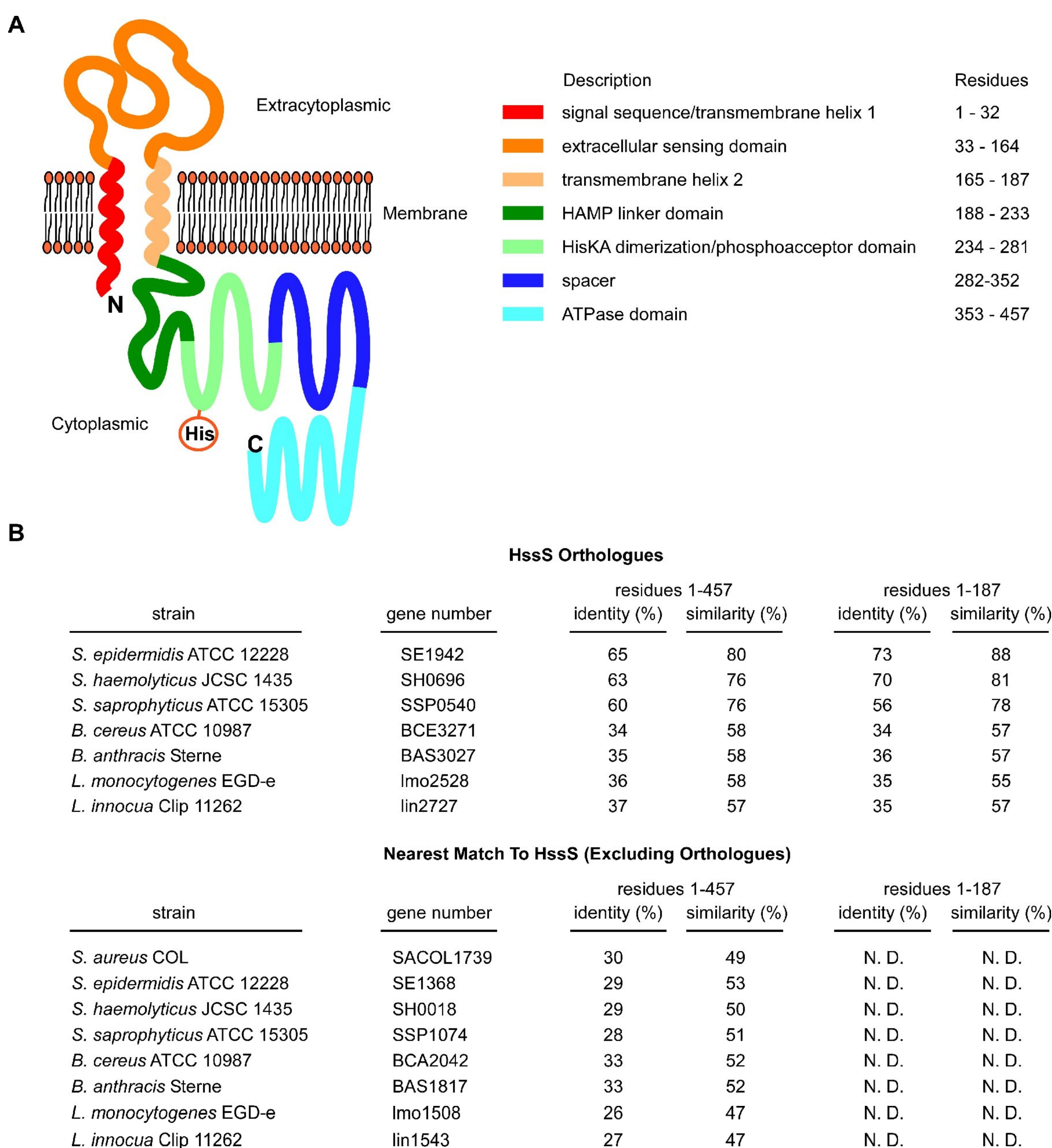
Supplementary Figures:



HssS Orthologues

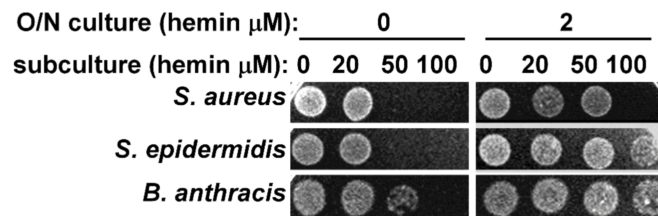
strain	gene number	residues 1-457 identity (%)	residues 1-457 similarity (%)	residues 1-187 identity (%)	residues 1-187 similarity (%)
S. epidermidis ATCC 12228	SE1942	65	80	73	88
S. haemolyticus JCSC 1435	SH0696	63	76	70	81
S. saprophyticus ATCC 15305	SSP0540	60	76	56	78
B. cereus ATCC 10987	BCE3271	34	58	34	57
B. anthracis Sterne	BAS3027	35	58	36	57
L. monocytogenes EGD-e	lmo2528	36	58	35	55
L. innocua Clip 11262	lin2727	37	57	35	57

Nearest Match To HssS (Excluding Orthologues)

strain	gene number	residues 1-457 identity (%)	residues 1-457 similarity (%)	residues 1-187 identity (%)	residues 1-187 similarity (%)
S. aureus COL	SACOL1739	30	49	N. D.	N. D.
S. epidermidis ATCC 12228	SE1368	29	53	N. D.	N. D.
S. haemolyticus JCSC 1435	SH0018	29	50	N. D.	N. D.
S. saprophyticus ATCC 15305	SSP1074	28	51	N. D.	N. D.
B. cereus ATCC 10987	BCA2042	33	52	N. D.	N. D.
B. anthracis Sterne	BAS1817	33	52	N. D.	N. D.
L. monocytogenes EGD-e	lmo1508	26	47	N. D.	N. D.
L. innocua Clip 11262	lin1543	27	47	N. D.	N. D.

Supplemental Figure 1. HssS is a unique sensor histidine kinase conserved across many Gram positive bacteria. A) The predicted membrane topology and domain organization of HssS. An N-terminal signal peptide was predicted by SignalP 3.0 and transmembrane helices were predicted by TMHMM 2.0. Domains represent predictions

based on CDART analysis. B) Comparison of HssS to potential orthologues (top) and the nearest non-HssS match in the indicated genomes. Full-length HssS (residues 1-457) and the predicted HssS sensing domain (residues 1-187) were aligned with the protein product of the given gene and the percent identity and similarity between the two were calculated.



Supplement Figure 2. The Hrt and Hss systems are conserved across several Gram positive bacteria. *S. aureus*, *S. epidermidis*, and *B. anthracis* were grown in TSB supplemented with 0 or 2 μM hemin and then subcultured for 2 hrs into TSB supplemented with increasing concentrations of hemin (μM) as indicated. The cultures were then serially diluted and plated on TSA plates.

Supplementary Methods

Creation of *S. aureus* strains inactivated for *hssR* or *hrtA*.

Briefly, sequences flanking *hrtA* were PCR-amplified using primers SAV2359-51-AttB1:GGGGACAAGTTTGTACAAAAAAGCAGGCT-TATTAGTGGTTTAGCTCAAGG and SAV2359-31-*XmaI*:TCCCCCCGGG-CCTTCTCCGAAATTTTTGACG for the upstream fragment and primers SAV2359-52-*XmaI*:TCCCCCCGGG-TTAGTGGCTTGTAAAGACGC and AV2359-32-AttB2:GGGGACCACTTTGTACAAGAAAGCTGGGT-TTTTCTCATACTTCTAGTCGC for the downstream fragment. Sequences flanking *hssR* were PCR-amplified using primers

SAV2361-51-AttB1:GGGGACAAGTTTGTACAAAAAAGCAGGCT-CTACCCCAATTATTGTTAGTG and SAV2361-31-*XmaI*:TCCCCCCGGG-CACCATAGCTATAAACTCCC for the upstream fragment and primers SAV2361-52-*XmaI*:TCCCCCCGGG-TGGGGCTATGATTATGAAGG and SAV2361-32-AttB2:GGGGACCACTTTGTACAAGAAAGCTGGGT-GCTCAGCTGCGTTGTAATG for the downstream fragment. The PCR fragments were then assembled into pCR2.1 (Invitrogen) and recombined into pKOR1 (1). Inactivation of *hrtA* and *hssR* genes was achieved by allelic replacement with pKOR1*ΔhrtA* and pKOR1*ΔhssR*, as described previously (1).

***Complementation of the ΔhrtA and ΔhssR mutant Staphylococci*.**

In the first PCR reaction primers P1A:CCCCGAATTCGCACCATAGCTATAAACTCC and P2A:CGACTAATGCCATATCGATTCACTTCTCCC were used to amplify the *hrtAB* promoter from *S. aureus* strain Newman genomic DNA. In the second PCR reaction primers P3A:GAATCGATATGGCATTAGTCGTTAAAG and P4A:CCCCGGATCCGCCACTAATCAGTTATTTTGC were used to amplify the *hrtA* gene from Newman genomic DNA. The PCR products were purified (Qiagen gel extraction kit) and a 1:300 dilution of purified reaction 1 and reaction 2 PCR products mixed and amplified using P1A and P4A as primers for PCR. This yielded an amplicon in which the *hrtAB* intergenic region promoter was fused to the *hrtA* gene. The *hrtAB* promoter-*hrtA* fusion was subcloned into pCR2.1 (Invitrogen). The insert was excised using restriction endonucleases *EcoRI* and *BamHI* and then ligated into pOS1 (2) cut with the same enzymes. Similarly, to make a complementation vector with the wildtype *hssR* gene, the *hssR* gene and its endogenous promoter were PCR amplified using primers SAV2361-5-*Pst*:CTGCAGCGATTCACTTCTCCCTATTTC and SAV2361-3-*BamHI*:GGATCCTCGCAATTCTAGCATAGAGTG. The *hssR* amplicon was then cloned

into pCR2.1 (Invitrogen). The insert was excised using restriction endonucleases *PstI* and *BamHI* and then ligated into pOS1 cut with the same enzymes. Both complementation vectors were then transformed into the corresponding isogenic mutant strains via electroporation resulting in the generation of ∆*hrtA/phrtA* and ∆*hssR/phssR*. As control, we electroporated empty pOS1 vector into wildtype (WT/pOS) and mutants strains (∆*hrtA/pOS* and ∆*hssR/pOS*). All vector containing stains were grown in media supplemented with 10 µg/ml of chloramphenicol.

Construction of a *hrtAB* Promoter-*xylE* Reporter

The *hrtAB* intergenic region was fused to the *xylE* structural gene by PCR-SOE. In the first reaction, primer P1B:CCCCGAATTCGCACCATAGCTATAAACTCC and P2B:CCTTTGTTCATATCGATTCACTTCTCC were used to amplify the *hrtAB* intergenic region from *S. aureus* strain Newman genomic DNA. In the second PCR reaction primers P3B:GTGAATCGATATGAACAAAGGTGTAATGCG and P4B:CCCGGATCCATACCATCAGGTCAGCACGG were used to amplify the *xylE* structural gene using pALC1639 template DNA (kindly provided by Dr. Ambrose Cheung) (3) and PCR product purified using a Qiagen gel extraction kit. For the PCR-SOE reaction, a 1:300 dilution of purified reaction 1 and reaction 2 PCR products was mixed and amplified by PCR using P1A and P4A. This yielded a construct in which the *hrtAB* intergenic region is fused to the *xylE* gene in such a manner that the position of the start codon for *xylE* corresponds to the position of the start codon for *hrtB* in the *hrtAB* locus. The *hrtAB* promoter-*xylE* fusion DNA was then subcloned into pOS1. As a control we also constructed a promoterless *xylE* vector. The *xylE* gene was amplified from pACL1639 template DNA using the primers P1C:CCCGAATTCATGAACAAAGGTGTAATGC and

P2C:CCCGGATCCATACCATCAGGTCAGCACGG and then cloned into the pOS1 vector.

RT-PCR

S. aureus cultures were grown O/N at 37 ^{o}C with shaking in 5 mls of RPMI containing 1 % casamino acids with or without different hemin concentrations. 1 ml of the O/N cultures was then sedimented and the bacterial pellet treated with RNAprotect Bacteria Reagent (Qiagen). Cells were then incubated with lysostaphin for 10 minutes at 37 ^{o}C to remove the bacterial cell wall. Total RNA was then isolated from bacterial protoplasts using the RNAeasy kit (Qiagen). Total RNA was examined via absorbance (Abs 260/280) and by agarose gel electrophoresis. 2 µg of total RNA were used for the reverse transcription reaction using M-MLV reverse transcriptase (Promega) and random hexamers primers (Applied Bbioscience). The RT-reaction product was then diluted 1:200 and used for a PCR reaction using specific primers for the *hrtA* transcript (ABC2-5-RT:TAAACAGCATCGTCCTAGTG and ABC-3-RT:CAAATAATCTTCGATCGTGTG) and the *16srRNA* transcript (16srRNA-5-RT:GCGAAGAACCTTACCAAATC and 16srRNA-3-RT:CCAACATATCACGACACG). PCR amplicons were then analyzed by agarose gel electrophoresis.

Growth Curve Assay

S. aureus cultures were grown overnight (O/N ~15 hrs) at 37 ^{o}C with shaking at 180 RPM in tryptic soy broth (TSB). Cultures were diluted 1:75 and inoculated into round-bottom 96-well plates in a final volume of 150 µl. Cultures were grown at 37 ^{o}C with aeration and bacterial growth was monitored by the increase in absorbance (O.D.$_{600}$) over time. For the hemin adaptation assays, the medium was supplemented with different hemin concentrations prior to bacterial inoculation. The results represent the

mean ± S.D. from triplicate determinations and the asterisks denote statistically significant differences as determined by Student's *t* test ($p \leq 0.05$).

Mouse Model of Infection

Seven to eight week old female BALB/c mice (Jackson Laboratories) were infected with 1×10^6 colony forming units by i.v. injection into the retro-orbital vein complex as described previously (4, 5). *S. aureus* wildtype, *ΔhrtA* or Δ*hssR* mutant strains were used in these infections. Four days after infection, mice were euthanized with CO_2. Spleen, livers, and kidneys were removed, analyzed for abscess formation, homogenized in PBS, and staphylococcal load determined by colony formation on tryptic soy agar (TSA). Ten or more mice were infected with each strain of *S. aureus*. Statistical analyses were performed using the Student's *t* test. Mouse infections were approved by Vanderbilt University's Institutional Animal Care and Use Committee (IACUC). All experiments conform to regulatory guidelines for animal infections.

Flow Cytometry

All antibodies and reagents for cell surface staining were purchased from BD Pharmingen, unless stated otherwise. Total erythrocyte-free spleen, kidney and liver lymphocytes and leukocytes of individual, age matched (7 weeks old) BALB/c females infected with *S. aureus* or uninfected as described above, were stained for four-color flow cytometric analysis. The following antibodies were used: anti-B220-FITC, anti-B220-APC, anti-B220-PerCP-Cy5.5, anti-CD11b-FITC, anti-CD11b-PerCP, anti-Ly6G-FITC, anti-CD8a-FITC, anti-CD11c-PE, anti-CD3e-PE, anti-CD4-PE, anti-CD19-PE, CD1d-tetramer-PE, anti-CD3e-PerCP-Cy5.5, anti-CD8a-PerCP-Cy5.5, anti-CD14-PE, anti-DX5-allophycocyanin, and anti-CD3ε-allophycocyanin. Invariant natural killer cells (iNKT; $CD3\varepsilon^+$CD1d $tetramer^+$) cells, NK cells ($CD3\varepsilon^-DX5^+$) cells, $CD4^+$ T cells

($CD3\varepsilon^+CD4^+CD8^-$), $CD8^+$ T cells ($CD3\varepsilon^+CD4^-CD8^+$), and granulocytes ($CD11b^+$/Ly6G+) were analyzed amongst electronically gated $B220^{neg}$ lymphocyte population. Dendritic cells (DC; $CD11c^+CD11b^-$) were analyzed amongst total, ungated cell population. Four-color flow cytometry was performed using FACSCalibur® instrument (Becton Dickinson), and the data were analyzed using FlowJo software (Treestar Inc).

Microarray studies

Total bacterial RNA was isolated from *S. aureus* strain Newman (Δ*hrtA*) grown to late-log phase in the absence or presence of 1 μM hemin. RNA was reverse transcribed, cDNA fragmented, 3' biotinylated and hybridized to commercially available *S. aureus* GeneChips® following the manufacturer's recommendations for antisense prokaryotic arrays (Affymetrix, Santa Clara, CA). GeneChips® were washed, stained and scanned, as previously described (6). Signal intensity values for each GeneChip® qualifier (predicted open reading frame or intergenic region) was normalized to the median GeneChip signal. Each condition was analyzed at least three times. Data from replicate samples were averaged and analyzed with GeneSpring GX software (Agilent Technologies, Santa Clara, CA), as previously described (6, 7). Differential expression was defined as ≥ 3-fold change in transcript titer (Student's *t*-test p-value of ≤ 0.05).

Supplemental References

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