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Metal ion acquisition in *Staphylococcus aureus*: overcoming nutritional immunity

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Abstract

Transition metals are essential nutrients to virtually all forms of life, including bacterial pathogens. In *Staphylococcus aureus*, metal ions participate in diverse biochemical processes such as metabolism, DNA synthesis, regulation of virulence factors, and defense against oxidative stress. As an innate immune response to bacterial infection, vertebrate hosts sequester transition metals in a process that has been termed “nutritional immunity.” To successfully infect vertebrates, *S. aureus* must overcome host sequestration of these critical nutrients. The objective of this review is to outline the current knowledge of staphylococcal metal ion acquisition systems, as well as to define the host mechanisms of nutritional immunity during staphylococcal infection.

Keywords

Staphylococcus aureus; Calprotectin; Iron; Manganese; Zinc; Copper

Introduction

Staphylococcus aureus is a remarkably successful human pathogen. It is the most common cause of skin and soft tissue infections [1, 2], endocarditis [3], and osteomyelitis [4] and a common cause of bloodstream infections, surgical site infections, and pneumonia. Invasive staphylococcal infections are a source of considerable morbidity and mortality [5]. Treatment of staphylococcal disease is complicated by the prevalence of antimicrobial-resistant strains. Penicillin-resistant *S. aureus* emerged in the 1940s [6], followed by the initial description of methicillin-resistant *S. aureus* (MRSA) in 1961 [7]. The development of subsequent anti-staphylococcal antibiotics has almost uniformly been followed by reports of resistance or treatment failure [8]. Additionally, attempts to design and implement effective staphylococcal vaccines have been unsuccessful thus far [9]. Also complicating the control of staphylococcal infection is the emergence of virulent, community-acquired MRSA (CA-MRSA) strains [10]. Because CA-MRSA infection can occur in those individuals with no known predisposing factors for acquisition of *S. aureus*, it is difficult to predict which populations are at the greatest risk.

The capability of *S. aureus* to cause disease is facilitated by production of a diverse array of virulence factors [11, 12]. Staphylococcal adhesins allow effective binding and colonization of host tissues. After colonization of host tissues, expression of secreted toxins and exoenzymes leads to degradation of host tissues, further tissue invasion, and metastasis to other sites. A subset of staphylococcal virulence factors counteracts the host immune response. This includes factors that destroy neutrophils or inhibit their activity [13–18], prevent activation of the complement cascade [19–22], disrupt phagocytosis [23–25], limit the efficacy of antimicrobial peptides [26–28], and interfere with or augment the function of T cells [29, 30]. The spectrum of human disease following staphylococcal infection is therefore a function of the success of the host immune system in controlling staphylococcal virulence. If the host immune system is effective in controlling staphylococcal infection, the clinical outcome is that of asymptomatic colonization or localized disease, such as a soft tissue abscess. If *S. aureus* succeeds in overcoming the host immune system, the result is invasive and disseminated disease. In this way, human infection by *S. aureus* represents a constant battle between host and pathogen.

The concept of nutritional immunity exemplifies the struggle between *S. aureus* and the human immune system [31]. Nutritional immunity describes the process whereby a host sequesters nutrients essential to bacterial growth, thus limiting the ability of invading pathogens to proliferate in the host environment. The most well characterized example of nutritional immunity during staphylococcal infection is iron sequestration, in which iron is maintained predominantly intracellularly or in complex with high-affinity host binding proteins [32]. The result of this sequestration is an extracellular iron concentration insufficient to support bacterial growth [33]. Since iron is necessary for bacterial proliferation, *S. aureus* must circumvent iron sequestration in order to successfully infect host tissues. Accordingly, staphylococci have evolved systems for the acquisition, processing, and detoxification of iron and iron-containing host molecules and proteins. Emerging evidence suggests that the principle of nutritional immunity extends to other transition metals, including zinc and manganese [34].

The objective of this review is to provide a comprehensive analysis of both the mechanisms by which *S. aureus* acquires transition metals, as well as the host mechanisms aimed at sequestration of these essential nutrients. We focus on the impact of metal ion acquisition on staphylococcal virulence and the host immune response to staphylococcal infection.

***S. aureus* overcomes nutritional immunity to obtain iron from the host**

Iron is a critical nutrient for both humans and pathogenic bacteria. In humans, iron-containing compounds participate in a number of important cellular processes including energy metabolism, cellular proliferation, DNA repair, and protection against oxidative stress [35–37]. However, iron is also essential to invading bacterial pathogens; therefore vertebrates sequester iron as an innate immune response against bacterial infection. Strict regulatory control of iron homeostasis also protects the host from damage associated with either iron overload or iron deficiency. Iron excess, as manifested in hemochromatosis or in individuals requiring chronic transfusions, results in tissue damage and organ dysfunction. Conversely, iron deficiency, encountered in patients with chronic inflammation or inadequate dietary intake, results in ineffective erythropoiesis [35].

A number of factors contribute to the sequestration of iron in the vertebrate host (Fig. 1). The iron content of vertebrates is mostly intracellular, either maintained within the iron storage protein ferritin, or complexed to the tetrapyrrole ring of heme. Heme is the major iron reservoir in vertebrates, accounting for up to 80% of total body iron [38]. The majority of vertebrate iron is therefore inaccessible to predominantly extracellular pathogens such as

S. aureus. Furthermore, any iron released into the extracellular environment is tightly bound to the high-affinity iron-scavenging glycoproteins transferrin and lactoferrin. Together, these mechanisms ensure that the amount of free extracellular iron that is available to invading pathogens is negligible. In response to infection, mammalian hosts can further limit iron availability through a number of mechanisms including decreased dietary iron absorption, reduced release of iron from macrophages, and release of apolactoferrin from neutrophil granules at the site of infection [32]. The importance of iron sequestration as an immune response to invading pathogens is illustrated by individuals with iron overload, who have an increased susceptibility to bacterial infection. For example, patients with hemochromatosis are more susceptible to infection by *Yersinia enterocolitica*, *Vibrio vulnificans*, and *Listeria monocytogenes* [39–42].

In order to successfully infect humans, bacteria must overcome iron limitation in host tissues. Pathogenic bacteria have evolved five primary mechanisms to circumvent host iron sequestration. The first mechanism, utilized by *Borrelia burgdorferi* and *Lactobacillus plantarum*, is to substitute manganese for iron in metal-containing enzymes, eliminating the cell's requirement for iron [43, 44]. A second mechanism is the production of secreted iron-binding compounds known as siderophores. Siderophores bind iron with high affinity and thus can effectively compete with extracellular iron sequestration by transferrin and lactoferrin. Third, bacteria utilize heme acquisition systems to obtain host iron. Bacterial heme acquisition systems involve cell surface receptors that recognize either free heme or heme bound to hemoproteins, a transport apparatus to move heme across the cell membrane into the bacterial cytoplasm, and enzymes that liberate iron from heme. A fourth mechanism used by bacteria to obtain iron is the expression of ferric or ferrous iron transporters. Finally, bacteria may express transferrin and lactoferrin receptors to obtain iron directly from high-affinity host proteins. To successfully infect humans, *S. aureus* utilizes siderophores and a heme acquisition system to overcome host iron sequestration (Fig. 1).

***S. aureus* produces siderophores to steal iron from transferrin**

S. aureus produces two polycarboxylate-type siderophores, staphyloferrin A and staphyloferrin B, which were initially isolated and characterized from *Staphylococcus hyicus* [45]. An additional staphylococcal siderophore, aureochelin, has been proposed but not further characterized [46]. Iron acquisition by staphyloferrin A and staphyloferrin B is regulated by the ferric uptake regulator (Fur) in response to environmental iron concentrations [47–49]. Fur is an iron-dependent transcriptional regulator that is conserved among Gram-positive and Gram-negative bacteria [50]. In iron-replete conditions, Fur binds a consensus DNA sequence (Fur box) upstream of Fur-regulated genes, leading to transcriptional repression. In iron-deplete conditions, Fur no longer binds to Fur boxes, and transcription of Fur-regulated genes can proceed. In this way, *S. aureus* can initiate a transcriptional program based on the amount of iron available in the environment [51].

Staphyloferrin A is a 479 Da molecule encoded by the *sfaABCD* gene cluster [47, 52]. Following iron complexation by staphyloferrin A, transport across the staphylococcal membrane is mediated by the ABC transporter HtsABC, in a process powered by a separate ATPase, PhuC (Fig. 1) [47, 53]. The name of the *htsABC* operon is derived from its initial characterization as a heme transport system [54]. Subsequent crystallographic studies of HtsA demonstrate that this protein clearly binds staphyloferrin A [55]. Staphyloferrin B is a 448 Da compound composed of L-2,3-diaminopropionic acid, 1,2-diaminoethane, and -ketoglutaric acid [56]. Staphyloferrin B is a product of the *sbnABCDEFGHI* operon, of which only SbnC, SbnE, SbnF, and SbnH are required for in vitro synthesis [57]. Transport of staphyloferrin B across the staphylococcal cell membrane is mediated by the staphylococcal iron-regulated transporter SirABC (Fig. 1) [58, 59]. The advantage of producing two siderophores is unclear, although there is precedence for this phenomenon in

other bacteria including *Pseudomonas aeruginosa* (pyoverdines and pyochelin) and *Bacillus anthracis* (bacillibactin and petrobactin). Concomitant inactivation of the genes responsible for the production of staphyloferrin A and staphyloferrin B results in severe growth limitation in iron-deplete media [47]. Inactivation of the *sbn* locus alone, but not the *sfa* locus, also yields a growth defect in iron-restricted media [47]. Thus, under in vitro conditions, staphyloferrin B seems to have a more prominent role in staphylococcal growth during iron limitation. Yet both staphyloferrin A and staphyloferrin B can remove iron from human holotransferrin to support *S. aureus* growth in iron-deplete conditions [60]. Whether or not staphyloferrin A and staphyloferrin B have distinct in vivo roles remains to be tested.

In addition to siderophore-mediated iron acquisition by staphyloferrin A and B, *S. aureus* has the capability to liberate iron from heterologous siderophores and mammalian catecholamine hormones. Brock and Ng demonstrated that *S. aureus* is capable of utilizing the hydroxamate-type siderophore desferrioxamine as an iron source [61]. Uptake of desferrioxamine is dependent on the *fhuCBG* operon, which encodes the membrane-spanning and ATPase components of a classical traffic ATPase [62]. Subsequent experiments identified FhuD1 and FhuD2 as the lipoprotein receptors for FhuCBG-mediated siderophore uptake [63]. The ability of *S. aureus* to utilize desferrioxamine as an iron source is clinically relevant given its use as a chelating agent, and previous reports that this compound enhances the growth and virulence of other bacterial and fungal pathogens [64–66]. *S. aureus* also has the capability to utilize mammalian catecholamine hormones as an iron source. Beasley et al. demonstrated that catecholamine hormones enhance the growth of siderophore-deficient *S. aureus* grown in the presence of human serum or transferrin [60]. Uptake of catecholamine iron is dependent on SstABCD, a previously described iron-regulated ABC transporter [67].

Iron acquisition by siderophores contributes to staphylococcal virulence

Siderophore-mediated iron acquisition is essential to the virulence of a number of bacterial pathogens. Inactivation of siderophore production reduces virulence in *B. anthracis* [68], *P. aeruginosa* [69, 70], *Y. enterocolitica* [71], *Legionella pneumophila* [72], and *Escherichia coli* [73], among others. A limited number of experiments have investigated the role of siderophore-mediated iron acquisition in *S. aureus*. Beasley et al. examined the virulence of *S. aureus* possessing mutations in either *sirA*, *hts*, *sbn*, *sfa*, *sst*, or combinations thereof [60]. Mutation of the loci encoding staphyloferrin A or staphyloferrin B alone does not lead to a statistically significant reduction in colony recovery from the organs of intravenously infected mice. This result is in contrast to previously published data in which mutation of *sbnE* significantly decreased colony recovery in a murine kidney abscess model [74]. Inactivation of either the catecholamine transporter (Sst) alone, both siderophore operons (*sfa* and *sbn*), or both siderophore transporters (Hts and Sir) yields a reduction in colony recovery from the hearts of systemically infected mice. Additionally, combined inactivation of *sfa*, *sbn*, and *sst* results in reduced colony recovery from the heart and liver, whereas combined inactivation of *sst*, *sirA*, and *hts* decreases colony recovery in heart, liver, and kidney. Mutation of the gene encoding FhuC, the ABC-type ATPase required for both staphyloferrin A and B uptake, results in decreased murine morbidity, but not colony recovery, in a kidney abscess model [53]. Collectively, these results suggest that siderophore-mediated iron acquisition is important for *S. aureus* virulence.

As bacterial pathogens have evolved to use siderophores to obtain host iron, so have vertebrate hosts evolved to counteract these processes. Siderocalin, also known as lipocalin-2 or neutrophil gelatinase-associated lipocalin, is a mammalian protein capable of binding bacterial siderophores. Siderocalin binds enterobactin, a catecholate siderophore produced by *E. coli*, resulting in bacteriostasis in iron-limited conditions [75, 76]. In keeping with this, siderocalin-deficient mice are more susceptible to *E. coli* infection [76, 77].

Subsequent studies revealed that siderocalin is also important for prevention of infection by *Klebsiella pneumoniae* and *Mycobacterium tuberculosis* [78–80]. Siderocalin production is induced by a number of stimuli, including TLR4 binding [76], bacterial colonization of the nasal mucosa [81], and ischemia [82]. Thus, production of siderocalin is a host strategy to sequester iron that has been earmarked for bacterial utilization. Not to be outdone, some bacterial pathogens have evolved mechanisms to circumvent siderocalin. *B. anthracis* produces two siderophores, bacillibactin and petrobactin [83]. Bacillibactin is readily bound by siderocalin, but petrobactin has a unique chelating subunit that precludes siderocalin binding [84]. *Salmonella* Typhimurium utilizes a similar strategy to evade siderocalin by producing a glycosylated derivative of enterobactin known as salmochelin [85]. These “stealth siderophores” are yet another example of bacterial subversion of innate immunity. Stealth siderophores have not been described in *S. aureus*.

***S. aureus* preferentially acquires iron from heme and hemoproteins**

Although siderophore-mediated iron acquisition promotes staphylococcal growth in iron-limited conditions in vitro and in vivo, the preferred iron source for *S. aureus* is heme. When *S. aureus* is grown in the presence of isotopically labeled iron complexed to either transferrin or heme, a fourfold to fivefold enrichment in the ratio of heme-iron to transferrin-iron is noted in bacteria as compared with the media [54]. Thus, *S. aureus* preferentially imports heme-iron over transferrin-iron.

In vertebrates, heme is the primary reservoir of iron, representing up to 80% of total body iron. This is in contrast to transferrin-bound iron, which represents less than 1% of total body iron [38]. Heme consists of a tetrapyrrole ring encircling an iron atom, and is produced in the bone marrow and liver. Heme is used as a prosthetic group for many enzymes, and these hemoproteins have diverse roles, including the transfer of electrons and redox reactions. The most abundant hemoprotein in vertebrates is hemoglobin, which consists of four heme moieties bound to polypeptide chains. One human erythrocyte contains approximately 280 million molecules of the hemoglobin tetramer [86]. Other hemoproteins include myoglobin, peroxidases, and cytochromes. Heme biosynthesis and catabolism must be carefully regulated, given the potential toxicity of heme and hemoprotein derivatives [87]. Hemoglobin liberated from erythrocytes is bound by the high-affinity binding protein haptoglobin and subsequently processed by the reticuloendothelial system [88]. Additionally, free heme released into plasma can be sequestered by albumin, hemopexin, and alpha-1-microglobulin [86, 88, 89]. These mechanisms protect the host from heme toxicity, while also limiting the availability of heme and hemoproteins to invading pathogens.

Given that heme is the major reservoir of iron in vertebrate hosts, it is not surprising that both Gram-positive and Gram-negative bacterial pathogens have evolved mechanisms to liberate iron from host heme [90]. Several classes of bacterial heme acquisition systems have been described [90, 91]. In *S. aureus*, heme acquisition is mediated by the iron-regulated surface determinant (Isd) system. The *isd* locus was initially identified during an examination of the *S. aureus* genome for homologues of the transpeptidase Sortase A, which functions to anchor proteins to the cell wall [92–94]. The only Sortase A homologue identified in *S. aureus*, Sortase B (*srtB*), is contained within an iron-regulated operon that also contains genes whose products were homologous to heme-binding proteins. Upstream of this operon are two additional genes divergently transcribed from independent promoters. Consensus Fur boxes are located upstream of each operon. Since the initial description of the *isd* locus, much has been learned regarding heme uptake by the Isd system. The Isd system is encoded by ten genes in five operons: *isdA*, *isdB*, *isdCDEFsrtBisdG*, *isdH*, and *orfXisdI* [95]. IsdB and IsdH are cell surface receptors for hemoglobin and hemoglobin-haptoglobin, respectively [96, 97]. Heme is passed from IsdB or IsdH to IsdA, also located

at the cell surface [98, 99]. IsdA transfers heme to IsdC, which mediates transfer across the cell wall [99, 100]. Alternatively, IsdH and IsdB may pass heme directly to IsdC or IsdE [98]. IsdDEF is an ABC transporter in which IsdE is a heme-binding lipoprotein, IsdD is an ATPase, and IsdF is a permease. Heme is passed from IsdC to IsdE and subsequently transported into the cytoplasm (Fig. 1) [98]. Interestingly, mutation of *isdDEF* decreases, but does not abolish, the ability of *S. aureus* to grow on heme, suggesting that there are IsdDEF-independent mechanisms of heme import [101]. Mutations in the *htsABC* locus also reduce heme acquisition by *S. aureus*, but the crystal structure of HtsA strongly suggests staphyloferrin A is the ligand [54]. It is therefore possible that HtsB and HtsC function with an as yet-unidentified lipoprotein receptor to transport heme across the cell membrane.

Once heme enters the staphylococcal cytoplasm, the tetrapyrrole ring is degraded to liberate free iron by two heme-oxygenases, IsdG and IsdI (Fig. 1) [102, 103]. Unlike human heme-oxygenases which degrade heme to iron and the chromophore biliverdin, IsdG and IsdI degrade heme to iron and a novel chromophore, staphylobilin [104]. Deletion of either *isdG* or *isdI* results in a decreased ability to utilize heme, indicating that these enzymes are not functionally redundant [105]. Overexpression of either IsdG or IsdI can complement an *isdGI* mutant, suggesting that the relative amount of each heme-oxygenase may affect heme utilization [105]. Both *isdG* and *isdI* are transcriptionally regulated by iron, such that maximal expression occurs in iron-limited conditions. IsdG is also post-transcriptionally regulated by heme, as protein stability is enhanced in the presence of heme [105]. These findings suggested a model whereby *S. aureus* can fine-tune its capacity for heme utilization based on the environmental conditions encountered.

Like eukaryotes, bacteria must carefully regulate intracellular heme concentrations to ensure that nutrient iron needs are met while preventing toxicity. In *S. aureus*, this is accomplished by mechanisms to both sense and alleviate heme toxicity. Staphylococci exposed to subinhibitory concentrations of heme can subsequently resist heme toxicity at lethal concentrations, suggesting an adaptive response to exogenous heme [106]. Proteomic analyses revealed that 21 *S. aureus* proteins are regulated by heme independently of iron [48]. Included among these proteins is the ATP-binding component of a putative ABC transporter that increases 45-fold in the presence of heme. The gene encoding this protein is adjacent to a predicted permease component of the transport system. Inactivation of these genes, designated heme-regulated transporter genes *hrtA* and *hrtB*, severely impairs the ability of *S. aureus* to grow on high concentrations of heme. These and subsequent experiments suggest that HrtAB is a transport system dedicated to the excretion of excess heme or a toxic heme metabolite [106, 107]. The strong heme-dependent regulation of *hrtAB* suggests that the expression of these genes is under the control of a dedicated transcriptional regulator. Consistent with this, two genes predicted to encode a two-component system are located immediately adjacent to the *hrtAB* locus on the staphylococcal chromosome. Bacterial two-component systems consist of a membrane-bound kinase that can transduce environmental stimuli to the second component, a response regulator which affects transcriptional changes. The two-component system upstream of *hrtAB*, subsequently named the heme sensor system (HssR and HssS), is required for the adaptive response to heme toxicity and activates transcription of *hrtAB* [106]. Thus, *S. aureus* senses heme through HssRS, increasing transcription of *hrtAB*, which leads to alleviation of heme toxicity. However, the exact ligand of HssS and the identity of the substrate exported by HrtAB have yet to be identified.

Heme acquisition is essential to the virulence of *S. aureus*

The ability of staphylococci to acquire heme and hemoproteins in vivo is facilitated by lysis of host erythrocytes by pore-forming toxins such as alpha-hemolysin (encoded by *hla*) (Fig. 1). Inactivation of alpha-hemolysin limits the virulence of *S. aureus* [108–114]. Moreover,

passive immunization with anti-Hla antibodies or active immunization with nontoxic Hla is protective in animal models of staphylococcal infection [108, 115]. After liberation of hemoproteins from host cells, the ability of *S. aureus* to utilize heme via the Isd system is critical for virulence. Several lines of evidence indicate that disruption of the Isd heme acquisition system at each step (surface receptor binding, transport across the cell wall, transport across the cell membrane, or release of iron from heme) leads to decreased virulence in animal models of staphylococcal infection. Mutation of *isdA*, *isdB*, and *isdC*, but not *isdH*, decreases bacterial recovery and organ abscesses in murine models of systemic infection [96, 116, 117]. Additional support for the role of IsdB in staphylococcal pathogenesis comes from experiments using mice expressing human hemoglobin. Compared to wild-type mice, mice expressing human hemoglobin are more susceptible to *S. aureus* infection, as evidenced by increased colony recovery from infected organs. IsdB binds human hemoglobin more readily than murine hemoglobin in vitro, and deletion of *isdB* abolishes the enhanced virulence of staphylococci infecting mice with human hemoglobin [118]. Collectively, these results suggest an important role for surface proteins of the Isd system in staphylococcal virulence, and have led to additional experiments investigating the vaccine potential of these proteins. IsdA and IsdB were identified as vaccine candidates using “reverse vaccinology” [119], and subsequent studies confirmed that immunization with these proteins is protective in murine models of infection [117, 120–123]. Additional human studies are needed to investigate the vaccine potential of Isd proteins. A phase one human clinical trial of the IsdB vaccine V710 is complete [124], and further trials are ongoing.

In addition to its role as a heme-binding protein, there is evidence that IsdA also contributes to staphylococcal pathogenesis through roles in both adhesion to host tissues and resistance to innate immune responses. IsdA binds both fibrinogen and fibronectin in vitro, indicating a potential role as an iron-regulated adhesin [125]. Consistent with this hypothesis, IsdA facilitates binding to human desquamated epithelial cells, and promotes nasal colonization in rats [122, 126, 127]. IsdA also plays a role in resistance to the host immune response. By decreasing staphylococcal cell hydrophobicity, IsdA increases the resistance of *S. aureus* to antimicrobial compounds produced by human sebum, as well as other antimicrobial peptides [128]. This is reflected in decreased survival of *S. aureus isdA* mutants on live human skin. Mutation of *isdA* also leads to increased susceptibility to hydrogen peroxide and to killing by human neutrophils [129]. Finally, IsdA protects against the bactericidal activity of lactoferrin [130]. Thus, the contribution of IsdA to staphylococcal virulence may extend beyond its role in heme acquisition.

Heme transport across the cell membrane is also required for staphylococcal virulence. Inactivation of either *htsB* or *htsC* significantly reduces heme acquisition and decreases bacterial recovery and abscess formation in mice infected systemically with *S. aureus* [54]. Similarly, concurrent inactivation of *htsA* and *isdE* leads to a statistically significant decrease in bacterial burden in the lung, heart and kidneys of systemically infected mice [131]. Upon entering the cytoplasm, heme is degraded by IsdG or IsdI to liberate iron, a process that also contributes to staphylococcal virulence. Mutation of either *isdI* or *isdG* results in decreased colony recovery from the hearts of systemically infected mice. In contrast, mutation of *isdG*, but not *isdI*, results in decreased recovery from infected kidneys [105]. These findings raise the possibility that IsdG and IsdI have distinct roles depending on the host environments encountered in vivo. The presence of two differentially regulated heme degrading enzymes could allow *S. aureus* to cope with changing iron and heme concentrations as infection progresses. During the early stages of infection, staphylococci may encounter iron-limiting conditions while not yet importing substantial amounts of heme. Under these conditions, IsdG is destabilized by the lack of intracellular heme, leaving IsdI to degrade the low levels of imported heme. After seeding and degradation of host

tissues, increased heme acquisition and the concomitant increase in intracellular heme levels would stabilize IsdG, increasing the overall capacity for heme degradation.

A surprising result was obtained when assessing the virulence of *S. aureus* strains inactivated for the heme sensing system and heme-regulated transporter. Rather than compromising virulence, *S. aureus* strains possessing mutations in *hrtA* or *hssR* grow to an average of two to three logs higher bacterial density in the livers of infected mice as compared with wild-type staphylococci [106]. This hepatic hypervirulence is the result of inhibition of a liver-specific immune response. Further experiments revealed that inactivation of HrtA leads to membrane damage which triggers increased expression and secretion of immunomodulatory factors [132]. The mechanism by which mutation of *hssR* leads to immunomodulation remains to be characterized, but disruption of heme sensing and detoxification systems clearly impact staphylococcal pathogenesis.

The role of manganese in staphylococcal infection

The most well characterized model of nutritional immunity during bacterial infection is sequestration of iron. However, sequestration of manganese is also an important facet of the innate immune response to staphylococcal infection [34]. Manganese is an essential nutrient for both eukaryotic and prokaryotic organisms. In humans, manganese participates in diverse cellular functions including detoxification of free radicals, metabolism, bone growth, and support of hemostasis [133]. Like iron, manganese must be carefully regulated to prevent cellular damage. Manganese intoxication occurs predominantly as a result of occupational exposure, particularly in the welding, mining, and smelting industries. Excessive manganese is neurotoxic, leading to clinical manifestations ranging from mild dystonia to a constellation of extra-pyramidal symptoms resembling Parkinson's disease [134]. The clinical manifestations of manganese deficiency are less clear. In a study of seven men fed manganese-deficient diets, a fleeting dermatitis developed in five subjects [135]. However, naturally occurring manganese deficiency has not been described in humans [133].

Bacteria, like humans, must acquire manganese and regulate its intracellular concentration. Manganese-dependent bacterial enzymes are necessary for myriad processes, including carbohydrate and amino acid metabolism, signal transduction, stringent response, and defense against oxidative stress [136]. Thus, the success of bacterial pathogens in human infection depends on the ability to obtain this critical nutrient from host tissues. Likewise, prevention or resolution of infection is aided by host mechanisms that sequester manganese from invading pathogens.

Bacterial acquisition of manganese is facilitated by high-affinity transporters

In 1995, a manganese transporter was characterized in the cyanobacterium *Synechocystis* 6803 [137]. The genes necessary for production of this transporter, named *mntABC* for manganese transporter, encode products with sequence similarity to the ABC superfamily of bacterial permeases. Further experiments confirmed MntABC as an ABC-type transporter consisting of a cytoplasmic ATP-binding protein (MntA), a transmembrane protein (MntB), and solute-binding protein (MntC). Homologues of MntABC have since been found to facilitate manganese uptake in a number of Gram-positive and Gram-negative pathogens. A second family of bacterial manganese transporters was identified during characterization of the *Bacillus subtilis* protein MntR, a manganese-modulated transcriptional regulator [138]. Que and Helmann discovered transposon-insertion mutants that alleviated the increased manganese susceptibility of an *mntR* mutant. The most commonly recovered insertions were in a gene (*mntH*) predicted to encode a proton-coupled metal ion transporter of the natural resistance-associated macrophage protein (Nramp) family. In a separate report, additional

MntH transporters were characterized in *E. coli*, *Salmonella Typhimurium*, *Burkholderia cepacia*, and *P. aeruginosa* [139]. Unlike mammalian Nramp proteins which mediate transport of a number of cations, bacterial Nramp transporters are highly selective for manganese [140].

Compared to the knowledge of bacterial manganese import systems, considerably less is known about how bacteria respond to toxic intracellular levels of manganese. In 1973, Fisher et al. utilized radiolabeled manganese to demonstrate both uptake and efflux of manganese by *B. subtilis* [141]. Almost four decades later, the first bacterial manganese efflux system was characterized. The *Streptococcus pneumoniae* gene *mntE* encodes an efflux system of the cation diffusion facilitator (CDF) family [142]. Nearly all bacterial genomes encode CDF family members, most of which are involved in efflux of zinc or other heavy metal cations [143]. In contrast, the MntE exporter in *S. pneumoniae* is specific for manganese. Whether other bacterial pathogens express dedicated manganese efflux systems is unknown.

The *S. aureus* genome encodes MntABC-type (*mntABC*) and Nramp-type (*mntH*) manganese transporters, both of which have been functionally characterized. Mutation of *mntA* in *S. aureus* results in a reduced growth rate in metal-depleted minimal media, a phenotype that is reversible upon addition of manganese. In contrast, mutation of *mntH* does not lead to an appreciable growth defect in metal-depleted media, suggesting that MntABC may be the dominant manganese transporter in *S. aureus* under in vitro conditions [144]. The transcriptional regulator MntR affects expression of both *mntABC* and *mntH*. In manganese-replete conditions, transcription of *mntABC* is repressed by MntR. Deletion of *mntR* leads to constitutive transcription of *mntABC* irrespective of manganese levels. In contrast, *mntH* transcription is reduced in the absence of MntR, suggesting that MntR may be a bifunctional regulator in *S. aureus*, as previously described for *B. subtilis* [138]. In addition to regulation by MntR, *mntABC* is negatively regulated by PerR, a manganese and iron-dependent Fur homologue that controls responses to oxidative stress [144, 145]. It is unknown if the regulation of manganese homeostasis in *S. aureus* involves an efflux system. The *S. aureus* genome encodes CDF family proteins, but those that have been characterized are predicted to have roles in zinc efflux [146, 147].

Aerobically growing microorganisms are at risk for toxicity from reactive oxygen species, and staphylococcal manganese acquisition is crucial for defense against oxidative stress. *S. aureus* produces two superoxide dismutases, SodA and SodM, which detoxify superoxide anion to hydrogen peroxide [148, 149]. Hydrogen peroxide can be further reduced to water and oxygen by the enzyme catalase. The activities of SodA and SodM are dependent on manganese [148, 149]. Manganese limitation therefore renders *S. aureus* more susceptible to oxidative stress (Fig. 2) [150]. Additionally, *S. aureus* employs superoxide dismutase-independent mechanisms to detoxify superoxide, a process that is also dependent on manganese [144].

Manganese acquisition contributes to the virulence of bacterial pathogens

Disruption of manganese acquisition reduces the virulence of a number of bacterial pathogens. The Nramp-type manganese transporter MntH is a critical virulence determinant for *Brucella abortus* [151]. Similarly, acquisition of manganese is required for intracellular survival and virulence of *Salmonella enterica* [152]. Disruption of MntABC in *Neisseria gonorrhoeae* reduces intracellular survival and biofilm formation [153]. Several other pathogens also require manganese uptake for infection, including *Enterococcus faecalis*, *S. pneumoniae*, and *Yersinia pestis* [154–156]. Inactivation of the manganese efflux protein MntE in *S. pneumoniae* reduces nasal colonization and bloodstream invasion in mice, despite an increased resistance to reactive oxygen species [142]. This suggests that the

ability to regulate intracellular concentrations of manganese may also be important for bacterial pathogenesis.

In *S. aureus*, mutation of *mntA*, *mntH*, or *mntR* in the laboratory strain 8325-4 does not lead to a significant reduction in bacterial recovery in a murine skin abscess model. In contrast, concurrent inactivation of *mntA* and *mntH* significantly decreases bacterial recovery in this model. Disruption of *mntA*, *mntH*, *mntR*, or both *mntA* and *mntH* also significantly reduces intracellular survival in human endothelial cells [144]. Additional evidence for the role of manganese acquisition in staphylococcal virulence came from studies of host factors that sequester manganese during infection.

Vertebrate hosts sequester manganese as an innate immune response to bacterial infection

Sequestration of manganese is an important facet of the innate immune response to bacterial infection. Two strategies utilized by vertebrate hosts to limit manganese availability to invading pathogens have been described. First, the availability of manganese to intracellular pathogens is limited by the cation transporter Nramp1. Nramp1 is an integral membrane protein expressed by professional phagocytes. Upon phagocytosis, Nramp1 is recruited to the phagosome, where it remains during the maturation of phagosomes into phagolysosomes [157]. Experiments using a fluorescent probe that is readily incorporated into the phagosome demonstrated that Nramp1 functions as a pH-dependent transporter capable of manganese efflux from the phagolysosome [158]. In this way, the availability of manganese is limiting for intracellular pathogens. Additional studies suggest that Nramp1 may also transport other cations such as iron and zinc [159, 160], and therefore may participate in limitation of multiple metals to intracellular pathogens. Consistent with this, disruption of Nramp1 in mice leads to increased susceptibility to the intracellular pathogens *Salmonella* Typhimurium, *Mycobacterium bovis*, and *Leishmania donovani* [161]. The importance of Nramp1 during human infection is suggested by studies linking certain polymorphisms in Nramp1 to an enhanced susceptibility to both *M. tuberculosis* and nontuberculous mycobacteria [162–164]. The precise mechanism by which Nramp1 polymorphisms enhance susceptibility to mycobacteria is unclear.

A second mechanism utilized by vertebrates to limit the availability of manganese to invading pathogens was discovered during a search for host factors that limit bacterial growth in abscesses [34]. One of the most abundant host proteins identified in tissue abscesses of mice infected with *S. aureus* was S100A8, a component of the heterodimeric protein calprotectin (also known as SA1008/SA1009, calgranulin A/B, or myeloid-related proteins 8/14). Calprotectin, which accounts for ~40% of the cytosolic protein pool of neutrophils [165–167], was originally identified based on its ability to inhibit the growth of a variety of fungal and bacterial pathogens in vitro [168, 169]. Calprotectin is also a component of neutrophil extracellular traps, fiber-like structures consisting of chromatin and neutrophil granules that bind to and facilitate killing of microorganisms [170, 171]. A number of immunomodulatory functions have been ascribed to calprotectin, including chemotactic activity for neutrophils and macrophages [166, 167], regulation of inflammation in response to vascular injury [172], amplification of the immune response via TLR4 binding [173, 174], induction of cell death [175, 176], and regulation of myeloid-derived suppressor cells [177, 178]. Previous in vitro studies suggested that the antimicrobial activity of calprotectin was related to its ability to chelate zinc, limiting the availability to pathogens [179–182]. Subsequent in vivo analyses revealed that calprotectin also limits bacterial growth in abscesses via chelation of manganese. Visceral abscesses in mice infected with *S. aureus* are essentially devoid of manganese and zinc. In contrast, abscesses in mice lacking calprotectin contain manganese levels equivalent to those of surrounding tissue, as well as significantly increased bacterial burdens compared with wild-type mice.

The concentration of zinc does not differ significantly between *S. aureus* abscesses in wild-type and calprotectin-deficient mice. Collectively, these results demonstrated that manganese limitation is an important innate immune response to staphylococci in tissue abscesses. *S. aureus* strains possessing mutations in *mntA* or *mntB* are more sensitive to the antibacterial effects of calprotectin, further supporting the importance of manganese acquisition to staphylococcal virulence [34].

A partial mechanistic explanation of the in vivo antibacterial effects of calprotectin has been described [150]. Treatment of *S. aureus* with calprotectin results in enhanced susceptibility to oxidative stress through inhibition of SodA and SodM. This renders calprotectin-treated *S. aureus* more susceptible to killing by neutrophils, which exert antimicrobial activity partly through the generation of reactive oxygen species (Fig. 2). Mutation of staphylococcal *sodA* and *sodM* results in decreased bacterial recovery from infected organs in a murine model of systemic infection. However, in calprotectin-deficient mice there is no difference in bacterial recovery between wild-type and *sodA**sodM*-inactivated *S. aureus*. Therefore, calprotectin-mediated inhibition of bacterial superoxide defenses is an important immune response to staphylococcal abscesses.

At least one bacterial species is capable of binding and inactivating calprotectin. *Fingoldia magna* is a Gram-positive anaerobic bacterium that is typically a human commensal, but is also capable of causing opportunistic infections [183]. A subset of clinical isolates of *F. magna* produce Protein L, which has domains that bind both immunoglobulins and calprotectin. *F. magna* strains that express Protein L are protected from calprotectin-mediated killing [184]. This is yet another example of bacterial circumvention of host immune responses aimed at limitation of nutrients. Whether or not *S. aureus* is capable of disrupting host-mediated manganese limitation is unknown. *S. aureus* produces an arsenal of factors that subvert neutrophil function, including pore-forming toxins, phenol soluble modulins, proteases, and molecules that inhibit chemotaxis [185]. It is possible that destruction of neutrophils and their antimicrobial contents allows *S. aureus* to avoid calprotectin-mediated manganese limitation. However, staphylococcal toxins such as the Pantone-Valentine leukocidin induce the formation of neutrophil extracellular traps, which are rich in calprotectin and thus could potentiate local manganese limitation [171, 186]. Further studies are necessary to determine if staphylococcal destruction of neutrophils is an effective subversion mechanism against calprotectin-mediated metal chelation. Nevertheless, the fact that *S. aureus* can proliferate readily in abscesses suggests the presence of systems dedicated to overcoming calprotectin-mediated manganese limitation.

The contribution of zinc to the host-pathogen interaction

Zinc is an essential nutrient for humans. Zinc deficiency occurs in several settings, including poor nutrition, mal-absorption, alcohol abuse, liver disease, and genetic disorders. The manifestations of zinc deficiency are numerous, and include a profound alteration of immune cell function. In an experimental human model, mild zinc deficiency resulted in a decreased CD4⁺/CD8⁺ T cell ratio, decreased IL-2 activity, and imbalance of TH₁ and TH₂ responses [187, 188]. Additional studies have demonstrated deleterious effects of zinc deficiency on all components of the human immune system, including neutrophils, natural killer cells, T and B lymphocytes, and monocytes [189]. Both human and animal studies have shown a correlation between zinc deficiency and enhanced susceptibility to infection. Zinc-deficient animals are more susceptible to a variety of viral, bacterial, fungal, and parasitic pathogens [190]. Conversely, human trials of zinc supplementation demonstrate protection against infectious diseases. In children, zinc supplementation reduces the duration and severity of acute diarrhea, and reduces the incidence of lower respiratory tract infection [191–193]. Zinc supplementation in patients with sickle cell disease reduces the incidence of

bacterial infections, including *S. aureus* pneumonia. In elderly adults, zinc supplementation significantly reduces the incidence of infection [194]. Taken together, these findings indicate a critical role for zinc in protection against infectious diseases.

Bacterial mechanisms of zinc homeostasis

Zinc is also an essential nutrient for bacterial pathogens. Zinc-binding proteins, which constitute an average of 5% of bacterial proteomes [195], participate in diverse processes such as regulation of virulence factors, metabolism, and inactivation of antibiotics. Zinc concentrations must be carefully regulated, as excess zinc can inappropriately bind to thiol groups of bacterial proteins and interfere with their function. In bacteria, zinc homeostasis is achieved through coordinated regulation of import and export. Bacteria express both high- and low-affinity zinc importers. ZnuABC is a high-affinity ABC-type zinc transporter best characterized in *E. coli*, consisting of a periplasmic binding protein (ZnuA), membrane permease (ZnuB), and ATPase (ZnuC) [196]. Zinc import systems homologous to Znu have been identified in several other bacterial pathogens [197]. The ZnuABC system is regulated by Zur, a metallo-regulatory protein of the Fur family, such that zinc import is repressed in zinc-replete conditions [196]. Zur has two regulatory metal binding sites, allowing for graded expression of zinc-responsive genes in accordance with zinc availability [198]. A low-affinity zinc transporter, ZupT, was identified in *E. coli* based on similarity to eukaryotic zinc transporters of the ZIP family [199]. However, the precise mechanism of zinc import by ZupT is unknown, as is its distribution among other bacterial genomes. Zinc export is essential for maintenance of homeostasis, and is achieved primarily through P-type ATPases which couple ATP hydrolysis to transport of zinc and other metals across the cell membrane. ZntA in *E. coli* mediates efflux of both zinc and cadmium [200]. Another zinc exporter (CzcCBA) was identified in *Ralstonia metallidurans*, and also mediates efflux of cobalt and cadmium [201]. Czc-type exporters have subsequently been postulated to mediate efflux of zinc in other bacteria, including *P. aeruginosa* and *S. aureus* [146, 202].

Efforts to identify the mechanisms of zinc import, export, and regulation in *S. aureus* have had mixed success. Both plasmid and chromosomally encoded zinc efflux proteins have been functionally characterized in *S. aureus*. Resistance to both zinc and cobalt is conferred by the chromosomally encoded *czrAB* operon, which encodes a metal-regulated transcriptional repressor (CzrA, also known as ZntR) and a CDF antiporter (CzrB, also known as ZntA) [146, 147, 203]. CadA is a P-type ATPase encoded by the *S. aureus* plasmid pI258 that mediates resistance to cadmium, lead, and zinc [204]. In contrast to these functionally characterized exporters, the proteins mediating zinc uptake in *S. aureus* have yet to be identified. Two genes upstream of a Zur homologue, named *mreA* and *mreB* for metal-responsive elements, were predicted to encode the ATP-binding and membrane permease components of an ABC transporter. It was postulated that *mreA* and *mreB* might encode elements of a zinc transporter based on sequence homology. However, there was no appreciable change in susceptibility to zinc limitation relative to wild type when *mreA* and *mreB* were inactivated [205]. Additional studies are needed to clarify staphylococcal mechanisms of zinc homeostasis.

Dynamic changes in zinc distribution occur in response to infection and inflammation

Imaging mass spectrometry revealed that staphylococcal murine abscesses are nearly devoid of zinc. Previous reports suggested that the antimicrobial function of calprotectin is mediated through zinc chelation, implying that calprotectin may be a mediator of zinc sequestration in staphylococcal abscesses. However, zinc levels within the staphylococcal abscess are unaffected by genetic defects in calprotectin production, raising the possibility that additional host factors contribute to zinc limitation in abscesses [34]. The acute-phase response to infection is known to alter zinc distribution in vertebrates. Administration of

human IL-1 to rats leads to decreased serum zinc concentrations with concomitant redistribution to various tissues such as the liver, bone marrow, and thymus [206]. Similarly, IL-6 administration to mice leads to hypozincemia and increased hepatic zinc concentration through upregulation of the zinc transporter Zip14 in hepatocytes [207, 208]. Limitation of zinc availability may be beneficial to controlling *S. aureus* infection, as several processes that contribute to staphylococcal virulence are zinc dependent, including biofilm formation and superantigen activity [209, 210]. However, the role of zinc acquisition in staphylococcal pathogenesis has yet to be determined in vivo.

Exploiting transition metals to battle bacterial pathogens: the example of copper

Copper is an important cofactor for a number of bacterial enzymes, but like other transition metals, it is toxic if concentrations are not carefully regulated. The precise mechanism by which copper homeostasis is achieved in *S. aureus* has not been fully elucidated. Exposure to copper induces expression of *copA* and *copZ*, which encode a P-type ATPase (CopA) and copper metalochaperone (CopZ), respectively. In *S. aureus*, CopA is involved in copper efflux, as evidenced by increased intracellular copper concentrations in a *copA* mutant [211]. Expression of *copA* is regulated by the copper-sensitive operon repressor (CsoR) [212, 213]. Copper binding by CsoR results in a conformational change, causing release of operator DNA and de-repression of the copper-sensitive operon. CopZ has not been functionally characterized in *S. aureus*. In *B. subtilis* and *L. monocytogenes*, CopZ interacts and exchanges copper with CopA, and is therefore predicted to protect bacteria from the inappropriate binding of copper to cytoplasmic proteins [214, 215]. Additional studies are needed to characterize copper homeostasis in *S. aureus*, and to assess the role of copper acquisition in virulence.

Disruption of copper homeostasis reduces the virulence of select bacterial pathogens. Deletion of *copA* in *S. pneumoniae* causes reduced virulence in a mouse pneumonia model [216]. Similarly, inactivation of a putative copper exporter (MctB) in *M. tuberculosis* significantly reduces bacterial burden in the lungs of infected guinea pigs [217]. Finally, mutation of the *P. aeruginosa* gene *cueA*, an ATPase involved in protection from copper toxicity, results in a 50-fold decrease in virulence in a lethal dose murine model [218]. Collectively, these results reveal that diverse bacterial pathogens encounter potentially toxic levels of copper in vivo, and invite the question of whether modulation of copper levels in host tissues might be an innate immune response to infection. The importance of copper to the vertebrate immune response is demonstrated by copper-deficient mice, which exhibit an impaired humoral immune response and enhanced susceptibility to a variety of bacterial pathogens [219, 220]. In contrast to iron, manganese, and zinc, the levels of copper increase in response to infection and inflammation [221, 222]. This effect is partially mediated by proinflammatory cytokines, which stimulate hepatic synthesis of ceruloplasmin, the major plasma copper-binding protein [223]. Inflammatory cytokines also mediate a copper-dependent antibacterial response in macrophages. In response to interferon-gamma, macrophages increase the expression of two copper transporters, CTR1 and ATP7A. ATP7A is trafficked to the phagosome where it imports bactericidal levels of copper. Conversely, RNAi-mediated depletion of ATP7A leads to increased bacterial survival in the phagosome [220]. Therefore, in addition to nutritional immunity mediated by metal ion sequestration, vertebrate hosts can increase metal ion concentration in certain environments to kill bacterial pathogens.

The toxicity of transition metals has been utilized in the development of new antimicrobials for multi drug-resistant *S. aureus*. For example, copper-coated surfaces exhibit rapid killing of MRSA as compared with brass and stainless steel [224]. Similarly, orthopedic implants

made of a titanium-copper alloy are antibacterial in a rabbit model of staphylococcal implant-associated infection [225]. Copper-containing biocides have shown promise as hand sanitization gels and environmental disinfectants [226, 227]. These preliminary studies reveal that copper-containing compounds are effectively antibacterial at concentrations that are not cytotoxic to human or animal cells. How exposure to toxic levels of copper kills *S. aureus* is unknown. In response to copper exposure, *S. aureus* initiates a transcriptional program characterized by induction of oxidative stress and misfolded protein responses, suggesting that copper toxicity results in increased oxidative stress and protein damage [228]. Like iron, copper can interact with hydrogen peroxide to generate damaging reactive oxygen species. *S. aureus* strains inactivated for the hydrogen peroxide-reducing enzymes KatA and AhpC are more sensitive to copper toxicity, further supporting oxidative stress as a mechanism for copper-mediated staphylococcal killing.

Imaging mass spectrometry, a powerful tool for the study of metal ion distribution during bacterial infection

The discovery of calprotectin's role in manganese sequestration during staphylococcal infection was facilitated by imaging mass spectrometry (IMS). IMS uses matrix-assisted laser desorption/ionization time of flight to directly analyze a variety of molecules in tissue sections. IMS has been used to analyze proteins, peptides, lipids, drugs, and metabolites [229]. Whereas sample preparation for conventional proteomic analyses results in disruption of tissue architecture, IMS allows for demonstration of sample distribution in intact tissues. Furthermore, IMS can be combined with additional imaging modalities such as magnetic resonance imaging to provide a three-dimensional co-registration of proteomic data with anatomy [230].

IMS was instrumental in the identification of calprotectin as an innate immune effector responding to staphylococcal abscesses [34]. Examination of mice infected with *S. aureus* by IMS revealed that calprotectin co-localized to abscessed tissue in a neutrophil-dependent manner. Given previous reports that calprotectin functions via metal chelation, laser-ablation inductively coupled plasma mass spectrometry (LA-ICPMS) was used to demonstrate metal ion distribution and concentrations in abscessed tissue. LA-ICPMS is a highly sensitive form of mass spectroscopy capable of quantifying elemental abundance within two dimensions. As discussed above, LA-ICPMS analysis of infected murine tissues allowed determination of manganese and zinc distribution in staphylococcal abscesses, and confirmed that manganese is limited in abscessed tissue. LA-ICPMS therefore allows for determination of both spatial distribution of metal ions as well as their absolute concentrations in a range of infected tissues. These technologies will help to determine whether calprotectin-mediated metal chelation is an immune response specific to staphylococci in abscesses, or is a more general immune strategy to limit bacterial growth in a diverse range of host tissues.

In addition to the study of the host responses to bacterial infection, IMS offers a number of exciting microbiologic applications. IMS can be used to image single microbial colonies, bacteria growing in biofilms, interactions between two different microbes, and poly-microbial communities [231]. The ability to characterize microbial physiology in vivo, together with the capability to assess host immune responses, provides an opportunity for comprehensive study of bacterial infections. For example, IMS analysis of experimental staphylococcal infection could be utilized to reveal microbial colonization factors facilitating adhesion, host proteins responding to colonization and tissue damage, changes in elemental distribution in response to infection, in vivo proteome analysis of biofilms, distribution of antibiotics in infected tissues, and bacterial proteomic changes in response to antimicrobial therapy. This paradigm could be applied to a number of different host tissues

and pathogens. These broad applications make IMS an exciting and powerful tool for study of bacterial infection.

Concluding remarks

The success of *S. aureus* as a human pathogen is facilitated by its ability to infect and survive within diverse host tissues. The ability to acquire nutrients from the host, including metal ions, is critical for staphylococcal infection. Despite innate immune mechanisms that limit the availability of transition metals, *S. aureus* can proliferate in vertebrate hosts through the expression of metal acquisition systems that successfully overcome nutritional immunity. Our understanding of the pathways that mediate iron acquisition in *S. aureus* has increased considerably. During infection, staphylococci sense low levels of iron in host tissues and respond by initiating a transcriptional program that includes expression of iron-acquisition systems. Iron acquisition is critical to *S. aureus* virulence, and therefore is an attractive target for the development of new antimicrobials and vaccines.

Emerging data suggest that host limitation of manganese is also an essential component of the immune response to staphylococcal infection. Calprotectin-mediated manganese sequestration limits *S. aureus* growth in abscesses, in part by disrupting manganese-dependent oxidative stress defenses. The expression of high-affinity manganese transporters allows *S. aureus* to overcome manganese sequestration and survive within the hostile environment of an abscess. Additional studies suggest that infection-induced redistribution of other metal ions such as copper and zinc also contribute to resistance to microbial infection. Unlike iron, manganese, and zinc, host copper concentrations increase in response to infection, suggesting that the human immune system may exploit the toxicity of copper as an innate immune mechanism against bacterial pathogens.

There are several questions yet to be answered regarding staphylococcal metal ion acquisition and the host mechanisms that limit metal availability. Thus far, the contribution of metal acquisition systems to staphylococcal virulence has been assessed primarily in defined organ abscesses. Although skin and soft tissue abscesses are common clinical manifestations of staphylococcal disease, infection of other tissues such as bone, lung, or heart may not be accompanied by formation of defined abscesses. Furthermore, the availability of essential metal nutrients may differ among host tissues, raising the possibility that the contribution of metal acquisition to staphylococcal pathogenesis may vary according to the route and primary focus of infection. For example, concomitant inactivation of both *isdB* and *isdH* leads to a significant reduction in bacterial recovery from the lungs, heart, and kidneys of mice infected intravenously with *S. aureus*. In contrast, when mice were infected intranasally with *S. aureus isdBH* mutants, there is no significant difference in bacterial recovery from the lungs in comparison to wild type [131]. Additional experiments are therefore needed to establish the importance of staphylococcal metal acquisition, and conversely host-mediated metal sequestration, for infection of diverse host tissues.

The kinetics of host-mediated metal sequestration during staphylococcal infection are unknown. LA-ICPMS studies reveal that mature staphylococcal abscesses are essentially devoid of manganese and zinc. However, the metal concentrations encountered by invading staphylococci prior to abscess formation have not been determined. An understanding of the role of staphylococcal metal acquisition during earlier phases of infection is valuable, as there may be opportunities for therapeutic intervention prior to dissemination and formation of destructive tissue lesions. Further experiments are also necessary to clarify the mechanisms of metal ion homeostasis in *S. aureus*. In contrast to our knowledge of *S. aureus* iron acquisition and homeostasis, little is known about how staphylococci procure and regulate the concentration of other transition metals such as copper and zinc. The

availability of sensitive imaging mass spectrometry methods will facilitate further study of the struggle for metals between host and bacterium. Determination of additional mechanisms by which staphylococci overcome nutritional immunity may generate new targets for antimicrobial design and vaccine development.

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Fig. 1.

S. aureus overcomes nutritional immunity to obtain iron from the host. **a** During infection, staphylococcal hemolysins lyse red blood cells to release hemoglobin, which can be further degraded to heme and free iron. **b** In response to infection, vertebrate hosts fortify iron sequestration. Free iron is bound by transferrin and lactoferrin, free heme by hemopexin, and hemoglobin by haptoglobin to limit iron availability to invading pathogens. **c** *S. aureus* produces two side-rophores, staphyloferrin A and staphyloferrin B, which bind iron with high affinity and allow competition with iron sequestration by lactoferrin and transferrin. Import of iron bound to staphyloferrin A and staphyloferrin B is mediated by HtsABC and SirABC, respectively, in processes powered by the FhuC ATPase. **d** The Isd system mediates acquisition of heme, the preferred iron source during *S. aureus* infection. IsdB and IsdH are cell-surface receptors for hemoglobin and hemoglobin-haptoglobin, respectively. IsdB and IsdH pass heme to IsdA, or alternatively to IsdC or IsdE, culminating in transport

across the cell membrane by IsdDEF. In the cytoplasm, heme is degraded by IsdG or IsdI to release iron

Fig. 2.

Calprotectin-mediated nutritional immunity of manganese and zinc. **a** In staphylococcal tissue abscesses, neutrophils release calprotectin, reactive oxygen species, and other antibacterial substances. **b** *S. aureus* acquires manganese via the manganese transporters MntABC and MntH. Zinc acquisition occurs via an undetermined mechanism. Manganese acquisition is critical for detoxification of superoxide anions via SodA and SodM, and thus contributes to resistance to killing by neutrophils and increases staphylococcal survival within abscesses. **c** Calprotectin binds manganese and zinc, limiting the availability of these critical nutrients in abscesses. The concomitant decrease in staphylococcal manganese and zinc acquisition cripples oxidative stress defenses and zinc-dependent processes, leading to decreased bacterial survival in the abscess.