

A Heme-responsive Regulator Controls Synthesis of Staphyloferrin B in *Staphylococcus aureus**[♦]

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Staphylococcus aureus possesses a multitude of mechanisms by which it can obtain iron during growth under iron starvation conditions. It expresses an effective heme acquisition system (the iron-regulated surface determinant system), it produces two carboxylate-type siderophores staphyloferrin A and staphyloferrin B (SB), and it expresses transporters for many other siderophores that it does not synthesize. The ferric uptake regulator protein regulates expression of genes encoding all of these systems. Mechanisms of fine-tuning expression of iron-regulated genes, beyond simple iron regulation via ferric uptake regulator, have not been uncovered in this organism. Here, we identify the ninth gene of the *sbn* operon, *sbnI*, as encoding a ParB/Spo0J-like protein that is required for expression of genes in the *sbn* operon from *sbnD* onward. Expression of *sbnD-I* is drastically decreased in an *sbnI* mutant, and the mutant does not synthesize detectable SB during early phases of growth. Thus, SB-mediated iron acquisition is impaired in an *sbnI* mutant strain. We show that the protein forms dimers and tetramers in solution and binds to DNA within the *sbnC* coding region. Moreover, we show that SbnI binds heme and that heme-bound SbnI does not bind DNA. Finally, we show that providing exogenous heme to *S. aureus* growing in an iron-free medium results in delayed synthesis of SB. This is the first study in *S. aureus* that identifies a DNA-binding regulatory protein that senses heme to control gene expression for siderophore synthesis.

Staphylococcus aureus is a formidable human pathogen capable of causing a wide range of opportunistic infections, including endocarditis, meningitis, and osteomyelitis, and it is currently the most frequent cause of bloodstream infections in Canada and the United States (1–5). Like the vast majority of all characterized organisms, with the exception of some lactobacilli and spirochetes (6, 7), *S. aureus* requires the use of iron to survive. Iron in a human host, however, is typically not free as it is bound within host proteins that serve essential host functions, and at the same time, it limits the amount of readily available iron to invading microbes (8, 9). This process of iron limi-

tation is referred to as host nutritional immunity and is an obstacle to the survival of microbes, including *S. aureus*.

To overcome this obstacle, *S. aureus* has evolved multiple sophisticated iron uptake mechanisms (10, 11). Because the predominant source of iron in the human body is iron bound to heme, representing greater than 70% of all intracellular iron, heme is an important source of iron during *S. aureus* infection (12). *S. aureus* acquires heme from hemoglobin using proteins of the iron-regulated surface determinant (Isd) pathway (13). This well characterized pathway involves a series of cell wall-anchored proteins, lipoproteins, membrane permeases, and cytosolic proteins. Together, these proteins function to bind hemoglobin, extract heme from hemoglobin, mobilize heme through the thick peptidoglycan layer to a membrane ABC transporter, and degrade heme once it enters the cytoplasm (10, 11, 14–16).

Representing less than 0.1% of iron in the body, an important extracellular source of iron is that which is bound by host glycoproteins transferrin and lactoferrin (9). Through the use of small iron-chelating molecules, referred to as siderophores, *S. aureus* is capable of removing iron from these glycoproteins and delivering it to the bacterial cell (10, 11). There are two predominant classes of siderophore synthesis pathways as follows: non-ribosomal peptide synthetase-dependent and non-ribosomal peptide synthetase-independent siderophore (NIS)³ syntheses (17). Non-ribosomal peptide synthetase siderophore synthesis has been well characterized for many siderophores, such as enterobactin (18). *S. aureus* produces two citrate-based siderophores: staphyloferrin A (SA) and staphyloferrin B (SB). As NIS siderophores, SA and SB are synthesized not in a step-wise fashion using large multidomain/modular proteins but instead are constructed through use of synthetase enzymes that form siderophores by sequential condensation reactions of alternating subunits of dicarboxylic acid with amino-alcohols, alcohols, and diamines (19–22).

The biosynthetic enzymes and efflux protein for SA are encoded from within the *sfa* (also known as *sfna*) locus (22, 23), where genes *sfaA*, *sfaB*, and *sfaC* form an operon, and *sfaD* is divergently transcribed to *sfaA*. SfaB and SfaD are NIS type A-like synthetases that facilitate the condensation of D-ornithine with two molecules of citrate (22). SfaC is a pyridoxal-

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³ The abbreviations used are: NIS, non-ribosomal peptide synthetase-independent siderophore; SA, staphyloferrin A; SB, staphyloferrin B; Fur, ferric uptake regulator; L-Dap, L-2,3-diamino-propionic acid; qPCR, quantitative real time PCR; cTMS, Chelex-100-treated Tris minimal succinate; RACE, rapid amplification of cDNA end; UV-Vis, UV-visible; EDDHA, ethylenediamine-*N,N'*-bis(2-hydroxyphenyl)acetic acid.

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phosphate-dependent amino acid racemase, and SfaA is an efflux pump that secretes SA into the extracellular milieu (22, 24).

The 9-gene *sbn* gene cluster encodes for SB biosynthesis and efflux, and these genes have been implicated in virulence in both the mouse bacteremic and the rat infective endocarditis models of *S. aureus* infection (25, 26). SbnA and SbnB perform the first step in SB synthesis with the formation of the precursor molecules L-2,3-diaminopropionic acid (L-Dap) and α -ketoglutarate from O-phospho-L-serine and L-glutamate (21). SbnG acts as a citrate synthase to convert acetyl-CoA and oxaloacetate into citrate (27, 28). L-Dap, citrate, and α -ketoglutarate are the constituents of SB. The siderophore synthetase enzymes SbnC, SbnE, and SbnF facilitate condensation reactions with these precursors to build the SB molecule, whereas SbnH is a decarboxylase that converts one of the two L-Dap molecules within SB into ethylenediamine (19). The function of the terminal gene product in the *sbn* operon, SbnI, is previously uncharacterized, and thus the foundation of this study was to determine the function of SbnI in the cell.

The regulation of genes that encode for proteins implicated in iron metabolism in *S. aureus* is controlled by the ferric uptake regulator (Fur), a transcriptional regulator found in many Gram-positive and -negative bacteria (29). Functioning as a dimer and with the use of metal cofactors, Fur binds to an AT-rich 19-bp palindromic consensus sequence referred to as the Fur box, located in the promoter region of regulated genes. Fur bound to the Fur box results in limited transcription of downstream genes (29, 30).

In the absence of Fur repression during growth in iron-starved conditions, some bacteria adopt additional regulatory mechanisms for fine-tuning gene expression of iron-regulated genes. In *Ralstonia solanacearum*, a soil-borne pathogen that also utilizes staphyloferrin B for iron acquisition, the synthesis of SB is negatively regulated by Fur in high iron concentrations and is also negatively regulated in low iron concentrations by PhcA (31). PhcA is an environmentally responsive transcription factor that responds to the presence of the autoinducer 3-hydroxypalmitic acid methyl ester, a molecule produced as a result of quorum sensing (31, 32). Thus PhcA is able to afford *R. solanacearum* as an additional level of control for siderophore synthesis that works in response to environmental cues. In *S. aureus*, however, it is unclear as to what regulates the expression of many genes implicated in iron metabolism when cells are growing in limited iron supply.

In this study, we report a novel heme-responsive regulatory protein, SbnI, that controls expression of the genes in the *sbn* operon and thus controls siderophore-mediated iron uptake. The *sbnI* mutant had an SB-dependent growth defect in iron-limited media, because of delayed and reduced production of SB, due to significantly decreased transcript levels for genes *sbnDEFGH* in the mutant. Electrophoretic mobility shift assays demonstrated that SbnI binds DNA upstream of a newly identified promoter within the *sbnC* coding region. Moreover, we show that SbnI is capable of binding to heme and heme binding inhibits DNA binding. We propose a model whereby SbnI is required for transcription throughout the SB biosynthetic operon and senses intracellular heme as a means to reduce SB

synthesis in favor of heme acquisition. This model supports the observations of Skaar *et al.* (12) who reported that heme is the preferred iron source for *S. aureus*.

Experimental Procedures

Bacterial Strains, Plasmids, and Growth Media—All bacterial strains and plasmids used in this study are listed and described in Table 1. Strains were stored in 15% glycerol stocks at -80°C and, prior to use, were streaked onto tryptic soy broth (TSB) (Difco) agar plates containing the appropriate antibiotic. Solid media were prepared with addition of 1.5% w/v Bacto agar (Difco). Concentrations of antibiotics used were as follows: 50 $\mu\text{g}/\text{ml}$ kanamycin and 100 $\mu\text{g}/\text{ml}$ ampicillin for *Escherichia coli* selection, 4 $\mu\text{g}/\text{ml}$ tetracycline, 5 $\mu\text{g}/\text{ml}$ chloramphenicol, and 3 $\mu\text{g}/\text{ml}$ erythromycin for *S. aureus* selection. *E. coli* strains were grown in Luria Broth (Difco), and *S. aureus* strains were grown in TSB or either Chelex-100-treated Tris minimal succinate (cTMS) or Roswell Park Memorial Institute (RPMI) (Gibco) medium for growth under iron restrictions. All solutions and media were made using water purified with the Milli-Q water purification system (Millipore).

Bacterial Growth Curves—Bacteria were cultured on TSB agar, and then several single isolated colonies were picked and inoculated into 2 ml of cTMS (flask/volume ratio of 10:1) and grown for ~ 8 h at 37°C with shaking at 220 rpm. The bacteria were then harvested, resuspended in fresh cTMS medium, and inoculated into 5 ml of cTMS medium (flask/volume ratio 10:1) to a starting A_{600} equivalent of 0.005.

Western Blotting—Rabbit polyclonal antisera recognized SbnI were generated by ProSci (Poway, CA) using the custom antibody production package number 1.

Cells were grown to an A_{600} of 1.0, and 3×10^9 colony-forming units were harvested and resuspended in 0.1 ml of lysis buffer (25 mM Tris-HCl, 50 mM glucose, 150 mM NaCl, 10 mM EDTA, pH 8.0, 5 μg of lysostaphin, $1 \times$ Laemmli buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.01% bromophenol blue)) and incubated at 37°C for 1 h and then boiled for 10 min before being run through a 12% polyacrylamide gel. Following electrophoresis, proteins were transferred to a nitrocellulose membrane following standard protocols. Primary anti-SbnI polyclonal antiserum was used at a dilution of 1:1000, and secondary antiserum (anti-rabbit IgG conjugated to IRDye 800; Li-Cor Biosciences, Lincoln, NE) was used at a 1:20,000 dilution. Membranes were scanned on a Li-Cor Odyssey Infrared Imager (Li-Cor Biosciences) and visualized using Odyssey Version 3.0 software.

RNA Isolation and qPCR—*S. aureus* RN6390 and H984 (RN6390 *sbnI*) were grown in quadruplicate cultures in 2 ml of cTMS medium for 8 h. Cultures were then harvested and subcultured at A_{600} 0.005 in 10 ml of cTMS and grown to mid-exponential phase ($A_{600} \sim 1.0$). Cells equating to an A_{600} of 3.0 were harvested for each culture, and RNA extraction was performed by E.Z.N.A[®] total RNA kit according to the manufacturer's instructions with the addition of 0.25 $\mu\text{g}/\text{ml}$ lysostaphin to the lysis solution. RNA purity was determined by agarose gel, and RNA concentration was determined by NanoDrop[®] ND-1000 UV-Vis spectrophotometer. cDNA preparation was performed using 500 ng of total cellular RNA reverse-tran-

TABLE 1

Bacterial strains, plasmids, and primers used in this study

The following abbreviations are used: Ap^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Ery^R, erythromycin resistance; Km^R, kanamycin resistance; Tet^R, tetracycline resistance; AUAP, abridged universal anchor primer; UAP, universal anchor primer.

Bacterial strains, plasmids, oligonucleotides	Description	Source or Ref.
<i>E. coli</i>		
DH5α	F-Φ ₈₀ dLacZΔM15 <i>recA1 endA1 nupG gyrA96 glnV44 thi-1 hsdR17</i> (<i>r_k⁻ m_k⁺</i>) λ ⁻ <i>supE44 relA1 deoR Δ(lacZYA-argF)</i> U169	Promega
BL21(DE3)	F ⁻ <i>ompT gal dcm lon hsdS_B (r_B⁻ m_B⁺)</i> λ (DE3 [<i>lacI lacIUV5-T7 gene 1 ind1 sam7 nin5</i>])	Novagen
<i>S. aureus</i>		
RN4220	<i>r_k⁻ m_k⁺</i> accepts foreign DNA	47
RN6390	Prophage-cured wild-type strain	48
H306	RN6390 <i>sirA::Km</i> ; Km ^R	25
H984	RN6390 <i>sbnI::Tet</i> ; Tet ^R	This study
H1312	H984/pSbnI; <i>sbnI</i> mutant complemented with <i>sbnI</i>	This study
H1313	H187 containing pCN51	This study
H1317	H984 containing pCN51	This study
H1324	RN6390 <i>sbnI</i> ; Tet ^R	23
H1448	RN6390 <i>hts::Tet</i> ; Tet ^R	23
Plasmids		
pET28a(+)	Overexpression vector for His ₆ -tagged proteins; Km ^R	Novagen
pCN51	Cadmium-inducible expression vector; Ery ^R	49
<i>psbnI</i> (pAB1)	pCN51- <i>sbnI</i> ; pCN51 vector for expression of <i>sbnI</i> ; Ery ^R	This study
pGylux	Promoterless <i>E. coli/S. aureus</i> shuttle vector for luminescence, Ap ^R , Cm ^R	33
pCM326	1479-bp <i>sbnA/B</i> cloned into pGylux; Ap ^R , Cm ^R (fragment A)	This study
pCM328	2322-bp <i>sbnA/B/C</i> cloned into pGylux; Ap ^R , Cm ^R (fragment B)	This study
pCM330	2389-bp <i>sbnC/D</i> cloned into pGylux; Ap ^R , Cm ^R (fragment C)	This study
pCM332	2395-bp <i>sbnD/E</i> cloned into pGylux; Ap ^R , Cm ^R (fragment D)	This study
Oligonucleotides		
Cloning of <i>sbnI</i> from <i>S. aureus</i> for pET28a(+)	GC AGC CAT ATG AAT CAT ATT GCT GAA CAT TTA A (forward) TTG TGT CTC GAG TCA TCA TAT TTC CCT CAA CAT (reverse)	
Cloning of <i>sbnI</i> from <i>S. aureus</i> for complementing vector (<i>psbnI</i>)	TTG AGC GGA TCC CTT AAG CCA TCC ACA TCC TG (forward) TTG CGC GAA TTC TCG CTA TCA ATA CCG TAA ATC (reverse)	
Cloning of fragment A	GCC TCC CCGGTCAATAAAAATATTTATGATTTACATGC (forward) GACTCCCGGGAACCTTGCTTCCATAACTGCAATT (reverse)	
Cloning of fragment B	GACTCCCGGGTAATTTAGGCATTGCGTTG (forward) GACTCCCGGGTGCACCACTAAAGG (reverse)	
Cloning of fragment C	GACTCCCGGGTGTCTGAACATTGCAG (forward) GACTCCCGGGAATGAACGGCGAATAC (reverse)	
Cloning of fragment D	GACTCCCGGGACTGACAGTACTTGT (forward) GACTCCCGGGCCTGTGTTACTAAAC (reverse)	
RT-PCR: <i>sbnA</i>	ACTTCTGGTAATTTAGGCATTGCG (forward) TGCATCAGGTTCTCAACCATTTC (reverse)	
RT-PCR: <i>sbnB</i>	CCTGAAAATGGACACATCGC (forward) CAAAAATAATGACGCCACTTGC (reverse)	
RT-PCR: <i>sbnC</i>	ATGAATGGGAAGTGGCTCG (forward) GCTAATGGATGAAATGGACGAT (reverse)	
RT-PCR: <i>sbnD</i>	CGTAGAAATACAGTTGTGGAGTGG (forward) AAATAAGCATACCGCAAACC (reverse)	
RT-PCR: <i>sbnE</i>	CGAATCCATTAGGGCAAACAG (forward) CTTAACATCGGTGAATCAGGC (reverse)	
RT-PCR: <i>sbnF</i>	TTTTATGCGATGGAAGGG (forward) CGTCGTTTCTACTTTATCTTTGTGCG (reverse)	
RT-PCR: <i>sbnG</i>	TCATTAAGTGTGGATATGGGTGC (forward) TTAGCCATCTCCATTGCATCAAG (reverse)	
RT-PCR: <i>sbnH</i>	CGCTCGCAATGCCAAAGATTC (forward) TAACGCCTATGCCACCAACAG (reverse)	
RT-PCR: <i>spoB</i>	AGAGAAAGACGGCACTGAAAACAC (forward) ATAACGACCCACGCTTGCTAAG (reverse)	
GSP1	GAAAGCTGATGTGCTGTTAG	
GSP2	GAGCCCGGGTCTAGACGCATACTTGAAGACGACAG	
AUAP	GATACCCGGGCCACGCGTTCGACTAGTAC	
UAP	GGCCACGCGTTCGACTAGTACGGGIIGGGIIG	

scribed using SuperscriptTM II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. For each qPCR, 1 μg of cDNA was amplified in a Rotor-Gene 6000 (Corbett Life Science) using the iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad). Gene expression for each sample was quantified in relation to *spoB* expression. A standard curve was generated for each gene examined.

Disc Diffusion Assays—Concentrated spent culture supernatants were prepared from 10-ml cultures of *S. aureus* RN6390. Culture supernatants were taken at 10, 12, 16, 20, 24, and 36 h after inoculation. Growth was assessed via *A*₆₀₀, and culture

densities between replicates and strains were normalized for each time point. Cells were harvested by centrifugation, and the supernatant was lyophilized for 12 h. Lyophilized supernatant was resuspended in 0.5 ml of sterile double-distilled H₂O. *S. aureus* cells, the *htsA* mutant strain or the *sirA* mutant strain, were seeded into TMS agar containing 10 μM EDDHA to achieve 1 × 10⁴ cells per ml. For each replicate, 10 μl of concentrated supernatant were applied to sterile paper discs that were then placed onto the plates containing the seeded reporter strains, and growth about the disc was measured after 24 and 48 h of incubation at 37 °C.

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Identification of Promoters—Four DNA fragments, spanning the *sbn* operon from *sbnA* to *sbnE*, were cloned into the promoterless shuttle vector pGylux (33) as follows: fragment A of 1479 bp (pCM326); fragment B of 2322 bp (pCM328); fragment C of 2389 bp (pCM330); and fragment D (pCM332) of 2395 bp. To measure luciferase activity, individual colonies from *S. aureus* carrying these plasmids were inoculated into TMS medium and incubated overnight at 37 °C. Cells were subsequently inoculated into cTMS with or without 1 μM FeCl_3 at an A_{600} 0.1 and incubated for 8 h. At that time, the A_{600} and luminescence were measured on a SynergyH4 Hybrid Reader (Biotek). Luminescence values were expressed as cps/ A_{600} .

Identification of a Transcription Start Site—To identify an initiation of transcription, we used rapid amplification of cDNA ends (RACE). The original mRNA was prepared from *S. aureus* RN6360 grown in cTMS, as per the protocol described for qPCR. First strand cDNA was synthesized by incubating for 50 min at 42 °C the following: 1 μg of RNA, 1 μM gene-specific primer 1 (GSP1), 50 μM dNTP, and 20 units of Superscript II reverse transcriptase (Invitrogen). The mRNA was removed by treating the synthesized cDNA with RNase mix (0.5 units of RNaseH and 50 units of RNaseT1) for 30 min at 37 °C. After purification of the cDNA with the BioArray cDNA purification kit (ENZO Life Sciences), it was C-tailed with 20 units of terminal transferase (Roche Diagnostics) and 0.5 mM dCTP as recommended by the supplier. The first PCR was performed using the universal anchor primer (Invitrogen) that will anneal to the C-tailed cDNA and an *sbnC*-specific primer GSP2. The second PCR (177 bp) was performed using the first PCR as a template, a primer that will anneal to the abridged universal anchor primer (Invitrogen) and the same specific primer GSP2. GSP2 and abridged universal anchor primer contain SmaI restriction endonuclease sites that were later used for cloning the second PCR product into pGEM7. The pGEM7 clones were later sequenced to determine the initiation of transcription.

Protein Expression and Purification—Recombinant SbnI protein was overexpressed and purified essentially as described previously (34). In brief, cells were grown to mid-log phase at 37 °C with aeration before the addition of 0.4 mM isopropyl β -D-thiogalactopyranoside; the cells were then grown for an additional 16 h at room temperature with shaking before harvesting. Cells were resuspended in 50 mM Hepes, 1 mM dithiothreitol (DTT), pH 7.4, prior to lysis at 25,000 p.s.i. in a Cell-Disruptor (Constants Systems Ltd.). Cell debris was pelleted by 15 min of centrifugation at 3000 rpm in a Beckman Coulter Allegra® 6R benchtop centrifuge that was followed by ultracentrifugation at 50,000 rpm in a Beckman Coulter Optima® L-900K ultracentrifuge for 45 min. The lysate was filtered with a 0.45- μm nylon filter (VWR) and then applied to a 1-ml His-Trap column (GE Healthcare) equilibrated with buffer A (50 mM Hepes, 1 mM DTT, and 10 mM imidazole, pH 7.4). The His₆-SbnI was eluted with a 0–80% concentration of buffer B (50 mM Hepes, 1 mM DTT, and 500 mM imidazole, pH 7.4). Protein was dialyzed in 50 mM Hepes, 1 mM DTT, pH 7.4, at 4 °C. Protein purification was determined by SDS-PAGE. Protein concentrations were determined through the Bradford assay using the Bio-Rad protein assay dye (Bradford) reagent concentrate (5 \times), and samples were read at A_{595} ; a standard

curve was made for each concentration determination with 1 mg/ml BSA.

Oligomerization Analysis—Size exclusion chromatography was performed using a Superdex 200 10/30 GL column (Amersham Biosciences) coupled to an FPLC system (Pharmacia). The column was equilibrated with 300 mM ammonium formate buffer, pH 7.4. SbnI samples, in 300 mM ammonium formate, were run in a 500- μl volume injected into the column and eluted at a flow rate of 200 $\mu\text{l}/\text{min}$. Protein was followed by absorption measurements at 280 nm. The column was calibrated with blue dextran (void volume), β -amylase from sweet potato (200 kDa), alcohol dehydrogenase from yeast (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase from bovine erythrocytes (29 kDa), and cytochrome *c* from horse heart (12.4 kDa).

Recombinant SbnI was dialyzed in 300 mM ammonium formate, pH 7.4, and 100 mM NaCl prior to sedimentation velocity ultracentrifugation analysis. For analysis of SbnI in reducing agent, buffer also contained 10 mM DTT added post-dialysis. The dialysis buffer was retained for use in the reference sector for all runs. Analytical ultracentrifugation was carried out in a Beckman XL-A analytical ultracentrifuge with a four-hole An-60Ti rotor and double-sector cells with Epon charcoal centerpieces. Centrifugation was carried out at 5 °C. A total of 60 absorbance measurements were taken at 280 nm at 5-min intervals at 0.002-cm radial steps and averaged over three readings. The rotor speeds were 35,000 rpm for SbnI in 100 mM NaCl and 45,000 rpm for SbnI in 20 mM DTT. Data were analyzed using non-linear regression in Sedfit software and fit to a $c(s)$ distribution to determine sedimentation coefficients corrected to 20 °C and in H_2O . All data were fit to a root mean square deviation equal to or less than 0.007.

DNA Binding Assays—Electrophoretic mobility shift assays (EMSA) were performed with purified SbnI and double-stranded, fluorescently labeled DNA. The DNA probe was amplified through PCR with IRDye® 700-labeled custom primers purchased from Integrated DNA Technologies (IDT®). Protein and DNA were incubated in a 25- μl volume that contained 120 ng of fluorescently labeled DNA, 50 mM Hepes, 240 $\mu\text{g}/\text{ml}$ bovine serum albumin (BSA), 15.2 $\mu\text{g}/\text{ml}$ poly[d(I-C)], and SbnI, at room temperature for 45 min. Samples were separated on 6% non-denaturing polyacrylamide gels in TBE buffer (10 mM Tris, 89 mM Tris borate, 2 mM EDTA, pH 8.3) at 120 V for 1.5 h. Competition EMSAs used 120 ng of labeled *sbnC* probe and 1200 ng of either unlabeled *sbnC* or *rpoB* probe. For EMSAs containing heme, a 4 mM heme stock was diluted to 250 μM in 50 mM Hepes, pH 7.4, just prior to use. For preparation of heme stock, see below.

Heme Titrations—Heme stocks were prepared as follows. Bovine heme (Sigma) was resuspended to 5 mM in 0.1 N NaOH (*i.e.* 0.00326 g/ml) and vortexed vigorously until in solution. The solution was sterilized by filtration through a 0.2-micron filter. A series of dilutions were prepared in 0.1 N NaOH, and scanning UV-Vis spectra were taken on the solution. The post-filtration concentration was determined on this solution using the molar extinction coefficient for hemin in 0.1 N NaOH of 58,400 $\text{cm}^{-1} \text{M}^{-1}$ at 385 nm (35). Heme solution was aliquoted and stored at -20 °C. Heme stocks were diluted just

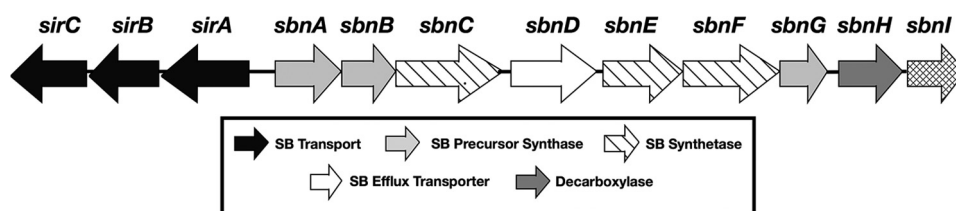


FIGURE 1. *sir-sbn* locus schematic. The *sbnI* gene is situated as the 9th and terminal gene of the operon.

prior to use in the buffer or media appropriate to the experiment.

Heme titrations, using UV-Vis spectroscopy, were performed using 1 μM SbnI and increasing concentrations of heme. SbnI was in 300 mM ammonium formate, pH 7.4, and incubated for 5 min at room temperature with heme concentrations ranging from 0.2 to 2 μM , with a 0.2 μM increase after each reading. Heme stocks (2.5 mM) were prepared from hemin chloride (Sigma) by adding NaOH, which were further diluted with 300 mM ammonium formate, pH 7.4, to a working concentration of 200 μM . UV-Vis spectra were recorded using Cary 50 Bio UV-Vis spectrophotometer with background correction for buffer and for each heme concentration. Triplicate samples were recorded.

ESI-MS was performed by using 50 μM recombinant SbnI, stored in 300 mM ammonium formate and 10 mM DTT, pH 7.4, and titrated with molar equivalents of heme. Heme was prepared in a 1 mM stock by dissolving 10 mg of hemin in concentrated NH_4OH neutralized with 10 mM ammonium formate, pH 6. The average incubation time for SbnI and heme was 3 min. Spectral data were recorded with Bruker Micro-TOF II (Bruker Daltonics, Toronto, Ontario, Canada) operated in the positive ion mode set for soft ionization. Scan parameters were set for 500–4000 m/z , and the spectra were collected for 1 min, minimum, and deconvoluted using the maximum entropy license for the Bruker Compass data analysis software.

Results

Requirement of Iron-regulated SbnI for SB Production in S. aureus—SB can be synthesized entirely *in vitro* using purified *sbn*-derived enzymes (19–21, 27). Indeed, SB can be synthesized from the activities of SbnA, SbnB, SbnC, SbnE, SbnF, SbnG, and SbnH when provided with *O*-phospho-L-serine, glutamate, oxaloacetate, and acetyl-CoA as substrates (19–21, 27). Despite its product seemingly having no essential enzymatic role in SB synthesis, *sbnI*, the ninth gene in the *sbn* operon (Fig. 1), is conserved in *S. aureus* strains, along with the rest of the *sbn* operon. This suggests it may play an important role in SB biosynthesis. Searches of the protein databases revealed that the protein shared limited sequence similarity with the DNA partitioning proteins ParB and SpoJ.

To assess the functional role, if any, of SbnI in SB synthesis, we knocked out *sbnI* by insertion of a tetracycline-resistance cassette into the middle of the gene. As part of our initial studies into the functional role of SbnI, we first confirmed its expression profile in WT *S. aureus*. A canonical Fur box was situated upstream of *sbnA*, and as such, expression of the genes within the operon is regulated by iron levels. qPCR was used to confirm that expression of the predicted terminal gene of the

operon, *sbnI*, is similarly regulated by iron levels (Fig. 2A). Western blot analysis, using anti-SbnI antisera, corroborated the qPCR findings, demonstrating that SbnI protein expression was controlled by iron (Fig. 2B). Western blots also confirmed that the *sbnI* insertion mutant (H984) lacked detectable expression of SbnI (Fig. 2B).

With the confirmed *sbnI::Tet* mutant strain, H984, growth kinetics were recorded, comparing the relative growth of the WT, H984, H984 + *psbnI*, and the Δsbn (*i.e.* complete operon knock-out) strains grown in RPMI 1640 medium, an iron-restricted, chemically defined medium. In this medium, and in comparison with the WT and complemented strains, H984 had a severe growth defect, similar to the growth defect observed for strain H1342, the Δsbn strain (Fig. 3). Because our previous work has shown that production of SA is repressed in RPMI 1640 medium (28), these results suggested that SbnI was critical for SB-mediated iron acquisition.

Because WT *S. aureus* strains have the ability to produce both SA and SB under conditions of iron starvation, and because their activity can be readily detected in bioassay experiments (20, 21, 27, 28), a disc diffusion bioassay was performed to examine SB production in RN6390 (WT), H984, and the complemented strain (H984 + *psbnI*). For these experiments, bacteria were grown in cTMS medium which, as opposed to RPMI 1640 medium, does not result in inhibition of SA or SB. As shown in Fig. 4, in this medium the growth was comparable among the three strains tested, throughout the duration of the experiment. Spent culture supernatants were taken at six designated time points, hours 10, 12, 16, 20, 24, and 36 post-inoculation. The supernatants were then applied onto sterile paper discs on agar seeded with the *sirA* or *htsA* mutant strains. The *sirA* strain is unable to uptake SB and thus relies on SA uptake for growth, whereas the *htsA* strain is unable to uptake SA, and thus its growth is reliant on SB. The data demonstrated that, compared with WT RN6390, H984 seemed to make detectable quantities of SA sooner in the growth cycle (Fig. 4A). However, more striking, and in agreement with data in Fig. 3, although RN6390 produced detectable quantities of SB by 10 h, we were unable to detect SB from the supernatants of H984 until at least the 24-h time point. This defect was complemented by supplying *sbnI* *in trans* (Fig. 4B). These results suggest that SbnI plays a critical role in the synthesis of SB, particularly at early time points, and that the absence of SbnI does not hinder SA production, but rather it may result in increased production of SA.

Expression of the SB Biosynthetic and Efflux Genes Requires SbnI—In accordance with the idea that SbnI affected synthesis of SB, despite having no clear enzymatic function in the

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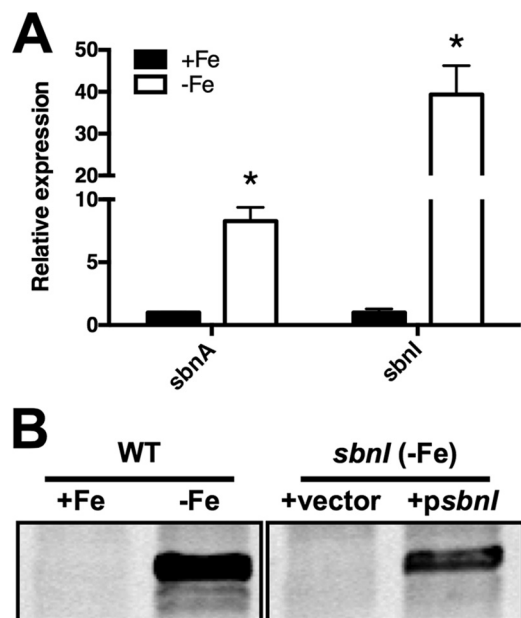


FIGURE 2. Expression of *sbnI* is regulated by iron. *A*, qPCR data showing significant increase in gene expression of *sbnA* and *sbnI* in WT RN6390 when grown in iron-restricted media versus iron-replete medium (*, $p < 0.01$). All strains were grown in cTMS with (+Fe, solid bars) or without (-Fe, clear bars) $50 \mu\text{M}$ FeCl_3 . Gene expression of strains grown in iron-replete medium is used as comparator and is arbitrarily set to 1. Statistics were performed using Student's unpaired *t* test. *B*, Western blot with α -SbnI antisera demonstrating iron-regulated control of SbnI. WT RN6390 cells were grown in cTMS medium with (+Fe) or without (-Fe) addition of $50 \mu\text{M}$ FeCl_3 (left panel). Immunoblotting also confirmed the lack of expression of SbnI in the *sbnI* mutant strain carrying pCNS1 (vehicle control; vector), and complementation of expression in the mutant carrying *psbnI* (carries *sbnI* gene).

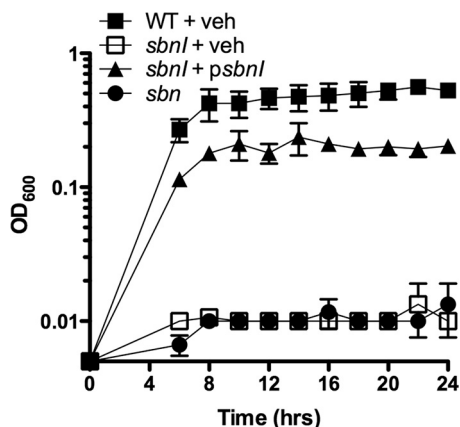


FIGURE 3. Growth kinetics of *S. aureus* strains in RMPI 1640 medium. RN6390 and derivatives were grown in RMPI 1640 medium incorporating $0.2 \mu\text{M}$ EDDHA to further enhance iron restriction. Vehicle control (*veh*) was empty pCNS1.

biosynthetic pathway (20, 21, 27), and given that SbnI shares low sequence similarity to the DNA-binding proteins ParB and Spo0J, we theorized that SbnI may function as a regulatory protein that impacted expression of SB biosynthetic genes. To evaluate this, RNA levels were measured for each of the genes throughout the *sbn* operon, comparing levels in WT to those in H984. Strikingly, these experiments revealed a dramatic drop (90–99% decrease) in RNA transcripts for genes *sbnD* through *sbnH* in H984 compared with RN6390 (Fig. 5).

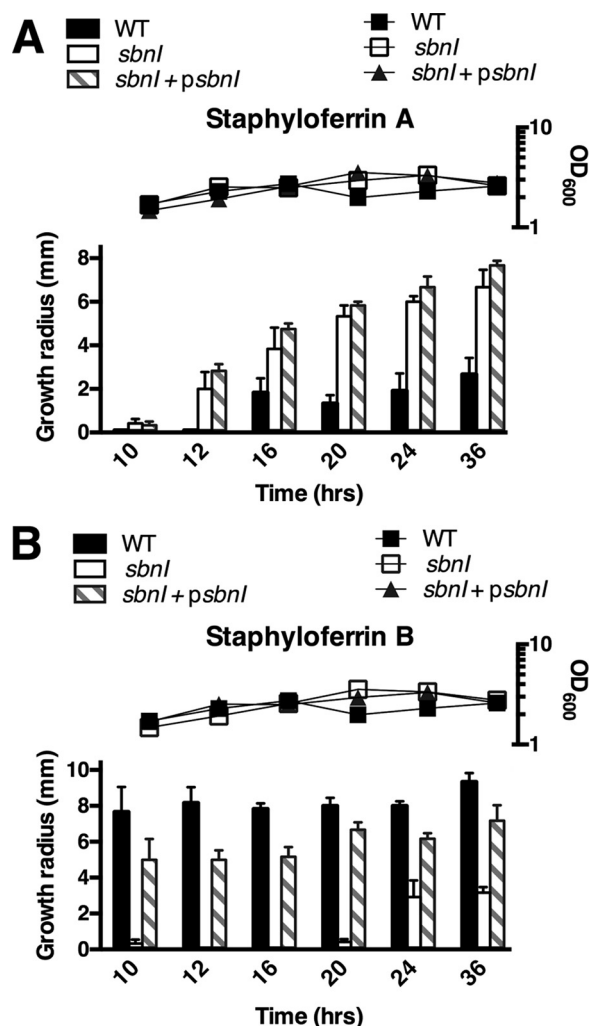


FIGURE 4. Disc diffusion assay demonstrating requirement of *sbnI* for SB production. RN6390 and derivatives were grown in cTMS medium with $0.1 \mu\text{M}$ EDDHA. Triplicate spent culture supernatants were analyzed at indicated time points post-inoculation. Supernatants were concentrated and resuspended in sterile distilled H_2O and placed onto sterile paper discs subsequently placed on cTMS agar seeded with either the *sirA* (A) or the *htsA* (B) strain. Growth around the disc was an indication of the presence of SA (A) or SB (B). All strains were grown in cTMS medium in which growth, as measured by OD_{600} (right y axis), was found to be comparable.

Identification of an Internal Promoter in the sbn Operon— Because SbnI had a drastic effect on the expression of genes downstream of *sbnC*, we theorized that SbnI may be regulating expression of genes downstream of *sbnC* by regulating the activity of a promoter internal to the operon. In an attempt to demonstrate this, we cloned several DNA fragments spanning the region from *sbnA* through *sbnE* upstream of the promoterless luciferase genes in the vector pGylux (Fig. 6A). The constructs were then introduced into strains RN6390 and H984, and the bacteria were grown in cTMS medium with or without added iron. As expected, these experiments showed that there was robust promoter activity in the fragment carrying the promoter region upstream of *sbnA*, and this activity was ~40% decreased in H984 (Fig. 6A), in agreement with the qPCR data in Fig. 5. Furthermore, in agreement with the presence of a canonical Fur box upstream of *sbnA*, the activity from the *sbnA* promoter was significantly ($p < 0.01$) attenuated when the cells

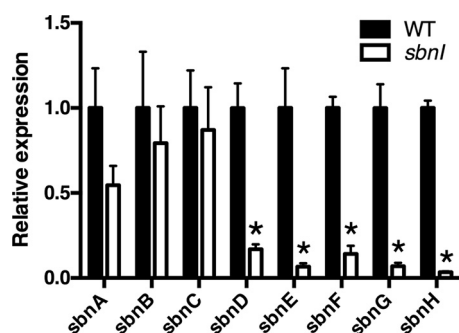


FIGURE 5. Mutation of *sbnI* results in altered expression throughout the *sbn* operon. RN6390 and its *sbnI*::Tet derivative were grown in cTMS with 0.1 μ M EDDHA, a medium in which growth was comparable between the two strains. qPCR was used to measure RNA levels for each gene, where WT gene expression was set to 1. Statistics were performed using the Student's unpaired *t* test. *, $p < 0.005$.

were grown in medium containing iron *versus* in the same medium but lacking iron (Fig. 6A). In addition to DNA fragment A, carrying the *sbnA* promoter region, we observed that DNA fragment C also demonstrated fairly strong promoter activity that was also significantly ($p < 0.01$) regulated by iron levels in the growth medium. Moreover, this activity was abrogated in H984, indicating that it was SbnI-dependent. This phenotype was complemented in H984 carrying *sbnI* in *trans* (data not shown).

RACE was performed for fragment C to elucidate the transcription start site present within the fragment. The experiment was repeated on multiple biological samples for robustness and demonstrated the presence of a confirmed transcription start site at position A82356 (based on genome *S. aureus* Newman), which is located 625 bp downstream of the start of the *sbnC* coding region (Fig. 6B).

SbnI Forms Dimers and Is Able to Bind DNA Region Upstream of *sbnD*—Based on data demonstrating the SbnI-dependent regulation of an internal promoter within the *sbn* operon, we next were interested in investigating whether SbnI controlled gene expression through direct binding to nucleic acids. However, because DNA-binding proteins frequently form dimers or oligomers, we investigated this property. His₆-tagged SbnI was purified by metal affinity chromatography (Fig. 7A) and used to evaluate the oligomerization status of the protein in solution. Size exclusion chromatography and sedimentation velocity-analytical ultracentrifugation determined that SbnI forms dimers and tetramers in non-reducing conditions but primarily monomers with limited dimer conformation under reducing conditions (*i.e.* in the presence of 10 mM DTT) (Fig. 7, B–D).

Electrophoretic mobility shift assays (EMSA) were performed with purified His₆-SbnI, in non-reducing conditions so as to promote oligomerization of the protein. Based on the luciferase assay and RACE data suggesting an internal promoter in the *sbnC* region, we sought to find a DNA fragment upstream of *sbnD* that could be bound by SbnI. Multiple EMSAs were performed using 250–300-bp fragments located within and upstream of the region identified as fragment C in Fig. 6. A 271-bp probe located 772 bp upstream of the transcription initiation site internal to the *sbnC* coding region was identified as a binding site for SbnI (Fig. 8). The inability of dena-

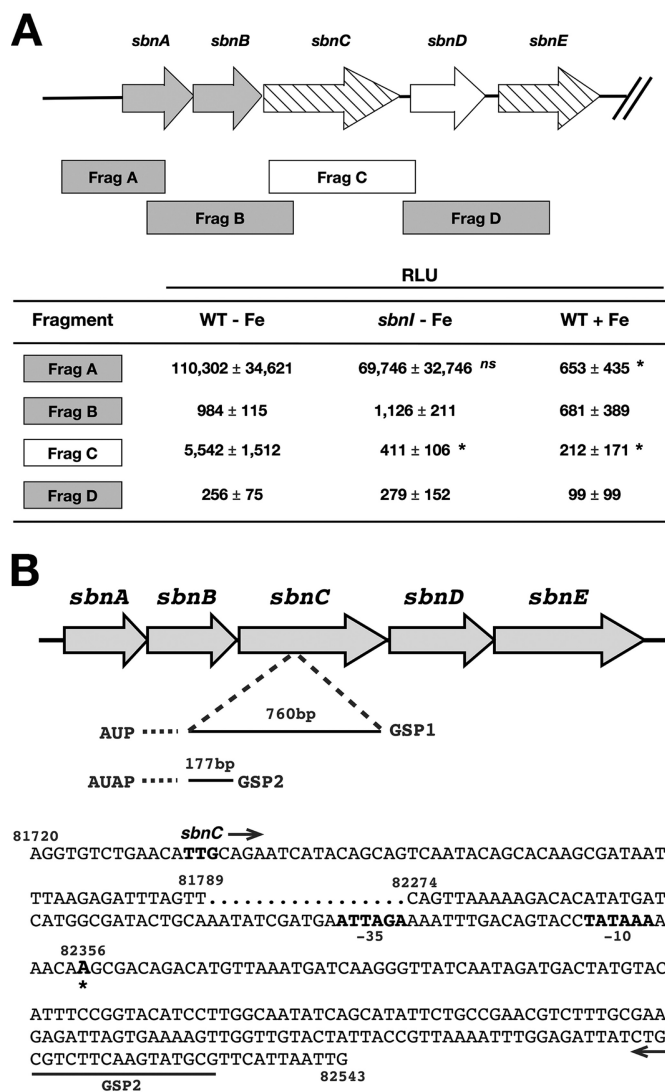


FIGURE 6. Identification of a promoter within the *sbnC* coding region. A, DNA fragments, spanning genes *sbnA*–*E*, were cloned into the pGylux vector, and luciferase activity was measured. Activity was expressed at *cpsA*₆₀₀. Statistical analyses were performed for each respective fragment, comparing the luciferase activity of either the *sbnI* strain without iron (*sbnI* – Fe) to WT without iron (WT – Fe) or WT with iron (WT + Fe) to WT without iron (WT – Fe). Statistics were performed using Student's unpaired *t* test; *, $p < 0.01$, *ns* = not significant. B, identification of initiation of transcription. Using the 5'RACE method, A81175 (based on *S. aureus* Newman genome sequence) was identified as the +1 site and is located 625-bp downstream of the *sbnC* start codon. Two PCR products of ~760 bp (AUP/GSP1) and 117 bp (abridged universal anchor primer/GSP2) were generated, and the 117-bp product was cloned into pGEM7 and sequenced. The locations of the primer GSP2 and the –10 and –35 sites are indicated.

tured protein (SbnI incubated for 10 min at 100 °C) and His₆-SbnG to bind this DNA fragment (Fig. 8, lanes 3 and 6, respectively) confirmed binding specificity of SbnI for this fragment, ruling out the possibility of nonspecific interactions with the IRDye-700 molecule. Binding specificity to this probe was confirmed by showing that excess unlabeled *sbnC* DNA fragment, but not excess nonspecific (*i.e.* *rpoB*) unlabeled DNA, could interfere with the binding of SbnI to the labeled fragment.

SbnI Binds Heme—The iron response regulator (Irr) protein of *Bradyrhizobium* spp. is a DNA-binding transcription factor regulating gene expression. The protein binds heme, which results in substantially decreased affinity for DNA (36, 37). This

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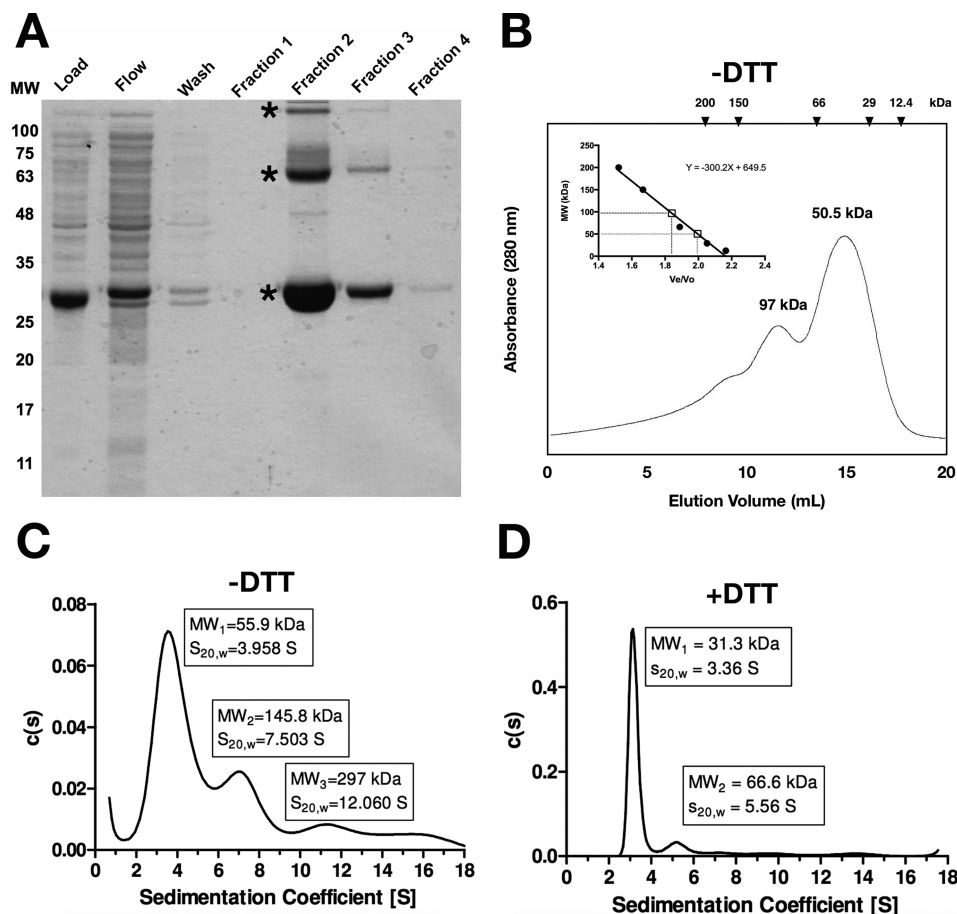


FIGURE 7. SbnI forms dimers and tetramers in solution. *A*, purification of recombinant His₆-SbnI. Various samples during the purification process are run through a polyacrylamide gel, starting with pre-column (*load*), flow-through (*flow*), wash fraction (*wash*), and elution fractions 1–4. The identity of the three bands labeled with an asterisk was confirmed as SbnI using MALDI. *B*, size exclusion chromatography of SbnI. Chromatogram represents size exclusion data of SbnI run in 300 mM ammonium formate, pH 7.4, at flow rate of 0.2 ml/min across Superdex 200 FPLC column. Elution volumes of known protein standards are shown at the top and are used to generate a standard curve (*inset*). *C* and *D*, sedimentation velocity-analytical ultracentrifugation was performed on SbnI run in 300 mM ammonium formate, pH 7.4, 100 mM NaCl. *C*, 10 mM DTT was included.

interaction is aided by an HXH motif located central to the Irr protein (38). Because SbnI contains the sequence HIHEH (*i.e.* tandem HXH motif) at its extreme N terminus, we investigated the possibility that, in similar fashion to Irr, SbnI was capable of an interaction with heme. Heme titration experiments were performed using both UV-Vis spectroscopy (Fig. 9A) and ESI-MS (Fig. 9B), and both techniques demonstrated that SbnI was indeed capable of binding to heme, with saturation occurring at approximately a 1.5:1 heme to protein ratio.

Heme Obviates the SbnI-DNA Interaction—Given that SbnI is capable of binding both DNA and heme, we next sought to determine whether the interaction of SbnI with heme would alter its DNA binding properties, as it does with the Irr protein (37). To examine this, EMSAs were repeated using SbnI protein that was pre-incubated with increasing concentrations of heme. As shown in Fig. 10, the ability of SbnI to bind the *sbnC* DNA fragment was diminished with increasing concentrations of heme. Moreover, at a 1:1 molar ratio of protein to heme, DNA binding was completely inhibited (Fig. 10).

Extracellular Heme Controls SB Synthesis—Although previous studies have demonstrated that *S. aureus* preferentially uses heme as an iron source (12), no studies have yet investigated the effect of heme on the synthesis of siderophores.

Moreover, our data showing that SbnI is required for SB synthesis, and that heme obviated the DNA-binding ability of SbnI, led us to investigate how exogenously supplied heme may affect SB production.

To investigate the effects of heme on SA and SB production, a disc diffusion assay was performed to determine the relative amounts of each siderophore produced during growth of WT RN6390 in cTMS medium with either 1 μ M FeCl₃ (WT + Fe) or 1 μ M heme (WT + heme). As described previously, the spent culture supernatants were spotted onto cTMS agar seeded with either *sir* or *hts* mutants to examine for the presence of SA or SB, respectively. As shown in Fig. 11A, the relative amounts of SA were comparable between supernatants from cultures grown in equivalent concentrations of FeCl₃ versus heme. However, in agreement with the results described above, spent supernatants from cultures grown in heme, versus FeCl₃, showed decreased amounts of SB during the early stages of growth, until at least the 16-h time point, a point when cultures were entering stationary phase. As opposed to the global suppressive ability of iron on iron-regulated genes, in a Fur-dependent fashion, our results indicate that heme specifically suppresses the synthesis of SB. We suggest that this regulation functions through heme's ability to inhibit DNA binding of

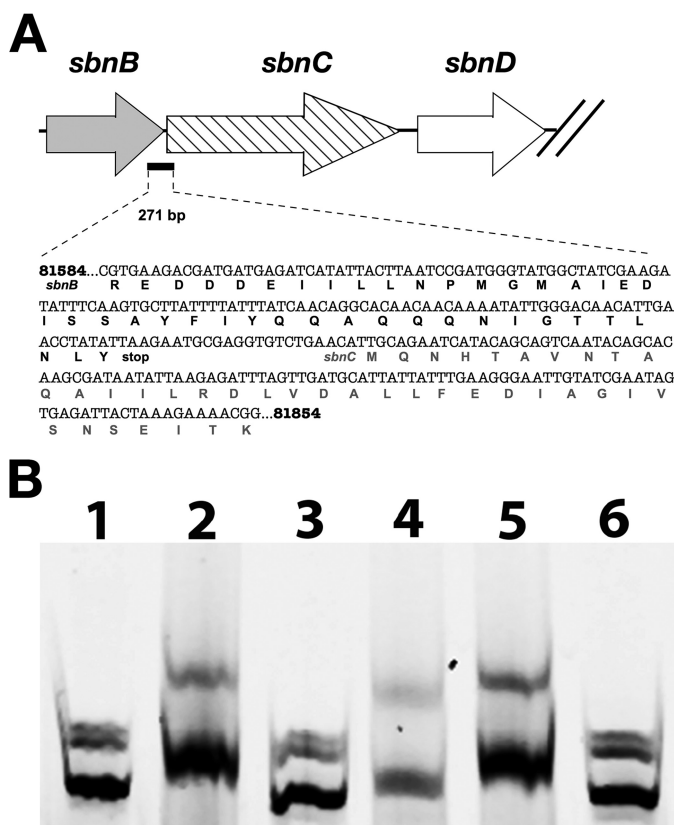


FIGURE 8. Identification of a DNA-binding site for SbnI. *A*, schematic identifying the location of a 271-bp DNA fragment that was used for the EMSA experiment illustrated in *B*. The base numbering is from genome sequence for *S. aureus* strain Newman. *B*, electrophoretic mobility shift assay demonstrating SbnI binding to DNA fragment shown in *A*. Each sample contained 120 ng of fluorescently labeled, 270-bp dsDNA, as well as the following: lane 1, DNA alone; lane 2, 25 μM SbnI; lane 3, 25 μM denatured SbnI; lane 4, 25 μM SbnI with 100:1 unlabeled *sbnC* probe to labeled probe; lane 5, 25 μM SbnI with 100:1 unlabeled *rpoB* probe to labeled probe; lane 6, 25 μM SbnI.

SbnI, whose function is required for transcription of genes within the *sbn* operon (*i.e.* SB biosynthesis/secretion genes).

Discussion

The capacity of *S. aureus* to establish an infection is reliant on its ability to acquire iron from the host. The expression of many genes encoding proteins involved in iron metabolism is “iron-regulated” through the activity of the global regulatory protein, Fur. Fur-mediated regulation of gene expression is widespread in both Gram-negative and Gram-positive bacteria (29, 30). Indeed, expression of the *sbn* operon is repressed by Fur during growth in iron-replete media. However, *S. aureus* is unlikely to encounter iron-replete conditions during infection of the host, given that the host actively sequesters iron from pathogens (39, 40). Moreover, because *S. aureus* possesses multiple iron acquisition strategies, all regulated at a minimum by Fur, additional mechanisms are likely to exist for fine-tuning the response of *S. aureus* to the availability of different iron chelates during iron starvation conditions. This is especially true in light of previous work demonstrating that *S. aureus* prefers to utilize heme as an iron source (12). Our identification of SbnI as a transcriptional regulator of *sbn* operon expression, in response to heme, is the first such identification of a molecular mechanism underpinning a manner by which *S. aureus* “senses” heme to control

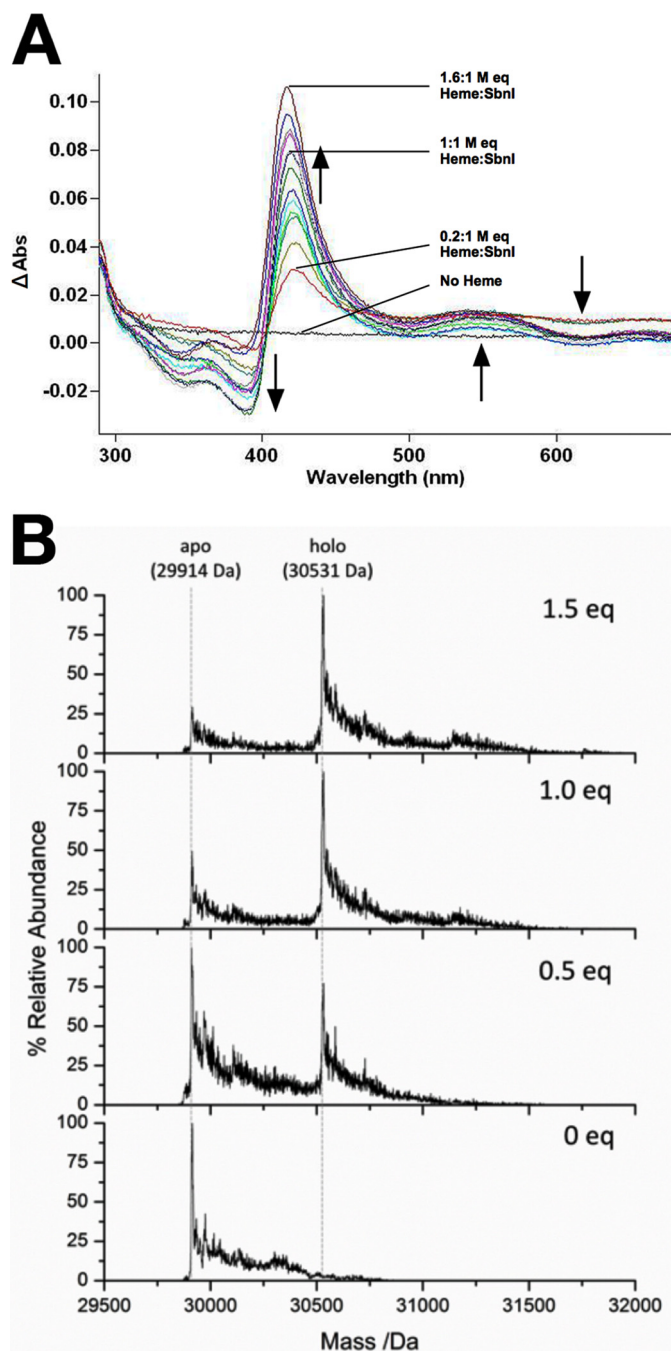


FIGURE 9. SbnI binds heme. *A*, UV-visible difference absorbance spectra recorded following addition of increasing concentrations of heme to 1 μM SbnI in 300 mM ammonium formate, pH 7.4. The absorption spectra were measured at 22 °C. The shift in spectral maxima coincident with the absorption spectrum of the protein-bound heme with increasing heme concentrations is indicated by arrows. The concentration of the heme at the end of the titration was 1.6 μM , where the increase in Soret peak is due to nonspecific heme stacking, as SbnI optimally binds heme at a 1:1 molar ratio heme/SbnI, as indicated by ESI-MS data shown in *B*. *B*, deconvoluted electrospray ionization mass spectral data of SbnI in 300 mM ammonium formate, pH 7.4, and 2 mM DTT as a function of increasing heme stoichiometry (0 mol eq (bottom) to 1.5 mol eq added heme (top)). The heme solution was freshly prepared prior to the measurement. The apo-SbnI has a mass of 29,914 Da. Heme binding to the holo-SbnI results in a new mass at 30,531 Da. The relative abundance of the apo-SbnI decreases as the heme concentration increases. The holo-SbnI with a single heme bound (mass 30,531 Da) is the major species even with excess heme (top).

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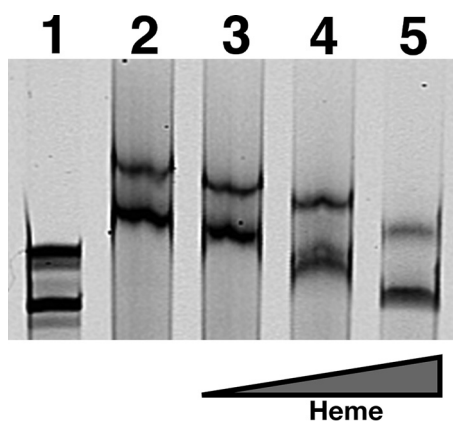


FIGURE 10. **Heme decreases affinity of SbnI for DNA.** EMSA was performed as described for Fig. 8. Each sample contained 120 ng of the fluorescently labeled, double-stranded *sbnC* probe. Lane 1, DNA alone; lane 2, 25 μM SbnI; lane 3, 25 μM SbnI with 0.2:1 molar equivalent of heme to SbnI; lane 4, 25 μM SbnI with 0.5:1 eq of heme to SbnI; lane 5, 25 μM SbnI with 1:1 eq of heme to SbnI.

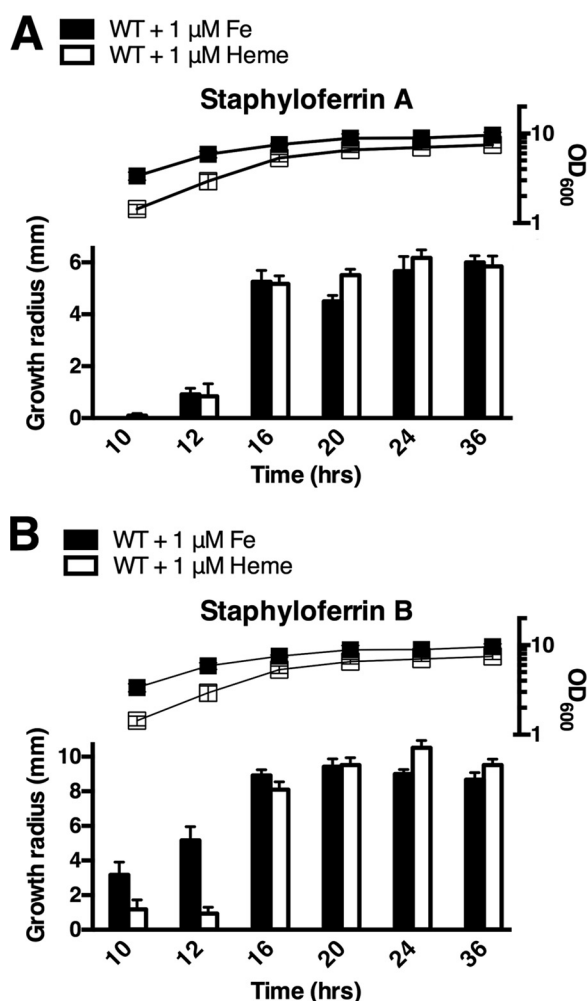


FIGURE 11. **Heme reduces staphyloferrin B production.** Disc diffusion assay was performed as described previously, and all strains were grown in cTMS with either the addition of 1 μM FeCl_3 or 1 μM heme. *A*, growth radius from SA secretion was comparable between WT + heme and WT + Fe for all time points. *B*, disc diffusion assay demonstrating reduced amount of SB in culture supernatants at 10 and 12 h of growth from WT grown in 1 μM heme when compared with WT grown in 1 μM FeCl_3 . Optical density of cultures at the various time points is plotted above the disc diffusion assay results (right *y* axis).

siderophore synthesis. The model provides some mechanistic insight in support of the observations of Skaar *et al.* (12), who reported that heme is the preferred iron source of *S. aureus*.

In this study, we show that SbnI is a regulatory protein with DNA and heme binding capabilities. SbnI regulates transcript levels of genes in the *sbn* operon, particularly genes including *sbnD* and downstream of *sbnD*. Thus, under iron starvation conditions, SbnI is required to allow expression of the gene encoding the SB efflux pump, SbnD, and essential biosynthetic genes, *sbnE–H*. This ultimately serves to increase production of SB but also to ensure that, once SB synthesis has been initiated by the activity of SbnA and SbnB enzymes (19–21), the efflux pump is expressed. This ultimately ensures that SB, and potentially its intermediates, does not accumulate in the cytoplasm. Regulatory control of efflux *versus* biosynthesis has been documented for the biosynthesis/efflux of the antibiotic actinorhodin in *Streptomyces coelicolor* (41). Ensuring expression of the efflux pump prior to synthesis of the end product ultimately serves to “protect” the cell from an overabundance of cytosolic antibiotics. SB is a high affinity iron chelator that, if located in high abundance in the cell cytosol, could negatively impact cellular function by binding to intracellular iron. Thus, the SbnI-dependent up-regulation of *sbnD* and downstream genes may serve to ensure expression of the efflux pump prior to complete synthesis of the SB molecule. The early steps of SB synthesis, which result in the formation of L-Dap by SbnA and SbnB (20, 21), may also be important for SbnI function, and we are currently investigating the possibility that SB intermediates, including L-Dap, work as co-regulators by binding with SbnI.

This is the first study to investigate the importance of SbnI in siderophore synthesis, and our data clearly implicate a role for SbnI in transcriptional control from regions within the *sbn* operon. The mechanisms underlying the function of SbnI in DNA binding and transcriptional control are areas of interest. The similarity of SbnI with ParB/Spo0J may provide some insight. The Spo0J protein has been demonstrated to bind DNA at disparate locations and, along with its ability to self-associate, can cause DNA bending within higher order nucleoprotein complexes (42). In the case of SbnI, there may be additional locations throughout the *sbn* operon that SbnI may bind in forming regulatory complexes that control transcription. These, along with the identification of exact nucleotide-binding sequences for SbnI, are priorities for our ongoing studies.

Along with demonstrating that SbnI is a regulatory protein of SB efflux and biosynthesis, we suggest that SbnI controls production of SB in response to heme because in this study we showed that SbnI is capable of binding to heme, and when complexed with heme it is unable to interact with nucleic acid, at least the region we identified within the *sbnC* coding region. In this manner, SbnI may represent a novel mechanism by which *S. aureus* regulates siderophore synthesis and utilization in response to the presence of heme (Fig. 12), perhaps as a sophisticated method to transition from SB-mediated iron uptake to heme-mediated iron uptake. In support of this hypothesis, we were able to demonstrate that providing extracellular heme to *S. aureus* resulted in a decrease in SB production but not SA production. Although we favor the hypothesis that this effect is mediated through SbnI, our data cannot as of yet conclusively

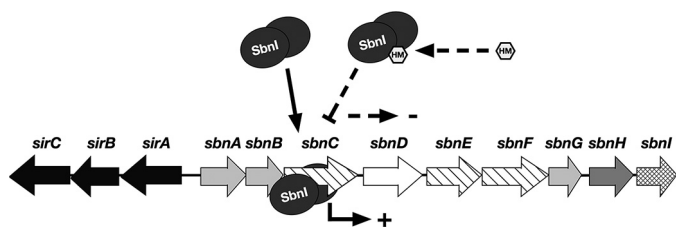


FIGURE 12. **Proposed model of SbnI-mediated gene regulation in response to heme.** In conditions of low iron and low heme, SbnI dimer binds to DNA within the *sbnC* coding region and promotes expression of genes *sbnD–H*. Under conditions of low iron and high heme, heme binds to SbnI, preventing binding of SbnI to DNA and thereby resulting in decreased expression of genes *sbnD–H*.

support this contention. When provided exogenous heme, it is difficult to determine how much of the heme is taken into the cell and remains intact and capable of binding to proteins like SbnI. Moreover, it is still unknown the role that intracellular heme biosynthesis plays in regulating metabolic processes such as siderophore biosynthesis. Ultimately, the ability to sense intracellular heme may be a mechanism whereby *S. aureus* conserves energy by not utilizing siderophore-mediated iron uptake unnecessarily when heme can be used as the primary iron source. Should heme no longer be accessible as an iron source, we hypothesize that “active” SbnI would allow transcription of downstream *sbn* genes and that pre-formed L-Dap in the cell would allow for a rapid transition to SB-mediated iron uptake. The fine-tuning of SB and heme uptake systems in *S. aureus* is of interest because both *sbn* and *isd* genes have been implicated in virulence in *S. aureus* models of infection (16, 25, 26, 43–46).

Author Contributions—D. E. H. conceived and coordinated the study. T. B. P. and M. J. S. performed and analyzed the experiments shown in Fig. 9. H. A. L. and C. L. M. performed experiments. H. A. L. and D. E. H. wrote the paper. All authors analyzed the results and approved the final version of the manuscript.

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