BioMetals (2006) 19:193–203
DOI 10.1007/s10534-005-4863-7

Iron acquisition and transport in *Staphylococcus aureus*

A.W. Maresso & Olaf Schneewind*
*Department of Microbiology, University of Chicago, CLSC Room 601, 920 E 58th Street, Chicago, IL 60637, USA; *Author for correspondence (Tel.: +1-773-834-9060; E-mail: oschnee@bsd.uchicago.edu)*

Received 12 October 2005; accepted 7 November 2005

Key words: *Bacillus anthracis*, heme, iron, *isd*, *isdC*, sortase, *Staphylococcus aureus*

Abstract

Pathogenic Gram-positive bacteria encounter many obstacles in route to successful invasion and subversion of a mammalian host. As such, bacterial species have evolved clever ways to prevent the host from clearing an infection, including the production of specialized virulence systems aimed at counteracting host defenses or providing protection from host immune mechanisms. Positioned at the interface of bacteria/host interactions is the bacterial cell wall, a dynamic surface organelle that serves a multitude of functions, ranging from physiologic processes such as structural scaffold and barrier to osmotic lysis to pathogenic properties, for example the deposition of surface molecules and the secretion of cytotoxins. In order to succeed in a battle with host defenses, invading bacteria need to acquire the nutrient iron, which is sequestered within host tissues. A cell-wall based iron acquisition and import pathway was uncovered in *Staphylococcus aureus*. This pathway, termed the *isd* or iron-responsive surface determinant locus, consists of a membrane transporter, cell wall anchored heme-binding proteins, heme/haptoglobin receptors, two heme oxygenases, and sortase B, a transpeptidase that anchors substrate proteins to the cell wall. Identification of the *isd* pathway provides an additional function to the already bountiful roles the cell wall plays in bacterial pathogenesis and provides new avenues for therapeutics to combat the rise of antimicrobial resistance in *S. aureus*. This review focuses on the molecular attributes of this locus, with emphasis placed on the mechanism of iron transport and the role of such a system during infection.

Introduction

Bacteria colonize nearly every environmental niche and the vastness of their inhabitation is testament of microbial adaptation and genomic plasticity. Whereas most bacteria inhabit environments that are foreign to humans, the habitat of several species does overlay with our own. Most of these interactions can be classified as symbiotic, where both participants receive a benefit from the relationship. In several instances, however, bacteria and their mammalian hosts engage in molecular warfare (Finlay & Cossart 1997). When this occurs, pathogenic bacteria yield weaponry to invade, suppress, and intoxicate their mammalian hosts (Casadevall & Pirofski 2004). These systems are diverse in structure and function, ranging from the upregulation of specific adhesion molecules to the assembly of specialized translocation systems which inject toxins or effectors into host cells (Finlay & Falkow 1997). Although many of these components are critical for bacterial survival, one of the most dynamic is the cell wall of Gram-positive bacteria (Ghuysen & Hakenbeck 1994). In many contexts, the cell wall lies at the interface of the interaction between a eukaryotic cell and the bacterial pathogen. Gram-positive pathogens have evolved complex protein transport systems that serve to subvert host physiology (Navarre & Schneewind 1999), including the binding and transport of the essential nutrient iron (Braun 2001; Wandersman & Delepelaire 2004). Recently, a novel cell wall based iron acquisition

system, the *isd* locus, was described in *S. aureus* (Skaar & Schneewind 2004). The characterization of this system has provided new insights into cell wall function and pathogen/host interactions.

S. aureus cell wall structure and function

The staphylococcal cell wall is composed of varying length glycan strands of repeating disaccharide *N*-acetylmuramic acid-(β1-4)-*N*-acetylglucosamine (MurNAc-GlcNAc) (Ghuysen & Strominger 1963; Ghuysen *et al.* 1965; Dmitriev *et al.* 2004). MurNAc is amide linked to alanine of the cell wall tetrapeptide [L-Ala-D-isoGln-L-Lys(NH_2-Gly_5)-D-Ala], which is tethered to neighboring peptidoglycan strands via an amide bond between the amine of the pentaglycine crossbridge (NH_2-Gly_5) and the carboxyl of D-alanine at position four of the cell wall peptide (Ghuysen *et al.* 1965; Tipper *et al.* 1965; Tipper & Strominger 1965; Tipper 1968) (Figure 1). In fully assembled, mature cell wall, a network of peptidoglycan strands and crosslinks gives rise to a single large macromolecule, whose three-dimensional lattice spans the circumference of bacterial cells (Ghuysen 1968; Labischinski & Maidhof 1994). Cell wall peptidoglycan is decorated with several diverse modifications, including polysaccharide, lipoteichoic acid, teichoic and teichuronic acids. The molecular structure, biosynthesis and functional properties of these modifications have been reviewed elsewhere (Robbins & Schneerson 1990; Fischer 1994; Neuhaus & Baddiley 2003).

Perhaps the most dynamic components of the cell wall are proteins that associate with the peptidoglycan (Navarre & Schneewind 1999). For example, the cell wall of *S. aureus* and other Gram-positive pathogens contain proteins involved in metabolism such as fructosidase (Burne & Penders 1994). Cell wall anchored proteins in other species are involved in conjugal transfer of DNA between two mating bacteria (Galli *et al.* 1990, 1992; Clewell 1993). Surface proteins of *Listeria monocytogenes* bind to host cell receptors and promote bacterial entry into host epithelial cells during infection (Gaillard *et al.* 1991; Dramsi *et al.* 1995). Thus, these factors lie directly at the interface of the host/pathogen interaction and have evolved for the subversion, modulation, and in many cases elimination of host adaptive and acquired immune responses. As examples for the latter strategies, staphylococcal protein A and streptococcal M proteins, respectively, bind the Fc portion of immunoglobulin as a means of evading opsono-phagocytosis (Moks *et al.* 1986; Herwald *et al.* 2004). Further, many Gram-positive pathogens adhere to host extracellular matrix using fibronectin binding proteins as an early step in the infectious process (Flock *et al.* 1987; Patti *et al.* 1994). Other cell wall proteins, such as C5a peptidase, cleave components of the complement system, thereby subverting the innate immune response such as complement mediated phagocytic killing (Chen & Cleary 1990). Collectively, these cell wall anchored proteins contribute to the disease-causing arsenal of Gram-positive pathogens (Foster & Höök 1998).

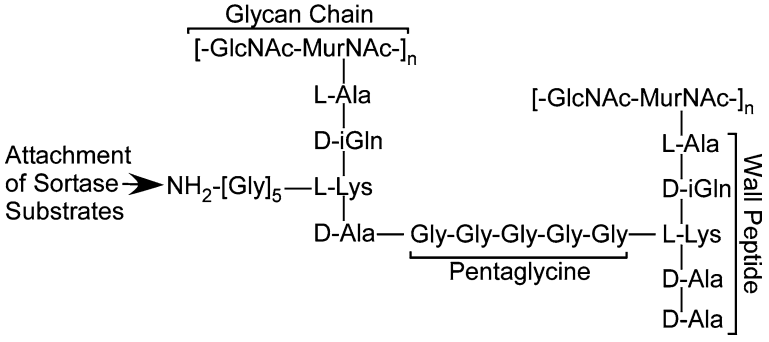

Figure 1. Structure of the peptidoglycan of *S. aureus*. The staphylococcal cell wall is composed of varying length glycan strands of repeating disaccharide *N*-acetylmuramic acid-(β1-4)-*N*-acetylglucosamine (GlcNAc-MurNAc). MurNAc is amide linked to alanine of the pentapeptide [L-Ala-D-iGln-L-Lys($Gly_{(5)}$)-D-Ala], which is crosslinked to the peptidoglycan through a pentaglycine crossbridge attached to lysine of the next pentapeptide. The free amine of the pentaglycine is the site of sortase-mediated anchoring of surface proteins.

Sortase A, a transpeptidase that anchors surface proteins to the cell wall

Many proteins destined to be displayed on the surface of Gram-positive pathogens are anchored to the cell wall by a class of enzymes termed sortases (Srt), transpeptidases which recognize a conserved LPXTG motif at the C-terminus of surface protein substrates (Schneewind *et al*. 1992; Mazmanian *et al*. 1999) (Figure 2). Approximately 176 homologs of SrtA, the sortase that was first described in *S. aureus* (Mazmanian *et al*. 1999), have been identified and can be grouped into five different classes (Pallen *et al*. 2001; Comfort & Clubb 2004; Ton-That *et al*. 2004; Boekhorst *et al*. 2005; Dramsi *et al*. 2005). More than 900 potential surface protein substrates in over 50 different species of Gram-positive bacteria have been revealed with bioinformatics approaches (Fischetti *et al*. 1990; Navarre & Schneewind 1999; Pallen *et al*. 2001; Comfort & Clubb 2004; Boekhorst *et al*. 2005). The importance of sortase in pathogenesis has been elucidated by the finding that SrtA of *S. aureus* displays a three-log reduction in virulence in tissue abscess mouse models of infection (Mazmanian *et al*. 2000; Jonsson *et al*. 2002). Sortase's ubiquitous distribution in many Gram-positive bacteria and its requirement for virulence, coupled to the fact that sortase is located in the envelope and accessible to inhibitors highlights these transpeptidases as potential targets for the development of antimicrobial therapeutics (Weiss *et al*. 2004).

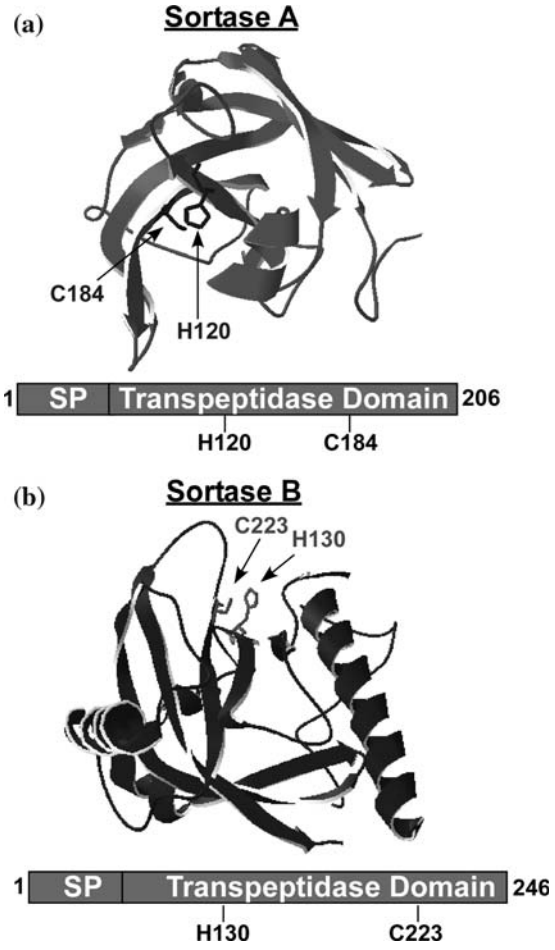

Figure 2. Crystal structures and functional domains of S. aureus SrtA and SrtB. *S. aureus* sortase A (a) and B (b) demonstrating active site amino acids C184 and H120 (a) and H130 and C223 (b) extending from a hydrophobic cleft that binds LPXTG (SrtA) or NPQTN (SrtB) recognition motifs. SrtA PDB ID = 1T2P, SrtB PDB ID = 1QWZ.

The C-terminal signature motif for sortase recognition is composed of a canonical sequence motif (LPXTG for sortase A), followed by a hydrophobic stretch of amino acids and positively charged residues (Schneewind *et al*. 1993). Although almost all Gram-positive microbes contain sortase A and LPXTG motif surface proteins, the motif can vary in amino acid composition. For example, leucine (L) in the first position is replaced with asparagine (N) and glycine at the fifth position is replaced with asparagine (N) in sortase B substrates (SrtB subfamily) or alanine (A) in substrates of subfamily four sortases (Comfort & Clubb 2004; Boekhorst *et al*. 2005). The current model for sortase-mediated anchoring suggests surface-displayed proteins are synthesized in the bacterial cytoplasm as a "P1" precursor which undergoes N-terminal signal peptide cleavage during translocation across the cytoplasmic membrane, thereby generating the "P2" precursor (Schneewind *et al*. 1993; Ton-That *et al*. 1998) (Figure 3). The sorting signal of surface-anchored proteins retains the polypeptide in the secretory pathway, in close proximity to sortase. Sortase then cleaves between the threonine (T) and the glycine (G) of the LPXTG motif to generate a thioacyl-enzyme intermediate between the active site cysteine residue of sortase (C184 in *S. aureus* SrtA) and the C-terminal threonine of the cleaved substrate (Navarre & Schneewind 1994; Ton-That *et al*. 1999; Ton-That *et al*. 2000;

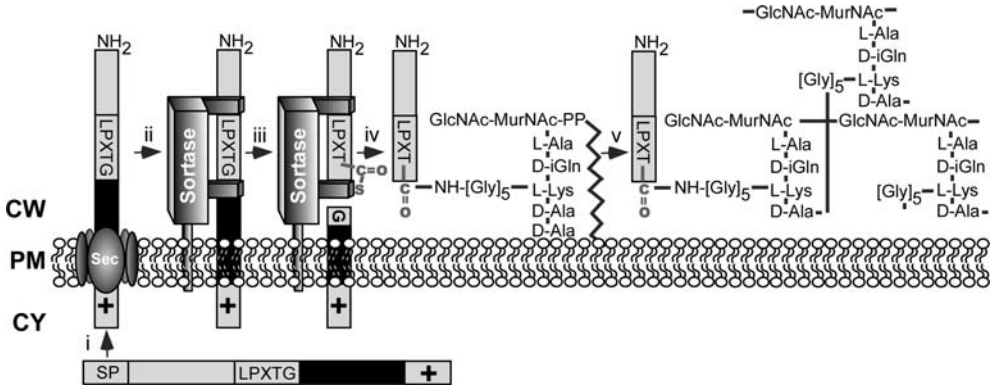

Figure 3. Model of sortase-mediated anchoring. (i, ii) Proteins to be surface anchored to the cell wall are initiated onto the Sec pathway by an N-terminal signal peptide (SP). (iii) Following cleavage of the LPXTG-recognition motif, a thioacyl-enzyme intermediate is formed between sortase and threonine of surface protein. (iv, v) Resolution of the thioacyl-enzyme occurs through nucleophilic attack of the amine group of the pentaglycine of lipid II to generate lipid II-linked surface protein, which is incorporated into the cell wall. The black bar and "+" represent the hydrophobic patch and poly-basic cluster, respectively, of amino acids which comprise the sorting signal.

Ton-That *et al*. 2002). The thioacyl-enzyme intermediate is resolved by the nucleophilic attack of the amine within pentaglycine crossbridges thereby forming an amide bond between surface protein and lipid II cell wall biosynthesis precursor ("P3" intermediate) (Perry *et al*. 2002). Transpeptidation and transglycosylation of cell wall biosynthesis incorporate lipid-linked P3 intermediates into the peptidoglycan to generate mature, cell wall-anchored surface protein (Schneewind *et al*. 1995; Ton-That *et al*. 1997 Navarre *et al*. 1998; Ton-That & Schneewind 1999).

Iron transport and the iron-responsive surface determinant (*isd*) system of *S. aureus*

Iron is essential for basic bacterial physiologic processes such as electron transport or nucleotide synthesis. However, free iron is virtually not available in host environments (Wandersman & Delepelaire 2004). Instead iron is complexed with other molecules, such as protoporphyrins (heme) or proteins (ferritin, transferrin, lactoferrin, hemoglobin, etc). Upon entry into a mammalian host, a bacterial pathogen must acquire iron from tissues to survive (Wandersman & Delepelaire 2004). *S. aureus* has evolved several different sophisticated iron uptake systems. Here we review these iron uptake systems by analyzing the regulation of gene expression, sequestration of target iron, bacterial iron transport, and release of iron from heme tretrapyrrol.

Regulation

Regulation refers to the concept that bacteria must be able to sense and appropriately respond to the need to acquire iron from the environment (Holmes 2000). This response operates at the level of gene expression for the factors that are responsible for this uptake, and includes transcriptional, translational, and post-translational regulatory mechanisms (Holmes 2000). In many bacterial pathogens, this feat is accomplished by the global determinant ferric uptake repressor (Fur) protein (Hantke 1981; Hantke 2001). *S. aureus* Fur was identified by PCR amplification of a region of the staphylococcal genome which has homology to Fur sequences from other Gram-positive bacteria (Xiong *et al*. 2000; Horsburgh *et al*. 2001). Purified Fur bound to the promoter regions of the ferrichrome-uptake system (*fhu*), an operon believed to code for two ferrichrome permeases and an ATP-binding protein (Xiong *et al*. 2000). Further, inactivation of *fur* and *perR*, a homologue of *fur*, led to a reduction in virulence in a mouse model of *S. aureus* infection (Horsburgh *et al*. 2001).

Sequestration

More than 95% of the iron in the human body is located within host cells, which poses a problem for extra-cellular pathogens like *S. aureus*. To circumvent this barrier to growth, *S. aureus* has evolved mechanisms to scavenge extracellular iron.

Sequestration refers to the binding of molecular iron or iron-complexed molecules by factors produced and secreted into the surrounding milieu by the bacterium. These factors include siderophores, low-molecular weight compounds that chelate iron with high affinity. In *S. aureus*, several siderophores have been identified, termed staphyloferrin A and B and aureochelin (Konetschny-Rapp *et al.* 1990; Drechsel *et al.* 1993; Courcol *et al.* 1997). Recently, an iron-regulated operon containing nine genes that function in the production of a novel siderophore has been described, termed *sbn* for siderophore biosynthesis. Mutation of one gene in this operon, *sbnE*, eliminated siderophore production and virulence in a mouse model of infection, indicating that this system is important in *S. aureus* infections (Dale *et al.* 2004a). In addition to siderophores, proteins anchored to the staphylococcal surface also act to sequester iron. The observation that transferrin, an iron-binding protein in extra-cellular host fluids, binds to staphylococcal transferrin-binding protein A (StbA, also known as the heme-binding protein IsdA (Mazmanian *et al.* 2003)), suggests that Gram-positive pathogens may elaborate iron sequestration mechanisms that differ from siderophore-mediated acquisition (Taylor & Heinrichs 2002).

Transport

Transport of iron from the surrounding milieu into the bacterium involves translocation of molecules across the 50–100 nm diameter of the cell wall envelope and cytoplasmic membrane of *S. aureus*. At least four *S. aureus* ABC transporter systems have been described: *sirABC*, *fhuABC*, *sitABC*, and *sstABC* operons (Morrissey *et al.* 2000; Cabrera *et al.* 2001; Dale *et al.* 2004b, c). ABC transporters are hypothesized to utilize ferric-siderophore binding receptors to translocate secreted siderophores complexed with iron into the bacterial cytoplasm. These receptors are tethered to the cytoplasmic membrane through a diacylglycerol moiety linked to their N-terminal amino acid (Hantke & Braun, 1973). In addition to lipoproteins functioning directly in siderophore transport, membrane ATPases are believed to provide the energy for the translocation of iron across the plasma membrane.

Release

As most iron in host tissues is stored by heme-binding proteins located within cells, staphylococci are faced with the problem of releasing iron sources via the secretion of hemolysins, membrane active toxins that penetrate and damage host cell membranes (Bernheimer *et al.* 1968; Jonsson *et al.* 1985; Dinges *et al.* 2000). Assuming that heme-iron is then captured and transported into staphylococci, how can the iron atom be released from heme tetrapyrrol for use in biosynthetic processes? Heme monoxygenases catalyze oxidative degradation of heme to biliverdin, CO_2, and iron (Maines 1997). This reaction is conserved between mammals and some bacterial species and serves different functions depending on the organism in question. The best characterized bacterial heme oxygenases are those of *Pseudomonas aeruginosa* and *Neisseria miningitidis* (Schmitt 1997; Zhu *et al.* 2000; Ratliff *et al.* 2001; Stojiljkovic & Perkins-Balding 2002). To date, two types of heme oxygenase systems have been described in Gram-positive pathogens, HmuO from *Corynebacterium diphtheriae*, the causative agent of diphtheria, and IsdG and IsdI from *S. aureus* (discussed below) (Wilks & Schmitt 1998; Wilks 2002; Skaar *et al.* 2004a; Wu *et al.* 2005).

Iron regulated surface determinants (Isd) of *S. aureus*

The recently identified iron-response surface determinant (*isd*) locus in *S. aureus*, comprises genes that are involved in each of the four aspects of iron acquisition briefly summarized above, and can therefore be used for the establishment of a paradigm for similar systems in other Gram-positive bacteria (Mazmanian *et al.* 2003).

The *isd* locus was discovered during a search of the *S. aureus* genome for SrtA homologs, which uncovered another sortase, SrtB (Mazmanian *et al.* 2002). The structural *srtB* gene is located within a cluster of eight genes and three transcriptional units, *isdA*, *isdB*, and *isdCDEF srtB isdG*). One of these genes, *isdC*, encodes a secreted polypeptide substrate of sortase B with a C-terminal sorting signal, including an NPQTN motif, hydrophobic domain and charged tail (Mazmanian *et al.* 2002). Fur binding sites are

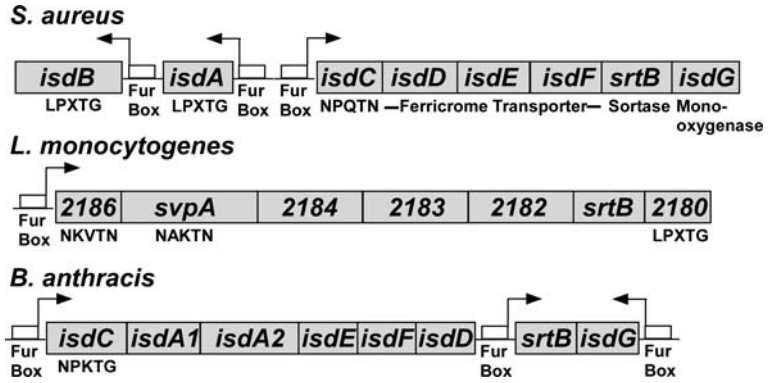

Figure 4. Genomic organization of the *isd* operon in *S. aureus*, *L. monocytogenes* and *B. anthracis*. The *S. aureus isd* operon is described in the text. The *Listeria monocytogenes isd* locus contains *isdC* and *isdA* homologs (*2186* and *svpA*, respectively), a ferrichrome transporter with homology to *isdDEF* (*2182-4*), a sortase (*srtB*), and an LPXTG-containing protein of unknown function (*2180*). The ORFs are described according to the *L. monocytogenes* nomenclature. The *Bacillus anthracis isd* locus contains eight open reading frames, including a sortase (*srtB*), *isdC* and *isdA* homologs, a ferrichrome transporter (*isdDEF*), and a monoxygenase (*isdG*). Fur boxes and sortase-recognition motifs are indicated.

located upstream of the three transcriptional *isd* units, and the expression of all of the genes in this locus is regulated by the availability of iron (Mazmanian *et al*. 2003). Further, several *isd* genes contain homology to proteins known to play a role in iron acquisition. Specifically, the *isd* locus encodes for a putative membrane transport system (IsdDEF), cell wall-anchored heme (IsdA and IsdC), hemoglobin (IsdB) and hemoglobin-haptoglobin (IsdH/HarA) binding proteins, and two monooxygenases (IsdG and IsdI) (Mazmanian *et al*. 2003) (Figure 4). Homologs of the *isd* locus are found in *Bacillus anthracis* and *Listeria monocytogenes*, suggesting that *isd*, or components of it, functions also in other Gram-positive pathogens (Bierne *et al*. 2004; Newton *et al*. 2005) (Figure 4). To investigate the role of SrtB in the function of this locus, a FLAG epitope-tagged version of IsdC was constructed and expressed in a *S. aureus* s*rtB* mutant strain (Mazmanian *et al*. 2002). Unlike wild-type *S. aureus*, IsdC-FLAG was not anchored to the cell wall in *srtB*$^{-}$ variants, suggesting that IsdC serves as a substrate of SrtB *in vivo*. A peptide bearing the sequence NPQTN (IsdC sorting signal) was cleaved by recombinant SrtB *in vitro* and *in vivo* between the threonine (T) and the asparagine (N) residues and the C-terminal threonine of IsdC is linked to pentaglycine cell wall crossbridges of staphylococcal peptidoglycan (Marraffini & Schneewind 2005). Unlike sortase A anchored surface proteins (IsdA, IsdB and IsdH), sortase B anchored IsdC is buried within the staphylococcal murein envelope and this unique position is presumably important for the passage of heme-iron across the cell wall (Marraffini & Schneewind 2005). Taken together, these data reveal that SrtB, just like SrtA, functions as a transpeptidase and catalyzes cleavage and cell wall anchoring of its IsdC protein substrate (Mazmanian *et al*. 2002). *srtB* expression in *S. aureus* was observed when *fur* was disrupted, demonstrating that *srtB* is regulated in an iron-dependent manner similar to other genes in the *isd* locus (Mazmanian *et al*. 2002).

Determination of the role of SrtB and SrtB-mediated anchoring in pathogenesis was investigated using a mouse model of renal abscess formation (Albus *et al*. 1991). In this experiment, mice were injected with 1×10^{7} colony forming units of isogenic *S. aureus* variants harboring deletions of the target gene(s), followed by a five to nine day incubation to allow for the infection to progress (Mazmanian *et al*. 2002). Homogenized kidneys were then assayed for abscess formation by incubation of homogenate on agar plates to observe colony formation, an indicator of relative bacterial load in each respective mouse tissue. Using this model, it was determined that loss of the *srtB* gene resulted in 10-fold reduction in the number of colony forming units isolated after a nine day infection as opposed to no effect seen at five days (Mazmanian *et al*. 2002). This reduction in the pathogenesis of *S. aureus srtB* mutants has been observed in other experimental systems,

indicating that SrtB and IsdC are involved in the persistence, rather than the establishment, of *S. aureus* infections (Jonsson *et al.* 2003; Weiss *et al.* 2004).

If the *isd* operon encodes for proteins involved in the uptake of heme-iron, one would expect components of *isd* to bind heme-iron. To test this hypothesis, IsdA, IsdB, IsdC, IsdD, IsdE and IsdG were purified as either $His_{(6)}$ or glutathione-*S*-transferase (GST) fusions, electrotransferred to membrane and incubated with hemin, followed by quantification of the amount of hemin bound per pmol purified protein. All *isd* proteins assayed bound hemin at levels ~5--15-fold higher than the control protein GST. Subsequent investigation revealed the IsdC--heme interaction was non-covalent and competitively inhibited by heme addition, suggesting that IsdC may function in the binding and passage of heme-iron across the cell wall envelope (Mazmanian *et al.* 2003).

In addition to heme acquisition, the *isd* locus encodes for proteins that bind other iron-containing molecules, such as the proteins hemoglobin and haptoglobin. Hemoglobin is the most abundant iron-binding protein in the blood and, when complexed with haptoglobin, constitutes the major iron-binding entity in the body. IsdH, also called HarA, binds hemoglobin and hemoglobin–haptoglobin complexes within the N-terminal part of the protein (Dryla *et al.* 2003).

A more detailed appreciation of the *isd* locus as an iron uptake system occurred with the investigation of IsdG and IsdI. Initial bioinformatics analysis suggested that IsdG and IsdI represented members of the ABM family of monooxygenases, first discovered in *Streptomyces* spp., and led to the hypothesis that IsdG and IsdI may be involved in the oxidative degradation of heme (Skaar *et al.* 2004a). Purified IsdG and IsdI bound heme and, in the presence of a suitable electron donor such as ascorbate or NADPH-cytochrome P450 reductase, these polypeptides catalyzed oxidative degradation of heme to biliverdin, a degradation product of mammalian heme oxygenases. Further, IsdI complemented the heme utilization defect of a *C. ulcerans* heme oxygenase mutant, suggesting IsdI performs heme degradation *in vivo* (Skaar *et al.* 2004a).

If *S. aureus* scavenges iron from multiple different biological sources, does this pathogen have a preference for one form of iron over another during infection? The growth phase-dependent preference for different iron sources of *S. aureus* was investigated with isotopically labeled hemin with ^{54}Fe and transferrin with ^{57}Fe by measuring the ratios $^{57}Fe/^{56}Fe$ (transferrin) to $^{54}Fe/^{56}Fe$ (heme) with inductively coupled plasma mass spectrometry (ICP-MS) (Skaar *et al.* 2004b). The data demonstrated that *S. aureus* preferentially utilizes heme-iron during growth in culture, and that significant amounts of heme-iron are incorporated into membrane factors, presumably electron transport factors (von Wachenfeldt & Hederstedt 1990). Inactivation of the *isd* locus and three other iron transport systems in *S. aureus* (SstABCD, SirABC, and FhuCBG), followed by $^{57}Fe/^{56}Fe$ to $^{54}Fe/^{56}Fe$ analysis, indicated these systems were not responsible for the observed heme-iron preference in *S. aureus* (Skaar *et al.* 2004b) This data suggested an unidentified heme-iron transport system was responsible for the observed preference. A combination of bioinformatics and iron transport analysis of the *S. aureus* genome revealed an uncharacterized iron transport system in *S. aureus* which was termed *htsABC* (heme transport system). Two of these genes, *htsB* and *C*, are hypothesized to be ABC transporter permeases and display sequence similarity to transport systems in *Yersinia enterocolitica* and *Corynebactrium diphtheriae*. Further, a Fur binding sequence is located just upstream of *htsA*, suggesting the *hts* system is regulated in an iron-dependent manner. Inactivation of *htsB* or *htsC* resulted in an increased ratio of transferrin to heme-iron, suggesting these genes function in the selective uptake of heme-iron. Investigation of the role of *htsB* and *htsC* in pathogenesis using the mouse model of abscess formation indicated both genes were important in disease progression in the liver and kidney. These experiments established the *hts* locus as a novel heme-iron acquisition system with pathogenic implications and provide an additional avenue towards the development of potential therapeutics to combat *S. aureus* infections (Skaar *et al.* 2004b).

Model for iron acquisition systems in the cell wall envelope of *S. aureus*

A functional model for the *isd* system in *S. aureus* is displayed in Figure 5. During the infectious

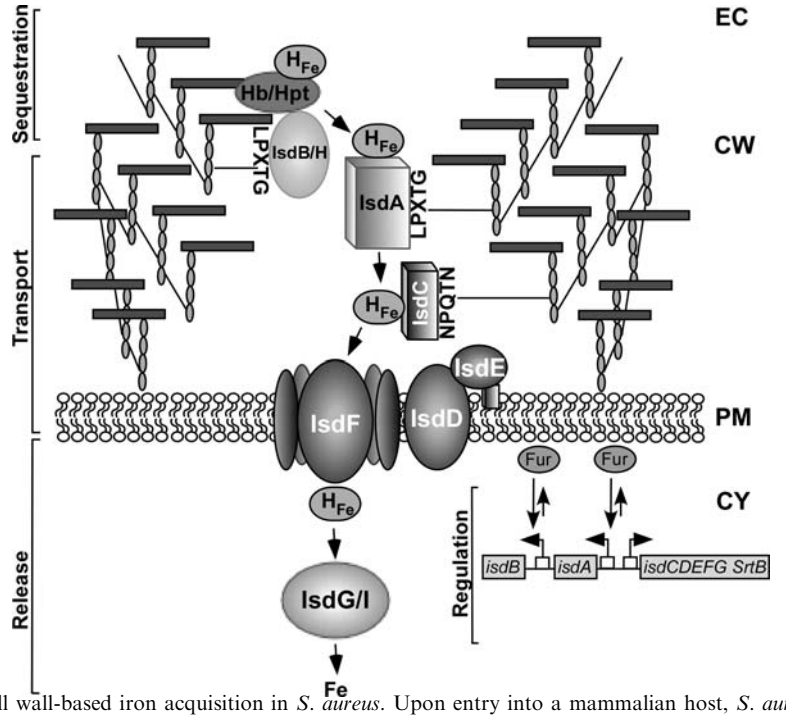

Figure 5. Model for cell wall-based iron acquisition in *S. aureus*. Upon entry into a mammalian host, *S. aureus* senses iron-deplete conditions and the global iron regulator Fur is removed from binding sites upstream of *isd* genes, leading to their expression. Isd proteins are segmented to their appropriate cellular locations. IsdB/H binds hemoglobin/haptoglobin (Hb/Hpt) and transfers heme (H_{Fe}) containing iron to IsdA, IsdC and finally the permease transport complex (IsdDEF) located in the plasma membrane. Once heme enters the bacterial cytoplasm, two monoxygenases (IsdG/I), catalyze the oxidative degradation of the heme to release iron, which is utilized for basic bacterial growth and physiology. EC = extracellular, CW = cell wall, PM = plasma membrane, CY = cytoplasm.

process, *S. aureus* senses iron-deplete conditions and the Fur repressor is removed from its binding site just upstream of the locus. The genes of the *isd* locus are expressed and their protein products direct to their appropriate location in the bacterial cells: IsdD, IsdE, IsdF and SrtB (membrane), IsdA, IsdB, IsdC, IsdH (cell wall), IsdG and IsdI (cytoplasm). Obviously, SrtA and SrtB are instrumental in the deposition of heme-iron transport factors in the cell wall envelope. In a still poorly understood process, heme is presumably liberated from heme-binding proteins such as hemoglobin (most likely bound to IsdB or IsdH) and transferred to IsdC. IsdC, being buried in the cell wall, is thought to assist in passage of heme iron across the cell wall envelope to the membrane transport system IsdDEF or HtsABC, which deliver heme into the cytoplasm of *S. aureus*. Oxidative degradation of heme by the monoxygenases IsdG and IsdI completes the acquisition process, allowing free iron to be utilized as a co-factor for enzymatic reactions.

Future directions

The model presented above generates more questions than answers. There are three stages in this model for which future experimentation would lead to a greater understanding of the mechanism of heme uptake through the *isd* system. The first stage deals with the removal of heme from heme-binding proteins such as hemoglobin. Do IsdB and IsdH function as hemophores that remove the compound from mammalian heme-binding proteins or are there other factors required for the removal of heme from its binding site? At what stage of infection and in what tissue does this process normally occur? The second major point refers to the transfer of heme across the cell wall and cytoplasmic membrane into the cytoplasm of Gram-positive pathogens. What are the structural determinants of IsdA, IsdB, IsdH, and IsdC that allow for the binding of heme? Do IsdB and IsdH form a conduit that allows heme to pass through cell wall envelope and, if so, how does IsdC

participate in this heme transfer conduit by occupying a unique position in the cell wall envelope? The last phase involves the exact role of the *isd* uptake system in *S. aureus* pathogenesis and whether this system, as opposed to other iron uptake systems, should be a target for the development of therapeutics against *S. aureus* infections. It would be interesting to know what relative contribution to pathogenesis each iron uptake system in *S. aureus* displays in a mouse model of infection. Further, is the *isd* system upregulated at all stages during the infectious process or are there additional levels of control? Although the answers to these questions may not be readily forthcoming, insights drawn from experimentation into these unknown areas will provide a greater understanding of not only *S. aureus* pathogenesis but also for infections caused by other Gram-positive organisms.

Acknowledgements

This work was supported by grants from the National Institutes of Health AI38897 and AI52474. O. Schneewind acknowledges membership within and support from the Region V "Great Lakes" Regional Center of Excellence in Biodefense and Emerging Infectious Diseases Consortium (GLRCE, National Institute of Allergy and Infectious Diseases Award 1-U54-AI-057153).

References

Albus A, Arbeit RD, Lee JC. 1991 Virulence studies of *Staphylococcus aureus* mutants altered in type 5 capsule production. *Infect Immun* **59**, 1008–1014.

Bernheimer AW, Avigad LS, Grushoff P. 1968 Lytic effects of staphylococcal alpha-toxin and delta-hemolysin. *J Bacteriol* **96**, 487–491.

Bierne H, Garandeau C, Pucciarelli MG, Sabet C, Newton S, Garcia-del Portillo F, Cossart P, Charbit A. 2004 Sortase B, a new class of sortase in *Listeria monocytogenes*. *J Bacteriol* **186**, 1972–1982.

Boekhorst J, de Been MW, Kleerebezem M, Siezen R. 2005 Genome-wide detection and analysis of cell wall-bound proteins with LPxTG-like sorting motifs. *J Bacteriol* **187**, 4928–4934.

Braun V. 2001 Iron uptake mechanisms and their regulation in pathogenic bacteria. *Int J Med Microbiol* **291**, 67–79.

Burne RA, Penders JE. 1994 Differential localization of the *Streptococcus mutans* GS-5 fructan hydrolase enzyme, FruA. *FEMS Microbiol Lett* **121**, 243–249.

Cabrera G, Xiong A, Uebel M, Singh VK, Jayaswal RK. 2001 Molecular characterization of the iron-hydroxamate uptake system in *Staphylococcus aureus*. *Appl Environ Microbiol* **67**, 1001–1003.

Casadevall A, Pirofski LA. 2004 The weapon potential of a microbe. *Trends Microbiol* **12**, 259–263.

Chen CC, Cleary PP. 1990 Complete nucleotide sequence of the streptococcal C5a peptidase gene of *Streptococcus pyogenes*. *J Biol Chem* **263**, 3161–3167.

Clewell DB. 1993 Bacterial sex pheromone-induced plasmid transfer. *Cell* **73**, 9–12.

Comfort D, Clubb RT. 2004 A comparative genome analysis identifies distinct sorting pathways in Gram-positive bacteria. *Infect Immun* **72**, 2710–2722.

Courcol RJ, Trivier D, Bissinger MC, Martin GR, Brown MR. 1997 Siderophore production by *Staphylococcus aureus* and identification of iron-regulated proteins. *Infect Immun* **65**, 1944–1948.

Dale SE, Doherty-Kirby A, Lajoie G, Heinrichs DE. 2004a Role of siderophore biosynthesis in virulence of *Staphylococcus aureus*: identification and characterization of genes involved in production of a siderophore. *Infect Immun* **72**, 29–37.

Dale SE, Doherty-Kirby A, Lajoie G, Heinrichs DE. 2004b Role of siderophore biosynthesis in virulence of *Staphylococcus aureus*: identification and characterization of genes involved in production of a siderophore. *Infect Immun* **72**, 29–37.

Dale SE, Sebulsky MT, Heinrichs DE. 2004c Involvement of SirABC in iron-siderophore import in *Staphylococcus aureus*. *J Bacteriol* **186**, 8356–8362.

Dinges MM, Orwin PM, Schlievert PM. 2000 Exotoxins of *Staphylococcus aureus*. *Clin Microbiol Rev* **13**, 16–34.

Dmitriev BA, Toukach FV, Holst O, Rietschel ET, Ehlers S. 2004 Tertiary structure of *Staphylococcus aureus* cell wall murein. *J Bacteriol* **186**, 7141–7148.

Dramsi S, Dehoux P, Lebrun M, Goossens P, Cossart P. 1995 Identification of four new members of the internalin multigene family of Listeria monocytogenes EGD. *Infect Immun* **65**, 1615–1625.

Dramsi S, Trieu-Cuot P, Bierne H. 2005 Sorting sortases: a nomenclature proposal for the various sortases of Gram-positive bacteria. *Res Microbiol* **156**, 289–297.

Drechsel H, Freund S, Nicholson G, Haag H, Jung O, Zahner H, Jung G. 1993 Purification and chemical characterization of staphyloferrin B, a hydrophilic siderophore from staphylococci. *Biometals* **6**, 185–192.

Dryla A, Gelbmann D, Gabain Avon, Nagy E. 2003 Identification of a novel iron regulated staphylococcal surface protein with haptoglobin–haemoglobin binding activity. *Mol Microbiol* **49**, 37–53.

Finlay BB, Cossart P. 1997 Exploitation of mammalian host cell functions by bacterial pathogens. *Science* **276**, 718–725.

Finlay BB, Falkow S. 1997 Common themes in microbial pathogenicity revisited. *Microbiol Mol Biol Rev* **61**, 136–169.

Fischer W. 1994 Lipoteichoic acid and lipids in the membrane of *Staphylococcus aureus*. *Med Microbiol Immunol* **183**, 61–76.

Fischetti VA, Pancholi V, Schneewind O. 1990 Conservation of a hexapeptide sequence in the anchor region of surface proteins from Gram-positive cocci. *Mol Microbiol* **4**, 1603–1605.

Flock JI, Fröman G, Jönsson K, Guss B, Signäs C, Nilsson B, Raucci G, Höök M, Wadström T, Lindberg M. 1987 Cloning and expression of the gene for a fibronectin-binding protein from *Staphylococcus aureus*. *EMBO J* **6**, 2351–2357.

Foster TJ, Höök M. 1998 Surface protein adhesins of *Staphylococcus aureus*. *Trends Microbiol* **6**, 484–488.

Gaillard J-L, Berche P, Frehel C, Gouin E, Cossart P. 1991 Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from Gram-positive cocci. *Cell* **65**, 1127–1141.

Galli D, Friesenegger A, Wirth R. 1992 Transcriptional control of sex-pheromone-inducible genes on plasmid pAD1 of *Enterococcus faecalis* and sequence analysis of a third structural gene for (pPD1-encoded) aggregation substance. *Mol Microbiol* **6**, 895–904.

Galli D, Lottspeich F, Wirth R. 1990 Sequence analysis of *Enterococcus faecalis* aggregation substance encoded by the sex pheromone plasmid pAD1. *Mol Microbiol* **4**, 895–904.

Ghuysen J-M. 1968 Use of bacteriolytic enzymes in determination of wall structure and their role in cell metabolism. *Bacteriol Rev* **32**, 425–464.

Ghuysen J-M and Hakenbeck R (Eds) . 1994 Bacterial cell wall. Amsterdam: Elsevier Science B.S.

Ghuysen J-M, Strominger JL. 1963 Structure of the cell wall of *Staphylococcus aureus*, strain Copenhagen. II. Separation and structure of the disaccharides. *Biochemistry* **2**, 1119–1125.

Ghuysen J-M, Tipper DJ, Birge CH, Strominger JL. 1965 Structure of the cell wall of *Staphylococcus aureus* strain Copenhagen. VI. The soluble glycopeptide and its sequential degradation by peptidases. *Biochemistry* **4**, 2245–2254.

Hantke K. 1981 Regulation of ferric iron transport in *Escherichia coli* K-12: fur not only affects iron metabolism. *Mol Gen Genet* **182**, 288–292.

Hantke K. 2001 Iron and metal regulation in bacteria. *Curr Opin Microbiol* **4**, 172–177.

Hantke K, Braun V. 1973 Covalent binding of lipid to protein. *Eur J Biochem* **34**, 284–296.

Herwald H, Cramer H, Morgelin M, Russel W, Sollenberg U, Norrby-Teglund A, Flodgaard H, Lindbom L, Bjorck L. 2004 M protein, a classical bacterial virulence determinant, forms complexes with fibrinogen that induce vascular leakage. *Cell* **116**, 367–379.

Holmes RK. 2000 Biology and molecular epidemiology of diphtheria toxin and the *tox* genes. *J Infect Dis* **181**, S156–167.

Horsburgh M, Ingham E, Foster SJ. 2001 In *Staphylococcus aureus*, Fur is an interactive regulator with PerR, contributes to virulence, and is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis. *J Bacteriol* **183**, 468–475.

Jonsson IM, Mazamanian SK, Schneewind O, Vendrengh M, Bremell T, Tarkowski A. 2002 On the role of *Staphylococcus aureus* sortase and sortase-catalyzed surface protein anchoring in murine septic arthritis. *J Infect Dis* **185**, 1417–1424.

Jonsson IM, Mazmanian SK, Schneewind O, Bremell T, Tarkowski A. 2003 The role of *Staphylococcus aureus* sortase A and sortase B in murine arthritis. *Microbes Infect* **5**, 775–780.

Jonsson P, Lindberg M, Haraldsson I, Wadstrom T. 1985 Virulence of *Staphylococcus aureus* in a mouse mastitis model: studies of hemolysin, coagulase, and protein A as possible virulence determinants with protoplast fusion and gene cloning. *Infect Immun* **49**, 765–769.

Konetschny-Rapp S, Jung G, Meiwes J, Zahner H. 1990 Staphyloferrin A: a structurally new siderophore from staphylococci. *Eur J Biochem* **191**, 65–74.

Labischinski H, Maidhof H. 1994 Bacterial peptidoglycan: an overview and evolving concepts. In: Ghuysen J-M, Hakenbeck R, eds. *Bacterial cell wall*. Amsterdam: Elsevier, pp. 23–38.

Maines MD. 1997 The heme oxygenase system: a regulator of second messenger gases. *Annu Rev Pharmacol Toxicol* **37**, 517–554.

Marraffini LA, Schneewind O. 2005 Anchor structure of staphylococcal surface proteins. V. Anchor structure of the sortase B substrate IsdC. *J Biol Chem* **280**, 16263–16271.

Mazmanian SK, Liu G, Jensen ER, Lenoy E, Schneewind O. 2000 *Staphylococcus aureus* mutants defective in the display of surface proteins and in the pathogenesis of animal infections. *Proc Natl Acad Sci USA* **97**, 5510–5515.

Mazmanian SK, Liu G, Ton-That H, Schneewind O. 1999 *Staphylococcus aureus* sortase, an enzyme that anchors surface proteins to the cell wall. *Science* **285**, 760–763.

Mazmanian SK, Skaar EP, Gasper AH, Humayun M, Gornicki P, Jelenska J, Joachimiak A, Missiakas DM, Schneewind O. 2003 Passage of heme-iron across the envelope of *Staphylococcus aureus*. *Science* **299**, 906–909.

Mazmanian SK, Ton-That H, Su K, Schneewind O. 2002 An iron-regulated sortase anchors a class of surface protein during *Staphylococcus aureus* pathogenesis. *Proc Natl Acad Sci USA* **99**, 2293–2298.

Moks T, Abrahmsen L, Nilsson B, Hellman U, Sjöquist J, Uhlén M. 1986 Staphylococcal protein A consists of five IgG-binding domains. *Eur J Biochem* **156**, 3577–3588.

Morrissey JA, Cockayne A, Hill PJ, Williams P. 2000 Molecular cloning and analysis of a putative siderophore ABC transporter from *Staphylococcus aureus*. *Infect Immun* **68**, 6281–6288.

Navarre WW, Schneewind O. 1994 Proteolytic cleavage and cell wall anchoring at the LPXTG motif of surface proteins in Gram-positive bacteria. *Mol Microbiol* **14**, 115–121.

Navarre WW, Schneewind O. 1999 Surface proteins of Gram-positive bacteria and the mechanisms of their targeting to the cell wall envelope. *Microbiol Mol Biol Rev* **63**, 174–229.

Navarre WW, Ton-That H, Faull KF, Schneewind O. 1998 Anchor structure of staphylococcal surface proteins. II. COOH-terminal structure of muramidase and amidase-solubilized surface protein. *J Biol Chem* **273**, 29135–29142.

Neuhaus FC, Baddiley J. 2003 A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in Gram-positive bacteria. *Microbiol Mol Biol Rev* **67**, 686–723.

Newton SM, Klebba PE, Raynaud C, Shao Y, Jiang X, Dubail I, Archer C, Frehel C, Charbit A.. 2005 The *svpA-srtB* locus of *Listeria monocytogenes*: *fur*-mediated iron regulation and effect on virulence. *Mol Microbiol* **55**, 927–940.

Pallen MJ, Lam AC, Antonio M, Dunbar K. 2001 An embarrassment of sortases – a richness of substrates. *Trends Microbiol* **9**, 97–101.

Patti JM, Allen BL, McGavin MJ, Höök M. 1994 MSCRAMM-mediated adherence of microorganisms to host tissues. *Annu Rev Microbiol* **48**, 89–115.

Perry AM, Ton-That H, Mazmanian SK, Schneewind O. 2002 Anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. III. Lipid II is an *in vivo* peptidoglycan substrate for sortase-catalyzed surface protein anchoring. *J Biol Chem* **277**, 16241–16248.

Ratliff M, Zhu W, Deshmukh R, Wilks A, Stojiljkovic I. 2001 Homologues of neisserial heme oxygenase in Gram-negative bacteria: degradation of heme by the product of the pigA gene of *Pseudomonas aeruginosa*. *J Bacteriol* **183**, 6394–6403.

Robbins JB, Schneerson R. 1990 Polysaccharide–protein conjugates: a new generation of vaccines. *J Infect Dis* **161**, 821–832.

Schmitt MP. 1997 Utilization of host iron sources by *Corynebacterium diphtheriae*: identification of a gene whose product is homologous to eukaryotic heme oxygenases and is required for acquisition of iron from heme and hemoglobin. *J Bacteriol* **179**, 838–845.

Schneewind O, Fowler A, Faull KF. 1995 Structure of the cell wall anchor of surface proteins in *Staphylococcus aureus*. *Science* **268**, 103–106.

Schneewind O, Mihaylova-Petkov D, Model P. 1993 Cell wall sorting signals in surface protein of Gram-positive bacteria. *EMBO* **12**, 4803–4811.

Schneewind O, Model P, Fischetti VA. 1992 Sorting of protein A to the staphylococcal cell wall. *Cell* **70**, 267–281.

Skaar EP, Gaspar AH, Schneewind O. 2004a IsdG and IsdI, heme degrading enzymes in the cytoplasm of *Staphylococcus aureus*. *J Biol Chem* **279**, 436–443.

Skaar EP, Humayun M, DeBord KL, Schneewind O. 2004b Iron source preference of *Staphylococcus aureus* infections. *Science* **305**, 1626–1628.

Skaar EP, Schneewind O. 2004 Iron-regulated surface determinants (Isd) of *Staphylococcus aureus*: stealing iron from heme. *Microb Infect* **6**, 390–397.

Stojiljkovic I, Perkins-Balding D. 2002 Processing of heme and heme-containing proteins by bacteria. *DNA Cell Biol* **21**, 281–295.

Taylor JM, Heinrichs DE. 2002 Transferrin binding in *Staphylococcus aureus*: involvement of a cell wall-anchored protein. *Mol Microbiol* **43**, 1603–1614.

Tipper DJ. 1968 Alkali-catalyzed elimination of D-lactic acid from muramic acid and its derivatives and the determination of muramic acid. *Biochemistry* **7**, 1441–1449.

Tipper DJ, Ghuysen J-M, Strominger JL. 1965 Structure of the cell wall of *Staphylococcus aureus*, strain Copenhagen. III. Further studies of the dissacharides. *Biochemistry* **4**, 468–473.

Tipper DJ, Strominger JL. 1965 Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-alanine. *Proc Natl Acad Sci USA* **54**, 1133–1141.

Ton-That H, Faull KF, Schneewind O. 1997 Anchor structure of staphylococcal surface proteins. I. A branched peptide that links the carboxyl terminus of proteins to the cell wall. *J Biol Chem* **272**, 22285–22292.

Ton-That H, Labischinski H, Berger-Bächi B, Schneewind O. 1998 Anchor structure of staphyococcal surface proteins. III. The role of the FemA, FemB, and FemX factors in anchoring surface proteins to the bacterial cell wall. *J Biol Chem* **273**, 29143–29149.

Ton-That H, Liu G, Mazmanian SK, Faull KF, Schneewind O. 1999 Purification and characterization of sortase, the transpeptidase that cleaves surface proteins of *Staphylococcus aureus* at the LPXTG motif. *Proc Natl Acad Sci USA* **96**, 12424–12429.

Ton-That H, Marraffini LA, Schneewind O. 2004 Protein sorting to the cell wall envelope of Gram-positive bacteria. *Biochim Biophys Acta* **1694**, 269–278.

Ton-That H, Mazmanian SK, Alksne L, Schneewind O. 2002 Anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. II. Cysteine 184 and histidine 120 of sortase A form a thiolate imidazolium ion pair for catalysis. *J Biol Chem* **277**, 7447–7452.

Ton-That H, Mazmanian SK, Faull KF, Schneewind O. 2000 Anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. I. Sortase catalyzed *in vitro* transpeptidation reaction using LPXTG peptide and NH_2-Gly_3 substrates. *J Biol Chem* **275**, 9876–9881.

Ton-That H, Schneewind O. 1999 Anchor structure of staphylococcal surface proteins. IV. Inhibitors of the cell wall sorting reaction. *J Biol Chem* **274**, 24316–24320.

Wachenfeldt Cvon, Hederstedt L. 1990 *Bacillus subtilis* 13-kilodalton cytochrome c-550 encoded by cccA consists of a membrane-anchor and a heme domain. *J Biol Chem* **265**, 13939–13948.

Wandersman C, Delepelaire P. 2004 Bacterial iron sources: from siderophores to hemophores. *Annu Rev Microbiol* **58**, 611–647.

Weiss WJ, Lenoy E, Murphy T, Tardio L, Burgio P, Projan SJ, Schneewind O, Alksne L. 2004 Effect of *srtA* and *srtB* gene expression on the virulence of *Staphylococcus aureus* in animal infection. *J Antimicrob Chemother* **53**, 480–486.

Wilks A. 2002 Heme oxygenase: evolution, structure, and mechanism. *Antioxid Redox Signal* **4**, 603–614.

Wilks A, Schmitt MP. 1998 Expression and characterization of a heme oxygenase (HmuO) from *Corynebacterium diphtheriae*. Iron acquisition requires oxidative cleavage of the heme macrocycle. *J Biol Chem* **273**, 837–841.

Wu R, Skaar EP, Zhang R, Joachimiak G, Gornicki P, Schneewind O, Joachimiak A. 2005 *Staphylococcus aureus* IsdG and IsdI, heme-degrading enzymes with structural similarity to monooxygenases. *J Biol Chem* **280**, 2840–2846.

Xiong A, Singh VK, Cabrera G, Jayaswal RK. 2000 Molecular characterization of the ferric uptake regulator, *fur*, from *Staphylococcus aureus*. *Microbiology* **146**, 659–668.

Zhu W, Wilks A, Stojiljkovic I. 2000 Degradation of heme in Gram-negative bacteria: the product of the *hemO* gene of *Neisseriae* is a heme oxygenase. *J Bacteriol* **182**, 6783–6790.