For the important human pathogen Staphylococcus aureus, host heme is a vital source of nutrient iron during infection. Paradoxically, heme is also toxic at high concentrations and is capable of killing S. aureus. To maintain cellular heme homeostasis, S. aureus employs the coordinated actions of the heme sensing two-component system (HssRS) and the heme regulated transporter efflux pump (HrtAB). HssRS-dependent expression of HrtAB results in the alleviation of heme toxicity and tempered staphylococcal virulence. Although genetic experiments have defined the role of HssRS in the heme-dependent activation of hrtAB, the mechanism of this activation is not known. Furthermore, the global effect of HssRS on S. aureus gene expression has not been evaluated. Herein, we combine multivariable difference gel electrophoresis with mass spectrometry to identify the heme-induced cytoplasmic HssRS regulon. These experiments establish hrtAB as the major target of activation by HssRS in S. aureus. In addition, we show that signaling between the sensor histidine kinase HssS and the response regulator HssR is necessary for growth of S. aureus in high concentrations of heme. Finally, we show that a direct repeat DNA sequence within the hrtAB promoter is required for heme-induced, HssR-dependent expression driven by this promoter and that phosphorylated HssR binds to this direct repeat upon exposure of S. aureus to high concentrations of heme. Taken together, these data establish the mechanism for HssRS-dependent expression of HrtAB and, in turn, provide a functional understanding for how S. aureus avoids heme-mediated toxicity.

Staphylococcus aureus is a commensal organism that, upon breeching surface colonization sites, is capable of infecting virtually any tissue of the human body. Hence, it is likely that staphylococci can sense and respond to distinct environmental cues and stressors, which change upon the transition from commensal colonizer to invading pathogen. Consistent with this supposition, S. aureus has been shown to alter gene or protein expression upon changes in cell density, pH, oxidative stress, nutrient and oxygen availability, as well as upon heat and cold shock and growth in the presence of the iron source heme (1–7). A primary mechanism by which S. aureus mediates these changes in gene expression is through the elaboration of two-component systems (TCS)4 (1). TCS typically sense signals through a membrane-localized histidine kinase, which undergoes autophosphorylation at a specific histidine residue upon recognition of a ligand or other appropriate signal. The phosphorylated histidine kinase then transphosphorylates an aspartate residue of its cognate response regulator (8). Phosphorylated response regulators bind to the promoters of the genes that they regulate, often leading to enhanced target gene expression. Through this mechanism, bacteria use TCS to translate external signals into changes in gene expression programs, facilitating responses to environmental stimuli. Although S. aureus has a number of characterized and uncharacterized TCS, the signals that activate the sensor kinases of these systems, the molecular details of phosphorelay, and the target DNA sites to which activated response regulators bind are incompletely defined (1).

S. aureus undergoes a dramatic alteration in protein expression in response to depletion of nutrient iron (6). This programmed response is necessitated by the role of iron as a cofactor for enzymes involved in many cellular processes, including nucleotide biosynthesis, aerobic respiration, and protection against reactive oxygen species (9, 10). The requirement for iron in essential biochemical processes makes it a nutrient that pathogenic bacteria must acquire in the context of infection (10). However, free iron is a limiting nutrient for invading bacteria due to the fact that most mammalian iron is sequestered by iron-binding proteins such as the iron transport protein transferrin, the iron storage protein ferritin, and the oxygen-carrying and -storing proteins hemoglobin and myoglobin. In these latter two proteins, iron is found stably chelated at the center of the porphyrin ring of heme and is thus inaccessible to most bacteria. Nevertheless, the fact that the majority of the

4 The abbreviations used are: TCS, two-component system; Isd, iron-regulated surface determinant; Hts, heme transport system; HssRS, heme sensor response regulator (R) and sensor kinase (S); HrtAB, heme-regulated ABC transporter ATPase (A) and permease (B); 2D-DIGE, two-dimensional difference gel electrophoresis.

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□ The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

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iron within the human body is in the form of heme establishes it as a potential source of iron for pathogenic bacteria (11).

*S. aureus* is able to satisfy its nutrient iron requirement by acquiring heme from hemoglobin, a process vital to staphylococcal infection (12–15). Staphylococcal heme acquisition is accomplished through cell wall-anchored, membrane-bound, and cytoplasmic proteins of the *Isd* and *Hts* loci (15). The concerted actions of the *Isd* and *Hts* systems free heme from hemoglobin, shuttle it across the bacterial cell wall, transport it through the membrane and into the cytoplasm, and cleave the porphyrin ring to release free iron (13, 15).

Heme is a challenging molecule for biological systems to metabolize due to its propensity to generate reactive oxygen species, damage membranes, and peroxidate lipids (16). Although heme as well as its oxidized form hemin represent a species, damage membranes, and peroxidate lipids (16).

Although heme as well as its oxidized form hemin represent a species, damage membranes, and peroxidate lipids (16). This adaptive response is facilitated by the sensing of heme through the two-component heme sensor system (HssRS) (17). Upon exposure of *S. aureus* to heme, HssRS increases transcription of the genes encoding the heme-regulated transporter efflux pump (HrtAB), a bicistronic ABC-type transporter involved in the alleviation of heme toxicity (17). Interestingly, *S. aureus* mutants in *hrtAB* display increased hepatic virulence and an amplified heme-induced secretion of virulence factors (17). This highlights the importance of heme sensing and adaptation in *S. aureus* pathogenesis. Although HssRS has been identified as a regulatory system that is critical to staphylococcal heme sensing and involved in pathogenesis, the complete hemin-dependent HssRS regulon has not been described. Furthermore, the mechanism by which HssRS activates *hrtAB* expression in a hemin-dependent manner has not been elucidated.

In this report, we investigate the mechanism of signaling and gene regulation by HssRS. We present evidence that *hrtAB* is the major target of HssRS upon exposure of *S. aureus* to heme. Furthermore, we show that signaling between the sensor histidine kinase HssS and the response regulator HssR is essential for the adaptive response to heme. We also demonstrate that phosphorylated HssR binds to a direct repeat DNA sequence within the *hrtAB* promoter when bacteria encounter exogenous heme. These studies directly connect HssRS with the adaptive response to heme and reveal the functional details of a newly identified *S. aureus* TCS. The conservation of *hrtAB* and *hssRS* across a variety of Gram-positive organisms that associate with vertebrate blood makes these findings applicable to numerous bacterial pathogens.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—** *S. aureus* strain Newman (20) and its derivatives were used in all experiments (see Table 1). Tryptic soy broth was used for the growth of *S. aureus*; for plasmid selection in *S. aureus*, chloramphenicol was used at a concentration of 10 μg/ml. Luria broth (genetic manipulations) and terrific broth (protein expression) were used for the growth of *Escherichia coli*; for plasmid selection in *E. coli*, ampicillin was used at a concentration of 100 μg/ml (21).

**Genetic Manipulations in *S. aureus*—** All plasmids were first electroporated into the restriction-deficient primary recipient RN4220 (22), after which they were electroporated into appropriate electrocompetent secondary recipients (23).
2D-DIGE—Two-dimensional difference gel electrophoresis (2D-DIGE) was performed on triplicate samples of cytoplasmic extract from S. aureus wild type or an isogenic ΔhssR mutant grown for 15 h at 37 °C with shaking at 180 rpm in the presence or absence of 5 μM hemin. Samples were prepared, labeled, and resolved, and individual protein features with altered expression patterns were identified as described (6).

Purification of HssR and HssS—The entire hssR open reading frame and the signaling domain of hssS (corresponding to amino acids 185–457) were cloned into the E. coli expression plasmid pET15b, making pET15b.hssR and pET15b.hssS for the expression of hexahistidine N-terminal fusions of both proteins. Pfu mutagenesis (24) was used to create expression constructs for the mutants HssR:D52A and HssS:H249A. pET15b.hssR:D52A and pET15b.hssS:H249A were verified by sequencing (Vanderbilt University DNA sequencing facility). For all protein expression, E. coli BL-21(DE3) harboring each plasmid was subcultured 1:100 from 15-h cultures into terrific broth at 37 °C with shaking at 225 rpm until the A 600 of the culture reached 0.3. Growth temperature was then switched to 16 °C for 1 h, and expression was induced by adding isopropyl-1-thio-D-galactopyranoside (0.2 mM). After an additional 20 h of growth at 16 °C, bacteria were harvested, and recombinant proteins were purified by nickel affinity chromatography using nickel-nitrilotriacetic acid Superflow (Qiagen) following the manufacturer’s recommendations. Purified proteins were dialyzed and stored at −20 °C.

In Vitro Phosphorelay—70 μl of HssS or HssS:H249A labeling reaction was prepared (50 mM Tris, pH 8.0, 5 mM MgCl2, 200 mM KCl, 0.2 mM dithiothreitol, 10% glycerol, 20 μM ATP, 5 μM HssS or HssS:H249A), 20 μCi of [γ-32P]ATP (Amersham Biosciences) was added to both labeling reactions, and samples were incubated at 37 °C. At 5, 15, and 45 min after the addition of radiolabeled ATP, 10-μl samples were removed and mixed with 2× SDS loading buffer. At 45 min, HssR or HssR:D52A were added to the appropriate reactions to a final concentration of 17.5 μM. 10-μl samples were taken 5, 15, and 45 min after the addition of HssR or HssR:D52A. Samples were loaded onto 15% polyacrylamide gels, and, after SDS-PAGE, gels were dried and analyzed using a PhosphorImager.

XylE Reporter Constructs/htrtAB Promoter Truncations and Mutations—Construction of plrtAB.xylE has been described previously (17). A reporter construct in which the xylE gene is controlled by the S. aureus lipoprotein diacylglycerol transferase (lgt) promoter (25) was made by removing an NdeI site from xylE by Pfu mutagenesis (24) and cloning xylE lacking an NdeI site into pOS1plgt (25) between the NdeI and BamHI sites of this vector, making pgt.xylE. Using phtrtAB.xylE as a template, five truncations were made in the hrtAB promoter by PCR amplification using 5 different 5′ primers, which anneal to sites within the hrtAB promoter and a 3′ primer matching the 3′-end of xylE. These promoter-xylE fragments were inserted into pOS1 (26), making plrtAB.xylE.T1–5. Mutations were confirmed by sequencing from the 5′-end of the hrtAB promoter to the 3′-end of xylE. Four residues within the direct repeat sequence upstream of the hrtAB start site were mutated by PCR SOEing (27). plrtAB.xylE with four direct repeat base mutations (plrtAB.xylE.DR) was confirmed by sequencing as above.

Complementation Constructs—A plasmid containing a copy of hssR under the control of its native promoter was created by PCR-amplifying hssR with its promoter using S. aureus Newman genomic DNA as a template and inserting amplified DNA into pOS1, making pssR. A plasmid containing a copy of hssS under the control of the lgt promoter was created by PCR-amplifying hssS and inserting amplified DNA into pOS1plgt, creating pssS. A C-terminal c-myc-tagged hssR was created by PCR-amplifying hssR from S. aureus genomic DNA using a 5′ primer within the hssR promoter and a 3′ primer matching the 3′-end of hssR and including the coding sequence for the c-myc epitope (EQKLISEEDL). Amplified DNA was inserted into pOS1, creating phssR-myc. A C-terminal c-myc-tagged hssS was created in the same manner as for hssR. Amplified DNA was inserted into pOS1plgt, generating phssS-myc. The hssR-myc D52N mutation and the hssS-myc H249A mutation were introduced as described above for the generation of E. coli expression constructs, generating phssR-myc:D52N and phssS-myc:H249A. Mutations were confirmed, and secondary mutations were ruled out by sequencing. Expression of tagged HssS and HssR was tested by preparing bacterial extracts and immunoblotting as follows. For HssR-Myc, 15-ml cultures of bacteria grown for 15 h were centrifuged, washed with wash buffer (50 mM Tris, pH 7.5, 150 mM NaCl), and lysed in 800 μl of lysis buffer (wash buffer containing two complete protease inhibitor tablet per milliliter (Roche Diagnostics)) by mechanical disruption using a FastPrep 24 (MP Biomedicals). For HssS-Myc, bacteria were grown and washed as above. Bacteria were then resuspended in TSM (100 mM Tris, pH 7.0, 500 mM sucrose, 10 mM MgCl2, 20 μg/ml lysostaphin) and incubated at 37 °C for 30 min, and protoplasts were harvested by centrifugation. Protoplasts were re-suspended in 800 μl of lysis buffer as above and sonicated. Triton X-100 was added to 1%, insoluble material was removed by centrifugation, and supernatant was removed for analysis. 30 μg of lysate from HssR-Myc or HssS-Myc expressing S. aureus was subjected to SDS-PAGE, transferred to nitrocellulose, and blotted with sc-789 polyclonal rabbit anti-c-Myc primary (Santa Cruz Biotechnology, Santa Cruz, CA) and AlexaFluor-680-conjugated anti-rabbit secondary (Invitrogen) antibodies. Membranes were dried and scanned using an Odyssey Infrared Imaging System (LI-COR Biosciences).

S. aureus Growth Kinetics—15-h cultures of S. aureus grown in the presence or absence of 2 μM hemin were diluted 1:75 to 150 μl of fresh medium with or without 10 μM hemin in triplicate on a 96-well round-bottom cell culture plate. Cells were grown at 37 °C with shaking at 180 rpm, and absorbance values were determined at the indicated times after inoculation. All spectrophotometry was performed using a Cary 50 MPR microplate reader coupled to a Cary 50 Bio UV-visible spectrophotometer (Varian, Inc.).

XylE Activity Assay—Appropriate strains were grown overnight at 37 °C with shaking at 180 rpm and were then subcultured into fresh media for 3 h. Bacteria were then dispensed into 1.5-ml Eppendorf tubes containing hemin dilutions and were grown for 2 h. Cytoplasmic extracts were prepared and reporter activity was determined as previously described (17).
HssRS Signaling and DNA Binding

Magnetic Bead Pulldown Assay—For capture of HssR-Myc from S. aureus extracts, S. aureus harboring phssR-myc was subcultured 1:100 from an overnight culture into 100 ml of Tryptic soy broth with or without 7 μM hemin. Bacteria were grown for 7 h at 37 °C with shaking at 180 rpm and were then pelleted, washed with 20 ml of 50 mM Tris, pH 7.5, 150 mM NaCl, and resuspended in 20 ml of TSM for 30 min. Bacterial protoplasts were then pelleted, resuspended in 10 ml of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 2 mM MgCl₂), and lysed by French press. Mechanically sheared salmon sperm DNA was added to lysates to a final concentration of 10 μg/ml. Bead-DNA complexes were prepared by adding 5 μg of biotinylated 300-bp hrtB coding sequence DNA or hrtAB promoter DNA prepared by PCR using a 5’-biotinylated primer to 500 μg of Dynabeads M-280 streptavidin (Dynal Biotech) in 400 μl of wash buffer and wash buffer (50 mM Tris, pH 7.5, 1 mM NaCl, 0.5 mM EDTA) and rotating at a rotisserie at room temperature for 15 min. DNA-bead complexes were washed three times in 1 ml of wash buffer, resuspended in 50 μl of lysis buffer, and added to 5 ml of S. aureus extract. Mixtures were rotated for 30 min at room temperature, and DNA-bead complexes were washed four times with 1 ml of lysis buffer and eluted with 50 μl of 50 mM Tris, pH 7.5, 500 mM NaCl. For oligonucleotide elution experiments, DNA-protein-bead complexes were prepared using extracts from hemin-treated S. aureus as above and were eluted in increasing concentrations (0.001, 0.01, 0.1, 1, and 12.5 μM) of double-stranded oligonucleotides dissolved in 50 mM Tris, pH 7.5, and prepared by allowing complementary 40-mer corresponding to the wild-type (AAAAACAAATTGTTCATATTGAGTTCATATTTCAACCTTAT) or mutant (AAAAACAATTGTCACTATTGAGTTCATATTTCAACCTTAT) hrtAB direct repeat to cool to room temperature from 90 °C. Samples from triplicate reactions were analyzed by SDS-PAGE and immunoblotting as described above. For quantification, band intensities were determined using Odyssey Infrared Imaging System software (LI-COR Biosciences). For each elution series, band intensities were adjusted for background intensity, summed, and each band intensity was expressed as a percentage of the sum. For pulldown assay of in vitro phosphorylated purified HssR, 200 μl of phosphorylation reaction was prepared by incubating 1 μM HssR or HssR:D52A in 50 mM Tris, pH 7.5, 50 mM KCl, 2 mM MgCl₂ with or without 20 μM potassium acetyl phosphate for 2 h at 37 °C. Samples were centrifuged at 13,000 rpm for 2 min to remove precipitated protein, and 10 μl of soluble protein was removed for SDS-PAGE analysis. 25 μl of bead-DNA complexes prepared as described above were added to 75 μl of soluble protein in 1 ml of 50 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 2 mM MgCl₂, 10 μg/ml sheared salmon sperm DNA. Samples were rotated on a rotisserie at room temperature for 15 min, after which beads were washed four times and eluted with 50 μl of 50 mM Tris, pH 7.5, 500 mM NaCl. Samples were analyzed by SDS-PAGE followed by immunoblotting using an anti-hexahistidine tag antibody as described for the detection of c-myc-tagged proteins.

RESULTS

hssRS Is Required for Hemin-induced Expression of HrtA—S. aureus uses hemin as a nutrient iron source, and hemin-iron acquisition is required for staphylococcal infection (12–14). Conversely, high concentrations of hemin (10 μM) kill staphylococci (17). However, staphylococci that have been pre-exposed to a sub-toxic concentration of hemin (2 μM) are able to grow when inoculated into medium containing 10 μM hemin (17). This adaptive response is mediated by the concerted actions of the HssRS TCS and the HrtAB efflux pump (17). Mutation of either hssS or hssR eliminates the ability of S. aureus to adapt to growth in the presence of a high level of hemin, a defect that can be complemented by providing wild-type copies of the corresponding genes in trans (data not shown and Ref. 17).

Upon exposure to 10 μM hemin, S. aureus induces an increase in the expression of HrtA, the ATPase component of the HrtAB efflux pump (6, 17). To test whether the TCS response regulator HssR is required for this hemin-induced increase in HrtA and to identify additional HssR-regulated proteins, we performed two-dimensional difference gel electrophoresis (2D-DIGE). Cytoplasmic extracts were prepared in triplicate from S. aureus wild type or ΔhssR grown in the presence or absence of 5 μM hemin, the highest hemin concentration in which ΔhssR is able to grow with wild-type kinetics. Proteins were labeled with amino-reactive fluorescent dyes, resolved on two-dimensional gels, and protein features with differential expression patterns were excised and identified by mass spectrometry (Fig. 1 and supplemental Table S1) (6).

Upon exposure of wild type to 5 μM hemin, HrtA increased in abundance by a factor of 2.44 (p = 0.024) (Fig. 1 and supplemental Table S1). This hemin-dependent increase in HrtA expression is less than what we have reported previously (6) because of the lower concentration of hemin used in the present experiment. Importantly, strains inactivated for hssR did not up-reg-
Histidine 249 of HssS and aspartate 52 of HssR are essential for HssRS phosphorelay. A, alignment of residues within the dimerization/phosphorylation domain from several well characterized bacterial histidine kinases. Phosphorelay histidine residues are in bold. The predicted phosphorelay histidine of HssS (His-249) is boxed. B, alignment of residues within the phosphoacceptor domain of response regulators, which engage in phosphorelay with the histidine kinases from A. Phosphoacceptor aspartate residues are shown in bold. The predicted phosphorelay aspartate of HssR (Asp-52) is boxed. C, Coomasie Blue stain of SDS-PAGE showing purified HssR and HssS intracellular domain and mutant HssR (D52A) and HssS intracellular domain (H249A). D, in vitro phosphorelay between HssS and HssR. HssS or mutant HssS:H249A were added to [γ-32P]ATP, and samples were collected at 5, 10, and 45 min after the start of labeling. Following 45 min of labeling, HssR or mutant HssR:D52A were added to HssS (vertical arrow) and samples were collected at 50, 60, and 90 min (5, 15, and 45 min after the addition of HssR or HssR:D52A). Proteins were separated by SDS-PAGE, and gels were exposed by autoradiography. E, quantification of HssS phosphorylation from triplicate experiments performed as described in D. Band intensities of HssS before and after the addition of HssR (diamonds), HssS before and after the addition of HssR:D52A (boxes), and HssS:H249A before and after the addition of HssR (circles) were determined by PhosphorImager analysis and converted to the amount of 32P-phosphorylated HssS (HssS-P) in picograms. Addition of HssR or HssR:D52A is indicated by a diagonal arrow. Asterisks above symbols denote statistically significant differences in HssS-P amount when compared with any other condition at that time point by Student’s t-test (p < 0.05). Error bars represent one standard deviation from the mean. F, quantification of HssS 32P-phosphorylation (HssS-P) as described for HssS in E. The x-axis represents time after the start of labeling (or 5, 15, or 45 min after the addition of HssR).

ulate HrtA expression upon exposure to hemin (1.02-fold increase, p = 0.92) (Fig. 1 and supplemental Table S1). These results support our previous observation that HssR is responsible for the hemin-dependent up-regulation of the hrtAB transcript (17). Furthermore, no cytoplasmic protein features other than HrtA were identified by 2D-DIGE analysis that required HssR for a hemin-dependent increase in expression (supplemental Table S1). Although HrtB is likely to be expressed in a hemin-dependent, HssR-dependent manner as judged by the localization of hrtB in a bicistronic operon with hrtA and the fact that the HrtAB transcript displays this expression pattern (17), HrtB is a membrane protein (data not shown) and is not present in the cytoplasmic fractions used in our 2D-DIGE experiment. A number of stress-related and metabolic proteins displayed changes in abundance only in ΔhssR exposed to hemin, consistent with previous reports demonstrating that HssR protects S. aureus from hemin toxicity (17). Based on these results, we conclude that hssR is necessary for the hemin-induced increase in HrtA and that, within the window of the S. aureus cytosolic proteome resolvable by 2D-DIGE, HrtA is the major target of activation by HssR upon exposure of S. aureus to hemin.

HssS Exhibits Autophosphorylation Activity and Phosphorelay to HssR in Vitro—HssS and HssR display significant sequence and domain similarity to bacterial TCS histidine kinases and response regulators, respectively (17). Upon receiving appropriate signals, bacterial histidine kinases undergo autophosphorylation at a specific histidine residue and subsequently phosphorylate an aspartate residue in the cognate response regulator through which they signal (8). To predict the HssR and HssS residues that participate in phosphorylrelay, the intracellular domain of HssS was aligned with the dimerization/phosphorylation domain from five characterized bacterial TCS histidine kinases (Fig. 2A) (PhoQ (28), EnvZ (29), CheA (30), YycG (31), and KdpD (32)). Likewise, HssR was aligned with the phosphoacceptor domain from five known bacterial TCS response regulators (Fig. 2B) (PhoP (33), OmpR (34), CheY (35), YycF (31), and KdpE (36)). Consistent with their assignment as members of TCS, a conserved histidine residue was identified in the predicted dimerization/phosphorylation domain of HssS (His-249), and a conserved aspartate residue was identified in the predicted phosphoacceptor domain of HssR (Asp-52) (Fig. 2A and B).

To determine whether HssS undergoes autophosphorylation and catalyzes transphosphorylation of HssR and to determine whether His-249 of HssS and Asp-52 of HssR are the signaling residues of HssRS, in vitro phosphorelay experiments were per-
HssRS Signaling and DNA Binding

HssRS Phosphorelay Is Required for the Adaptation of S. aureus to Hemin Toxicity in Vivo—To test whether phosphorelay between HssS and HssR is required by S. aureus for adaptation to growth in the presence of a high concentration of hemin, we tested HssS and HssR phosphorelay mutants for functionality in vivo. Full-length C-terminal epitope-tagged versions of wild-type HssS and HssR as well as their respective phosphorelay site mutants were cloned into an S. aureus expression vector and transformed into S. aureus ΔhssS and ΔhssR. S. aureus strains were pre-adapted to hemin by overnight growth in the presence of 2 μM hemin and were subcultured into medium without hemin or medium containing 10 μM hemin. After overnight growth in 2 μM hemin, S. aureus wild type adapts to growth in 10 μM hemin, whereas in ΔhssS growth is arrested (Fig. 3A). Expression of a C-terminal epitope-tagged HssS-Myc in trans in ΔhssS restored hemin adaptation. However, HssS-Myc: H249A was not able to complement the hemin-sensitive phenotype of ΔhssS (Fig. 3A). This inability to complement is not due to poor expression of HssS-Myc: H249A, because the level of expression of this protein in S. aureus is similar to that of wild-type HssS-Myc as detected by immunoblot (Fig. 3B). In similar experiments, an epitope-tagged HssR-Myc was able to complement the hemin-sensitive phenotype of ΔhssR (Fig. 3C).

FIGURE 3. Residues essential for HssRS phosphorelay are required for adaptation of S. aureus to hemin toxicity. A, sensitivity of S. aureus ΔhssS expressing HssS: H249A to hemin toxicity. S. aureus wild type harboring an empty vector (wt + p, solid squares), ΔhssS harboring an empty vector (ΔhssS + p, solid circles), ΔhssS harboring phssS-myc (ΔhssS + phssS-myc, solid diamonds), and ΔhssS harboring phssS-myc: H249A (ΔhssS + phssS-myc: H249A, empty diamonds) were grown overnight in 2 μM hemin and were subcultured into medium lacking hemin (solid lines) or containing 10 μM hemin (dashed lines). A_{600} readings were taken on triplicate cultures at the indicated times after inoculation and averages ± one standard deviation are plotted. B, anti-c-myc immunoblot (I.B.: α-myc) of protoplasts from S. aureus strains described in A. Immunoblot is representative of results obtained from three independent experiments. Asterisk denotes a consistently observed cross-reactive band. C, sensitivity of S. aureus ΔhssR expressing HssS-RD52N to hemin toxicity. S. aureus wild-type harboring an empty vector (wt + p, solid squares), ΔhssR harboring an empty vector (ΔhssR + p, solid circles), ΔhssR harboring phssR-myc (ΔhssR + phssR-myc, solid diamonds), and ΔhssR harboring phssR-myc: D52N (ΔhssR + phssR-myc: D52N, empty diamonds) were grown and analyzed as described in A. D, anti-c-myc immunoblot (I.B.: α-Myc) of cytoplasmic fractions of S. aureus strains described in C. The immunoblot is representative of results obtained from three independent experiments.
HssR-Myc:D52A due to poor expression of HssR-Myc:D52A in S. aureus, was not able to complement despite significant expression (Fig. 3D). Taken together with the data presented in Fig. 1, these data suggest that functional signaling between HssS and HssR is necessary for the regulation of hrtAB from S. aureus promoter and previously published results, the enzyme XylE. Consistent with the hemin-induced up-regulation of S. aureus promoter upon exposure to wild-type S. aureus to hemin (Fig. 4A) (17). hrtAB promoter activity also occurs in a manner that is dependent on hssR (Fig. 4A), an observation that is consistent with the dependence on hssR for hemin-induced expression of HrtA (Fig. 1). Furthermore, hssR-dependent expression driven by the hrtAB promoter is dose-responsive with respect to hemin, with reporter activity detectable at 460 nM hemin and reaching a plateau at close to 5 μM hemin (Fig. 4A). Importantly, the lack of hrtAB promoter-driven expression of XylE in ΔhssR is not due to an inability of ΔhssR to synthesize XylE when grown in the presence of hemin, as evidenced by identical levels of XylE activity at all concentrations of hemin tested from wild-type and ΔhssR harboring a plasmid that constitutively expresses XylE (Fig. 4A).

To identify candidate DNA sequences upstream of hrtAB required for HssR-dependent expression, we aligned the hrtAB promoter sequences from eight different species of Gram-positive bacteria that contain potential orthologues of the HssRS and HrtAB systems (Fig. 4C) (17). This analysis revealed a perfectly conserved direct repeat sequence within the predicted hrtAB promoter of S. aureus, S. epidermidis, Bacillus cereus, and B. anthracis. Although less well conserved in the hrtAB promoters of the Listeria and other Staphylococci, certain bases are invariant across genera. A greater degree of variation exists in the bases between and outside of the repeats, suggesting that this direct repeat sequence is critical for functionality of the HssRS/HrtAB systems. Because TCS response regulators are known to bind to direct repeat sequences (37), we hypothesized that this direct repeat sequence is the cis-acting element with which HssR interacts.

To test whether the direct repeat upstream of hrtAB is necessary for promoter activation, a series of truncation mutants within the hrtAB promoter were constructed within the context of the hrtAB promoter-xylE reporter construct and tested for hemin-induced reporter activity. Truncation of the hrtAB promoter up to the direct repeat sequence had no effect on promoter activity compared with the full-length promoter (Fig. 4D). However, removal of half or all of the direct repeat

A Direct Repeat Sequence Upstream of hrtAB Is Necessary for Hemin-dependent Promoter Activation—The successful reconstruction of phosphorelay between HssS and HssR strongly suggests the assignment of this system as a staphylococcal TCS. Furthermore, the demonstration that HssR is required for HrtA expression upon exposure of S. aureus to hemin implicates hrtAB as an HssR target gene. By extension, it is likely that HssR binds to promoter sequences upstream of the hrtAB operon upon exposure of staphylococci to hemin to regulate transcription. To test this hypothesis, we first established a reporter assay that monitors the hrtAB promoter-driven expression of the enzyme XylE. Consistent with the hemin-induced up-regulation of HrtA and previously published results, the hrtAB promoter drives expression of XylE upon exposure of wild-type S. aureus to hemin (Fig. 4A) (17). hrtAB promoter activity also occurs in a manner that is dependent on hssR (Fig. 4A), an observation that is consistent with the dependence on hssR for hemin-induced expression of HrtA (Fig. 1). Furthermore, hssR-dependent expression driven by the hrtAB promoter is dose-responsive with respect to hemin, with reporter activity detectable at 460 nM hemin and reaching a plateau at close to 5 μM hemin (Fig. 4A). Importantly, the lack of hrtAB promoter-driven expression of XylE in ΔhssR is not due to an inability of ΔhssR to synthesize XylE when grown in the presence of hemin, as evidenced by identical levels of XylE activity at all concentrations of hemin tested from wild-type and ΔhssR harboring a plasmid that constitutively expresses XylE (Fig. 4A).

To identify candidate DNA sequences upstream of hrtAB required for HssR-dependent expression, we aligned the hrtAB promoter sequences from eight different species of Gram-positive bacteria that contain potential orthologues of the HssRS and HrtAB systems (Fig. 4C) (17). This analysis revealed a perfectly conserved direct repeat sequence within the predicted hrtAB promoter of S. aureus, S. epidermidis, Bacillus cereus, and B. anthracis. Although less well conserved in the hrtAB promoters of the Listeria and other Staphylococci, certain bases are invariant across genera. A greater degree of variation exists in the bases between and outside of the repeats, suggesting that this direct repeat sequence is critical for functionality of the HssRS/HrtAB systems. Because TCS response regulators are known to bind to direct repeat sequences (37), we hypothesized that this direct repeat sequence is the cis-acting element with which HssR interacts.

To test whether the direct repeat upstream of hrtAB is necessary for promoter activation, a series of truncation mutants within the hrtAB promoter were constructed within the context of the hrtAB promoter-xylE reporter construct and tested for hemin-induced reporter activity. Truncation of the hrtAB promoter up to the direct repeat sequence had no effect on promoter activity compared with the full-length promoter (Fig. 4D). However, removal of half or all of the direct repeat

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**Figure 4.** A direct repeat sequence within the hrtAB promoter is necessary for hemin-dependent promoter activation. A, hssR is required for activation of the hrtAB promoter in a hemin-dependent, dose-responsive manner. S. aureus wild-type and ΔhssR harboring a plasmid in which the reporter gene xylE is under the control of the hrtAB promoter (wt + phtAB.xylE, solid squares; ΔhssR + phtAB.xylE, solid diamonds) or a plasmid in which xylE is under the control of the constitutive lgt promoter (wt + phtAB.xylE, empty squares; ΔhssR + phtAB.xylE, empty diamonds) (25) were grown in triplicate in the presence of the indicated concentration of hemin. XylE specific activity was determined as described under “Experimental Procedures.” Error bars are shown for all points and in most cases are too small to be seen. B, a schematic diagram of the hrtAB promoter showing the direct repeat (light gray), predicted TATA box (dark gray), predicted ribosome binding site (RBS, black), and the hrtAB and hssRS coding sequences. Numbers indicate base position with respect to the hrtB start codon. C, alignment of the direct repeat within the hrtAB promoter from several Gram-positive bacteria. Residues in bold occur at the given position in more than 50% of the examined sequences and match the S. aureus direct repeat. Asterisks above residues indicate absolute conservation across the sequences examined. D, hrtAB promoter truncation and mutation analyses using the phtAB.xylE reporter construct from A. Sequences within the hrtAB promoter were deleted up to the direct repeat, half way through the direct repeat, up to the predicted TATA box, up to the predicted ribosome binding site (RBS, black), and the hrtAB and hssRS coding sequences. Numbers indicate base position with respect to the hrtB start codon. C, alignment of the direct repeat within the hrtAB promoter from several Gram-positive bacteria. Residues in bold occur at the given position in more than 50% of the examined sequences and match the S. aureus direct repeat. Asterisks above residues indicate absolute conservation across the sequences examined. D, hrtAB promoter truncation and mutation analyses using the phtAB.xylE reporter construct from A. Sequences within the hrtAB promoter were deleted up to the direct repeat, half way through the direct repeat, up to the predicted TATA box, up to the predicted ribosome binding site (RBS, black), and the hrtAB and hssRS coding sequences. Numbers indicate base position with respect to the hrtB start codon. C, alignment of the direct repeat within the hrtAB promoter from several Gram-positive bacteria. Residues in bold occur at the given position in more than 50% of the examined sequences and match the S. aureus direct repeat. Asterisks above residues indicate absolute conservation across the sequences examined.
sequence completely eliminated hemin-induced reporter activity even in the presence of a fully inducing concentration of hemin (Fig. 4D). Furthermore, induction of the hrtAB promoter by hemin was eliminated by the mutation of four bases that are absolutely conserved within the direct repeat sequences of all organisms analyzed. Promoters containing any of the listed alterations to the direct repeat sequence displayed reporter activity at comparable levels to those of hrtAB promoters lacking a predicted TATA box or ribosome binding site. We conclude that the direct repeat sequence within the hrtAB promoter is controlled.

**HssR Binds to the Direct Repeat within the hrtAB Promoter**

To test whether HssR-Myc specifically associates with the direct repeat sequence within the hrtAB promoter, HssR-Myc bound to hrtAB promoter DNA-complexed beads was eluted in a competitive elution experiment. Double-stranded 40-mer oligonucleotides were prepared that correspond to the hrtAB promoter direct repeat or a direct repeat mutated in the four conserved residues essential for hemin-induced promoter activity (Fig. 4D). Bound proteins were sequentially eluted with increasing concentrations of double-stranded oligonucleotides and were detected by SDS-PAGE followed by immunoblotting against the epitope tag of HssR-Myc (Fig. 6A). Although double-stranded oligonucleotides containing a wild-type direct repeat eluted HssR-Myc from the hrtAB promoter at concentrations as low as 0.1 μM, oligonucleotides with a mutant direct repeat did not elute HssR-Myc at concentrations up to 12.5 μM, a result that is reproducible across multiple experiments (Fig. 6B). Taken together, these results suggest that HssR binds to the direct repeat sequence within the hrtAB promoter to induce expression of HrtAB when *S. aureus* senses hemin.

**DISCUSSION**

Gene regulation is one of the most widely studied areas of *S. aureus* pathogenesis. To date, six different staphylococcal TCS histidine kinases and their cognate response regulators have been characterized: AgrAC, SaerS, SrrAB/SrhRS, ArlRS, LytRS, and YycGF (38–46). Although these systems have been identified and confirmed to play important roles in gene regulation, the signals that are recognized and the molecular mechanisms responsible for signal transduction have only been well characterized in a few cases (38–46). Herein, we have reconstituted the signal transduction system responsible for HssR-mediated activation of hrtAB expression upon exposure of *S. aureus* to hemin...
systems, which result in the rapid amassing of heme by staphylococci (14). Heme damages cells by catalyzing the formation of reactive oxygen species; bacteria that encounter and import heme must have mechanisms to respond to or metabolize this reactive molecule (15). *S. aureus* responds to heme through the sensing of heme by HssRS, which induces the expression of HrbAB, an efflux pump involved in the alleviation of heme-mediated toxicity (Fig. 7) (6, 17).

The mechanism by which HssS senses heme is unknown. It is possible that the predicted extracytoplasmic domain of HssS directly binds to heme, resulting in the activation of HssS. The lack of similarity between the sensing domain of HssS and any known heme-binding protein as well as the paucity of candidate heme-binding residues within this domain (data not shown) raise the possibility that recognition of heme by HssS may occur by a novel binding mechanism. Alternatively, recognition of heme by HssS might occur by an indirect mechanism. Although we have not found a molecule or condition other than growth in the presence of heme which results in activation of HssRS (data not shown), it is nonetheless possible that activation of HssS by heme is indirect and that, instead of heme, HssS recognizes a by-product of hemin-mediated toxicity.

We demonstrate here that binding of phosphorylated HssR to hrtAB promoter DNA is sensitive to alterations in the direct repeat sequence to which it binds. In addition, the hrtAB direct repeat is conserved across numerous Gram-positive bacteria (Fig. 4). Taken together, these observations suggest that this site is required for HssR-mediated activation systems.
of the hrtAB system across genera. A consensus sequence for HssR (GTTCATATT(N)\textsubscript{2}GTTCATATT) can be predicted by comparing the hrtAB direct repeat across all available bacterial genomes. Using genomic analyses, we have been unable to identify an S. aureus gene or operon other than hrtAB, which contains this perfect direct repeat within its promoter (data not shown). However, an HssR consensus site containing 3–4 mismatches can be detected in the predicted promoter region of 14 S. aureus genes. 3 of these 14 with potential roles in the pathogenesis of S. aureus include SAV1553 (the superoxide dismutase sodA), SAV1159 (a predicted fibrinogen-binding protein precursor), and SAV2644 (a predicted autolysin) (data not shown). Although 2D-DIGE analyses did not reveal protein features other than HrtA that are up-regulated in a hemin-dependent, HssR-dependent manner, it remains a possibility that additional S. aureus genes are regulated by HssR upon exposure of staphylococci to high concentrations of heme.

S. aureus exists as both a commensal of the skin and anterior nares and as an invading pathogen in the blood and deeper body tissues. Inhabiting these diverse environments likely requires significant proteomic elasticity on the part of S. aureus; however, the molecular cues that alert the bacterium to its surroundings have not been well defined. As a cofactor of myoglobin and hemoglobin, heme is an abundant molecular marker of muscle tissue and blood, and invasive S. aureus are therefore likely to have considerable exposure to host heme sources during infection. In contrast, the absence of a significant myocyte or erythrocyte population in the healthy nasal epithelium prevents exposure of commensal S. aureus to significant levels of heme. These suppositions lead to the possibility that heme is a molecular marker of internal host tissue that allows S. aureus to sense when it has breached the host epithelium. Consistent with this hypothesis, we have shown that S. aureus strains activated for either hssR or hrtA exhibit altered virulence in a mouse model of systemic infection (17). hssRS and hrtAB orthologues can be found in many species of Gram-positive bacteria, including S. epidermidis, S. saprophyticus, Listeria monocytogenes, Bacillus anthracis, and B. cereus. Interestingly, hssRS or hrtAB orthologues are not found within the genome of B. subtilis (data not shown), an organism that is not commonly considered to be a pathogen or a saprophyte, and hence one that is unlikely to require systems that sense and respond to vertebrate molecules. The conservation of hrtAB/hssRS across numerous Gram-positive bacteria suggests that HssRS-mediated heme sensing may be a conserved host-sensing strategy employed by organisms that come into contact with vertebrate blood.

This study is one of the first examples of the in vitro reconstitution of a S. aureus TCS. Furthermore, it represents the characterization of signaling and DNA binding events important for the functioning of one of the few bacterial TCS that is responsible for sensing an abundant host molecule. Understanding the mechanisms by which bacterial pathogens recognize host molecules will provide insight into the ways in which pathogenic bacteria sense and respond to the host environment. These studies may also provide avenues for the design of novel therapeutic agents that either interfere with or augment bacterial signaling in ways that attenuate the virulence of the pathogen.

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