Staphylococcus aureus Transporters Hts, Sir, and Sst Capture Iron Liberated from Human Transferrin by Staphyloferrin A, Staphyloferrin B, and Catecholamine Stress Hormones, Respectively, and Contribute to Virulence[▽]

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Staphylococcus aureus is a frequent cause of bloodstream, respiratory tract, and skin and soft tissue infections. In the bloodstream, the iron-binding glycoprotein transferrin circulates to provide iron to cells throughout the body, but its iron-binding properties make it an important component of innate immunity. It is well established that siderophores, with their high affinity for iron, in many instances can remove iron from transferrin as a means to promote proliferation of bacterial pathogens. It is also established that catecholamine hormones can interfere with the iron-binding properties of transferrin, thus allowing infectious bacteria access to this iron pool. The present study demonstrates that *S. aureus* can use either of two carboxylate-type siderophores, staphyloferrin A and staphyloferrin B, via the transporters Hts and Sir, respectively, to access the transferrin iron pool. Growth of staphyloferrin-producing *S. aureus* in serum or in the presence of holotransferrin was not enhanced in the presence of catecholamines. However, catecholamines significantly enhanced the growth of staphyloferrin-deficient *S. aureus* in human serum or in the presence of human holotransferrin. It was further demonstrated that the Sst transporter was essential for this activity as well as for the utilization of bacterial catechol siderophores. The substrate binding protein SstD was shown to interact with ferrated catecholamines and catechol siderophores, with low to submicromolar affinities. Experiments involving mice challenged intravenously with wild-type *S. aureus* and isogenic mutants demonstrated that the combination of Hts, Sir, and Sst transport systems was required for full virulence of *S. aureus*.

Iron is an essential nutrient for almost all forms of cellular life. In aerobic environments, the metal exists predominantly as Fe(III), which at neutral pH has a solubility of approximately 1×10^{-9} M (52). Freely available iron therefore exists at concentrations well below the threshold to support microbial growth. Iron in animal tissues is further sequestered by high-affinity transport and storage proteins, such that the amount remaining free in circulation is extremely limited. In this context, host factors that sequester iron can be considered key components of innate immunity.

The primary iron sequestration factor in vertebrate serum is transferrin, a glycoprotein featuring two Fe(III) binding domains with high affinities for Fe(III) (K_d [dissociation constant], approximately 10^{-22} M) (1). In spite of this potent barrier against infection, septicemia is the 10th leading cause of mortality in the United States reported by the Centers for Disease Control and Prevention (29). Bacterial survival and proliferation in blood frequently involve strategies for scavenging iron from transferrin. These often employ siderophores, i.e., secreted, low-molecular-weight, high-affinity iron chelators, which contribute to the virulence of many bacterial pathogens (reviewed in reference 23). In addition to pathogen-generated molecules such as siderophores, the bacteriostatic

potential of serum may also be compromised by host hormone levels (8, 18–21, 39, 54). Catecholamine stress hormones, including epinephrine and norepinephrine, can interact with transferrin-bound Fe(III) and promote its reduction to Fe(II), for which transferrin has little affinity (54). Catecholamine hormones have previously been shown to form 2:1 and 3:1 complexes with iron(III) (30). Bacteria equipped with catechol siderophore uptake systems could feasibly import Fe(III)-catecholamine₃ complexes as “pseudosiderophores” for growth under iron-restricted conditions, as recently demonstrated for *Bacillus subtilis* and *Escherichia coli* (40).

Invasive infection by the opportunistic pathogen *Staphylococcus aureus* can result in syndromes including endocarditis and necrotizing pneumonia (42, 43). Given its high virulence potential, it is of interest to define mechanisms by which this bacterium scavenges host iron sources. *S. aureus* has a system to acquire iron from heme via the secretion of toxins and hemolysins that provide access to hemoglobin, followed by the actions of several *S. aureus* proteins that function in an overall process which includes the extraction of heme from hemoglobin and the eventual internalization of heme, followed by the extraction of iron from the porphyrin ring (38, 45, 60, 64, 65).

S. aureus is also capable of growing on transferrin as a sole source of iron (41, 50). The molecular genetics and biochemistry for the synthesis of two *S. aureus* siderophores, staphyloferrin A (SA) and staphyloferrin B (SB), were recently characterized (7, 9). Following capture of extracellular iron, the staphyloferrins are recognized by the highly specific receptors HtsA (SA) and SirA (SB), and at least iron, if not the iron-

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siderophore complex, is actively imported into the cytosol through permeases (3, 7, 24, 25). Furthermore, it has been shown that genomic inactivation of the staphyloferrin biosynthesis loci *sfa* (SA) and *sbh* (SB) eliminates siderophore output and severely curtails *S. aureus* growth in animal serum (3), prompting the question of whether the target of staphylococcal siderophores in serum is transferrin. In addition to the SA and SB siderophore import systems, *S. aureus* also carries the iron-regulated FhuBGC₂-D2/D1 transporter, with specificity for Fe(III)-hydroxamates (56, 57). One additional iron-regulated transporter, SstABCD, was described as a putative siderophore transporter based on sequence similarities to catechol transport systems in Gram-negative and Gram-positive bacteria (44), but it has not yet been characterized for substrate specificity.

This study describes the impact of staphyloferrins and catecholamines on the growth of *S. aureus* in the presence of human serum or transferrin and demonstrates their importance to *S. aureus* pathogenesis.

MATERIALS AND METHODS

Ethics statements. Human blood was obtained from healthy volunteers. Informed consent was obtained from all individuals, in compliance with the Office of Research Ethics at the University of Western Ontario. For animal infections, all protocols were reviewed and approved by the University of Western Ontario's Animal Use Subcommittee, a subcommittee of the University Council on Animal Care.

Bacterial strains, plasmids, and culture media. Bacterial strains and plasmids are summarized in Table 1. All bacteria were cultured at 37°C unless otherwise indicated. *E. coli* strains were grown in Difco Luria-Bertani broth (BD Diagnostics, Sparks, MD). For genetic manipulations, *S. aureus* strains were grown in Difco tryptic soy broth (TSB) (BD Diagnostics). For subsequent experiments, *S. aureus* strains were grown, as specified below, in Tris-minimal succinate broth (TMS) (59); TMS treated for 24 h at 4°C with 10% (wt/vol) Chelex-100 resin (Bio-Rad, Hercules, CA) prior to addition of postautoclaving nutrients (C-TMS); or an 80:20 mixture of C-TMS and human serum. For the latter, fresh sera from healthy human donors were separated from blood cells by centrifugation at $2,000 \times g$ for 20 min at 4°C, and complement was deactivated by incubation at 55°C for 1 h. Solid media were prepared by incorporating 1.5% (wt/vol) low-iron Difco Bacto agar (BD Diagnostics) to the specified medium. For selection of plasmids and recombinant alleles, antibiotics (BioShop, Burlington, Ontario, Canada) were added to the following concentrations: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; chloramphenicol, 5 µg/ml; erythromycin, 3 µg/ml; and lincomycin, 20 µg/ml. All media were made with water purified through a Milli-Q water purification system (Millipore, Billerica, MA). All glassware was treated overnight in 0.1 M HCl and rinsed thoroughly with Millipore-filtered water to remove residual contaminating iron.

Genetic manipulations. Standard DNA manipulations were performed essentially as described by Sambrook et al. (53). Restriction endonucleases, DNA-modifying enzymes, nucleotides, and PwoI DNA polymerase were purchased from Roche Diagnostics (Laval, Quebec, Canada) and New England BioLabs (Mississauga, Ontario, Canada). Plasmid DNA was purified using Qiagen QIAprep plasmid spin columns (Santa Clarita, CA) as described by the manufacturer. Plasmid purification from *S. aureus* included a 30-min pretreatment of cells in P1 buffer containing lysostaphin (Sigma-Aldrich, Oakville, Ontario, Canada). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and are described in Table 1.

***sstABCD* deletion and complementation.** The *sstABCD* operon and flanking regions were PCR amplified from strain RN6390 and cloned into plasmid pBAD24 by use of the restriction enzymes SalI and XbaI. The majority of the operon was excised via PvuII digestion, leaving flanking DNA sequences at the 5' region of *sstA* (upstream of nucleotide 250) and the 3' region of *sstD* (downstream of nucleotide 976). A Klenow polymerase-blunted BamHI restriction fragment containing an erythromycin resistance cassette was prepared from plasmid pDG646 and cloned into the PvuII restriction site. The Δ *sstABCD::Em* knockout allele was excised using the restriction enzymes SalI and XbaI and cloned into vector pAUL-A-Km, generating pSB10. This was passaged through *S. aureus* RN4220 (a restriction-deficient, modification-proficient strain) before

being introduced into *S. aureus* RN6390 by electroporation. Recipient strain RN6390 was cultured at 30°C to mid-log phase. The temperature was shifted to 42°C for a further 16 h, followed by plating of the bacteria onto TSB containing erythromycin and lincomycin. Resistant colonies were screened for kanamycin sensitivity, indicating a loss of the pSB10 backbone with allelic replacement of the *sstABCD* operon by the Δ *sstABCD::Em* construct. The chromosomal mutation was confirmed using PCR with primers external to the DNA sequences involved in mutagenesis. The Δ *sstABCD::Em* allele was mobilized into *S. aureus* Newman recipients by use of phage 80α as described previously (48). For complementation, the *sstABCD* operon and flanking regions were PCR amplified and cloned into plasmid pLI50 by use of the restriction enzymes SalI and XbaI, creating pSB5. This was passaged through RN4220 into recipient Newman strains via electroporation.

Bacterial growth in liquid culture. *S. aureus* growth curves were generated using a Bioscreen C plate reader (Oy Growth Curves, Finland). Prior to plate inoculation, strains were grown in glass tubes for 12 h in TMS broth and then subcultured and grown for 12 h in TMS broth chelated with 100 µM 2,2'-dipyridyl (Sigma-Aldrich). Cells were pelleted by centrifugation, washed twice in sterile 0.9% saline solution, and diluted 1:100 into 200- or 250-µl aliquots of TMS, C-TMS, or 80:20 C-TMS-human serum. Amendments to culture media included 10 µM human holotransferrin (~60% iron saturated) (Sigma-Aldrich), 50 µM or 200 µM catecholamine hormone [DL-norepinephrine hydrochloride, (–)-epinephrine, dopamine hydrochloride, or L-3,4-dihydroxyphenylalanine (L-DOPA)] (Sigma-Aldrich), and FeCl₃ (10 or 100 µM). L-DOPA and (–)-epinephrine were dissolved in 10 mM hydrochloric acid. Ampicillin (100 µg/ml) (for *E. coli*) or chloramphenicol (5 µg/ml) (for *S. aureus*) was incorporated into the growth medium of strains harboring plasmid pLI50 or derivatives. Plates were incubated with constant shaking at medium amplitude. Optical density (OD) was recorded every 15 min, although for graphical clarity, figures have been edited to display values every 2 h.

Siderophore CAS assays. Quantification of siderophore output from *S. aureus* strains was performed by testing the iron-binding activity of culture supernatants, using a chrome azurol S (CAS) shuttle solution (55) as described previously (3); supernatant siderophore units were normalized to culture optical density.

Siderophores. Ferric enterobactin and ferric salmochelin S4 were purchased from EMC Microcollections. Petrobactin was a kind gift from the laboratory of D. Sherman (University of Michigan). 2,3-Dihydroxybenzoic acid (DHBA) was purchased from Sigma, and deferoxamine (Desferal) was obtained from the London Health Sciences Centre. Bacillibactin (BB) was purified from *B. subtilis* strain HB5800 as described previously (22), with modifications. Briefly, *B. subtilis* was grown in enterobacterial minimal culture medium (47), with previously described modifications (6), for 48 h. Cells were removed by centrifugation, and the culture supernatant was acidified to pH 3 with HCl and then extracted three times with 200-ml volumes of ethyl acetate. Pooled ethyl acetate fractions were dried over Na₂SO₄, filtered, and dried in a rotary evaporator. Residue was dissolved in 1 ml of methanol and added dropwise to 50 ml of stirred ether. The precipitate was pelleted by centrifugation, air dried, and resuspended in dimethyl sulfoxide. The concentration of Fe-BB was calculated spectrophotometrically using the extinction coefficient ($\epsilon_{490} = 4,700 \text{ M}^{-1} \text{ cm}^{-1}$) as described previously (22).

Plate bioassays. The ability of siderophores and catecholamines to promote the iron-restricted growth of *S. aureus* on agar plates was assessed using agar plate-based disk diffusion bioassays performed as previously described (3).

Purification of SstD and binding assays. A region of the *sstD* gene encoding the soluble portion of SstD (i.e., downstream of the lipobox motif) was PCR amplified and cloned into plasmid pGEX-2T-TEV by use of BamHI and SmaI restriction sites, generating plasmid pJB1 in *E. coli* strain BL21λ(DE3). For overexpression of SstD–glutathione S-transferase (SstD-GST), *E. coli* cells were grown at 30°C to mid-log phase, induced with 0.4 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG), and grown for another 16 h. Cells were collected by centrifugation and ruptured using a French press. Insoluble matter and cellular debris were removed following centrifugation at $5,000 \times g$ for 15 min and then at $164,000 \times g$ for 60 min. Filtered supernatant was passaged over a 5-ml GSTrap FF column (GE Healthcare, Piscataway, NJ) and eluted in buffer containing 50 mM Tris and 10 mM reduced glutathione, pH 8.0. SstD-GST was digested with recombinant hexahistidine-tagged tobacco etch virus (TEV) protease overnight at 4°C. GST and uncleaved SstD-GST were removed by a second passage over the GSTrap FF column, and TEV protease was removed by passage over a 1-ml HisTrap column (GE Healthcare), using binding buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 10 mM imidazole) and elution buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 500 mM imidazole). Purified SstD was dialyzed against 20 mM Tris, pH 8.0, 150 mM NaCl, and stored at –80°C. SstD concentration was calculated using a Bio-Rad protein assay (Bio-Rad, Mississauga, Ontario, Canada).

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

Bacterial strain, plasmid, or oligonucleotide	Description or sequence ^a	Source or reference
Strains		
<i>E. coli</i> strains		
BL21λ(DE3)	F [−] <i>ompT hsdS_B(r_B[−] m_B[−]) dcm gal λ(DE3)</i>	Novagen
DH5α	φf80d <i>lacZ</i> Δ <i>M15 recA1 endA1 gyrAB thi-1 hsdR17</i> (r _K [−] m _K [−]) <i>supE44 relA1 deoR Δ(lacZYA-argF)U169 phoA</i>	Promega
<i>B. subtilis</i> strain		
HB5800	Bacillibactin-producing strain	49
<i>S. aureus</i> strains		
RN4220	Prophage-cured laboratory strain; r _K [−] m _K ⁺ ; accepts foreign DNA	31
RN6390	Prophage-cured laboratory strain	51
Newman	Sequence type 8; wild-type clinical isolate	15
USA300 (LAC)	Sequence type 8; community-acquired MRSA	B. Kreiswirth
USA400 (MW2)	Sequence type 1; community-acquired MRSA	B. Kreiswirth
MSSA476	Sequence-type 1; community-acquired MSSA	B. Kreiswirth
H803	Newman <i>sirA</i> ::Km; staphyloferrin B transport mutant	14
H1074	Newman Δ <i>fluCBG</i> ::Em; staphyloferrin transport mutant (ATPase function); hydroxamate siderophore ABC transporter mutant	62
H1262	Newman Δ <i>htsABC</i> ::Tc; staphyloferrin A transport mutant	3
H1497	Newman <i>sirA</i> ::Km Δ <i>htsABC</i> ::Tc; staphyloferrin transport mutant	3
H1331	Newman Δ <i>sbnABCDEF GHI</i> ::Tc; staphyloferrin B-deficient strain	3
H1665	Newman Δ <i>sfaABCsfaD</i> ::Km; staphyloferrin A-deficient strain	3
H1666	Newman Δ <i>sbnABCDEF GHI</i> ::Tc Δ <i>sfaABCsfaD</i> ::Km; staphyloferrin-deficient strain	3
H2221	Newman Δ <i>sstABCD</i> ::Em; catechol transport mutant	This study
H2224	Newman Δ <i>sstABCD</i> ::Em <i>sirA</i> ::Km Δ <i>htsABC</i> ::Tc	This study
H2228	Newman Δ <i>sstABCD</i> ::Em Δ <i>sbnABCDEF GHI</i> ::Tc Δ <i>sfaABCsfaD</i> ::Km	This study
Plasmids		
pAUL-A-Km	Temperature-sensitive <i>E. coli-Staphylococcus</i> suicide shuttle vector (Km ^r)	62
pBAD24	<i>E. coli</i> cloning vector (Ap ^r)	28
pDG646	<i>E. coli</i> vector containing the <i>ermC</i> gene (Ap ^r)	27
pGEX-2T-TEV	<i>E. coli</i> vector for overexpression of recombinant proteins with TEV protease-cleavable glutathione S-transferase tags (Am ^r)	58
pJB1	<i>E. coli</i> pGEX-2T-TEV derivative vector for overexpression of SstD with a TEV-cleavable GST tag (Am ^r)	This study
pLI50	<i>E. coli-Staphylococcus</i> shuttle vector (Cm ^r)	32
pSB5	pLI50-derived complementation vector for <i>sstABCD</i> under the control of its native operator region (Cm ^r)	This study
pSB10	pAUL-A-Km derivative containing <i>ermC</i> flanked by DNA homologous to 5'- and 3'-flanking regions of <i>sstABCD</i> (Km ^r Em ^r) for mutagenesis of <i>sst</i>	This study
pSB11	pET28a(+) derivative encoding N-terminally hexahistidine-tagged soluble portion of SstA (Km ^r)	This study
Primers		
Primers for cloning of <i>sstABCD</i> into pBAD24 for mutagenesis		
Forward	AAAAGTCGACGGAATCACTGAAGATGTG (SalI)	
Reverse	GGGGTCTAGAGGTGAACATCCAAAGGAATCGTA (XbaI)	
Primers for cloning of <i>sstABCD</i> into pLI50 for complementation		
Forward	AAAAGTCGACGGAATCACTGAAGATGTG (SalI)	
Reverse	CCCCTCTAGACAATGATTAAGACCTTTAACCAT (XbaI)	
Primers for cloning of <i>sstD</i> (codons 28 to 343) into pGEX-2T-TEV for protein overexpression		
Forward	TTGGATCCCAATCAAAATCAGAACTAAAGG (BamHI)	
Reverse	CCTTTAACCATTGTTCCCTCTTT (blunt ended)	

^a Restriction sites in sequences are underlined, and the restriction enzyme is given in parentheses.

Ligand binding experiments. SstD was adjusted to 1 μM in 100 mM NaCl, 10 mM Tris, pH 8.0. Equimolar bovine serum albumin (Sigma-Aldrich) was used as a protein negative control. Ligand stocks were added at 2-fold concentration increments ranging between 0 and 40 μM. Ligands included enterobactin, salmochelin, petrobactin, bacillibactin, 2,3-DHBA, norepinephrine, epinephrine, dopamine, L-DOPA, and deferoxamine. For ferration, ligands were incubated for 5 min at room temperature with FeCl₃ at a ratio of 1:3 (Fe:catecholamines) or 1:1 (Fe:siderophores). Ligand affinity was measured at room temperature by

intrinsic tryptophan fluorescence quenching in a Cary Eclipse instrument (Agilent Technologies). Excitation was performed at 280 nm, and fluorescence was detected at 345 nm, using an excitation slit width of 5 nm and an emission slit width of 5 nm. For ferrated catechol siderophores, fluorescence data were corrected for nonspecific tryptophan quenching by ligands after analogous titration with 1 μM *N*-acetyl-tryptophanamide, as described previously (67). The volume of starting protein solutions was 500 μl, and data were corrected for changes in fluorescence due to changes in sample volume due to ligand additions. Fluores-

cence data were fitted to nonlinear regression analysis using a one-site binding model, and data were analyzed using Microsoft Excel and graphed using GraphPad Prism.

Generation of anti-SstD antisera and Western blotting. SstD was purified as described above. Polyclonal antibodies recognizing SstD were generated in New Zealand White rabbits by ProSci Inc. (Poway, CA), using procedures outlined in their custom antibody production package 1.

For analysis of SstD expression in *S. aureus* whole-cell lysates, strains were grown to mid-log phase in TMS containing 20% human serum. Cells from approximately 1.5 ml of culture were pelleted by centrifugation and incubated for 30 min at 37°C after resuspension in 100 μ l cell wall digestion buffer (0.3 g/liter raffinose, 50 μ M Tris-Cl [pH 7.5], 145 mM NaCl, 5 mM iodoacetamide, 0.1 mM phenylmethylsulfonyl fluoride, and 1 μ g lysostaphin [Sigma-Aldrich]). Total protein concentrations were calculated using the Bio-Rad protein assay following the manufacturer's instructions. Samples were boiled for 10 min, and sample volumes normalized to contain 10 μ g total protein were resolved by SDS-polyacrylamide gel electrophoresis (12% acrylamide resolving gel) and then transferred to a 45- μ m nitrocellulose membrane according to standard protocols (53). Detection of SstD on nitrocellulose was performed after the following steps: 12 h of blocking at 4°C in phosphate-buffered solution (PBS) containing 20% horse serum (Sigma-Aldrich) and 10% (wt/vol) skim milk; 2 h of exposure to the primary antibody at room temperature in PBS containing 0.05% Tween 20 and 2% (wt/vol) skim milk (1:7,500 dilution of rabbit antiserum); and 1 h of exposure to anti-rabbit IgG antibody conjugated to IRDye-800 (Li-Cor Biosciences) at room temperature in PBS-0.05% Tween-2% skim milk (1:10,000 dilution of antibody). Fluorescence was imaged using a Li-Cor Odyssey infrared imager (Li-Cor Biosciences).

Murine systemic model of infection. Seven-week-old female immunocompetent BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA) and housed in microisolator cages. Bacteria were grown to mid-log phase (OD_{600} of approximately 1.0) in TSB, pelleted by centrifugation, and washed twice in 0.9% saline. Bacterial saline suspensions were administered via 100- μ l tail vein injections (5×10^6 to 7×10^6 CFU/injection). Ninety-six hours following challenge, mice were euthanized via intraperitoneal injection of pentobarbital. Kidneys, livers, and hearts were excised and placed in a phosphate-buffered solution containing 0.1% (vol/vol) Triton X-100. Organs were homogenized for 10 s, and bacterial loads were calculated following serial dilution of the suspension and drop plating on TSB agar plates. Data are presented as $\log_{10}$ CFU recovered per organ.

An infection study was also performed on mice (as described above) carrying surgically implanted epinephrine dispensers as a method to evaluate the contribution of elevated catecholamine levels to the progression of staphylococcal disease. Forty-eight hours prior to *S. aureus* challenge, mice were anesthetized with isoflurane gas and administered 1 mg/kg of body weight of the analgesic meloxicam (Boehringer Ingelheim) in 400 μ l of saline solution via intraperitoneal injection. The right flank of each mouse was shaved and disinfected with a routine three-step scrub. A dorsoventral incision was made near the shoulder, and a region of skin orienting toward the hips was undermined. Individual mice were implanted with one Alzet 2001 osmotic pump (dispensing rate of 1 μ l/h; Durect Corp.) loaded with 1 mg/ml epinephrine in 0.8% buffered saline, pH 4 (Bioniche Life Sciences, Inc.). The drug dosage and osmotic pump model were selected based on a previous report (66). pH-adjusted sterile saline-loaded pumps were inserted for mice in a drug-free control group. Incisions were closed with sutures. Twenty-four hours prior to infection, mice were administered a second dose of meloxicam. Mice were monitored daily for symptoms of adverse reactions to the surgical procedure, prior to and during the staphylococcal sepsis trial; none were noted.

Computer analyses and statistics. DNA sequence analysis and oligonucleotide primer design were performed using the Vector NTI Suite 7 software package (Informax, Inc.). K_a values were calculated as previously described (58), and all curves were plotted using GraphPad Prism (GraphPad Software, La Jolla, CA). *In vivo* data were analyzed by Student's unpaired *t* test. *P* values of <0.05 were considered to indicate statistical significance.

RESULTS

Staphyloferrins permit growth on human serum and human transferrin. *S. aureus* assembles and secretes two polycarboxylate-type siderophores, staphyloferrin A and staphyloferrin B (SA and SB, respectively) (7, 9). These molecules are the products of enzymatic activity encoded by the genomic loci *sfa*

FIG. 1. Growth of *S. aureus* Newman and derivatives in Chelex-treated Tris-minimal succinate medium containing either 20% human serum (A) or 10 μ M human holotransferrin (holo-hTf) (B). The iron-restricted growth observed was dependent on high-affinity iron acquisition, since supplementation of either medium with 100 μ M $FeCl_3$ (inset) obviated any growth differences between strains. All data points represent average values for at least three independent biological replicates, and error bars indicate the corresponding standard deviations from the means.

and *sbn*, respectively. Deletions of individual loci had mild to imperceptible effects on growth of *S. aureus* in mammalian serum, while inactivation of both the *sfa* and *sbn* loci resulted in a mutant severely restricted for growth (3), presumably due to an inability to extract iron from serum transferrin. The current investigation confirms and extends these findings by demonstrating that human serum can also support the growth of *S. aureus* strains producing at least one of the two staphyloferrins, while a mutant incapable of producing either is severely restricted for growth (Fig. 1A). More importantly, human holotransferrin is sufficient to promote otherwise iron-

restricted growth of *S. aureus* strains producing at least one staphyloferrin siderophore (Fig. 1B), suggesting that iron extraction from holotransferrin occurs through two redundant mechanisms. It is noteworthy that we consistently observed that the *sbm* mutant, not the *sfa* mutant, had an extended lag phase in the presence of serum (Fig. 1A) (3) and not in Tris-minimal succinate medium with holotransferrin, suggesting that a serum component partially interferes with staphyloferrin A-mediated iron acquisition. In either culture medium, the siderophore-deficient growth defect could be compensated for by addition of FeCl_3 (Fig. 1A and B, insets).

Catecholamine hormones promote growth of staphyloferrin-deficient *S. aureus* in the presence of human serum or transferrin. Catecholamines interact with Fe(III) in transferrin and promote its reduction to Fe(II) (54), effectively removing iron from this important innate immune protein. As a result, catecholamine hormones have been shown to enhance growth of pathogenic bacteria in serum (2, 19, 39, 54). It is also known that they can stimulate the growth of *S. epidermidis* on transferrin (36, 46), although a similar effect was not noted for *S. aureus* (46). In this investigation, and in agreement with a previous study by Neal et al. (46), neither of four catecholamine hormones, norepinephrine, epinephrine, dopamine, and L-DOPA, added at a concentration of 50 μM , enhanced the growth of wild-type *S. aureus* on human serum or human holotransferrin (Fig. 2A and B). At the same concentration, however, they did enhance the growth of the staphyloferrin-deficient strain (Fig. 2A and B). In serum-containing medium, growth promotion equivalent to that conferred by staphyloferrin production could be achieved at a catecholamine concentration of 200 μM (Fig. 2C). Catecholamine-stimulated growth promotion was negligible in TMS in the absence of holotransferrin, ruling out the possibility that the commercially obtained molecules were preloaded with iron (data not shown).

The *sstABCD* operon is involved in transport of catecholamine-liberated transferrin iron. *S. aureus* culture supernatants test negative for catechol siderophores (11), and the *sbm* *sfa* mutant tests negative for production of any siderophores (3). Nevertheless, the *S. aureus* genome encodes numerous known or putative iron-regulated ABC transporters without biosynthetic loci for their corresponding substrates, such as the Fhu system for uptake of hydroxamate siderophores (56, 57). Pertinently, the proposed model for the 3:1 molar complex of a catecholamine hormone with Fe(III) in solution (54) resembles the hexadentate coordination provided by bacterial catechol siderophores such as enterobactin. Following the observation that catecholamine hormones stimulate growth in serum in the absence of siderophores, it was of interest to determine the role of transporters with potential to receive catechol ligands.

The *S. aureus* *sst* operon (NWMN_0702_0705) is iron regulated *in vitro* and *in vivo* and encodes a membrane-tethered lipoprotein, SstD (44). SstD is a member of the class III substrate binding protein family, a family which includes the *S. aureus* Fe(III) -staphyloferrin receptors HtsA (25) and SirA (24) and the heme-binding IsdE protein (26). BLAST analyses uncovered shared sequence identity between SstD and many other annotated iron-ligand receptor proteins, especially receptors for catechol siderophores, including enterobactin, petrobactin, anguibactin, and vibriobactin. Given this observa-

FIG. 2. In the presence of human serum or transferrin, catecholamines stimulate the growth of staphyloferrin-deficient *S. aureus*. Growth of *S. aureus* Newman and derivatives was measured in TMS medium containing either 20% human serum (A and C) or 10 μM human holotransferrin (B). Additions to the media were as follows: Fe, FeCl_3 ; NE, DL-norepinephrine; E, epinephrine; D, dopamine; and LD, L-DOPA. The numbers indicate final concentrations (μM). All data points represent average values for at least three independent biological replicates, and error bars indicate the corresponding standard deviations from the means.

tion, we investigated the role of *sstABCD* in catechol and catecholamine iron uptake.

To assess the role of the Sst transporter in iron acquisition in *S. aureus*, an *sst* mutant was required, so we chose to delete the

FIG. 3. Western immunoblot for detection of expression of SstD in *S. aureus*. The indicated strains were grown in TMS medium containing 20% human serum. Further experimental details are outlined in Materials and Methods. The SstD lipoprotein of approximately 38 kDa is identified.

entire operon from the *S. aureus* chromosome and replace it with an erythromycin resistance cassette. Figure 3 shows a Western blot obtained using anti-SstD antisera that demonstrates the lack of detectable SstD expression in the mutant strain and conserved expression of SstD in a range of commonly used laboratory and clinical strains, including three community-acquired strains, namely, two methicillin-resistant *S. aureus* (MRSA) strains and one methicillin-susceptible *S. aureus* (MSSA) strain.

Compared to its isogenic wild-type parent, the $\Delta sstABCD$ mutant was not compromised for growth in human serum containing catecholamines (Fig. 4A). However, this mutation coupled with *sfa* and *sbm* mutations rendered this staphyloferrin-deficient mutant insensitive to the growth-promoting effects of catecholamines in the presence of either human serum (Fig. 4A) or human transferrin (Fig. 4B), even at catecholamine concentrations as high as 200 μ M (Fig. 4C). The mutant phenotype was fully complemented by expression of wild-type *sstABCD* in *trans* (Fig. 4C).

SstABCD is required for growth promotion by catechol-type siderophores. To assess the role of Sst in capturing Fe(III)-catechol siderophores, growth promotion assays were performed by adding ferrated catechols to paper disks that were then placed onto TMS agar plates seeded with bacteria. While enterobactin, salmochelin S4, petrobactin, bacillibactin, and DHBA promoted the growth of laboratory and clinical *S. aureus* strains, as well as strains bearing *sfa* and *sbm* deletions, strains bearing *sfa*, *sbm*, and *sst* mutations were incapable of using enterobactin, bacillibactin, and DHBA and were severely compromised for growth using petrobactin and salmochelin S4 (data not shown). We attribute the latter result to intake of petrobactin and salmochelin S4 via a combination of the Sst transporter and other, as yet unknown transporters.

SstD has high affinity for iron-loaded catecholate/catecholamine ligands. Fluorescence quenching assays were used to measure the affinity of purified SstD (Fig. 5A) for catecholamines and catechol siderophores. Titration with iron-free hormone or siderophore ligands showed no quenching of the Trp/Tyr fluorescence of SstD (data not shown). The fluorescence of bovine serum albumin, a protein negative control, was not quenched with any of the ligands tested (data not shown). SstD fluorescence was quenched by all four ferrated catecholamines tested (Fig. 5B) and by ferrated catechol siderophores (Fig. 5C). Interactions were specific for catecholamine and catechol ligands, as ferrated deferoxamine, a hydroxamate, did not result in fluorescence quenching. Dissociation constants are reported in Table 2.

FIG. 4. Catecholamine-dependent growth stimulation of staphyloferrin-deficient *S. aureus* in medium containing either human serum (A and C) or transferrin (B) requires the SstABCD transporter. For panels B and C, although all tested catecholamines promoted growth of an Sst-proficient *S. aureus* strain equivalent to that with norepinephrine, for clarity, the results are graphed only for norepinephrine. In panel C, the *sstABCD* operon, expressed from plasmid pSB5 with the endogenous iron-regulated *sst* promoter, complemented the Δsst growth deficiency of staphyloferrin-deficient *S. aureus* in serum in the presence of catecholamines. pLI50 is the vehicle control, and additions to the media were as follows: Fe, FeCl_3 ; NE, DL-norepinephrine; E, epinephrine; D, dopamine; and LD, L-DOPA. The numbers indicate the final concentration (μ M). All data points represent average values for at least three independent biological replicates, and error bars indicate the corresponding standard deviations from the means.

FIG. 5. SstD binds ferrated catecholamines and catechol siderophores. (A) SstD was purified in preparation for ligand binding studies; see Materials and Methods for details. Fluorescence quenching was used to determine binding affinities of SstD for ferrated catecholamines (B) and ferrated catechol siderophores (C). E, epinephrine; NE, norepinephrine; D, dopamine; LD, L-DOPA; EB, enterobactin; S4, salmochelin S4; DHBA, 2,3-dihydroxybenzoic acid; BB, bacillibactin; PB, petrobactin.

Hts, Sir, and Sst transporters contribute to virulence. The frequently used murine sepsis model of *S. aureus* infection was used to evaluate the relative and combined contributions of siderophore biosynthesis, siderophore transport, and catecholamine iron acquisition genes *in vivo*. Bacterial processes disrupted via mutation included catechol iron uptake (*sst*), staphyloferrin biosynthesis (*sbm sfa*), and staphyloferrin uptake

(*sirA hts*). Furthermore, the effects of combined mutations in catechol iron uptake and staphyloferrin biosynthesis (*sbm sfa sst*) or staphyloferrin uptake (*sirA hts sst*) were tested. Groups of immunocompetent BALB/c mice were infected intravenously with 5×10^6 bacteria, and bacterial loads in target organs were enumerated at 96 h postinjection. Single-locus deletions for either staphyloferrin biosynthesis or uptake did not yield statistically significant reductions of bacterial counts in any organ (data not shown), while deletion of *sst* alone or deletion of the *sfa-sbm* or *hts-sir* combination did yield significant reductions in heart colonization (Fig. 6). Combined inactivation of *sst* with staphyloferrin biosynthesis resulted in a marked decrease in heart and liver colonization, but combining *sst* with the staphyloferrin uptake mutant resulted in an even larger drop in CFU recovered from the heart and liver and yielded the lowest average bacterial burden in the kidneys of all mutants tested (Fig. 6).

In an attempt to further assess whether *sst* mutants were unable to respond to catecholamines *in vivo*, we used wild-type Newman and its isogenic *sst* mutant in challenge experiments with mice that had surgically implanted pumps that delivered, throughout the 4 days, a constant amount of adrenaline (epinephrine) into each animal. The results (data not shown) did not identify any further difference in infectivity between the wild type and the *sst* mutant in comparison to the results shown in Fig. 6.

Siderophore production continues in the absence of transport, further restricting iron availability. The stronger *in vivo* attenuation observed for siderophore transport mutants than for siderophore biosynthetic mutants prompted us to ask the following question: in the absence of transport, are staphylococcal siderophores still synthesized? While not yet documented for *S. aureus*, the phenomenon is seen in other bacteria, including *E. coli* (12), *Bordetella* (4), *Pseudomonas* (63), and *Rhizobium* spp. (34). Where siderophore production continues in the absence of transport, the growth medium represents a more chelated environment to the bacteria. To begin to assess this for *S. aureus*, we observed that when strains were grown in unchelated TMS broth (i.e., no exogenous chelator added), growth defects were noted for mutants lacking both staphyloferrin transporters (*sir hts*) or the ATPase required to energize both transporters (*fluC*) (3, 62). This growth defect was not apparent for a mutant unable to produce staphyloferrins (*sbm sfa*) (Fig. 7A), presumably because the bacteria were able to access unchelated iron from the medium by using

TABLE 2. K_d values for SstD-ferric catecholamine and SstD-ferric catechol complexes

Ligand	Mean K_d (μ M)	SD	% Remaining fluorescence
Norepinephrine	1.07	0.54	69.0
Epinephrine	1.65	0.08	48.6
Dopamine	0.49	0.22	53.0
L-DOPA	1.44	0.27	36.0
Bacillibactin	1.21	0.14	38.7
2,3-DHBA	1.62	0.27	42.7
Enterobactin	0.29	0.06	40.0
Petrobactin	1.62	0.25	28.0
Salmochelin	0.35	0.20	59.7

FIG. 6. Contributions of siderophore biosynthesis and transport to *S. aureus* infection in immunocompetent BALB/c mice. Experimental details are found in Materials and Methods. Strains evaluated are as indicated, and bacterial burdens in organs were evaluated 4 days following challenge. Each symbol represents an individual mouse, and groups of 10 mice were challenged. Each horizontal line indicates the average $\log_{10}$ CFU/organ for the group. Statistically significant data, determined by Student's *t* test ($P < 0.05$), are shown for comparisons of groups of mice infected with mutant bacteria versus those infected with wild-type bacteria, unless otherwise indicated.

lower-affinity uptake systems. Further analyses showed that the transport mutants strongly enhanced siderophore secretion relative to their isogenic wild-type parental strains or biosynthesis mutant counterparts, as measured using CAS reagent (Fig. 7B). Bioassays using *sir* and *hts* transport mutants as reporter strains revealed that both staphyloferrins were present in supernatants of transport mutants (data not shown).

FIG. 7. Staphyloferrin production continues in the absence of the ability to transport staphyloferrins. (A) Growth curves for *S. aureus* Newman and derivatives in TMS medium that was not treated with Chelex 100. (B) Chrome azurol S reagent was used to assay culture supernatants for siderophore output throughout growth (shown in panel A) at the indicated time points. All data points represent average values for at least three independent biological replicates, and error bars indicate the corresponding standard deviations from the means.

DISCUSSION

The preferred iron source for *S. aureus* during infection is considered to be heme (61). In spite of this, inactivating components of heme uptake reduces fitness only partially in virulence assays (37, 65), suggesting either alternate mechanisms for heme uptake or a contribution of alternate host iron reservoirs to growth of invasive *S. aureus*. Transferrin, a key iron-scavenging protein in serum, has been shown to sustain the growth of *S. aureus* *in vitro* (33, 50). This is the first molecular approach to characterize the genetic factors involved in heme-independent growth in the presence of human serum or the serum component transferrin. Previous work revealed that production of either of two chemically distinct but functionally redundant staphyloferrin siderophores was necessary and sufficient to promote growth in animal serum in the absence of other iron ligands (3). This study finds the same paradigm to hold true for proliferation in human serum. More importantly, it was demonstrated that either staphyloferrin A or staphyloferrin B could remove iron from human holotransferrin to support *S. aureus* growth.

A key theme of the *S. aureus* iron uptake strategy is the blending of redundancy and complexity in targeting specific iron ligands. Here we uncovered the molecular basis for an alternate mechanism for holotransferrin iron extraction, ob-

served only in the absence of staphyloferrin production. This second mechanism capitalizes on the recently characterized phenomenon of holotransferrin iron liberation through complex formation with mammalian catecholamine stress hormones (2, 5, 10, 19, 54). Catecholamine iron was shown to promote the growth of *Bacillus* and *E. coli* on holotransferrin at concentrations comparable to those tested in this study (5, 40). This role was characterized for strains mutated to inactivate production of the catecholate siderophores bacillibactin (*Bacillus*) and enterobactin (*E. coli*). Uptake was dependent on either organism's catechol siderophore ABC transporter (5, 40, 54). Due to the overshadowing contribution of staphyloferrins (Fig. 1), characterizing the contribution of catecholamines to iron uptake from serum or transferrin was made possible only after constructing and characterizing two whole-locus siderophore biosynthesis knockout strains. Catecholamine iron uptake was subsequently shown to be mediated by a distinct transporter, SstABCD, previously implicated in the *S. aureus* adaptive response to low-iron environments (44).

A large reservoir of plasma catecholamines is found in the venous and arterial circuitry between mesenteric organs, kidneys, and the liver (16). While the catecholamine concentrations tested in this study (50 to 200 μ M) have been alluded to as physiologically or therapeutically relevant (54), it is unlikely that catecholamines approach micromolar concentrations in bulk plasma, even after gastric surgery (16). Nevertheless, in concert with siderophore activity, they may subvert the bacteriostatic effects of transferrin to promote sufficient proliferation in the bloodstream for evasion of phagocytic immune cells prior to colonization of organs. This highlights the necessity for precaution prior to therapeutic administration of catecholamine hormones. More importantly, catecholamines may be found in elevated levels in microenvironments surrounding wounds where nervous damage has occurred. Other research has noted increases in indigenous bacterial gut flora following localized destruction of noradrenergic neurons (35). In addition to providing a source of iron, these wounds provide an epithelial breach through which opportunistic bacteria may enter the bloodstream. The opportunistic coopting of other organisms' siderophores may provide *S. aureus* with a competitive advantage in heterogeneously colonized host niches such as the nares or the gastrointestinal tract. Also, little is known about the dissemination of enteric catechol siderophores from the gut flora, and it is possible that these may be found in sufficient quantities to contribute to growth of invasive *S. aureus*.

Previously, inactivation of *sst* was shown to make no significant contribution to bacterial survival in a rat intraperitoneal cage model of infection (44). In the current study, *sst* inactivation on its own also failed to have an impact on virulence within typically characterized murine organs (e.g., kidneys and liver) but did significantly decrease virulence in the absence of siderophore transport. Surprisingly, *sst* inactivation was sufficient to significantly decrease colonization of the mouse heart. This finding is significant, as *S. aureus* is a leading cause of infective endocarditis (17). It is noteworthy that we failed to identify any further difference in bacterial loads between wild-type Newman and its isogenic *sst* mutant for target organs of mice receiving an exogenous supply of epinephrine. Among several possible explanations, this enforces the idea that for *S.*

aureus, iron acquisition *in vivo* is a complex, multifactorial process involving several different mechanisms of iron capture from host sources.

In previous work, it was demonstrated that inactivation of one of the staphyloferrin B synthetases alone caused decreased *S. aureus* virulence (13). The present study used staphyloferrin mutants created by deleting entire gene loci encoding all enzymes necessary for siderophore biosynthesis. In contrast to single gene mutations, this has the benefit of not resulting in the production of intracellular siderophore intermediates that may affect the physiology of the bacterium. Using *sfa* and *sbm* whole-locus deletion mutants in this study, we failed to demonstrate that these mutations, on their own, result in a significant drop in bacterial burden compared to that for wild-type Newman in the organs of mice. This can be attributed to the continued production of one of the staphyloferrins, which was demonstrated to be sufficient to allow growth in serum and in the presence of holotransferrin. Only when *S. aureus* was incapable of producing both staphyloferrin siderophores (i.e., the *sfa sbm* double mutant) was there a significant effect on the virulence of *S. aureus*.

The K_d of SstD for ferrated catecholamine and catechol siderophore ligands, in the micromolar range (Table 2), provides an explanation for the critical involvement of the Sst transporter in utilization of these iron chelates. The K_d values determined in this study are also in close agreement with that previously determined for the *B. subtilis* FeuA protein and the ferric-norepinephrine complex (1.6 μ M) (40). Interestingly, of all ligands examined, SstD had the highest affinity for the enteric siderophores enterobactin and salmochelin S4, suggesting that under certain favorable conditions, these ligands might be viable iron sources for *S. aureus in vivo*. The K_d values of SstD for its ligands, in the micromolar range, contrast with the K_d values in the nanomolar range that were determined for HtsA and SirA for their cognate ligands, staphyloferrin A and staphyloferrin B (24, 25). In line with the micromolar range of affinities of *E. coli* FhuD for several hydroxamate ligands, this might reflect a sacrifice in ligand affinity in lieu of greater ligand diversity.

Our findings demonstrate that inactivation of three transporters, namely, Sir, Hts, and Sst, inhibits utilization of transferrin-iron. Combined with the lack of inhibitory feedback on siderophore production, this strain may enhance the bacteriostatic potential of its milieu through the secretion of nonutilizable iron chelators. This phenomenon may underlie the reduced fitness of the *sst hts sir* transporter mutant relative to that of the *sst sfa sbm* mutant in the murine infection model used in this study. In combination, therefore, these lipoproteins may be worthy candidates for inclusion in a multivalent antistaphylococcal vaccine, wherein the effectiveness of antibodies would rely upon inhibiting transporter function.

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