Iron-Source Preference of *Staphylococcus aureus* Infections

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Although bacteria use different iron compounds in vitro, the possibility that microbes distinguish between these iron sources during infection has hitherto not been examined. We applied stable isotope labeling to detect source-specific iron by mass spectrometry and show that *Staphylococcus aureus* preferentially imports heme iron over transferrin iron. By combining this approach with computational genome analysis, we identified *hts* (heme transport system), a gene cluster that promotes preferred heme iron import by *S. aureus*. Heme iron scavenging by means of *hts* is required for staphylococcal pathogenesis in animal hosts, indicating that heme iron is the preferred iron source during the initiation of infection.

Bacterial nutrient-uptake assays traditionally provide a sole nutrient source and rely on measurements of microbial growth as indicators of usage (*1*). This experimental strategy imposes pressure on bacterial populations to acquire nutrients for survival and does not provide information regarding the preferred nutrient sources of microbes. We developed an assay that provides multiple stable isotope–labeled nutrient compounds and allows independent tracking of each compound by its isotope label. The assay provides a growth medium containing elemental nutrients in which each compound is prepared from a different separated stable isotope tracer, which is then tracked through metabolic pathways by isotopic analysis of cultured cells or cellular fractionations by inductively coupled plasma mass spectrometry (ICP-MS). We applied this technique to iron (Fe) acquisition in *S. aureus*.

The ability to sequester iron is a primary defense mechanism against bacterial infection. In response, during human infections, bacterial uptake systems specific for host iron sources such as heme Fe and transferrin Fe combine to obtain this nutrient (*2*, *3*). These systems can take the form of receptors specific for heme Fe or transferrin Fe, or secreted siderophores that are capable of removing iron from human transferrin (*2*, *3*). Transferrin Fe accounts for less than 1% of the body's total iron, whereas heme Fe can represent greater than 80% (*4*), leading us to speculate that the abundance of heme Fe in humans may select for bacterial pathogens that preferentially acquire iron from this source. We used isotope-labeled nutrients to track simultaneous uptake of heme Fe and transferrin Fe and to determine whether *S. aureus* has a preferred iron source. Natural terrestrial iron is composed of four stable isotopes present in fixed relative proportions, 5.85% [^{54}Fe], 91.75% [^{56}Fe], 2.12% [^{57}Fe], and 0.28% [^{58}Fe], and mass-dependent fractionations of iron by microbial biochemical and inorganic redox processes have been studied and are much less than 1% (*5*–*7*). Thus, tracer compounds made up exclusively of the minor isotopes of iron can be followed in biological systems by monitoring changes in cellular iron isotope ratios by ICP-MS. For analysis, we obtained hemin and transferrin samples consisting almost exclusively of ^{54}Fe and ^{57}Fe, respectively (*8*). Isotopic labeling did not affect the ability of *S. aureus* to use these compounds as iron sources for growth (*9*).

To determine whether *S. aureus* exhibits source-dependent iron preference, we first serially passaged bacteria in low-iron medium until a decrease in growth rate indicative of iron starvation was observed (*10*). Iron-starved bacteria were subcultured into chemically defined medium and supplemented with equimolar amounts of [^{54}Fe] hemin and [^{57}Fe] transferrin, the exact ratio of which was confirmed by ICP-MS (*11*) (Fig. 1A). Natural Fe was present as a ubiquitous contaminant in all experiments, therefore all measured ratios reflect a combination of natural Fe (dominated by ^{56}Fe, 91.75%) and isotopically labeled Fe. Analysis of bacteria removed throughout the growth of the culture revealed as much as fourfold to fivefold enrichment in the ratio of heme Fe to transferrin Fe as compared

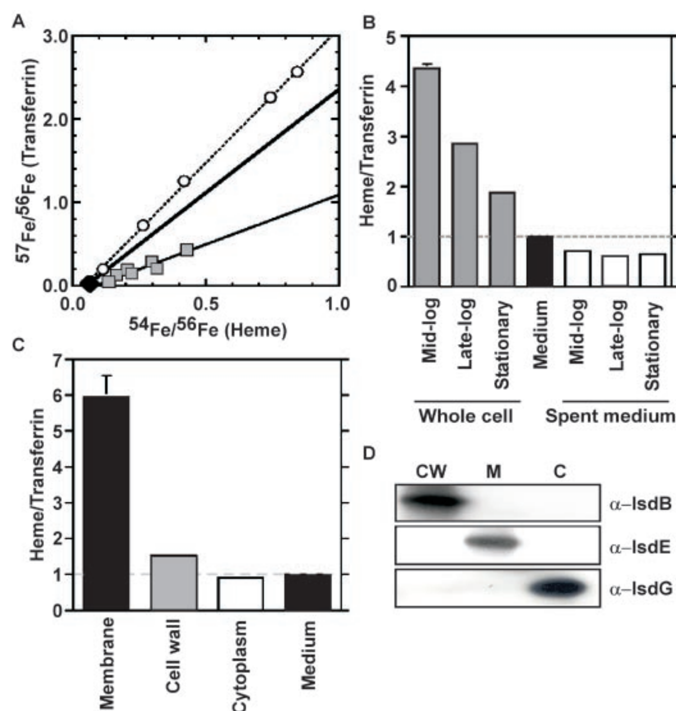

Fig. 1. Growth phase–dependent preference for heme Fe uptake in *S. aureus*. (**A**) ICP-MS measurement of iron isotope ratios in *S. aureus* cultures. The *x* and *y* axes indicate the measured ^{54}Fe/^{56}Fe (heme preference) and ^{57}Fe/^{56}Fe (transferrin preference) ratios, respectively. The natural isotopic composition of iron is shown by the black diamond. The heavy line indicates the ratio of iron isotopes present in the growth medium supplemented with [^{54}Fe] hemin and [^{57}Fe] transferrin. The squares represent bacterial samples and the circles represent spent medium samples. Each point represents an individual sample taken at different time points during three separate experiments. (**B**) Time course of iron uptake by *S. aureus*. Isotopic composition presented as ratios of heme Fe:transferrin Fe calculated from the ^{54}Fe/^{56}Fe and ^{57}Fe/^{56}Fe ratios measured by ICP-MS by correcting for the contributions to both ^{54}Fe and ^{57}Fe from natural iron, and accounting for the presence of ^{56}Fe in both tracers. Bacterial samples were taken at mid-log (9 hours), late-log (12 hours), and stationary (24 hours) phase. (**C**) Measurement of Fe isotope ratios in subcellular fractions (cytoplasm, cell wall, and membranes) from late-log cultures (12 hours) presented as the ratio of heme Fe to transferrin Fe. (**D**) Immunoblots of IsdB, IsdE, and IsdG as a fractionation control (*19*). CW, cell wall; M, membrane; C, cytoplasm. Error bars show mean + SE for all measurements but are usually too small to be seen.

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with that from the nutrient medium (Fig. 1, A and B). The heme Fe preference was further reflected in measurable heme Fe depletion from the spent media in the growth culture (Fig. 1, A and B). The intracellular ratio of heme Fe to transferrin Fe decreased as bacterial cells progressed through the growth of the culture (Fig. 1B), suggesting growth phase–dependent changes in heme Fe usage. Although bacterial removal of [^{54}Fe] hemin from the medium is clearly evident, there is little difference between the isotopic composition of the spent media from mid-log, late-log, and stationary phases. Thus, bacterial removal of [^{54}Fe] hemin from the medium is not responsible for the observed growth phase–dependent decrease in heme Fe preference. Together, these results not only demonstrate that *S. aureus* preferentially uses [^{54}Fe] hemin over [^{57}Fe] transferrin but are consistent with a growth phase–dependent up-regulation of transferrin Fe or siderophore Fe acquisition systems.

To determine the subcellular localization pattern of heme Fe and transferrin Fe, we removed late-log–phase bacterial cells from isotopically labeled medium. The bacterial cells were fractionated into three subcellular compartments (membrane, cell wall, and cytoplasm), and the abundance and isotopic ratio of iron in each compartment was determined by ICP-MS. The data presented in Fig. 1C show that the iron isotope ratios varied across subcellular fractions, suggesting that iron partitions differentially inside the bacterial cell. The magnitude of the observed variations in the Fe isotope ratio of the subcellular fractions is considerably greater than natural fractionations (*5–7*), requiring an explanation entirely in terms of the source of iron used. The ratio of heme Fe to transferrin Fe in the staphylococcal cytoplasm was less than that of the growth medium. This suggests that when heme is present, transferrin is primarily sorted to the cytoplasm, presumably for iron storage or use in cytoplasmic metalloproteins. The ratio of heme Fe to transferrin Fe in the cytoplasmic membrane was fivefold enriched relative to the culture medium, suggesting that iron obtained from heme may be preferentially sorted to this compartment. This degree of fractionation on the basis of isotope mass alone may be due to either the sorting of intact tetrapyrrol to the membrane or the selective membrane segregation of iron that has been removed from exogenously acquired heme. On the basis of the observation that the gram-positive cytoplasmic membrane is the primary site of heme-binding proteins such as cytochromes (*12*), it seems plausible that iron-containing tetrapyrrol may be sorted intact to the membrane and assembled as a cofactor for redox active proteins.

The assay provides a useful method to identify genes responsible for nutrient preferences by measuring changes in the intracellular isotopic ratio that occur upon gene inactivation. Analysis of the complete *S. aureus* genome identifies seven putative membrane transport systems possessing homology to known adenosine triphosphate–binding cassette (ABC)–type iron transporters, four of which have been previously studied (Fig. 2A). Staphylococcal siderophore transporter (SstABCD) (*13*), staphylococcal iron regulated (SirABC) (*14*), and ferric hydroxamate uptake (FhuCBG) (*15*) are thought to be involved in siderophore Fe transport, whereas iron-regulated surface determinants (IsdDEF) is involved in heme Fe transport from scavenged hemoglobin (*16*). Genetic inactivation of *isdDEF* does not inhibit growth on heme Fe as a sole iron source (*16*), implying that the primary heme Fe membrane transporter has yet to be identified. To address the role of these transport systems in heme Fe and transferrin Fe usage, each predicted iron transport system was inactivated through insertional mutagenesis. The mutagenesis strategy was designed so as to abrogate the expression of at least one gene in each transport system. Inactivation of *isdDEF*, *sirABC*, *fhuCBG*, *sav0609-10*, or *sav1554-1557* did not alter the heme preference of *S. aureus*. Inactivation of *sstABCD* resulted in a strain exhibiting a similar iron preference to that of the wild type at 9 hours (Fig. 2B). However, samples taken at 12 hours revealed an increase in heme preference (Fig. 2C). This observation is consistent with the role of SstABCD in siderophore Fe acquisition and supports the results presented in Fig. 1, suggesting temporal regulation of iron acquisition systems.

An alternate set of mutations in a previously uncharacterized transport system, which we have named *htsABC* (heme transport system), exhibited a unique isotopic profile. Inactivation of either *htsB* or *htsC* resulted in an increased ratio of transferrin Fe to heme Fe as compared with that of the wild type at various time points throughout the growth curve (Fig. 2, B and C), demonstrating that inactivation of HtsABC significantly reduces staphylococcal heme Fe acquisition. Sequence analysis showed that HtsB and HtsC are ABC transporter permeases representing the closest homologs in the *S. aureus* genome to HemU (*17*) and HmuU (*18*), the heme transport system permeases of *Yersinia enterocolitica* and *Corynebacterium diphtheriae*, respectively. A nucleotide sequence closely resembling the canonical staphylococcal Fur box (*19*), the site to which the iron-dependent repressor Fur binds (*20*), is located eight nucleotides upstream of the predicted start codon for HtsA (*9*), implying that the Hts system is activated under low-iron conditions. These observations suggest that the application of stable isotope tracking to strains mutant in predicted nutrient transporters successfully identified a previously unrecognized iron-regulated heme-transport system responsible for the heme Fe preference of *S. aureus*.

The strong preference for heme Fe exhibited by *S. aureus* implies a role for heme

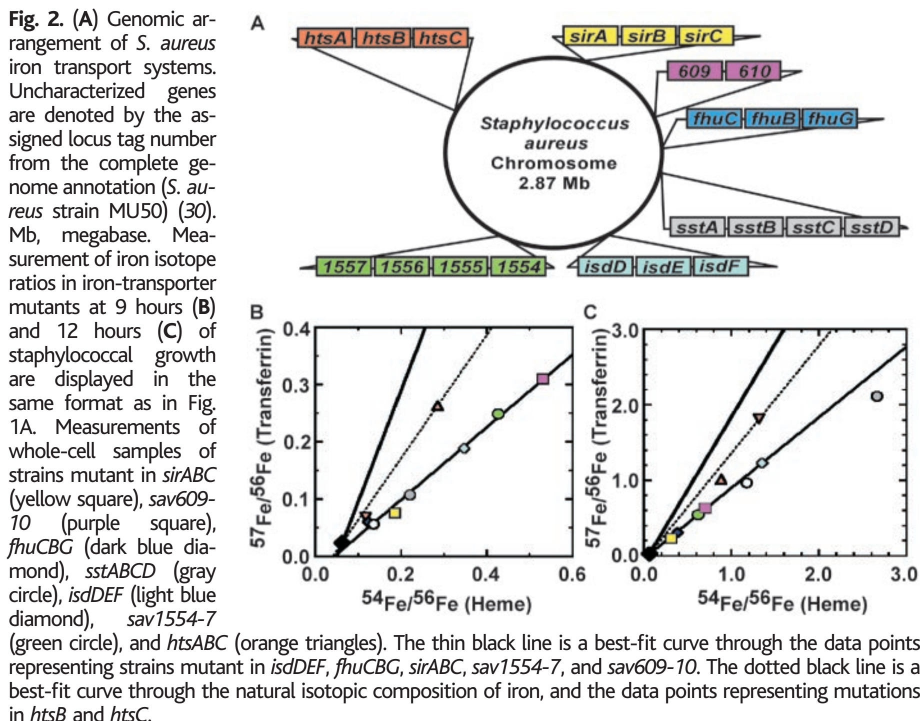

Fig. 2. (**A**) Genomic arrangement of *S. aureus* iron transport systems. Uncharacterized genes are denoted by the assigned locus tag number from the complete genome annotation (*S. aureus* strain MU50) (*30*). Mb, megabase. Measurement of iron isotope ratios in iron-transporter mutants at 9 hours (**B**) and 12 hours (**C**) of staphylococcal growth are displayed in the same format as in Fig. 1A. Measurements of whole-cell samples of strains mutant in *sirABC* (yellow square), *sav609-10* (purple square), *fhuCBG* (dark blue diamond), *sstABCD* (gray circle), *isdDEF* (light blue diamond), *sav1554-7* (green circle), and *htsABC* (orange triangles). The thin black line is a best-fit curve through the data points representing strains mutant in *isdDEF*, *fhuCBG*, *sirABC*, *sav1554-7*, and *sav609-10*. The dotted black line is a best-fit curve through the natural isotopic composition of iron, and the data points representing mutations in *htsB* and *htsC*.

Fe scavenging during infection. *S. aureus* infection of *Caenorhabditis elegans*, a nematode that uses heme proteins for oxygen transport (*21*), leads to intestinal multiplication of invading microbes, distension of the worm digestive tract, and staphylococcal killing of animals within 2 to 4 days (*22*, *23*) (Fig. 3A). Infection of *C. elegans* with *S. aureus* variants lacking either the *htsB* or the *htsC* gene caused a significant reduction in the ability of staphylococci to kill infected worms (Fig. 3A).

S. aureus abscess formation in various organ tissues of intravenously infected mice more closely resembles human infections (*24*, *25*). Twenty-four hours after intravenous infection, mice inoculated with *S. aureus* strains inactivated for *htsB* or *htsC* did not exhibit any of the overt signs of disease associated with wild-type infection (*9*). Examination of the organs of mice 96 hours postinfection demonstrated abscess formation in virtually all kidneys from mice infected with wild-type *S. aureus* (Fig. 3B), whereas abscesses were not detected in mice infected with *htsB* and *htsC* mutant mice (Fig. 3C). Additionally, there was a large reduction in the number of *htsB* and *htsC* mutant staphylococci in the kidneys and livers of infected mice as compared with that of the wild type (Fig. 3, D and E). The liver is the primary iron storage organ of mammals and the site of heme recycling through circulating hemehemopexin and hemoglobin-haptoglobin complexes (*26*). The severe defects in liver abscess formation of *S. aureus* $\Delta(htsB)$ or $\Delta(htsC)$ strains identify the acquisition of heme Fe at the site of heme recycling as a previously unknown pathogenic strategy of staphylococci. During infection, the *S. aureus* Hts system likely acquires additional heme Fe through hemolysin-mediated erythrocyte destruction and the subsequent release of hemoglobin (*27*). Previous work has revealed that *S. aureus* strains mutant in a siderophore synthesis operon display normal infection kinetics until 6 days after infection, at which time a substantial decrease in animal-associated bacterial counts is observed (*28*). Thus, early during infection staphylococci appear to satisfy their iron requirements through the most abundant iron source, hemoproteins. Upon depletion of hemoproteins, or once bacteria have occupied niches in the host environment that are devoid of hemoproteins, siderophores assume a vital function for the acquisition of iron from nonheme sources.

The inhibition of bacterial iron uptake has long been considered a promising area of research toward the creation of novel therapeutic options (*29*). Identifying the nutrient sources preferentially utilized during infection allows us to refine drug design strategies against the primary acquisition systems of bacteria.

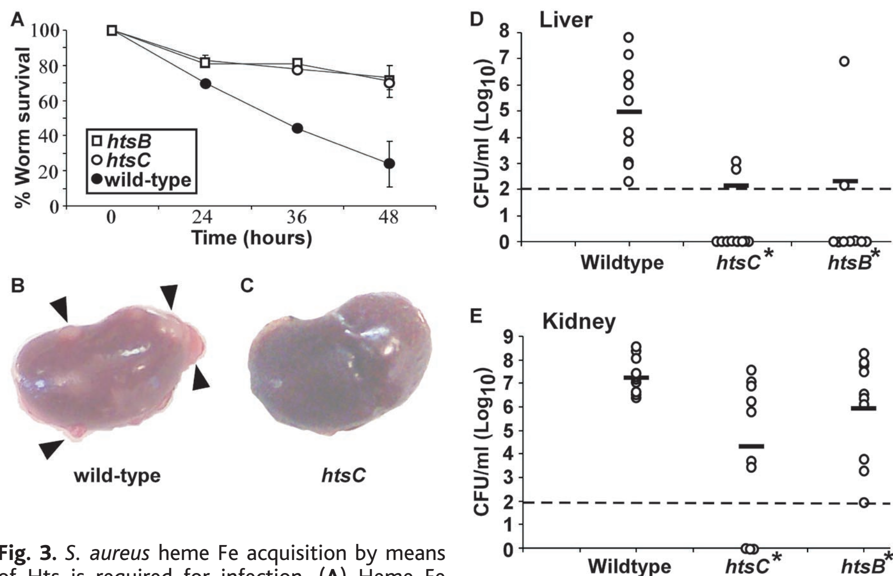

Fig. 3. *S. aureus* heme Fe acquisition by means of Hts is required for infection. (**A**) Heme Fe acquisition through the Hts system is required for *C. elegans* intestinal infection and *S. aureus* Newman (wild-type)–mediated host killing within 48 hours. Error bars show mean ± SD. Photographs of kidneys from Balb/c mice infected with wild-type (**B**) or isogenic *htsC* mutant *S. aureus* (**C**). Arrowheads mark staphylococcal abscesses. *S. aureus* multiplication in infected organs, liver (**D**), or kidney (**E**), was measured by tissue homogenization, dilution, and colony formation on agar medium. Each symbol represents data from one infected animal. The limit of detection is indicated as a dashed line, and the horizontal line denotes the mean of the log of the colony forming units. Asterisks denote statistically significant differences from the wild type as determined by a Student's *t* test ($P \leq .05$). CFU, colony forming units.

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8. The isotopic composition of [^{54}Fe] hemin (±SE) was 90.7 ± 0.6% [^{54}Fe], 8.99% [^{56}Fe], 0.184% [^{57}Fe], and <0.13% [^{58}Fe]. The isotopic composition of [^{57}Fe] transferrin was 0.051% [^{54}Fe], 5.035% [^{56}Fe], 94.4 ± 0.4% [^{57}Fe], and 0.497% [^{58}Fe].
9. E. P. Skaar, M. Humayun, T. Bae, K. L. DeBord, O. Schneewind, data not shown.
10. Natural iron is a ubiquitous source of iron in bacterial culturing experiments and is traced by the ^{56}Fe (91.75%) isotope. The availability of this iron can be limited during culture experiments by the addition of an aqueous phase iron chelator, but this was found to selectively remove iron from transferrin, and therefore no chelator was used in the present experiments. The potential sources of natural iron in our experiments include the natural iron stores of the bacteria, rust introduced by handling procedures and as a laboratory contaminant, and iron leached from the walls of containers used to handle the analytical solutions.
11. Materials and methods are available as supporting material on *Science* Online.
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Supporting Online Material

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Materials and Methods
References

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