D). The marked stimulatory effect of SynGUN4a-Deutero preincubations on Mg-chelatase activity (Fig. 3D) and the similar Deutero affinities of SynGUN4a and ChlH (Fig. 4F) (*15*) argue that SynGUN4a promotes Mg-chelatase–substrate interactions. In the presence of SynGUN4a, the activity of *Synechocystis* Mg-chelatase appears qualitatively more similar to that of Mg-chelatase from crude chloroplast fractions, which possess a 30- to 160-fold lower Proto Michaelis constant (K_m) than *Synechocystis* Mg-chelatase (*12*, *16*, *17*). GUN4 probably contributes substantially to the relatively low Proto K_m values reported in crude chloroplast fractions, and without GUN4, higher plants probably do not effectively use low concentrations of Proto or synthesize Mg-Proto at high rates. SynGUN4a bound Mg-Deutero more tightly than Deutero (Fig. 4, E and F), and the SynGUN4a K_d value for Mg-Deutero binding was substantially lower than the 2.43 ± 0.46 μM K_d value reported for ChlH (*15*). Because SynGUN4a has a relatively high affinity for Mg-Deutero, we suggest that SynGUN4a may participate in product release and/or a separate function that involves binding the product of Mg-chelatase.

Mg-chelatase has been suggested to associate with the chloroplast inner envelope, but Mg-chelatase has not been definitively localized within the chloroplast (*18*). Because we purified a ChlH-GUN4 complex from thylakoid membranes (Fig. 2C), we suggest that a fraction of Mg-chelatase may associate with thylakoids. Although more work is required to unambiguously define the composition of the envelope and thylakoid GUN4 complexes, it seems likely that both thylakoid and envelope GUN4 complexes contain Mg- chelatase subunits. The heterogeneity of the GUN4 complexes extracted from thylakoid membranes (Fig. 2B) and the large size difference between the thylakoid and envelope complexes (Fig. 2B) may reflect the unstable interactions among Mg-chelatase subunits (*11*) or the association of a ChlH-GUN4 complex with other envelope proteins that might have catalytic or signaling functions.

The large pool of free GUN4 in the stroma (Fig. 2B) suggests additional functions for GUN4. For example, the tetrapyrrole-binding activity of GUN4 might protect Proto and Mg-Proto from catabolic enzymes, help target Mg-Proto to downstream chlorophyll biosynthetic pathway enzymes, or help protect plants from photooxidative damage. Proto and Mg-Proto interactions with O_2 produce reactive oxygen species in bright light, and Mg-Proto accumulates transiently at dawn (*19*). The photosensitivity of *gun4-2* (Fig. 1E) and the absence of *GUN4* homologs in species that carry out anoxygenic photosynthesis support a role for GUN4 in photoprotection. However, we cannot rule out the possibility that the pool of free GUN4 is due to unstable ChlH-GUN4 interactions.

The precise role of GUN4 in plastid-to-nucleus signaling is not clear, because GUN4 both stimulates signal synthesis and binds the signal. Mg-Proto is thought to exit the plastid and interact with cytosolic signaling pathways (*4*–*6*). This model seems reasonable because related molecules (such as mitochondrial heme precursors, heme, phytochromobilin, and chlorophyll degradation products) are exported from plastids (*20*–*23*) and because tetrapyrrole trafficking is likely monitored by the cell. GUN4 may promote Mg-Proto export by stimulating Mg-Proto synthesis and/or by recruiting Mg-Proto to the plastid envelope.

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Supporting Online Material

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Passage of Heme-Iron Across the Envelope of *Staphylococcus aureus*

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The cell wall envelope of Gram-positive pathogens functions as a scaffold for the attachment of virulence factors and as a sieve that prevents diffusion of molecules. Here the *isd* genes (iron-regulated surface determinant) of *Staphylococcus aureus* were found to encode factors responsible for hemoglobin binding and passage of heme-iron to the cytoplasm, where it acts as an essential nutrient. Heme-iron passage required two sortases that tether Isd proteins to unique locations within the cell wall. Thus, Isd appears to act as an import apparatus that uses cell wall–anchored proteins to relay heme-iron across the bacterial envelope.

The envelope of Gram-positive bacteria is the site of interaction between microbes and their host environment during infection (*1*). The envelope is composed of the murein sacculus and attached polysaccharides, teichoic acids, and cell wall proteins (*1*, *2*). Murein is assembled from precursor molecules that generate glycan strands and peptide cross bridg-

es, enclosing the bacterial protoplast inside a single cell-wall macromolecule (*3*–*5*). To investigate the mechanisms of transport across the cell wall, we have analyzed the function of the *isd* locus (Fig. 1A) (*6*). *isdA* and *isdB* encode proteins with an LPXTG (*7*) motif–containing sorting signal (*6*), an element that targets polypeptides for linkage to the cell wall, which is catalyzed by sortase A (*srtA*) (*8*–*10*). *isdC* encodes a polypeptide with an NPQTN motif sorting signal and is attached to murein by sortase B, the structural gene for which (*srtB*) also resides within the *isd* locus (Fig. 1A) (*6*). The *isdD* gene product appears to be inserted in the plasma membrane (*6*). *isdE* is predicted to encode a heme-binding lipoprotein, whereas *isdF* specifies a membrane protein with an adenosine triphosphate–binding cassette (*6*). IsdE and IsdF may function to import heme-bound iron across the plasma membrane, because they display homology to bacterial heme transporters (*11*). The product of *isdG* presumably resides in the staphylococcal cytoplasm. We hypothesize that staphylococci scavenge heme-bound iron during infection and transport this molecule by means of anchored surface proteins across the cell wall envelope.

All three transcriptional *isd* units contain a Fur box (Fig. 1A), a DNA sequence to which the ferric uptake repressor (Fur) binds and inhibits transcription when the iron concentration is high (*12*, *13*). The concentration of free iron in human body fluids is low (10^{-18} M) (*14*), suggesting that *isd* genes are expressed during infection but not during growth in laboratory media containing iron. When *S. aureus* strain Newman was grown in the presence of the iron-chelating agent 2,2′-dipyridyl (*15*), expression of *isd* genes increased (Fig. 1B). The addition of ferrous sulfate ($FeSO_4$) abolished expression of the *isd* locus, suggesting that iron, but not any other divalent cation, was responsible for this phenotype (Fig. 1B).

To determine whether Isd proteins are displayed on the bacterial surface, we treated staphylococci with proteinase K (*16*). Only IsdB and a fragment of IsdA were digested by proteinase K (Fig. 1C). Removal of the cell wall envelope with lysostaphin allowed proteinase K degradation of all Isd proteins examined. Thus, IsdB and a portion of IsdA were displayed on the bacterial surface, whereas IsdC, IsdE, SrtA, and SrtB were buried in the cell wall. To detect the covalent linkage of proteins to the cell wall, we cleaved peptidoglycan with murein hydrolases and measured the mobility of staphylococcal enterotoxin B (Seb) fusions by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (*17*). Lysostaphin cut at the pentaglycine cross bridge, the anchoring point of staphylococcal surface proteins (*18*), and released Seb-IsdA, Seb-IsdB, and Seb-IsdC as species with uniform mobility on SDS-PAGE (Fig. 1D). Mutanolysin cleaved the glycan strands and released Seb-IsdA and Seb-IsdB as a spectrum of fragments with linked cell-wall subunits (*19*). In contrast, mutanolysin released Seb-IsdC with uniform mobility (Fig. 1D), suggesting that sortase B–anchored IsdC is not attached to cross-linked murein.

Purified glutathione *S*-transferase or polyhistidyl fusion proteins Gst-IsdA, His-IsdB, Gst-IsdC, Gst-IsdE, and His-IsdG, but not the control proteins Gst or His-tagged SA1071, absorbed light at 408 nm, a characteristic of polypeptides containing heme (e.g., hemoglobin) (*20*). To determine whether Isd proteins bind heme, purified proteins were electrotransferred to filter membrane, incubated with heme-iron, and stained (Fig. 2, A and B). Gst-IsdA, His-IsdB, Gst-IsdC, Gst-IsdD, Gst-IsdE, and His-IsdG bound heme-iron, whereas Gst alone or SA1071 did not. Quantification of the data revealed that His-IsdG bound more heme-iron than did Gst-IsdA, His-IsdB,

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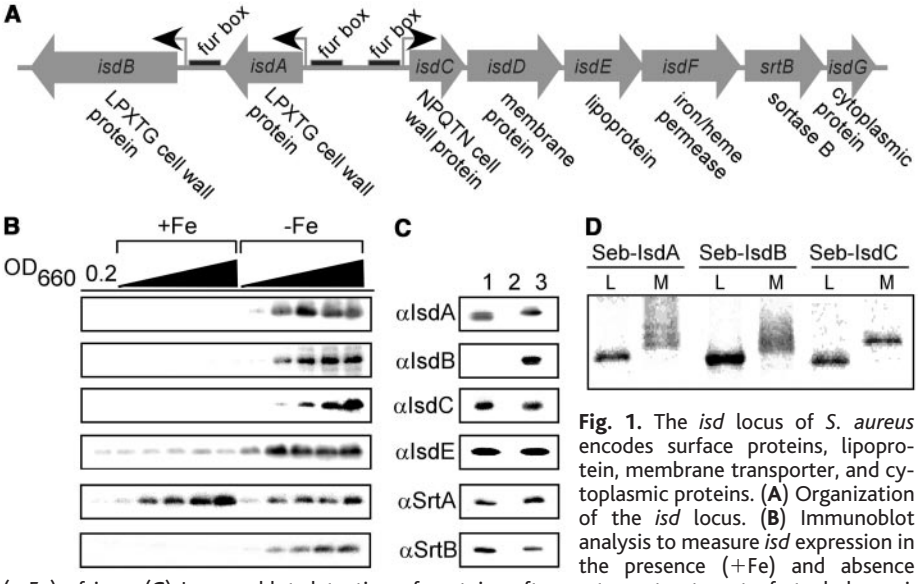
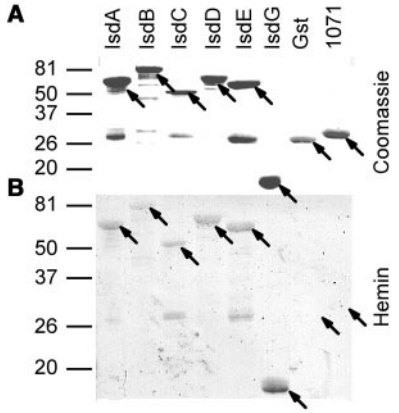


Fig. 1. The *isd* locus of *S. aureus* encodes surface proteins, lipoprotein, membrane transporter, and cytoplasmic proteins. (**A**) Organization of the *isd* locus. (**B**) Immunoblot analysis to measure *isd* expression in the presence (+Fe) and absence (−Fe) of iron. (**C**) Immunoblot detection of proteins after protease treatment of staphylococci. Lane 1, proteinase K treatment followed by lysostaphin digestion of cell wall; lane 2, lysostaphin digestion followed by proteinase K; lane 3, lysostaphin digestion without proteinase. (**D**) Cell wall was cut with lysostaphin (L) or mutanolysin (M), and pulse-labeled Seb-IsdA, Seb-IsdB, and Seb-IsdC were immunoprecipitated and analyzed by SDS-PAGE.



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Protein	Hemin (units/pmol)	StdDev
IsdA	6.01	(0.45)
IsdB	5.88	(1.44)
IsdC	9.18	(1.22)
IsdD	8.96	(2.41)
IsdE	10.88	(2.96)
IsdG	18.37	(5.09)
Gst	1.17	(0.85)
1071	1.60	(0.88)

Fig. 2. Heme-iron binds to IsdA, IsdB, IsdC, IsdD, IsdE, and IsdG. (**A**) Coomassie-stained SDS-PAGE to separate purified Gst-IsdA, His-IsdB, Gst-IsdC, Gst-IsdD, Gst-IsdE, His-IsdG, and Gst alone and a His-tagged *S. aureus* protein (1071). Arrows denote the full-length polypeptide and numbers indicate the migration of molecular standards (in kD). (**B**) Proteins in (A) were electrotransferred to a polyvinylidene difluoride membrane, incubated with heme-iron, and stained with chemiluminescent reagent. Gst and His-tagged 1071 were used as a negative control. (**C**) The amount of heme-iron bound per picomole of full-length protein (in arbitrary units) was determined.

Gst-IsdC, Gst-IsdD, or Gst-IsdE (Fig. 2C).

The nature of heme-iron binding to Gst-IsdC was investigated by polypeptide denaturation with 8 M urea, which released heme-iron as detected by decreased absorption at 408 nm (Fig. 3, A and B) (*16*). Gst-IsdC sedimented with heme-agarose beads, and this interaction was inhibited by the addition of soluble heme (Fig. 3C). Hemoglobin is the most abundant heme-binding protein in mammals (*20*), and it seems plausible that staphylococci scavenge iron by extracting heme from captured hemoglobin. Indeed, purified His-IsdB bound human hemoglobin in a dose-dependent and saturable manner, whereas Gst-IsdA and Gst-IsdC did not (Fig. 3D). The hemoglobin receptor IsdB encompasses an NH_2-terminal domain that is absent from other Isd proteins. Three heme-binding proteins, IsdA, IsdB, and IsdC, harbor a heme-binding domain with amino acid sequence similarity; however, this domain is distinct from the heme-binding domains of IsdD, IsdE, and IsdG.

If staphylococci deposit hemoglobin and heme-binding surface proteins in the cell wall, what is their contribution to the passage of heme-iron across the envelope? To address this question, we examined whether staphylococci require iron or heme-iron for growth (*16*). Growth media were depleted of divalent cations (zinc, magnesium, manganese, calcium, and iron) with Chelex, and metal-ion concentrations were measured by inductively coupled plasma mass spectrometry (*16*, *21*, *22*). The human clinical isolate *S. aureus* Newman failed to grow at iron concentrations below 300 nM (Fig. 4A); however, supplementing media with heme-iron restored growth (Fig. 4A). Deletion of the *isdA* or *isdF* genes did not significantly alter the ability of mutant staphylococci to grow in the presence of heme-iron. This observation is consistent with the finding that the staphylococcal genome encodes four different heme-iron ABC transporters (*12*) and at least four surface proteins with similar heme-binding domains: IsdA, IsdB, IsdC, and IsdH. The gene for *isdH*, encoding an LPXTG motif–type surface protein, is located outside the *isd* locus. We constructed an *S. aureus* mutant with a knockout mutation of the *isdC* gene; however, this deletion had a polar effect and reduced expression of other *isd* genes in this operon (fig. S1). We used a strain containing a deletion of the sortase B (*srtB*) gene, the enzyme that anchors IsdC to the cell wall envelope, to assess the involvement of IsdC in heme acquisition. Inactivation of *srtB* caused a significant reduction, and deletion of the sortase A (*srtA*) gene, alone or in combination with *srtB*, abolished staphylococcal growth. Although the surface proteins IsdA, IsdB, and IsdH appear to be essential for growth with heme-iron as the sole iron source, these factors were not required for growth in the presence of $FeSO_4$.

Staphylococci are capable of de novo heme synthesis; however, the passage of heme-iron across the envelope presumably provides iron for bacterial enzymes involved in oxidative phosphorylation. We used [^{55}Fe]heme to measure the passage of heme-iron (*16*). As a consequence of natural loss of ^{55}Fe from the porphyrin ring, some of the iron in our [^{55}Fe]heme preparation is not associated with heme. We incubated staphylococci with a large excess

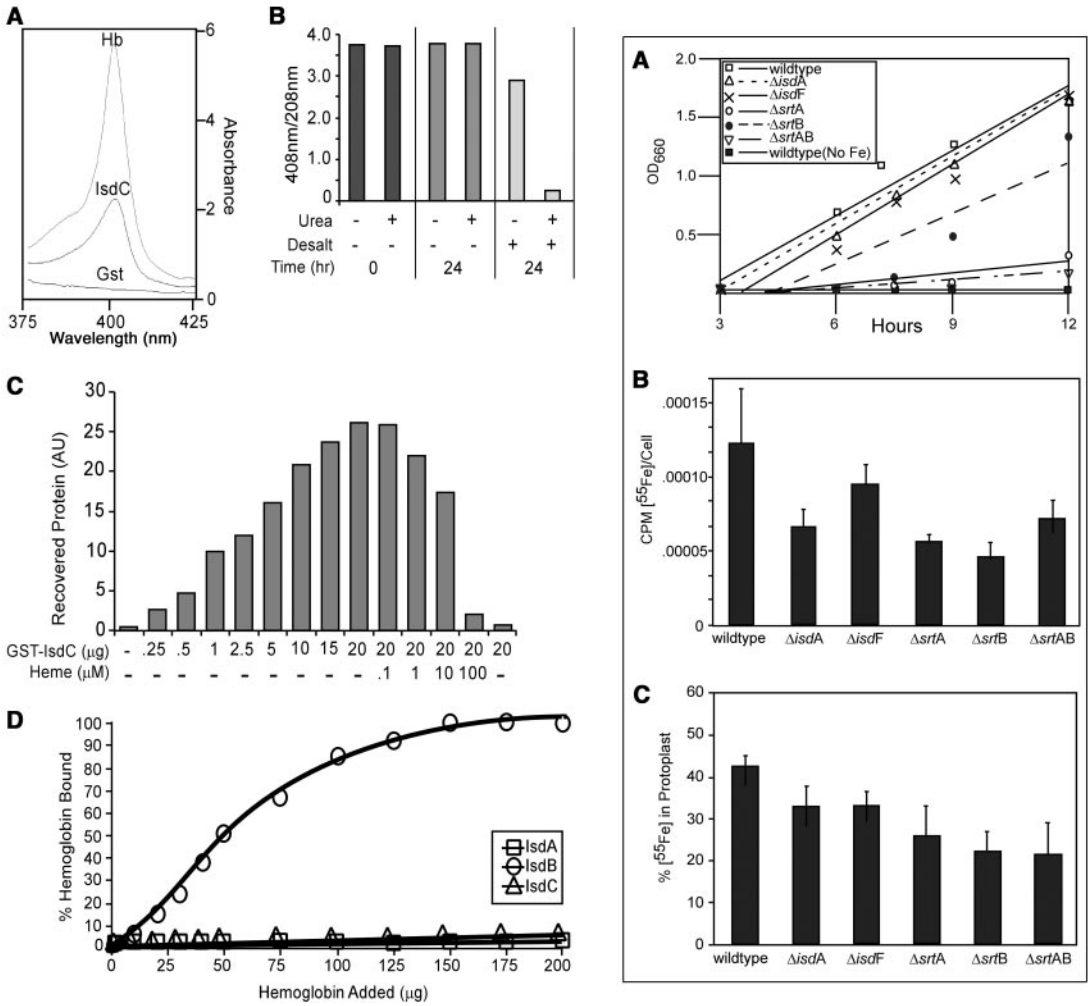

Fig. 3 (left). The *isd* locus encodes heme-binding proteins. (**A**) Spectrophotometric analysis of purified Gst-IsdC and hemoglobin (Hb) at 408 nm, the absorbance of heme. (**B**) Denaturation with 8 M urea, 24-hour incubation at room temperature, and desalting suggest a noncovalent interaction between heme-iron and Gst-IsdC. (**C**) Gst-IsdC binds heme-agarose beads in a dose-dependent manner and incubation with heme-iron abolishes this binding. (**D**) His-IsdB, but not Gst-IsdA or Gst-IsdC, binds human hemoglobin in a dose-dependent manner. **Fig. 4 (right).** *S. aureus* requires sortases and Isd proteins to internalize heme-iron. (**A**) Growth of *S. aureus* Newman in iron-free medium is restored when heme-iron is added. Deletion of the sortase A (*srtA*), sortase B (*srtB*), *isdA*, and *isdF* genes and their effect on the growth of mutant staphylococci in heme-iron–containing growth medium. (**B**) Inactivation of *isdA*, *isdF*, *srtA*, or *srtB* leads to a decrease in the amount of [^{55}Fe]heme-iron associated with cells of *S. aureus* as measured in scintillation counts per minute of ^{55}Fe. (**C**) Inactivation of *isdA*, *isdF*, *srtA*, or *srtB* decreased the amount of [^{55}Fe]heme that enters the staphylococcal cytoplasm.

of [^{55}Fe]heme-iron. At time intervals, heme-iron binding and envelope passage were quenched, followed by centrifugation and scintillation counting of the bacterial sediment. The amount of [^{55}Fe]heme cosedimentation increased over 5 to 10 min and then leveled off (fig. S2). Both live and heat-killed staphylococci were capable of sedimenting [^{55}Fe]heme-iron, consistent with the view that appreciable amounts of [^{55}Fe]heme are bound to staphylococcal receptors without passage to the bacterial cytoplasm. If so, deletion of surface protein or sortase genes may cause a substantial decrease in staphylococcal heme-iron binding. Indeed, deletion of *isdA*, *srtA*, *srtB*, or *srtA* and *srtB* caused a reduction in [^{55}Fe]heme cosedimentation (Fig. 4B). To distinguish binding from bacterial uptake of heme-iron, we treated staphylococci with lysostaphin and harvested the resulting protoplasts by centrifugation. In wild-type staphylococci, about 44% of all [^{55}Fe]heme is located in the protoplasts. The fraction of [^{55}Fe]heme in the protoplast was lower in mutants lacking sortase genes, *isdA*, or *isdF* (Fig. 4C). Thus, the *isd* locus of *S. aureus* is required for a portion of the binding and passage of heme-iron across the cell wall envelope into the cytoplasm. Nevertheless, other surface proteins and ABC transporters participate in similar heme-iron uptake pathways.

We suggest that *S. aureus* acquires iron during infection by first binding hemoglobin on the bacterial surface (IsdB). Heme is removed from hemoglobin (IsdA and IsdB) and transferred to cell wall (IsdC) and membrane translocation factors (IsdD, IsdE, and IsdF). IsdG, the cytoplasmic heme-iron binding protein, may be involved in removing iron from heme. Two sortases tether IsdA and IsdB as well as IsdC to the cell wall, and it appears that IsdC may harbor a unique anchor structure. The LPXTG anchor structure permits staphylococci to use IsdA and IsdB as receptors on the bacterial surface, whereas the NPQTN structure may allow for heme-iron passage across the cell wall. *S. aureus* likely acquires heme-bound iron during infection through the secretion of hemolysins (*23*), which disrupt the plasma membrane of erythrocytes to release hemoglobin (*24*). *Listeria monocytogenes* and *Bacillus anthracis* express Isd proteins, sortases, and hemolysins, suggesting that similar mechanisms of iron acquisition exist in other Gram-positive pathogens (*25*).

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Supporting Online Material

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Materials and Methods

Figs. S1 and S2

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