Molecular basis for the evolution of species-specific hemoglobin capture by pathogenic *Staphylococcus*

Jacob E. Choby^{1,2,3}, Hanna B. Buechi⁴, Allison J. Farrand¹, Eric P. Skaar^{1,2*}, Matthew F. Barber^{4*}

¹ Department of Pathology, Microbiology, and Immunology, Vanderbilt University Medical Center, Nashville, TN, 37232, USA

² Vanderbilt Institute for Infection, Immunology, and Inflammation, Vanderbilt University Medical Center, Nashville, TN, 37232, USA

³ Graduate Program in Microbiology and Immunology, Vanderbilt University, Nashville, TN, 37232, USA

⁴ Institute of Ecology and Evolution, University of Oregon, Eugene, OR, 97403, USA

*Correspondence to eric.skaar@vanderbilt.edu and mfbarber@uoregon.edu

21 **ABSTRACT**

22 Metals are a limiting resource for pathogenic bacteria and must be scavenged from host proteins.
 23 Hemoglobin provides the most abundant source of iron in the human body and is required by several
 24 pathogens to cause invasive disease. However, the consequences of hemoglobin evolution for bacterial
 25 nutrient acquisition remain unclear. Here we show that the α - and β -globin genes exhibit strikingly parallel
 26 signatures of adaptive evolution across simian primates. Rapidly evolving sites in hemoglobin correspond
 27 to binding interfaces of IsdB, a bacterial hemoglobin receptor encoded by pathogenic *Staphylococcus*
 28 *aureus*. Using an evolution-guided experimental approach, we demonstrate that divergence between
 29 primates and staphylococcal isolates governs hemoglobin recognition and bacterial growth.
 30 Reintroducing putative adaptive mutations in α - or β -globin proteins is sufficient to impair *S. aureus*
 31 binding, providing a mechanism for the evolution of disease resistance. These findings suggest that
 32 bacterial hemoprotein capture has driven repeated evolutionary conflicts with hemoglobin during primate
 33 descent.

34 **INTRODUCTION**

35 Animals possess a variety of molecular factors that effectively sequester essential metals from
 36 invasive microbes, contributing to an innate immune function termed nutritional immunity (1, 2). Iron, as
 37 a critical cofactor for many host and bacterial enzymes, has provided the paradigm for our current
 38 understanding of nutritional immunity. Since the discovery of iron limitation by egg white ovotransferrin
 39 in the 1940s (3), mechanisms underlying nutritional immunity and bacterial iron scavenging have been
 40 the subject of intense study (4). Many vertebrate-associated bacteria encode high affinity uptake systems
 41 targeting heme, an abundant iron-containing porphyrin cofactor (5).

42 The most abundant source of heme-iron in the mammalian host is hemoglobin, which mediates
 43 oxygen transport within circulating erythrocytes. The predominant adult hemoglobin consists of a tetramer
 44 containing two α -globin and two β -globin protein subunits, each of which binds a single heme molecule

45 for coordination of oxygen. The Gram-positive bacterium *Staphylococcus aureus* is well-adapted to the
 46 human host and is a leading cause of skin and soft tissue infections, endocarditis, osteomyelitis, and
 47 bacteremia (6). In order to acquire iron during infection, *S. aureus* has evolved a high-affinity hemoglobin
 48 binding and heme extraction system, termed the *iron regulated surface determinant* (Isd) system (7).
 49 Following the lysis of proximal erythrocytes via secreted bacterial toxins, released hemoglobin is captured
 50 by receptors at the *S. aureus* cell surface (8, 9). The Isd system of *S. aureus* in part consists of cell-wall
 51 anchored IsdB and IsdH, which bind hemoglobin and haptoglobin-hemoglobin, respectively (9, 10).

52 We and others have shown that IsdB is the primary hemoglobin receptor for *S. aureus* and critical
 53 for pathogenesis in murine models (9, 11-13). Additionally, IsdB is highly expressed in human blood (14)
 54 and a promising vaccine target (15), underscoring its importance in human disease. IsdB extracts heme
 55 from hemoglobin, and heme is subsequently passed across the cell wall and into the cytoplasm for
 56 degradation by the heme oxygenases IsdG and IsdI, liberating iron (16-20). Underscoring the importance
 57 of IsdB for pathogenesis, heme is the preferred iron source of *S. aureus* during murine infection (21). The
 58 cell-wall anchored IsdABCH proteins share between one and three NEAT (*near transporter*) domains for
 59 coordination of hemoglobin or heme. IsdB NEAT1 binds hemoglobin while NEAT2 binds heme, tethered
 60 by an intervening linker (22). Consistent with adaptation of *S. aureus* to colonize and infect humans, we
 61 previously found that *S. aureus* IsdB binds human hemoglobin more effectively than mouse hemoglobin,
 62 the common laboratory animal used to model *S. aureus* infection (13). These results suggest that
 63 hemoglobin variation among mammals could dictate effective heme acquisition by *S. aureus* and other
 64 Gram-positive bacteria.

65 Previous work has demonstrated that pathogens can promote rapid adaptation of host immunity
 66 genes through repeated bouts of positive selection (23-25). While adaptation during such evolutionary
 67 conflicts can take many forms, theoretical and empirical studies concur that an elevated rate of
 68 nonsynonymous to synonymous substitutions in protein-coding genes is indicative of recurrent positive
 69 selection (26, 27). To date, most empirical studies of host-pathogen ‘arms races’ have focused on viruses

(28-31). Recently we showed that the transferrin family of iron-binding proteins has undergone extremely rapid evolution in primates at protein surfaces bound by iron-acquisition receptors from Gram-negative bacteria (32, 33). These findings are consistent with a long-standing evolutionary conflict for nutrient iron, whereby mutations in iron binding proteins that prevent bacterial scavenging protect the host from infection and are favored by natural selection. While these studies have expanded our understanding for how pathogens shape the evolution of host genomes, they also raise the question of whether other components of nutritional immunity might be subject to similar evolutionary dynamics.

In addition to its role as the principal bloodstream oxygen transporter, hemoglobin has provided an important biological model for diverse areas of the life sciences. Elegant studies have illustrated how hemoglobin variation underlies multiple instances of adaptation to high altitudes in diverse vertebrate taxa (34-37). Hemoglobin alleles have also likely been subject to balancing selection in human populations, where mutations that produce sickle-cell disease also confer resistance to severe malaria (38) and have reached high frequencies in regions where malaria is endemic. Despite its long history of study, the consequences of hemoglobin evolution for vertebrate nutritional immunity remain unclear. In the present study we set out to investigate the evolution of hemoglobin family proteins in primates and determine whether primate hemoglobin evolution impacts the ability to sequester heme-iron from bacterial pathogens.

RESULTS

Parallel signatures of positive selection in primate hemoglobins at the LsdB binding interface

To investigate how natural selection has shaped hemoglobin diversity in simian primates, orthologs of the α - and β -globin genes were cloned and Sanger sequenced from primate cell lines as well as compiled from publically-available databases. In total, 27 α -globin and 30 β -globin orthologs were assembled for phylogenetic analyses using the PAML and HyPhy software packages (Figure 1A, Materials and Methods), which use nonsynonymous and synonymous substitution rates to infer

signatures of positive selection. Because globin genes have been shown to undergo gene conversion which can distort inferred phylogenetic relationships, all analyses were performed using both a well-supported species tree as well as gene trees generated using PhyML. All tests detected significant evidence of positive selection acting on both α - and β -globin using both species and gene phylogenies (Supplemental Data). Multiple iterations repeatedly identified two sites in α - and β -globin exhibiting strong signatures of positive selection (Figure 1A). It was immediately apparent that these rapidly-evolving sites localized to similar regions of the α - and β -globin proteins, specifically the N-terminal A helix and the hinge region between the E and F helices (Figure 1B). In fact, the two sites exhibiting signatures of selection in the α - and β -globin A helices are at homologous positions. These parallel signatures of selection between α - and β -globin could indicate that a similar selective pressure has driven this divergence between primate species. To investigate whether bacterial heme scavenging receptors could be one such pressure, rapidly evolving sites were mapped onto a recently solved co-crystal structure between human hemoglobin and the LsdB protein from *S. aureus*. Notably, all four residues are localized to the LsdB binding interface, in close proximity to the NEAT1 domain (Figure 1C). Together these findings indicate that primate globins have undergone rapid divergence at specific sites proximal to the binding interface with Gram-positive bacterial hemoglobin receptors.

Figure 1. Parallel signatures of positive selection among primate hemoglobins at the bacterial IsdB binding interface. (A) Species phylogenies for α-globin and β-globin orthologs analyzed (left) with alignments from representative species across hominoid (orange), Old World monkey (purple) and New World monkeys (brown). Amino acid sites under positive selection were identified in α-globin and β-globin (blue arrows) using PAML with both species and gene trees (posterior probabilities >0.95). (B) Amino acid alignment of human α-globin and β-globin proteins. The position of conserved helices A-H are shown, and identity between globins is noted as conserved (*), highly similar (:), and similar (.). (C) The residues of α-globin (red) and β-globin (salmon) under positive selection (blue spheres) at the interface of hemoglobin capture by *Staphylococcus aureus* IsdB (gray) (PDB:5VMM).

Primate hemoglobin variation dictates *S. aureus* binding and heme-iron acquisition

To assess how hemoglobin divergence among primates impacts recognition by *S. aureus*, recombinant hemoglobin from human, white-cheeked gibbon, baboon, talapoin, and marmoset were

purified, providing broad representation from our phylogenetic dataset. An established biochemical assay was used to measure binding of hemoglobin by *S. aureus*, in which *S. aureus* cells recognize recombinant human hemoglobin as well as hemoglobin purified from blood in an LsdB-dependent manner (Supplemental Figure 1). *S. aureus* exhibited significantly reduced binding of baboon and marmoset hemoglobin to the cell surface (Figure 2A). It was noted that binding patterns do not strictly match predictions based on host phylogeny, suggesting discrete large-effect substitutions in hemoglobin may contribute disproportionately to recognition by *S. aureus*. We next determined the ability of primate hemoglobins to support growth of *S. aureus* as the sole iron source. Consistent with whole-cell binding data, hemoglobins that were bound by *S. aureus* with low affinity were unable to support optimal bacterial growth, indicating that the capability to bind hemoglobin is a measure of the ability to utilize hemoglobin as an iron source (Figure 2B). Together these results demonstrate that variation among primate globins dictates bacterial hemoglobin capture and heme-dependent growth.

Figure 2. Primate hemoglobin variation dictates *S. aureus* binding and heme-iron acquisition. (A) *S. aureus* binds hemoglobin with species specificity. Iron-starved *S. aureus* was incubated with 10 μ g/ml purified recombinant hemoglobin from representative species. Hemoglobin bound to the surface of *S. aureus* was eluted and analyzed by SDS-PAGE; relative hemoglobin abundance was measured by densitometry analysis (Image J) and compared to human hemoglobin for each replicate. (B) LsdB preference of hemoglobin binding correlates with the ability of *S. aureus* to utilize hemoglobin as the sole iron source. Growth of *S. aureus* in iron-deplete medium with 2.5 μ g/ml of purified recombinant hemoglobin as the sole iron source. Shown is the final growth yield of *S. aureus* after 48 hours. For A

and B, graphed are the means of four independent experiments in biological triplicate $\pm$ SEM, ** $p < 0.005$; *** $p < 0.0005$ by two-way ANOVA with Sidak correction for multiple comparisons.

Species-specific diversity in α -globin restricts heme scavenging by *S. aureus*.

The identification of rapidly evolving sites at the LsdB binding interface in both α -globin and β -globin suggest that natural selection in primate globins has been driven by antagonistic evolutionary conflicts with related families of bacterial receptors during primate divergence. Mechanistically, these findings also suggest that both globins contribute to *S. aureus* species-specific hemoglobin capture. We therefore exploited the enhanced binding of human hemoglobin relative to baboon to examine the role of each globin subunit in this biochemical interaction. The ability of *S. aureus* to bind chimeric hemoglobins was measured, which revealed that both globins contribute to species-specificity (Figure 3A), as chimeras containing either human α - or β -globin were bound more effectively than baboon hemoglobin. However, α -globin appears to have a greater effect on human-specific capture, as the α -human β -baboon chimera was bound significantly better than α -baboon β -human. Focusing on phylogenetic variation at the protein binding interface, α -globin T8 and N78 are both proximal to the NEAT1 domain of LsdB (Figure 3B, 3C). Mutagenesis of the N-terminal alpha helix of human α -globin revealed that substituting the Lys of baboon at position 8 significantly reduced binding by *S. aureus* (Figure 3D). Additionally, substituting A5D, T8K, and N9H in human α -globin, which converts this 7 amino acid region to that of baboon, leaves *S. aureus* binding indistinguishable from that of baboon hemoglobin. These results demonstrate that the N-terminal helix of α -globin makes a major contribution to *S. aureus* hemoglobin specificity. Next, the relative importance of the rapidly evolving N78 residue in α -globin was assessed, which lies N-terminal to the sixth alpha helix (Figure 3B). Substitution of N78 to glutamine (present in baboon, talapoin, and other Old World primates) or to histidine, reduced binding of human hemoglobin (Figure 3E). Thus, substitutions at multiple residues in α -globin that exhibit signatures of repeated positive selection disrupt the ability of *S. aureus* to recognize human hemoglobin.

Figure 3. Species-specific diversity in α -globin restricts heme scavenging by *S. aureus*. (A) *S. aureus* binding of human, baboon, and chimeric hemoglobins demonstrates that both globins contribute to binding by LsdB. (B) Species phylogenies and sequence alignments surrounding positions exhibiting signatures of positive selection in α -globin. (C) Residues 8 and 78 of human α -globin (red) interact closely with LsdB (gray) (PDB:5VMM). (D) The T8 residue in α -globin contributes to LsdB recognition between human and baboon hemoglobin as assessed by LsdB binding of mutagenized hemoglobin. (E) The N78 residue of human α -globin contributes to species specificity by LsdB. For A, D, E, graphed are the means of 3-4 independent experiments with 2-3 biological replicates $\pm$ SEM, ns: no significance, * $p < 0.05$, ** $p < 0.005$; *** $p < 0.0005$ by two-way ANOVA with Sidak correction for multiple comparisons.

β -globin divergence contributes to *S. aureus* hemoglobin binding.

S. aureus was capable of binding α -baboon β -human chimeric hemoglobin with higher affinity than baboon hemoglobin (Figure 3A), signifying that β -globin also contributes to *S. aureus* species-specific hemoglobin capture. Therefore, the contribution of rapidly evolving residues in β -globin to this binding interaction were investigated (Figure 4A). Both S9 and A76 interact closely with the NEAT1 domain of LsdB (Figure 4B). The effect of substituting human β -globin S9 and A76 with residues found in other primate species analyzed in this work was systematically tested, which revealed that A76 is particularly important for binding by *S. aureus* (Figure 4C). Notably, baboon hemoglobin displays phylogenetic variation at both position 9 and 76, suggesting that these residues may contribute to the inability of LsdB to bind baboon hemoglobin. This variance might also explain the binding affinity differences between human hemoglobin and the α -human β -baboon chimera, observed in Figure 3A. As for α -globin, no single residue substitution improved binding by *S. aureus* LsdB, consistent with the idea that LsdB has specifically adapted to bind human hemoglobin. Taken together with earlier data, residues at the LsdB interface of both α -globin and β -globin contribute to recognition of hemoglobin by *S. aureus*. This is consistent with the NEAT1 domain of multiple LsdB monomers engaging in hemoglobin capture by binding both α - and β -globins, as observed in the co-crystal structure (22).

IsdB diversity among related staphylococcal strains impacts primate-specific hemoglobin capture.

Given the observed differences in *S. aureus* binding between diverse primate hemoglobins, we considered how genetic variation in IsdB might impact this interaction. The IsdB NEAT1 subdomain Q162R-S170T is critical for hemoglobin recognition and is completely conserved among more than three thousand *S. aureus* clinical isolates (11). Therefore, IsdB variation among congeneric *S. argenteus* and *S. schweitzeri* was assessed. These recently diverged taxa (Figure 5A) are both primate-associated and, unlike most other staphylococci, encode IsdB. We measured the ability of IsdB from *S. argenteus* and *S. schweitzeri* to bind hemoglobin by expressing them ectopically in *S. aureus*. Consistent with their overall high sequence identity, *S. schweitzeri* and *S. argenteus* IsdB bind primate hemoglobin with a similar pattern of species preference as *S. aureus* (Figure 5B). However, both the IsdB of *S. schweitzeri* and *S. argenteus* display reduced binding of talapoin hemoglobin, and *S. argenteus* IsdB does not bind marmoset significantly worse than human. These data indicate that variation among IsdB impacts species-specific hemoglobin capture.

Figure 5. IsdB diversity among related staphylococcal strains impacts primate-specific hemoglobin capture. (A) Maximum likelihood phylogeny of the DNA gyrase A protein from representative staphylococci generated using PhyML. *M. caseolyticus* was included as an outgroup. The similarity of IsdB in *S. argenteus* and *S. schweitzeri* relative to *S. aureus* is shown at right. Bootstrap values above 80 are indicated. (B) The IsdB of *S. aureus*, *S. schweitzeri*, and *S. argenteus* demonstrate a common species preference for hemoglobin binding when expressed ectopically in *S. aureus*. Graphed are the means of three independent experiments with 3 biological replicates $\pm$ SEM, * $p < 0.05$; **** $p < 0.0001$ by two-way ANOVA with Sidak correction for multiple comparisons.

IsdB NEAT1 domain diversity among staphylococci modulates human hemoglobin recognition

Closer examination of the Q162R-S170T region of IsdB NEAT1 revealed variation between related staphylococci, but no variation in the critical heme-binding region of NEAT2 (Figure 6A). This region of NEAT1 closely interacts with the N-terminal helices of either α -globin and β -globin, in close proximity to both discrete sites bearing signatures of adaptive evolution in α -globin and β -globin (Figure 6B). To determine the functional consequences of variation in this NEAT1 domain, Q162 and S170T were mutagenized in *S. aureus* IsdB to mimic the sequence of *S. schweitzeri* and *S. argenteus*. Variations at both of these positions reduced affinity for human hemoglobin, showing that in the context of *S. aureus* IsdB, Q162 and S170 are required for high affinity hemoglobin binding (Figure 6C).

Figure 6 LsdB NEAT1 domain diversity among staphylococci modulates human hemoglobin recognition. (A) An alignment of the NEAT1 subdomain critical for hemoglobin binding shows variation among staphylococcal LsdB, while no variation is observed for the NEAT2 subdomain required for heme binding. (B) The Q162-S170 subdomain of NEAT1 (cyan) is proximal to helices containing T8 and N78 of α -globin (red). (C) The NEAT1 subdomain of *S. aureus* is required for high affinity human hemoglobin binding. Plasmid encoded LsdB was mutagenized and the impact of variation at the terminal residues of the NEAT1 subdomain was assessed by hemoglobin binding. Graphed are the means of three independent experiments with 3 biological replicates $\pm$ SEM, ** $p < 0.005$, *** $p < 0.0005$ by two-way ANOVA with Sidak correction for multiple comparisons.

DISCUSSION

In this work we report that recurrent positive selection acting on primate α - and β -globin proteins restricts hemoglobin binding and nutrient acquisition by pathogenic *S. aureus*. Estimations of divergence in the *Staphylococcus* genus have been lacking, however the Kloos hypothesis (39) contends that staphylococci have coevolved with their mammalian hosts over long evolutionary timescales. In support of this concept, primate-specificity among staphylococci has been reported including *S. aureus*, *S. epidermidis*, and *S. warneri*, as well as avian (*S. gallinarum*), equine (*S. equorum*), and others. Indeed, it has been proposed that the canine-associated *S. pseudointermedius* diverged from *S. aureus* simultaneously with the divergence of Primate and Carnivora orders (40). Most staphylococci are commensal organisms, while *S. aureus* is uniquely adapted to infect deep tissue and cause disease. As such, the LsdB system is only encoded by *S. aureus* and closely related primate-associated staphylococci. By narrowing our analysis of hemoglobin evolution to primates, we are thus able to assess specific biological features of primate-associated staphylococci.

An outstanding question in the study of *S. aureus* evolution has been determining the selective pressures responsible for human-specific virulence factors. *S. aureus* asymptomatically colonizes the anterior nares of approximately one third of the human population, yet is capable of causing a wide range of invasive diseases. While some bacterial colonization factors have been implicated in pathogenesis, many virulence factors have evolved highly specific targets that are not obviously involved in nasal colonization (41). Additionally, transmission has been proposed to be a selective pressure that enhanced

265 the virulence of *S. aureus* relative to another human skin commensal, *S. epidermidis* (42). As such, we
 266 cannot definitively conclude that LsdB evolution has been driven by selection during invasive disease. It
 267 is also likely that variation across LsdB of *S. aureus*, *S. argenteus*, and *S. schweitzeri* may be the results
 268 of antigenic variation to evade the immune system. By focusing on the hemoglobin binding pocket of
 269 LsdB, we have been able to pinpoint critical variation for hemoglobin specificity.

270 Our phylogenetic analyses revealed strikingly parallel signatures of positive selection between
 271 the α - and β -globin genes across primates. In particular, rapidly evolving sites in the α - and β -globin A-
 272 helices are predicted to be homologous based on predicted protein alignments. Our results suggest that
 273 these correlations reflect selection in response to NEAT domain-containing bacterial receptors like LsdB
 274 with conserved globin binding sites. A well-established body of literature has shown that other selective
 275 pressures play an important role in patterns of hemoglobin polymorphism and divergence across
 276 vertebrates, including adaptation to high altitude and malaria resistance (34, 38). It is therefore possible
 277 that signatures of selection detected in our study have been driven by pressures other than nutritional
 278 immunity. Nonetheless, our empirical results demonstrate that variation in hemoglobins at discrete sites
 279 has important functional consequences for bacterial iron acquisition.

280 Previous studies have illustrated how mutations in hemoglobin coding or regulatory regions can
 281 have highly deleterious effects on heme binding, oxygen affinity, and protein stability (43, 44). In addition
 282 to aforementioned sickle-cell alleles, dozens of hemoglobin mutations in humans have been reported
 283 that contribute to genetic disease, including anemia and thalassemia (45). Thus, despite identifying
 284 particular sites that are highly divergent among primates, much of the globin gene content is constrained
 285 due to purifying selection. In future work it would be useful to determine how variation among primate
 286 globins impacts other endogenous biochemical functions, such as heme binding and oxygen affinity.
 287 Such insights could improve our fundamental understanding of hemoglobin biology and the mechanisms
 288 underlying human hemoglobinopathies.

In conclusion, this work illustrates how rapid, site-specific hemoglobin variation restricts heme acquisition by the prominent human pathogen *S. aureus*. These findings provide a fundamental new perspective on vertebrate globin evolution, highlighting nutritional immunity as a selective pressure that could strongly impact divergence and natural selection. Future studies will assist in illuminating how these combinations of adaptive mutations contribute to hemoglobin function and host physiology. Understanding the genetic and molecular determinants of bacterial pathogenicity is critical for developing new antimicrobial treatment strategies, particularly as major pathogens like *S. aureus* continue to develop resistance to existing antibiotics. Combining comparative genetics with molecular experimentation in turn provides not only a historical perspective of host-microbe evolutionary conflict but also mechanistic insights on modern human infectious disease.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.E.C, E.P.S, M.F.B; Investigation, J.E.C., H.B.B., A.J.F, M.F.B; Writing-original draft, J.E.C, E.P.S, M.F.B.; Writing-reviewing and editing, J.E.C., H.B.B., A.J.F., E.P.S., M.F.B; Visualization, J.E.C, M.F.B; Supervision, E.P.S., M.F.B., Funding acquisition, E.P.S., M.F.B.

COMPETING INTERESTS

The authors declare no competing interests.

MATERIALS AND METHODS

Bacterial strains

311 For *E. coli* strains, LB agar and broth (Fisher, Hampton, NH) were routinely used and grown at
 312 37°C. For selection of pHUG21, 12.5 µg/ml of carbenicillin (Fisher) was used; for selection of pHb0.0, 5
 313 µg/ml of tetracycline hydrochloride (Alfa Aesar, Haverhill, MA) was used, and for selection of pOS1 P_{lgt},
 314 50 µg/ml of carbenicillin was used. *Staphylococcus* strains were grown at 37°C using tryptic soy agar
 315 and broth (Fisher), except when noted throughout. For selection of pOS1 P_{lgt} 10 µg/ml chloramphenicol
 316 (Fisher) was used. Strains were streaked to agar from stocks stored at -80°C two days prior to each
 317 experiment.

318 ***Hemoglobin cloning and genetic manipulation***

319 We compiled a subset of α- and β-globin sequences from GenBank, as well as cloned α-globin
 320 orthologs from cDNA derived from primate cell lines and β-globin orthologs from primate genomic DNA.
 321 Species obtained from GenBank: olive baboon, bonobo, white-headed capuchin, chimpanzee, Angolan
 322 colobus, northern white-cheeked gibbon, green monkey, human, crab-eating macaque, common
 323 marmoset, Sumatran orangutan, rhesus macaque, golden snub-nosed monkey, and squirrel monkey.
 324 The α-globin orthologs cloned from cDNA: African green monkey, black-and-white colobus, white-handed
 325 gibbon, Western lowland gorilla, Francois' leaf monkey, black crested mangabey, white-faced marmoset,
 326 Nancy Ma's night monkey, patas monkey, proboscis monkey, Allen's swamp monkey, talapoin, and
 327 Wolf's guenon. The β-globin orthologs cloned from genomic DNA: crested macaque, drill, Bolivian red
 328 howler monkey, pigtailed macaque, black-crested mangabey, Nancy Ma's night monkey, patas monkey,
 329 white-faced saki, island siamang, black snub-nosed monkey, Allen's swamp monkey, talapoin, Spix's
 330 saddle-back tamarin, dusky titi, Wolf's guenon, and common woolly monkey. Primate cell lines were
 331 purchased from the Coriell Institute for Medical Research (Camden, NJ).

332 Primate hemoglobin cDNA was cloned into pHb0.0 using Gibson assembly (New England Biolabs
 333 [NEB], Ipswich, MA). In general, each α- and β-globin gene cDNA was amplified from template (above)
 334 using Phusion 2X Master Mix (Thermo, Waltham, MA) with primers that also had homology to pHb0.0.

335 Because of cDNA sequence homology, some primers were used for multiple species. pHb0.0-human
 336 was digested with *PacI* (NEB) and *HindIII*-HF (NEB) and the double-digested vector was isolated by gel
 337 purification (Qiagen, Germantown, MD). PCR products were assembled with digested pHb0.0,
 338 transformed to DH5 α , re-isolated by mini-prep (Thermo) and were confirmed by sequencing (GeneWiz,
 339 South Plainfield, NJ) with pHb0.0_for/pHb0.0_rev. Globin cDNA was amplified for assembly as follows:
 340 white-cheeked gibbon α -globin-primers AF327/328, white-cheeked gibbon β -globin-primers AF329/330,
 341 baboon α -globin-primers AF331/332, baboon β -globin-primers AF329/330, talapoin α -globin-primers
 342 AF327/328, talapoin β -globin-primers AF329/330, marmoset α -globin-primers AF333/334, and marmoset
 343 β -globin-primers AF329/335.

344 Chimeric hemoglobins were prepared by subcloning the α -globins. To enable digestion with *XbaI*
 345 (NEB) (which is sensitive to *dam* methylation) pHb0.0-human and pHb0.0-baboon were transformed and
 346 re-isolated from *E. coli* K1077 (*dam*⁻ *dcm*⁻). The α -globin from each plasmid was excised by digestion
 347 with *XbaI* (NEB) and *PacI* (NEB) and the α -globin and double-digested pHb0.0 containing β -globin were
 348 separately isolated by gel purification (Qiagen). Human α -globin was ligated into pHb0.0 containing
 349 baboon β -globin and baboon α -globin was ligated (T4 ligase; NEB) into pHb0.0 containing human β -
 350 globin. Chimeras were confirmed by sequencing (GeneWiz) with pHb0.0_for/pHb0.0_rev.

351 pHb0.0-human was mutagenized using QuikChange Site-Directed Mutagenesis Kit (Agilent,
 352 Santa Clara, CA) to create changes in the α -gene: T8K (primers AF289/290), T8K;N9H (primers
 353 JC112/113; using pHb0.0-human α T8K as template), and A5D;T8K;N9H (primers JC114/115; using
 354 pHb0.0-human α T8K;N9H as template). Changes in the human β -gene were as follows: S9N (primers
 355 AF303/304), S9A (primers AF309/310) A76N (primers AF313/314), and A76T (primers AF311/312).
 356 Residues in pHb0.0-baboon α -gene were mutagenized: K8T (primers JC194/195), K8T;H9N
 357 simultaneously (primers JC116/117), and D5A (primers JC118/119; using pHb0.0-baboon α K8T;H9N as
 358 template).

359 **Phylogenetic analyses**

360 Hemoglobin DNA sequence alignments were performed using MUSCLE. Input phylogenies were
 361 based upon supported species relationships (46) as well as maximum-likelihood gene phylogenies
 362 generated using PhyML with SPR topology search and 1000 bootstraps for branch support (47) .
 363 Substitution models were selected based on the ProtTest algorithm (48). Tests for positive selection were
 364 performed using codeml from the PAML software package with the F3X4 codon frequency model.
 365 Likelihood ratio tests (LRTs) were performed by comparing pairs of site-specific models (NS sites): M1
 366 (neutral) with M2 (selection), M7 (neutral, beta distribution of $dN/dS < 1$) with M8 (selection, beta
 367 distribution, $dN/dS > 1$ allowed). Additional tests which also account for synonymous rate variation and
 368 recombination, including FUBAR, FEL, and MEME, were performed using the HyPhy software package
 369 via the Datamonkey server (49, 50). Sites under positive selection were mapped onto three-dimensional
 370 molecular structures using Chimera (51) (<http://www.cgl.ucsf.edu/chimera/>).

371 The staphylococcal DNA gyrase gene tree was generated using PhyML with 1000 bootstraps as
 372 above. *M. caseolyticus* DNA gyrase was included as an outgroup. The similarity of IsdB in *S. argenteus*
 373 and *S. schweitzeri* relative to *S. aureus* is shown at right.

374 **Recombinant purification of hemoglobin**

375 Hemoglobin expression strains [BL21(DE3) pHUG21 pHb0.0] were streaked to LB agar
 376 containing 12.5 µg/ml carbenicillin and 5 µg/ml tetracycline hydrochloride. Single colonies were
 377 inoculated into 5 ml of LB broth supplemented with 12.5 ug/ml carbenicillin and 5 ug/ml tetracycline
 378 hydrochloride and grown for 14 h at 37°C with shaking. This culture was used to inoculated 1:500 into
 379 1.5 L of LB with 12.5 ug/ml carbenicillin, 5 ug/ml tetracycline hydrochloride, 100 µM hemin (prepared
 380 fresh at 10 mM in 0.1 M NaOH; Sigma St. Louis, MO), and 50 µg/ml of the iron chelator ethylenediamine-
 381 di(o-hydroxyphenylacetic acid (EDDHA [LGC Standards, Teddington, UK; solid added directly to
 382 medium) in a 2.8 L Fernbach flask. Cultures were grown at 37°C until OD₆₀₀ reached 0.6-0.8. The

expression of hemoglobin was induced with 40 µg/ml IPTG (RPI, Mount Prospect, IL). After 16 h post-induction, cells were collected by centrifugation. The cell pellet was resuspended in PBS containing 10 mM imidazole (Fisher), 5 mM MgCl₂ (Sigma), 1 Roche Protease inhibitor table (Fisher), and a few mg of lysozyme (Thermo) and deoxyribonuclease from bovine pancreas (Sigma). The cell pellet resuspended with rocking for 20 min following incubation on ice. Cells were lysed using an Emulsiflex (Avestin, Ottawa, CA) then cell lysate was clarified by ultracentrifugation (60 min at 17,000 g). Cell lysate was applied to a Ni-NTA resin (Qiagen), to which hemoglobin binds, washed with PBS with 10 mM imidazole. Hemoglobin was eluted with PBS with 500 mM imidazole. The hemoglobin-containing eluate was dialyzed twice sequentially in PBS at 4°C. Purified hemoglobin was filter sterilized with a 0.45 micron filter and stored in aliquots in liquid nitrogen. Hemoglobin concentration was measured with Drabkin's reagent (Sigma) using human hemoglobin as a standard.

Whole cell hemoglobin binding assay

S. aureus strains were streaked on tryptic soy agar (containing 10 µg/ml chloramphenicol for strains carrying plasmids) and grown at 37°C for 24 h. Single colonies were used to inoculate 3 ml of RPMI containing 1% cas-amino acids and 0.5 mM 2,2'-dipyridyl (Acros/Fisher) to induce expression of chromosomal *isdB* or 10 µg/ml chloramphenicol (for strains carrying plasmids with constitutive *isdB* expression). After 14-16 h of growth at 37°C with shaking, 2 OD₆₀₀ units were collected by centrifugation in a 1.5 ml Eppendorf tube. The cell pellet was resuspended with 1 ml PBS or PBS containing recombinant hemoglobin. 10 µg/ml (chromosomal *IsdB*) or 2.5 µg/ml (plasmid-borne *IsdB*) of hemoglobin was used. The cells were incubated with hemoglobin or PBS for 30 min at 37°C with shaking, then cells were collected by centrifugation at 4°C. Cells were washed thrice with 1 ml ice-cold PBS, centrifuging at 4°C. After the final wash, the cells were resuspended in 30 µl 0.5 M Tris pH 8.0 (Fisher) containing 4% SDS (Fisher) and heated at 90°C for 5 min to remove surface bound proteins. Cells were collected by centrifugation, and eluate was added to 6X loading buffer and heated at 90°C for 5 min. Samples were subjected to 12 or 17.5% SDS-PAGE and silver stained (GE, Boston, MA). Quantification was performed

by densitometric analysis with Image J (NIH). Because of variation in stain intensity across gels, all comparisons were made within the same gel, and relative density was calculated for each biological replicate within the same gel; the comparison was either to human hemoglobin or wildtype IsdB, depending on assay. Additionally, PBS only samples and *S. aureus* $\Delta isdB::erm$ were used to verify that hemoglobin binding in this assay is IsdB dependent as previously observed [Supplemental Figure 1] (11, 13).

Growth with hemoglobin as sole iron source

S. aureus Newman WT was streaked to tryptic soy agar and allowed to grow for 24 h at 37°C. A few colonies were used to inoculate 5 ml of RPMI (Corning, Corning, NY) supplemented with 1% cas-amino acids (Fisher) and 0.5 mM of EDDHA (prepared fresh in ethanol). After growth to stationary phase at 37°C with shaking, approximately 16 h, 4 μ l of culture was inoculated into 196 μ l of medium in a 96 well plate and OD₆₀₀ at 37°C with shaking was monitored over time using a BioTek plate reader. Medium was RPMI containing 1% cas-amino acids that had been stripped of cations with Chelex 100 (Sigma) according to manufacturer's instructions, filter sterilized, and supplemented with 25 μ M ZnCl₂, 25 μ M MnCl₂, 100 μ M CaCl₂, and 1 mM MgCl₂ (all from Fisher) to restore non-iron cations, 1.5 mM EDDHA to chelate any remaining free iron, and 2.5 μ g/ml of recombinant purified hemoglobin as the sole iron source. Growth using hemoglobin as a sole iron source is IsdB dependent (11, 13, 22).

Cloning of isdB

The full length coding sequences of IsdB were amplified from genomic DNA using Phusion 2X High-Fidelity Master Mix (Thermo); cells were treated with 20 μ g of lysostaphin (AMBI Products, Lawrence, NY) and DNA was isolated with Wizard genomic DNA extraction kit (Promega, Madison, WI). *S. aureus isdB* (NWMN_1040) was amplified using primers JC343/344, *S. schweitzeri isdB* (ERS140239_01018) using primers JC218/219, and *S. argenteus isdB* (SAMSHR1132_09750) using primers JC216/217. Each primer pair included homology to pOS1 P_{Igt} digested with NdeI and BamHI-HF

(NEB), and PCR products were ligated to pOS1 P_{Igt} with Hi-Fi assembly (NEB), transformed into *E. coli* DH5 α and re-isolated by miniprep (Thermo). All plasmids were sequence confirmed by Sanger sequencing (Genewiz). Plasmids were transformed into RN4220 by electroporation, re-isolated, and transformed into *S. aureus* Newman $\Delta isdB::erm$ by electroporation.

***S. aureus* *IsdB* site-directed mutagenesis**

pOS1 $P_{Igt}isdB_{aureus}$ was subjected to site-directed mutagenesis by PCR with Q5 Site Directed Mutagenesis (NEB). Primer pairs with desired mutation were used to create Q162R (primers JC315/316), S170T (primers 317/318) and Q162R;S170T (primers JC319/320). PCR products were transformed to DH5 α . Plasmids were isolated and subjected to Sanger sequencing with primers JC228/229 (Genewiz) to identify successful incorporation of the desired mutation. Plasmids were transformed into RN4220 by electroporation, re-isolated, and transformed into *S. aureus* Newman $\Delta isdB::erm$ by electroporation.

QUANTIFICATION AND STATISTICAL ANALYSIS

Specific statistical details for each experiment can be found in the corresponding figure legend. Data analysis and statistical tests were performed in Prism 6 (Graphpad).

Table S1. Strains and plasmids

Bacterial Strain	Source
<i>Escherichia coli</i> DH5 α	Laboratory stock, Thermo Fisher
<i>Escherichia coli</i> BL21(DE3) pHUG21	Douglas Henderson (University of Texas of the Permian Basin); (52)
<i>Staphylococcus aureus</i> RN4220	(53)
<i>S. aureus</i> Newman	Laboratory stock; (54)
<i>S. aureus</i> Newman $\Delta isdB::erm$	(7)
<i>S. argenteus</i> MSHR1132	DSMZ
<i>S. schweitzeri</i> FSA084	DSMZ
Plasmid	Source

pHb0.0-human	John Olson (Rice University); (52)
pHb0.0-white-cheeked gibbon	This paper
pHb0.0-baboon	This paper
pHb0.0-talapoin	This paper
pHb0.0-marmoset	This paper
pHb0.0- $\alpha_{\text{human}}\beta_{\text{baboon}}$	This paper
pHb0.0- $\alpha_{\text{baboon}}\beta_{\text{human}}$	This paper
pHb0.0-human α T8K	This paper
pHb0.0-human α A5D;T8K;N9H	This paper
pHb0.0-baboon α K8T	This paper
pHb0.0-baboon α D5A;K8T;H9N	This paper
pHb0.0-baboon β S9N	This paper
pHb0.0-baboon β S9A	This paper
pHb0.0-baboon β A76N	This paper
pHb0.0-baboon β A76T	This paper
pOS1 P _{Igt}	(55)
pOS1 P _{Igt} <i>isdB</i> _{aureus}	This paper
pOS1 P _{Igt} <i>isdB</i> _{schweitzeri}	This paper
pOS1 P _{Igt} <i>isdB</i> _{argenteus}	This paper
pOS1 P _{Igt} <i>isdB</i> _{aureus} Q162R	This paper
pOS1 P _{Igt} <i>isdB</i> _{aureus} S170T	This paper
pOS1 P _{Igt} <i>isdB</i> _{aureus} Q162R;S170T	This paper

447

448 **Table S2. Oligonucleotides used in this study**

Primer name	Sequence	Description
AF327	TTCAC TAAGGAGGTTAATTAATGGTGCTGTCTCCTGC C	Forward primer for white-cheek gibbon and talapoin α -globin
AF328	TCTAGATCATTACGGTATTTGGAGGTCAGC	Reverse primer for white-cheeked gibbon and talapoin α -globin
AF329	TACCGTTAATGATCTAGATAAGGAGGTAAATATATG	Forward primer for white-cheeked gibbon, baboon, talapoin, and marmoset β -globin

AF330	GAGCCTTTCGTTTTATTTAAGCTTCATTAGTGGTACTTGTGG	Reverse primer for white-cheeked gibbon, baboon, and talapoin β -globin
AF331	TTCACTAAGGAGGTTAATTAATGGTGCTGTCTCCTGAC	Forward primer for baboon α -globin
AF332	TCTAGATCATTAACGGTATTTGGAGGTCAG	Reverse primer for baboon α -globin
AF333	TTCACTAAGGAGGTTAATTAATGGTGCTGTCTCCCGC	Forward primer for marmoset α -globin
AF334	TCTAGATCATTAACGGTATTTGGAGGTCAGCAC	Reverse primer for marmoset α -globin
AF335	GAGCCTTTCGTTTTATTTAAGCTTCATTAGTGGTACTTGTGAG	Reverse primer for marmoset β -globin
AF289	CTGTCTCCGGCCGATAAAAAGAACGTTAAAGCTGCTTGG	Forward primer to create T8K in human α -globin
AF290	CCAAGCAGCTTTAACGTTCTTTTTATCGGCCGGAGACAG	Reverse primer to create T8K in human α -globin
JC112	TCTCCGGCCGATAAAAAGCACGTTAAAGCTGCTTGGGT	Forward primer to create N9H in human α -globin using pHb0.0-human α T8K as template
JC113	ACCCCAAGCAGCTTTAACGTGCTTTTTATCGGCCGGAGAGA	Reverse primer to create N9H in human α -globin pHb0.0-human α T8K as template
JC114	TAATTAATGCTGTCTCCGGACGATAAAAAGCACGTTAA	Forward primer to create A5D in human α -globin using pHb0.0-human α T8K;N9H as template
JC115	TTTAACGTGCTTTTTATCGTCCGGAGACAGCATTAAATTA	Reverse primer to create A5D in human α -globin using pHb0.0-human α T8K;N9H as template
JC194	CTGTCTCCTGACGACAAGACACACGTCAAGGCCGCCGTGGGGT	Forward primer to create K8T in baboon α -globin
JC195	ACCCCAAGGCCGCTTGACGTGTGTCTTGTCTGTCAGGAGACAG	Reverse primer to create K8T in baboon α -globin
JC116	CTGTCTCCTGACGACAAGACAAACGTCAAGGCCGCCGTGGGGT	Forward primer to create K8T;H9N simultaneously in baboon α -globin
JC117	ACCCCAAGGCCGCTTGACGTGTGTCTTGTCTGTCAGGAGACAG	Reverse primer to create K8T;H9N simultaneously in baboon α -globin
JC118	ATGGTGCTGTCTCCTGCCGACAAGACAAACGTC	Forward primer to create D5A in baboon α -globin using pHb0.0-baboon α K8T;H9N as template
JC119	GACGTTTGTCTTGTCTGGCAGGAGACAGCACCAT	Reverse primer to create D5A in baboon α -globin using pHb0.0-baboon α K8T;H9N as template α -globin
AF303	CTGACTCCGGAAGAAAAAACGCGGTTACTGCTCTG	Forward primer to create S9N in baboon β -globin
AF304	CAGAGCAGTAACCGCGTTTTTTCTTCCGGAGTCAG	Reverse primer to create S9N in baboon β -globin
AF309	CTGACTCCGGAAGAAAAAGCCGCGGTTACTGCTCTG	Forward primer to create S9A in baboon β -globin

AF310	CAGAGCAGTAACCGCGGCTTTTCTTCCGGAGTCAG	Reverse primer to create S9A in baboon β -globin
AF313	GCTTTCTCTGACGGTCTGAATCACCTGGACAACCTG	Forward primer to create A76N in baboon β -globin
AF314	CAGGTTGTCCAGGTGATTCAGACCGTCAGAGAAAGC	Reverse primer to create A76N in baboon β -globin
AF311	GCTTTCTCTGACGGTCTGACTCACCTGGACAACCTG	Forward primer to create A76T in baboon β -globin
AF312	CAGGTTGTCCAGGTGAGTCAGACCGTCAGAGAAAGC	Reverse primer to create A76T in baboon β -globin
pHb0.0_for	CGACTGGAAAGCGGGCAG	Forward sequencing primer for pHb0.0
pHb0.0_rev	GCATTGTTAGATTTTCATACAG	Reverse sequencing primer for pHb0.0
JC343	AAATACAATTGAGGTGAACATATGATGAACAAACAGC AAAAAG	Forward primer to clone <i>S. aureus isdB</i> into pOS1 P_{Igt} by Hi-fi assembly
JC344	AAACACTACCCCCTTGTGGATCCTTAGTTTTTACGT TTTCTAGGTAATAC	Reverse primer to clone <i>S. aureus isdB</i> into pOS1 P_{Igt} by Hi-fi assembly
JC218	AAATACAATTGAGGTGAACATATGATGAACAAACAGC AAAAAG	Forward primer to clone <i>S. schweitzeri isdB</i> into pOS1 P_{Igt} by Hi-fi assembly
JC219	AAACACTACCCCCTTGTGGATCCTTAGTTTTTACGT TTTCTAGGTAATAC	Reverse primer to clone <i>S. schweitzeri isdB</i> into pOS1 P_{Igt} by Hi-fi assembly
JC216	AAATACAATTGAGGTGAACATATGATGAACAAACAGC AAAAAG	Forward primer to clone <i>S. argenteus isdB</i> into pOS1 P_{Igt} by Hi-fi assembly
JC217	AAACACTACCCCCTTGTGGATCCTTAGTTTTTACGT TTTCGAGG	Reverse primer to clone <i>S. argenteus isdB</i> into pOS1 P_{Igt} by Hi-fi assembly
JC317	TTATGCAAGTACTGTAAACCTG	Forward primer, creates S170T in pOS1 $P_{Igt}isdB_{aureus}$
JC318	TGATAAACTGTTGAGTTCC	Reverse primer, creates S170T in pOS1 $P_{Igt}isdB_{aureus}$
JC315	AGATGGAAGTACAGTTTTATCATTATG	Forward primer, creates Q162R in pOS1 $P_{Igt}isdB_{aureus}$
JC316	TTCTTTTTTCATTTCAAAATCAATTG	Reverse primer, creates Q162R in pOS1 $P_{Igt}isdB_{aureus}$
JC319	TTATGCAAGTACTGTAAACCTGCTAGAGTTATTTTC	Forward primer, simultaneously creates Q162R;S170T in pOS1 $P_{Igt}isdB_{aureus}$
JC320	TGATAAACTGTCTAGTTCCATCTTTCTTTTTCATTTTC	Reverse primer, simultaneously creates Q162R; S170T in pOS1 $P_{Igt}isdB_{aureus}$
JC228	TAAGAAGAGATGTAAGAGTAGGG	pOS1 P_{Igt} forward sequencing primer
JC229	GGGGGAAACACTACCCCCTTG	pOS1 P_{Igt} reverse sequencing primer

450 SUPPLEMENTAL INFORMATION

451

452 **Supplemental Figure 1. The whole cell hemoglobin (Hb) binding assay allows for IsdB-dependent,**
453 **species-specific quantification of bound recombinant Hb.** A representative silver-stained SDS-
454 PAGE gel used to assess binding of hemoglobin to the surface of *S. aureus*. 100 ng of purified
455 recombinant Hb was loaded (lane 2) to demonstrate apparent molecular weight in this gel. The apparent
456 hemoglobin band is specific to Hb, as it is not visible when *S. aureus* is incubated with PBS alone (lane
457 3). *S. aureus* binds human hemoglobin purified recombinantly from *E. coli* (lane 4) as well as Hb purified
458 from blood (lane 6). Binding of Hb purified from mouse blood (lane 7) is significantly diminished, as first
459 reported in (13) Binding of Hb is dependent on IsdB, as demonstrated in lanes 8,10,11. An approximately
460 equivalent amount of *S. aureus* was used, as demonstrated by equal loading of non-Hb specific bands
461 across the top of the gel. Samples in lanes 5 and 9 are not discussed here.

462

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