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

Holly A. Laakso‡, Cristina L. Marolda‡, Tyler B. Pinter§1, Martin J. Stillman§, and David E. Heinrichs‡¶2

From the Departments of ‡Microbiology and Immunology and §Chemistry, ‡Centre for Human Immunology, University of Western Ontario, London, Ontario N6A 5C1, Canada

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‡Recipient of a postgraduate scholarship-doctoral from the Natural Sciences and Engineering Council.

§To whom correspondence should be addressed. Tel.: 519-6613984; Fax: 519-661-3499; E-mail: deh@uwo.ca.

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/SpolI-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 sbnl mutant, and the mutant does not synthesize detectable SB during early phases of growth. Thus, SB-mediated iron acquisition is impaired in an sbnl 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 limitation 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 (Ish) 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 that 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 stepwise 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 sfia) 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 N-ornithine with two molecules of citrate (22). SfaC is a pyridoxal-

3The abbreviations used are: NIS, non-ribosomal peptide synthetase-indepen-dent siderophore; SA, staphyloferrin A; SB, staphyloferrin B; Fur, ferric uptake regulator; L-Dap, L-2,3-diamino-propionic acid; qPCR, quantitative real time PCR; tCMS, Chelex-100-treated Tris minimal succinate; RACE, rapid amplification of cDNA end; UV-Vis, UV-visible; EDDHA, ethylenedia-mine-N,N’-bis(2-hydroxyphenylacetic 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 1,2,3-diaminopropionic acid (1-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 sideroaphore 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 PchA (31). PchA is an environmentally responsive transcription factor that responds to the presence of the autoinducer 3-hydroxypalmitic acid methyl ester, a molecule produced as a byproduct of bacterial growth (20). 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 μg/ml kanamycin and 100 μg/ml ampicillin for Escherichia coli selection, 4 μg/ml tetracycline, 5 μg/ml chloramphenicol, and 3 μg/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 A600 equivalent of 0.005.

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

Cells were grown to an A600 of 1.0, and 3 × 109 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, 1X Laemmli buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromphenol 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 1:10,000 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 A600 0.005 in 10 ml of cTMS and grown to mid-exponential phase (A600 ~1.0). Cells equating to an A600 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 μg/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<sup>R</sup>, ampicillin resistance; Cm<sup>R</sup>, chloramphenicol resistance; Ery<sup>R</sup>, erythromycin resistance; Km<sup>R</sup>, kanamycin resistance; Tet<sup>R</sup>, tetracycline resistance; AUAP, abridged universal anchor primer; UAP, universal anchor primer.

| Bacterial strains, plasmids, oligonucleotides | Description | Source or Ref. |
|---------------------------------------------|-------------|---------------|
| *E. coli*                                    |             |               |
| DH5α                                        | F- lacZΔM15 recA1 endA1 supG gyrA96 galV44 thi-l ilvR17 (r<sup>n</sup> m<sup>n</sup>)<sup>+</sup> supE44 relAI deoR (lacZΔZYA-argF)U169 | Promega |
| BL21(DE3)                                    | F- ompT 1 lac dcm lon sbn<sup>A</sup> (r<sup>n</sup> m<sup>n</sup>)<sup>+</sup> (DE3 [lacIUV5-T7 gene 1 ind1 sam7 mini]) | Novagen |
|                                            |             |               |
| *S. aureus*                                  |             |               |
| RN2220                                      | r<sup>n</sup> m<sup>n</sup> accepts foreign DNA | 47 |
| RN6390                                      | Prophage-cured wild-type strain | 48 |
| H306                                        | R6390 sbn<sup>I</sup>: Tet<sup>R</sup> | 25 |
| H984                                        | R6390 sbn<sup>I</sup>: Tet<sup>R</sup> | This study |
| H3132                                       | H984/pSbn<sup>I</sup>; sbn mutant complemented with sbn<sup>I</sup> | This study |
| H3133                                       | H187 containing pCN51 | This study |
| H3137                                       | H984 containing pCN51 | This study |
| H3124                                       | R6390 sbn<sup>I</sup> | 23 |
| H4148                                       | R6390 hts<sup>1</sup>: Tet<sup>R</sup> | 23 |

**Plasmids**

| pET28a<sup>(+)</sup>                          | Overexpression vector for His<sub>S</sub>-tagged proteins; Km<sup>R</sup> | Novagen |
| pCN51                                        | Cadmium-inducible expression vector; Ery<sup>R</sup> | 49 |
| pSbn<sup>I</sup> (pAB1)                       | pCN51-sbn<sup>I</sup>; pCN51 vector for expression of sbn<sup>I</sup>; Ery<sup>R</sup> | This study |
| pGlylux                                      | Promoterless E. coli/S. aureus shuttle vector for luminescence, Ap<sup>R</sup>, Cm<sup>R</sup> | 33 |
| pCM326                                       | 1479-bp sbn<sup>A/B</sup> cloned into pGlylux; Ap<sup>R</sup>, Cm<sup>R</sup> (fragment A) | This study |
| pCM328                                       | 2322-bp sbn<sup>A/B</sup>C cloned into pGlylux; Ap<sup>R</sup>, Cm<sup>R</sup> (fragment B) | This study |
| pCM330                                       | 2389-bp sbn<sup>C/D</sup> cloned into pGlylux; Ap<sup>R</sup>, Cm<sup>R</sup> (fragment C) | This study |
| pCM332                                       | 2395-bp sbn<sup>D/E</sup> cloned into pGlylux; Ap<sup>R</sup>, Cm<sup>R</sup> (fragment D) | This study |

**Oligonucleotides**

| Cloning of sbn<sup>I</sup> from *S. aureus* for pET28a<sup>(+)</sup> | GC AGC CAT ATG AAT CAT ATT GCT GAA CAT TTA A (forward) |               |
| Cloning of sbn<sup>I</sup> from *S. aureus* for complementing vector (pSbn<sup>I</sup>) | TGG TGT TTC GAG TCA TCA TAT TTC CCT CAA CAT (reverse) |               |
| Cloning of fragment A | GCC TCC CGGGTCAATAAAATTTATGATTTACATGC (forward) |               |
| Cloning of fragment B | GACTCCCGGGTCTGTTACTAAAC (reverse) |               |
| Cloning of fragment C | TAACGCCTATGCCACCACCAAG (reverse) |               |
| Cloning of fragment D | GCTGTTTCTACTTTCAACCATTTTC (reverse) |               |
| RT-PCR: sbn<sup>A</sup> | ACTTTTATGGAACTCTTGTACCAATACCG (forward) |               |
| RT-PCR: sbn<sup>B</sup> | CCTGAAAATGGGACACATCGC (forward) |               |
| RT-PCR: sbn<sup>C</sup> | CAAAATATAGCGCCACCTTGAC (forward) |               |
| RT-PCR: sbn<sup>D</sup> | GTTCAATGTGATGAAATGGGACGAT (forward) |               |
| RT-PCR: sbn<sup>E</sup> | GAGTTTCTAGGCGGAAACACG (forward) |               |
| RT-PCR: sbn<sup>F</sup> | GTTACCGGGTTGAGATTG (forward) |               |
| RT-PCR: sbn<sup>G</sup> | GTAATATAGCGCTTCATG (forward) |               |
| RT-PCR: sbn<sup>H</sup> | TACGGCGATATCC (forward) |               |
| RT-PCR: rpoB | AGGAGAAAATGGAATTGAC (forward) |               |

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Discussed using Superscript<sup>TM</sup> 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 a standard curve 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<sub>600nm</sub> 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 H<sub>2</sub>O. *S. aureus* cells, the htsA mutant strain or the sirA mutant strain, were seeded into TMS agar containing 10 μm EDDHA to achieve 1 × 10<sup>4</sup> 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 pGlylux (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 FeCl₃ at an A₆₀₀ 0.1 and incubated for 8 h. At that time, the A₆₀₀ and luminescence were measured on a SynergyH4 Hybrid Reader (Biotek). Luminescence values were expressed as cps/A₆₀₀.

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 for 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 Smal 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 His₅-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×), and samples were read at A₅₉₅, 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 μl/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 centrepieces. 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 H₂O. 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 μg/ml bovine serum albumin (BSA), 15.2 μg/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 usual. 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 cm⁻¹ M⁻¹ at 385 nm (35). Heme solution was aliquoted and stored at −20 °C. Heme stocks were diluted just
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 heme 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 NH₄OH 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 Spo0J.

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 + psbnl, 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 + psbnl). 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 htaA mutant strains. The sirA strain is unable to uptake SB and thus relies on SA uptake for growth, whereas the htaA 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
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).

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 promoter-less luciferase genes in the vector pGlylux (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...
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 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. His$_6$-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 (EMSAs) were performed with purified His$_6$-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 denatured protein (SbnI incubated for 10 min at 100 °C) and His$_6$-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 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.
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 mM FeCl₃ (WT/Fe) or 1 mM heme (WT/heme). As described previously, the spent culture supernatants were spotted onto cTMS agar seeded with *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...
SbnI, whose function is required for transcription of genes within the sbn operon (i.e. SB biosynthesis/secrection 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 iron availability.
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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 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.

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
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