 10 μ M EDDHA). We did observe, however, that at moderate levels of iron restriction (i.e., TMS supplemented with 1 μ M EDDHA), H672 and H686 grew nearly as well as the wild type. The supernatants of mutants grown under these conditions did react positively in CAS assays, but we were unable to detect staphylobactin in culture supernatants. As well, *S. aureus* RN6390 grew significantly better under severe iron restriction than *S. aureus* Newman did, and the former seemed to produce higher levels of siderophore activity as measured by CAS assays. It is plausible that *S. aureus* RN6390 produces an additional siderophore(s) that Newman lacks and that they are produced under moderate levels of iron restriction. The significantly longer lag period of Newman versus RN6390 in growth assays under conditions of severe iron restriction (Fig. 4B) supports this argument. Alternatively, there may be differences in the regulation of staphylobactin production between the two strains. For example, the levels of iron restriction needed for expression of *sbn* genes or the amount of staphylobactin produced, may be different in Newman than in RN6390. Other research groups have reported differences in the levels of siderophore produced by different members of the staphylococci (5, 16). Our results support the suggestion that expression of the *sbn* genes is important in situations of severe iron starvation in culture and, most certainly, is important in vivo since an *sbnE* mutant was attenuated in mice. Although the *sbnE* mutant was unable to survive for any period beyond 5 days in vivo, it is interesting that an average of 6×10^6 bacteria could still be recovered from kidneys at day 5 of the infection. Although it appears that the bacteria were actively being cleared, this observation supports the notion that siderophore production may not be required for the establishment of an infection but most certainly appears to be required for prolonged survival of *S. aureus* in

vivo. *S. aureus* may be able to scavenge various different sources of host iron (e.g., heme and hemoglobin) during the establishment of an infection, and indeed, *S. aureus* does possess the ability to bind heme and hemoglobin (17).

Attempts to isolate readily detectable quantities of staphyloferrin A and staphyloferrin B from *S. aureus* RN6390 and Newman were unsuccessful even when the culture media were supplemented with DL-ornithine and 2,3-diaminopropionic acid, precursors in the synthesis of staphyloferrin A and staphyloferrin B, respectively. This was surprising since previous studies have demonstrated staphyloferrin A and staphyloferrin B production by several different strains of *S. aureus* and several CoNS. Staphylobactin, with an *m/z* of 822—significantly larger than that of staphyloferrin A (*m/z* = 480), staphyloferrin B (*m/z* = 448), or the structurally uncharacterized aureochelin (*m/z* = 577)—may represent a novel structure. It is also feasible that one of the staphyloferrin molecules may comprise a part of the structure of staphylobactin. Further characterization of this molecule is obviously required to address this question.

The *sbnABCDEFGHI* operon (SA0112 to SA0120 in *S. aureus* N315 and SAV0116 to SAV0124 in *S. aureus* Mu50) was identified in searches of several *S. aureus* genomes for coding regions whose products shared significant similarity with siderophore biosynthetic genes in other bacteria. The operon is present in all completed and ongoing genome sequencing projects, and we could detect *sbn* genes in all *S. aureus* strains used in this study. We were unable to detect the presence of *sbn* genes, using low-stringency hybridization techniques, in CoNS species. We also could not identify homologs of any of the *sbn* genes in the genome sequences of *S. epidermidis* RP62A and *S. epidermidis* ATCC 12228. Since a previous investigation demonstrated the presence of the staphyloferrins in *S. epidermidis* strains (18), this lends further support to the idea that the *sbn* operon is responsible for the production of a siderophore not previously identified in the staphylococci. Our results suggest that the CoNS, generally less pathogenic than *S. aureus* due in large part to a relative lack of virulence factors, would appear to lack the ability to produce staphylobactin. As noted in this study, the ability to produce this siderophore, synthesized via expression of the *sbn* operon, correlates with enhanced virulence of *S. aureus* in a murine kidney abscess model and may, therefore, represent another key determinant that dictates differences in the virulence of CoNS versus *S. aureus*.

An operon homologous to the *S. aureus sbnABCDEFGHI* locus was identified in the genome sequence of *R. solanacearum*, specifically on the 2.1-Mb megaplasmid present in this bacterium. While the G+C contents of the two operons are quite different, reflecting the codon bias of the two organisms (72 mol% for the *R. solanacearum* locus; 37 mol% for *sbn* in *S. aureus*), the organization of predicted genes within the two loci and the similarities between the respective predicted proteins are striking. Given the different niches occupied by the two bacteria (primarily humans for *S. aureus* versus the soil for *R. solanacearum*), it is quite interesting to speculate as to how this locus might have been acquired by, or how it evolved in, these two different bacteria.

A consensus Fur box is centered approximately 60 bp upstream of *sbnA*, and our data support a role for Fur in the

transcriptional regulation of the *sbm* operon. The Fur box sequence shares 17 of 19 bp with the *S. aureus* consensus Fur box as described previously (13). Divergently transcribed from the *sbm* operon is *sirABC*, an iron-regulated operon whose products are similar to ABC transporters involved in the uptake of iron(III) siderophores (12). The *sir* and *sbm* operons are separated by 230 bp that possesses two putative Fur box sequences for the transcriptional control of each operon. The predicted *sirA* gene product is a 36-kDa lipoprotein that shares similarity with a periplasmic iron(III) siderophore-binding protein in *E. chrysanthemi*, while SirB and SirC, sharing significant similarity with CbrB and CbrC, respectively, are likely to encode transmembrane permease components of an ABC transporter. The proximity of the *sbm* and *sir* operons lead us to speculate that the SirABC proteins are involved in the transport of staphylobactin, a hypothesis we are currently testing.

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