Signal peptides direct surface proteins to two distinct envelope locations of *Staphylococcus aureus*

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Surface proteins of Gram-positive bacteria are covalently linked to the cell wall envelope by a mechanism requiring an N-terminal signal peptide and a C-terminal LPXTG motif sorting signal. We show here that surface proteins of *Staphylococcus aureus* arrive at two distinct destinations in the bacterial envelope, either distributed as a ring surrounding each cell or as discrete assembly sites. Proteins with ring-like distribution (clumping factor A (ClfA), Spa, fibronectin-binding protein B (FnbpB), serine-aspartate repeat protein C (SdrC) and SdrD) harbour signal peptides with a YSIRK/GS motif, whereas proteins directed to discrete assembly sites (*S. aureus* surface protein A (SasA), SasD, SasF and SasK) do not. Reciprocal exchange of signal peptides between surface proteins with (ClfA) or without the YSIRK/GS motif (SasF) directed recombinant products to the alternate destination, whereas mutations that altered only the YSIRK sequence had no effect. Our observations suggest that *S. aureus* distinguishes between signal peptides to address proteins to either the cell pole (signal peptides without YSIRK/GS) or the cross wall, the peptidoglycan layer that forms during cell division to separate new daughter cells (signal peptides with YSIRK/GS motif).

The EMBO Journal (2008) 27, 2656–2668. doi:10.1038/emboj.2008.185; Published online 18 September 2008

Subject Categories: membranes & transport; microbiology & pathogens

Keywords: crosswall; signal peptides; *Staphylococcus aureus*; surface protein; YSIRK/GS motif

Introduction

The murein sacculus of Gram-positive bacteria is a single large macromolecule, assembled from peptidoglycan precursor molecules and disassembled by lytic enzymes that exert an effect at division sites (Higashi *et al*, 1967; Strominger, 1968; Yamada *et al*, 1996; Baba and Schneewind, 1998). The murein sacculus serves as scaffold for covalent linkage of many different biological polymers, including carbohydrates, teichoic acids and proteins (Armstrong *et al*, 1959; Sanderson

et al, 1962; Munoz *et al*, 1967; Schneewind *et al*, 1995). Cell wall-anchored products are displayed on the bacterial surface endowing each microbe with unique attributes that, among many different physiological functions, enable host infection (Navarre and Schneewind, 1999). An important feature of the murein sacculus is its exoskeletal nature and rigid enclosure of cells that would otherwise burst due to osmotic pressure (Salton, 1952). Many molecules move around or through the murein sacculus; however, once linked to peptidoglycan, compounds remain immobilized until liberated by lytic enzymes (Marraffini *et al*, 2006).

Sortases catalyse the covalent anchoring of proteins to the cell wall envelope and recognize surface protein substrates through C-terminal LPXTG motif sorting signals (Schneewind *et al*, 1992, 1993; Mazmanian *et al*, 1999). On cleavage of the polypeptide precursor between the threonine and glycine residues of the LPXTG motif, sortase forms an acyl enzyme intermediate with the C-terminal threonine (Navarre and Schneewind, 1994; Ton-That and Schneewind, 1999; Ton-That *et al*, 2000). Nucleophilic attack of the amino group of cell wall cross-bridges, pentaglycine in *Staphylococcus aureus* (Matsushashi *et al*, 1965), generates the amide bond that links the C-terminal end of surface proteins to the bacterial cell wall envelope (Navarre *et al*, 1998; Ton-That *et al*, 1998; Perry *et al*, 2002). Recently, we asked whether anchored proteins are distributed uniformly over the surface of *S. aureus* (DeDent *et al*, 2007). Using immunofluorescence and confocal laser scanning microscopy, a ring-like distribution of protein A, the well-known immunoglobulin (Ig)-binding factor (Jensen, 1958), was detected on the staphylococcal surface (DeDent *et al*, 2007). In staphylococci where anchored proteins had been removed with protease, deposition of newly synthesized protein A occurred at 2–4 sites, which expanded until a ring-like distribution was achieved (DeDent *et al*, 2007).

Impetus for our current work is the report of Carlsson *et al* (2006) that surface proteins of *Streptococcus pyogenes* are directed to two different locations in the bacterial envelope by mechanisms that involve specific signal peptides. Classic experiments by Cole and Hahn revealed the division septum as the site of envelope deposition for M protein (Cole and Hahn, 1962; Hahn and Cole, 1963), Lancefield's antiphagocytic factor and molecular basis for typing of group A streptococci (Lancefield, 1928). In contrast, fibronectin-binding protein (protein F) (Hanski and Caparon, 1992) is deposited at the streptococcal cell poles (Carlsson *et al*, 2006). These localization patterns can be changed when signal peptides for M protein, which carries the YSIRK/GS motif, and protein F, which lacks this motif, are exchanged (Carlsson *et al*, 2006). YSIRK/GS motif signal peptides were first described for staphylococcal lipases, secreted glycerol ester hydrolases (Rosenstein and Götz, 2000). The sequence motif is otherwise found in signal peptides of cell

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Received: 3 March 2008; accepted: 25 August 2008; published online: 18 September 2008

wall-anchored surface proteins (Tettelin *et al.*, 2001). Remarkably, many surface proteins of streptococci and staphylococci, but not those of bacilli, clostridia or actinomycetales, carry YSIRK/GS motif signal peptides, indicating that acquisition of distinct types of signal peptides may be restricted to these genera. Sequenced genomes of *S. aureus* reveal 21 surface proteins with sorting signals, 13 of which harbour signal peptides with the YSIRK/GS motif. Earlier studies revealed that the presence or absence of YSIRK/GS motif signal peptides does not affect sortase-mediated anchoring of surface proteins but impacts the rate of signal peptide processing (Bae and Schneewind, 2003). However, this work left unresolved whether staphylococci can distinguish between signal peptides and direct surface proteins to discrete locations in the bacterial envelope.

Results

Signal peptides of staphylococcal surface proteins

Amino-acid sequences of signal peptides from staphylococcal surface proteins were aligned according to their predicted or known signal peptidase cleavage sites (Figure 1). The YSIRK/GS motif is positioned 18–20 residues upstream of the signal peptide cleavage site in 13 of these molecules. It is noted that the number of residues between the N-terminal formyl-methionine and the YSIRK/GS motif varies between each surface protein, whereas the spatial distance between the sequence motif and the signal peptidase cleavage site appears conserved and may be of biological significance. All motif sequences examined span 12 residues, including YSIRK, followed by a 3-residue spacer with 1 hydrophobic residue at the third position, glycine (G), a 2-residue spacer with polar and hydrophobic residues, and serine (S). Most of the residues between the second serine of the YSIRK/GS motif and the signal peptidase cleavage site are hydrophobic in

nature, in agreement with the signal peptide properties of these domains (Emr *et al.*, 1978; von Heijne, 1992). We observed some sequence variation in the tyrosine (Y, replaced with either phenylalanine or histidine), serine (glycine or alanine) and lysine (arginine) residues of the YSIRK peptide, whereas the IR and GS residues are absolutely conserved. A second group of signal peptides from seven different surface proteins lacked the YSIRK/GS motif (Figure 1).

Distribution of ClfA and SasF on the staphylococcal surface

Antibodies were raised against purified recombinant *S. aureus* surface protein A (SasA), D (SasD), F (SasF), K (SasK), clumping factor A (ClfA), serine-aspartate repeat protein C (SdrC) and D (SdrD), as well as fibronectin-binding protein B (FnbpB). Antibody binding to proteins on the staphylococcal surface was detected with Alexafluor 647-conjugated secondary antibodies and fluorescence microscopy (red staining; Figure 2). Our experiments used the Δ spa mutant strains SEJ1 (derived from RN4220) or SEJ2 (derived from strain Newman) to avoid antibody binding to protein A (Stranger-Jones *et al.*, 2006). Unlike streptococci, where cell division planes lie parallel (Swanson and McCarty, 1969), *S. aureus* divides perpendicular to previous division planes (Tzagoloff and Novick, 1977). Because of incomplete cell wall separation, differential interference contrast microscopy revealed single cells, diplococci or grape-like clusters of staphylococci (Figure 2) (Giesbrecht *et al.*, 1998). BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene)-vancomycin, a glycopeptide that binds with high affinity to D-Ala-D-Ala pentapeptide precursors of peptidoglycan synthesis (Walsh, 1993), was used to reveal the bacterial murein sacculus and cross wall, a layer of newly synthesized peptidoglycan that separates dividing staphylococcal daughter cells (green staining; Figure 2) (Schuhardt *et al.*, 1969; Giesbrecht *et al.*, 1998).

Figure 1 Signal peptides of *Staphylococcus aureus* surface proteins. The N-terminal signal peptides of *S. aureus* surface proteins containing a C-terminal cell wall sorting signal were aligned at their predicted signal peptidase cleavage sites (A/X). The signal peptides of 13 surface proteins harbour the YSIRK/GS motif. Identical residues are highlighted in yellow and summarized as *, conserved residues marked in grey or summarized with a colon (:). The YSIRK/GS motif was absent in the remaining seven proteins; following alignment, no characteristic sequence feature could be identified in these signal peptides. The signal peptide of SasA was abbreviated; the sequence RQKAFHDSLANKETRVRLYKSGKNWVKSGIKEIEMFKIMGLP was omitted at the position indicated by two backslashes (\).

Figure 2 Distribution of ClfA and SasF in the cell wall envelope of staphylococci. (A) *S. aureus* strains SEJ1 (Δspa), ACD1 (Δspa , *clfA::ermB*) and SEJ2 (Δspa) (Supplementary Table 1) were viewed with differential interference contrast (DIC) or fluorescence microscopy using BODIPY-vancomycin (green) or ClfA-specific antibodies followed by secondary goat anti-rabbit conjugated to Alexafluor 647 (red). ClfA is secreted through a signal peptide that harbours the YSIRK/GS motif (+ YSIRK). (B) *S. aureus* strains SEJ1 (Δspa), ACD2 (Δspa , *sasF::ermB*) and SEJ2 (Δspa) were viewed with DIC or fluorescence microscopy using BODIPY-vancomycin (green) or SasF-specific antibodies followed by secondary goat anti-rabbit conjugated to Alexafluor 647 (red). SasF is secreted through a signal peptide that does not harbour the YSIRK/GS motif (–YSIRK). Two-dimensional two-colour and DIC images were acquired sequentially with separate laser lines and merged using the ImageJ software.

Antibodies against ClfA detected a ring-like or hemispherical distribution of this protein on the surface of staphylococci (Figure 2A). Similar to staining reported for protein A (DeDent *et al*, 2007), distribution of ClfA for both SEJ1 and SEJ2 appeared irregular, and its ring-shaped contour in dividing cells was typically positioned perpendicular to vancomycin-stained cross walls (Figure 2A). As a control, *bursa aurealis* insertion into *clfA* (*clfA::ermB*) abrogated ClfA antibody binding, thereby revealing the specificity of this reagent. In contrast to ClfA, surface distribution of SasF assumed a much more discrete, punctate staining pattern, typically one, two or three locations in the staphylococcal envelope in both strains SEJ1 and SEJ2, whereas a ring-like contour surrounding the bacterial cell was not observed (Figure 2B). Sites of SasF deposition were found at different locations in the cell wall envelope but did not appear to colocalize with the cross wall.

Surface distribution of proteins with and without YSIRK/GS signal peptides

We sought to examine the universality of the observed surface distribution patterns for proteins with or without YSIRK/GS motif signal peptides. FnbpB, SdrC and SdrD each harbour a YSIRK/GS motif signal peptide. A ring-like or hemispherical surface distribution was observed for each of the three proteins (Figure 3A). The immune-fluorescent signals displayed for SdrD were very strong; this may in part conceal the uneven distribution of this protein over the bacterial surface (Figure 3A). The latter phenomenon was more readily detectable in fluorescence images of staphylococci stained with FnbpB- and SdrC-specific antibodies, which also assumed ring-shaped or hemispherical surface distribution of fluorescent signals (Figure 3A). Signal peptides of SasA, SasD and SasK lack the YSIRK/GS motif (Figure 1). The envelope of staphylococci that had been incubated with SasA-, SasD- or SasK-specific antibodies displayed discrete, punctate fluorescent signals (Figure 3B). In images from all three experi-

ments, we observed one, two or three surface protein deposits within the bacterial envelope; however, there were also many cells without detectable signals (Figure 3B). We presume that the frequent absence of surface protein deposits is due to the acquisition of two-dimensional fluorescence images; surface protein deposits residing underneath staphylococcal cells are most likely not detectable with this technology. As controls, *bursa aurealis* insertion mutations in *fnbpB*, *sasA*, *sasD*, *sasK*, *sdrC* or *sdrD* abrogated immunoreactive signals of corresponding antibodies, thereby demonstrating their specificity (data not shown). Thus, surface proteins that are secreted through YSIRK/GS motif signal peptides (*Spa*, *ClfA*, *FnbpB*, *SdrC* and *SdrD*) are distributed in a ring-like manner in the staphylococcal envelope and perpendicular to cross wall, that is, the cell division plane. In contrast, surface proteins that are secreted by signal peptides without a YSIRK/GS motif are found in discrete, punctate deposits in the cell wall envelope.

Reconstruction of surface protein deposits in the staphylococcal envelope

Following labelling of staphylococci with BODIPY-vancomycin and antibodies, serial images in optical planes separated by approximately 60 nm along the *z* axis were collected by sequential laser scanning confocal microscopy. These series were then used to generate three-dimensional fluorescence reconstructions of surface proteins in the murein sacculus. We first acquired images for large clusters of staphylococci to reveal protein deposits in bacteria where cell wall envelopes had not yet been completely separated. Figure 4A represents a summary image for such a staphylococcal cluster. ClfA staining of staphylococcal clusters revealed the ring-shaped distribution of surface proteins secreted through YSIRK/GS motif signal peptides as well as the perpendicular pattern of protein rings in neighbouring cells (Figure 4A). The three-dimensional reconstruction of the acquired data can be appreciated when the summary image is rotated (Supplementary

Figure 3 Distribution of surface proteins in the cell wall envelope of staphylococci. (A) *S. aureus* strain SEJ1 (Δspa) was analysed with differential interference contrast (DIC) or fluorescence microscopy using BODIPY-vancomycin (green) or specific antibodies (SdrD, SdrC and FnbpB) followed by secondary goat anti-rabbit conjugated to Alexafluor 647 (red). SdrD, SdrC and FnbpB are secreted through signal peptides that harbour the YSIRK/GS motif (+YSIRK). (B) *S. aureus* strain SEJ1 (Δspa) was viewed with DIC or fluorescence microscopy using BODIPY-vancomycin (green) or specific antibodies (SasA, SasD and SasK) followed by secondary goat anti-rabbit conjugated to Alexafluor 647 (red). SasA, SasD and SasK are secreted through signal peptides that do not harbour the YSIRK/GS motif (–YSIRK). Two-dimensional two-colour and DIC images were acquired sequentially with separate laser lines and merged using the ImageJ software.

Movie 1). As a control, ClfA antibody staining of Δspa , *clfA::ermB* mutant staphylococci detected only background fluorescence. Three-dimensional image reconstruction of staphylococcal clusters stained with anti-SasF revealed the discrete, circumscribed nature of protein deposits derived from signal peptide precursors without the YSIRK/GS motif (Figure 4A; Supplementary Movie 2). Control experiments with Δspa , *sasF::ermB* mutant staphylococci revealed occasional background fluorescence signals with SasF antibodies.

A series of confocal images of single staphylococcal cells with ClfA deposits confirmed the hemispherical or ring-like distribution of this surface protein (Figure 4B). These images also identified foci of abundant ClfA deposition within the ring of surface proteins, in agreement with earlier observations generated for protein A (DeDent *et al*, 2007). Our working model (*vide infra*) predicts that YSIRK/GS motifs containing surface proteins are deposited at specific sites, the location of which in the murein sacculus may change as bacteria grow and expand their cell wall envelope. Acquisition of similar images for staphylococci stained with SasF-specific antibody identified three discrete envelope deposits for proteins secreted without YSIRK/GS motif signal peptides (Figure 4B). These foci, however, are not connected by ring-like or hemispherical distributions of this surface protein, as is observed with ClfA and protein A (Figure 4B).

Two destinations for staphylococcal surface protein secretion

The distribution patterns of surface proteins with or without YSIRK/GS motif signal peptides in the staphylococcal envelope appear distinctly different. Staining for individual proteins, however, does not establish whether the foci of surface protein deposition are identical for ClfA and SasF. In other words, we sought to determine whether the destiny of

surface protein secretion in staphylococci is determined by the presence of specific signal peptides. To address this question, antibodies against ClfA (+YSIRK) were conjugated to Alexafluor 488 (green), whereas antibodies against SasF (–YSIRK) were conjugated to Alexafluor 647 (red). Staphylococci were stained with both antibodies and samples subjected to sequential laser scanning confocal microscopy and optical sections of ~ 120 nm through bacterial samples were acquired. Figure 5A depicts summary images of deconvoluted three-dimensional reconstructions for stacked confocal images. Although abundant foci of ClfA and SasF deposition were detected in the staphylococcal envelope, the vast majority of these fluorescent signals did not colocalize. As a control, double labelling of staphylococci for ClfB and SdrD, two surface proteins with YSIRK/GS signal peptides, revealed their colocalization (Supplementary Figure 1).

Single staphylococcal cells were also subjected to sequential laser scanning confocal microscopy. Optical sections along the *z* axis for two exemplary cells are displayed in Figure 5B. The sites of ClfA and SasF deposition in the staphylococcal cell wall envelope were distinct in each case examined. In some cells, deposition sites for ClfA and SasF appear to be positioned adjacent to one another (cell 1), whereas in others (cell 2), they appeared to be juxtaposed or perpendicular to one another (Figure 5B).

Dynamics of surface protein ring formation

To monitor the deposition of surface protein and formation of ring-like assemblies, staphylococci were treated with trypsin, a protease that removes surface-exposed proteins such as protein A, ClfA and SasF from the bacterial envelope, thereby abolishing antibody binding to the bacterial surface. Protease was quenched with inhibitor and cells were incubated in fresh media at 37°C for growth and anchoring of surface

Figure 4 Confocal laser microscopy reveals the three-dimensional distribution of ClfA and SasF on the staphylococcal surface. **(A)** *S. aureus* strains SEJ1 (Δspa), ACD1 ($\Delta spa, clfA::ermB$) or ACD2 ($\Delta spa, sasF::ermB$) were labelled with BODIPY-vancomycin (green) as well as ClfA- or SasF-specific antibodies followed by secondary goat anti-rabbit conjugated to Alexafluor 647 (red). Confocal microscopy was performed and z-series was acquired with 70-nm increments. Three-dimensional (3D) projections were created from stacks using ImageJ software. For 3D rotation of assembled images, see Supplementary Movies 1 and 2. **(B)** Representative single cell series for *S. aureus* SEJ1 labelled with BODIPY-vancomycin (green) and either ClfA- or SasF-specific antibodies (red).

proteins. Deposition of newly synthesized ClfA and SasF in the staphylococcal envelope was monitored by fluorescence microscopy. Immediately following trypsin treatment, no fluorescent signal was detected for ClfA or SasF (data not shown). At 20 min following trypsin treatment, fluorescent signals were detected in the staphylococcal envelope for both ClfA and SasF, revealing localized deposition of newly synthesized surface protein (Figure 6). It is noted that ClfA

and SasF were deposited at different sites in the bacterial envelope, as their red and green fluorescent signals were spatially restricted, but not colocalized. SasF appeared at the perimeter of diplococcal cells, and very rarely on single cells. ClfA was found almost exclusively at or near the cross wall of diplococcal cells, and rarely, if at all, on single cells (Supplementary Figure 5). Incubation for 40 min led to increased deposition of ClfA at the cross wall, which in

Figure 5 ClfA and SasF do not colocalize in the staphylococcal envelope. (A) ClfA and SasF antibodies were conjugated to Alexafluor 488 (α ClfA, green) or Alexafluor 647 (α SasF, red) and used to label *S. aureus* SEJ1 (Δ spa). Confocal microscopy was performed and z-series was acquired with 120-nm increments. Three-dimensional projections were created from stacks using the ImageJ software. (B) Representative single-cell DIC images and confocal microscopy z-series for staphylococci labelled with α ClfA (green)- and α SasF (red)-specific antibodies.

some cases expanded to form ring-like structures surrounding staphylococci. In contrast, areas of SasF deposition remained primarily at the cell periphery (Figure 6). At the 40-min time point, we observed occasional colocalization of ClfA and SasF in the bacterial envelope. Nevertheless, discrete SasF-specific fluorescent signals were distinguishable. Thus, ClfA and SasF appear to be deposited at unique sites in the staphylococcal envelope. In addition, the foci of ClfA, but not those of SasF deposition, are expanded to form ring-like assemblies of surface proteins at the cross wall.

Signal peptides address proteins to their final destination in the envelope

In agreement with the streptococcal model (Carlsson *et al*, 2006), we presumed that staphylococcal signal peptides may address surface proteins to their final destination, either a ring-like distribution or a focal assembly in the staphylococcal envelope. To test this, we transformed Δ spa, *clfA::ermB* or Δ spa, *sasF::ermB* mutant strains with recombinant plasmids expressing either *clfA* (pClfA) or *sasF* (pSasF). Immunofluorescence microscopy experiments revealed focal assemblies of SasF in the envelope of Δ spa, *sasF::ermB* (pSasF) cells and ring-like or spherical distributions of ClfA on the surface of Δ spa, *clfA::ermB* (pClfA) cells (Figure 7A). These results suggest that plasmid complementation with the wild-type gene *in trans* not only restored the expression defects of *sasF* or *clfA* mutants but also promoted trafficking of surface proteins to their proper destination. The signal

peptides of SasF and ClfA were exchanged and plasmid-encoded recombinant proteins, SP_{SasF}-ClfA and SP_{ClfA}-SasF, were expressed in Δ spa, *clfA::ermB* or Δ spa, *sasF::ermB* mutant staphylococci. Immunofluorescence microscopy revealed ring-like or spherical distribution of SasF-specific signals in Δ spa, *sasF::ermB* (pSP_{ClfA}-SasF) cells (Figure 7B). In contrast, ClfA deposition was restricted to discrete focal assemblies in the envelope of Δ spa, *clfA::ermB* (pSP_{SasF}-ClfA) cells (Figure 7B). Taken together, these results indicate that the signal peptides of ClfA and SasF are necessary and sufficient for delivery of surface proteins to two distinct locations in the staphylococcal cell wall envelope.

Expression of surface protein genes from multi-copy plasmids results in increased abundance of polypeptides in the bacterial envelope (Figure 7). To determine whether surface protein trafficking occurs also with genetic constructs that are inserted in single copy into the staphylococcal genome, we utilized the integration plasmid pCL55 (Lee *et al*, 1991). Wild-type *sasF* and a *sasF* variant with the *clfA* signal peptide were cloned into the integration plasmid pCL55 (pCL55-SP_{ClfA}-SasF). Following transformation and plasmid integration, *S. aureus* cells were treated with trypsin, protease quenched and bacterial growth was initiated by dilution into fresh media at 37°C. Sample aliquots were stained with primary labelled antibody to SdrD (green) or SasF (red) and viewed by fluorescence microscopy 20 and 40 min following trypsin treatment. *S. aureus* with wild-type

Figure 6 Deposition of newly synthesized ClfA and SasF in the cell wall envelope following trypsin treatment of staphylococci. **(A)** Staphylococci were treated with trypsin, which abolished all ClfA- and SasF-specific surface protein labelling in the bacterial envelope. At timed intervals, 20 or 40 min, following dilution of bacteria into fresh growth media, culture aliquots were labelled with α ClfA (green, conjugated to Alexafluor 488) or α SasF (red, conjugated to Alexafluor 647). DIC images as well as confocal laser microscopy z-series in 120-nm increments were acquired. Three-dimensional projections were created from stacks using the ImageJ software. **(B)** Representative single-cell DIC images and confocal microscopy z-series for staphylococci labelled with α ClfA (green)- and α SasF (red)-specific antibodies after 20 or 40 min of growth following trypsin treatment.

sasF and the empty pCL55 vector control (*Δspa* (pCL55)) displayed SasF at 20 and 40 min primarily in the periphery of dividing cells, with only rare signals in the proximity of the cell division septum (Figure 8). SdrD, on the other hand, was found primarily at or near the cross wall at 20 min and at both the cross wall and periphery at 40 min (Figure 8). In contrast, staphylococci expressing SasF with the ClfA signal peptide (+ YSIRK/GS motif) (*Δspa*, *sasF::ermB* (pCL55-SP_{ClfA}-SasF)) generated immunofluorescent signals for both SdrD and SasF that localized to the cross wall of dividing staphylococci at 20- and 40-min incubation intervals (Figure 8). Surface protein colocalization on SP_{ClfA}-SasF-expressing cells, though not complete, was markedly increased as compared with cells expressing wild-type SasF. Similar to what has been reported for protein F with the M protein signal peptide in group A streptococci (Carlsson *et al*, 2006), SP_{ClfA}-SasF was also found in the periphery of cells (Figure 8).

To examine the contribution of the YSIRK/GS motif in surface protein trafficking, we generated alanine amino-acid

substitutions in any one motif residue encoded by the *clfA* gene inserted through pCL55 into the staphylococcal genome; however, mutant proteins displayed no defect in deposition (ring-like distribution and deposition at the cross wall, data not shown). We therefore generated a variant with a scrambled YSIRK sequence (but leaving the G/S segment intact) and inserted the variant into the staphylococcal genome. Again, we observed no phenotype in immunofluorescence microscopy experiments (Supplementary Figure 2), indicating that the YSIRK/GS signal peptide, but not the YSIRK sequence alone, is responsible for directing ClfA into a ring-like surface distribution and deposition within the envelope at the cross wall.

Contributions of specialized secretion systems to surface protein trafficking

YSIRK/GS motif containing signal peptides harbour two positively charged amino acids, RK or RR, similar to substrates recognized by the twin-arginine translocase (TAT) pathway, which is capable of secreting fully folded

Figure 7 Signal peptides address staphylococcal surface proteins to their proper location in the cell wall envelope. (A) *S. aureus* strains ACD1 ($\Delta spa, clfA::ermB$) and ACD2 ($\Delta spa, sasF::ermB$) were transformed with plasmids to generate ACD3 ($\Delta spa, clfA::ermB, pClfA$) and ACD4 ($\Delta spa, sasF::ermB, pSasF$). Staphylococci were viewed with differential interference contrast (DIC) or fluorescence microscopy using BODIPY-vancomycin (green), ClfA- or SasF-specific antibodies followed by secondary goat anti-rabbit conjugated to Alexafluor 647 (red). (B) *S. aureus* strains ACD1 and ACD2 were transformed with plasmids to generate ACD5 ($\Delta spa, clfA::ermB, pSP_{SasF-ClfA}$) and ACD6 ($\Delta spa, sasF::ermB, pSP_{ClfA-SasF}$). Staphylococci were viewed with DIC or fluorescence microscopy using BODIPY-vancomycin (green), ClfA- or SasF-specific antibodies followed by secondary goat anti-rabbit conjugated to Alexafluor 647 (red).

polypeptides across the cytoplasmic membrane of bacteria (Berks *et al*, 2003). To test whether or not the TAT pathway is involved in the secretion or localized deposition of surface proteins, we viewed staphylococci harbouring a *bursa aurealis* insertion into the *tatC* gene ($\Delta spa, tatC::ermB$) by immunofluorescence microscopy. When compared with Δspa staphylococci, we observed no differences in ClfA deposition in the *tatC* mutant strain (Supplementary Figure 3).

Similar to *Streptococcus gordonii*, *S. aureus* encodes two accessory secretion genes, designated *secA2* and *secY2* (Bensing and Sullam, 2002; Baba *et al*, 2007). *S. gordonii*

secY2 and *secA2* are required for the secretion and functional display of GspB, a surface protein homologue of *S. aureus* SasA (Mazmanian *et al*, 2001; Bensing and Sullam, 2002). However, mutations that affect *secA2* and *gtfA*, encoding a glycosyl transferase that modifies GspB, cause the increased surface display of non-glycosylated GspB, in agreement with a model whereby SecA2 is involved in substrate recognition of a single polypeptide after it has been modified by glycosylation (Bensing and Sullam, 2002; Takamatsu *et al*, 2004; Bensing *et al*, 2007). To examine the possibility that the two accessory secretion genes,

Figure 8 YSIRK/GS signal peptides direct cell wall-anchored proteins to the septum and cross wall. (A) *S. aureus* strain ACD2 ($\Delta spa, sasF::ermB$) was transformed with the integration plasmid pCL55 encoding wild-type SasF (pCL55-SasF) or SasF bearing the ClfA signal peptide (pCL55-SP_{ClfA}-SasF) to generate strains ACD8 ($\Delta spa, sasF::ermB$, pCL55-SasF) and ACD9 ($\Delta spa, sasF::ermB$, pCL55-SP_{ClfA}-SasF). Staphylococci were treated with trypsin, which removed all SasF-specific surface protein labelling in the bacterial envelope. At timed intervals, 20 and 40 min, following dilution of bacteria into fresh growth media, aliquots of staphylococcal cultures were labelled with α SdrD (green, conjugated to Alexafluor 488) or α SasF (red, conjugated to Alexafluor 647). DIC images as well as confocal laser microscopy z-series in 120-nm increments were acquired. Images were deconvoluted using the Huygens™ software. Three-dimensional projections were created from deconvoluted stacks using the ImageJ software. Representative wide-field images are shown. (B) Representative images of diplococci from the experiment described in (A).

secA2 and *secY2* are required for the secretion of surface proteins, we used *bursa aurealis* insertions into *secY2* (*secY2::ermB*) or in *secA2* (*secA2::ermB*). *Bursa aurealis* insertion into *secY2* abolished the surface display of SasA (Figure 9), but did not affect the deposition of other surface proteins, such as SdrD or SasF (Supplementary Figure 4). In contrast, *secA2::ermB* increased the surface display of SasA (Figure 9); however, this mutation also caused no phenotype for the deposition of other surface proteins (data not shown).

Discussion

Cell wall-anchored surface proteins with YSIRK/GS motif signal peptides have been reported in streptococci (*S. pyogenes*, *S. agalactiae*, *S. faecalis* and *S. pneumoniae*) and

staphylococci (*S. aureus* and *S. epidermidis*), but not in *Listeria monocytogenes*, bacilli (*Bacillus subtilis*, *B. anthracis* and *B. cereus*), clostridia (*Clostridium tetani* and *C. perfringens*) or nocardia (*Corynebacterium diphtheriae*, *Actinomyces naeslundii*, *Streptomyces coelicolor*) (Rosenstein and Götz, 2000; Tettelin *et al*, 2001; Bae and Schneewind, 2003). When comparing the morphology of these microbes, we noticed that the majority of YSIRK/GS motif signal peptides occur in Gram-positive bacteria with round spherical cell shapes (cocci). This report, as well as earlier work from Gunnar Lindahl's laboratory, examined the function of YSIRK/GS motif signal peptides in the delivery of surface proteins to unique locations in the cell wall envelope of cocci (Carlsson *et al*, 2006). For streptococci, M protein (carrying the YSIRK/GS motif signal peptide) is secreted at the cell division site (Hahn and Cole, 1963), whereas protein F

Figure 9 Contribution of *secA2* and *secY2* towards the surface display of SasA. *S. aureus* strains SEJ1 (Δspa), ACD22 (Δspa , *sasA::ermB*), ACD19 (Δspa , *secA2::ermB*) or ACD20 (Δspa , *secY2::ermB*) were analysed by immunofluorescence microscopy with α SasA antibodies followed by Alexafluor 647-conjugated secondary antibody and staining with BODIPY-conjugated vancomycin and compared with captured DIC images. See text for details and Supplementary Figure 4 for SasF and ClfA staining of *secY2* mutant staphylococci.

(signal peptide without motif) is preferentially secreted at the cell pole (Carlsson *et al*, 2006). In staphylococci, five polypeptides with YSIRK/GS motif signal peptides (protein A, ClfA, SdrC, SdrD and FnbpB) are distributed in a ring-like manner in the vicinity of the cell division site. Four surface proteins without the YSIRK/GS motif (SasA, SasD, SasF and SasK) are deposited at discrete focal assemblies in the envelope that, however, do not overlap with the ring-like distribution of proteins secreted with the YSIRK/GS motif.

Pioneering studies on staphylococcal morphology used electron microscopy and revealed the cross wall, a thick layer of newly synthesized peptidoglycan separating adjacent daughter cells (Giesbrecht *et al*, 1976; Touhami *et al*, 2004). Cross wall synthesis is preceded by FtsZ ring formation and cytokinesis, that is, the segregation of chromosomes into daughter cells, which is also accompanied by FtsZ constriction and cell separation (Bi and Lutkenhaus, 1991; Lutkenhaus, 1993). Unlike the elongated growth of a rod-shaped microbe along its cylindrical axis (de Boer *et al*, 1990), the overall size of staphylococcal cells does not

increase prior to cross wall separation (Touhami *et al*, 2004). These findings imply that newly replicated chromosomes and cross wall, in short, two newly formed daughter cells, are all accommodated within a murein sacculus that has not expanded following the last round of cell separation. Once the cross wall has been cleaved by lytic enzymes (autolysin, Atl) (Oshida *et al*, 1995; Yamada *et al*, 1996), the separating daughter cells immediately assume similar size and shape as older cells already engaged in cell division (Touhami *et al*, 2004).

These observations provide an important base for our working model on protein targeting to the cell wall envelope (Figure 10). Once formed, murein sacculi of staphylococcal cells cannot expand unless the cross wall of dividing daughter cells is cleaved. We and others did not observe half-cells, hemispherical staphylococci with one flat surface (Giesbrecht *et al*, 1998). It therefore seems reasonable to assume that the bacterial osmotic pressure expands the shape of dividing daughter cells immediately to form round spheres (Touhami *et al*, 2004). Bulk *de novo* cell wall synthesis occurs mostly at

Figure 10 Model for surface protein trafficking in staphylococci. Newly synthesized peptidoglycan is assembled in the cross wall and, once daughter cells have been separated, this compartment is exposed as staphylococcal envelope. YSIRK/GS motif signal peptides address surface proteins to the cross wall of staphylococci, whereas conventional signal peptides direct proteins to peripheral secretion sites at the cell pole. Protein secretion sites accessed by conventional signal peptides by default lead to protein transport into extracellular milieu. Sortase-mediated anchoring of proteins with conventional signal peptides is thought to immobilize proteins in the immediate vicinity of staphylococcal secretion sites.

the cross wall, which represents about half of the murein sacculus of a future daughter cell (Briles and Tomasz, 1970; Pinho and Errington, 2003). Proteins deposited in the cross wall initially cannot be displayed on the bacterial surface; the cross wall compartment, including the space between two daughter cells, is secluded from the environment by murein sacculi. If so, protein traffic to the cross wall is probably difficult to measure with immunofluorescence microscopy, as antibodies may be unable to penetrate murein sacculi.

Considering the unique attributes of cell division in staphylococci, we propose that the destiny of surface protein traffic through YSIRK/GS motif signal peptides resides in the vicinity of future cell division sites and within the cross wall. Massive synthesis of peptidoglycan within the cross wall may ensure that assembly sites for surface protein deposition are mobile within the peptidoglycan layer as the cross wall expands, first within the division plane and then following separation of daughter cells in spherical staphylococci. Such presumed protein traffic to the cross wall would be unable to promote protein secretion into the extracellular medium, unless daughter cells have already separated. In agreement with this conjecture, the cross wall pathway appears to be engaged by YSIRK/GS motif signal peptides and may be largely restricted to surface proteins. The alternative secretion pathway, travelled by substrates with conventional signal peptides, by default leads across the murein sacculus into the extracellular medium. This pathway is engaged by surface proteins that get deposited in the cell wall as discrete foci surrounding these secretion sites.

Carlsson *et al* (2006) generated substitutions in the YSIRK/GS motif of M protein and measured polypeptide targeting to the cell wall envelope by immune-electron microscopy. Signal peptide variants with YAARK/GS or YSAAK/GS motifs continued to direct proteins to the cell division site, similar to wild-type M protein (Carlsson *et al*, 2006). We have also examined the amino-acid requirement of the YSIRK/GS motif for cross wall secretion. Single amino-acid substitutions of each conserved residue of the YSIRK/GS motif (data not shown) or a scrambled YSIRK sequence, all failed to abolish surface protein localization. Thus, although YSIRK/GS signal peptides are clearly sufficient to address surface proteins to the cross wall, the YSIRK sequence may not be required for this mechanism and another, discrete feature of these signal peptides must provide for surface protein traffic.

In summary, we report that staphylococci address surface proteins to discrete locations in the bacterial envelope by mechanisms requiring recognition of specific signal peptides. Protein traffic to discrete sites in the cell wall envelope of staphylococci seems a fascinating problem, the future focus of which is the unravelling of genes and mechanisms responsible for directing surface proteins to alternate envelope sites. Conservation of the YSIRK/GS motif in signal peptides of several bacterial species suggests that these mechanisms could apply to different microbes.

Materials and methods

Bacterial strains, media and growth conditions

S. aureus strains were grown in tryptic soya broth (TSB) at 37°C to mid-log phase (optical density at 600 nm (OD₆₀₀) of 0.5), unless otherwise stated. *Escherichia coli* DH5α was grown in Luria-Bertani broth at 37°C (Hanahan, 1983). The Δspa mutant strains SEJ1, a derivative of RN4220 (Kreiswirth *et al*, 1983), and SEJ2, a variant of strain Newman (Duthie and Lorenz, 1952), have been described earlier (Stranger-Jones *et al*, 2006; Gründling and Schneewind, 2007). Surface protein mutants harbouring the *bursa aurealis* mariner transposon insertions were obtained from the Phoenix (ΦNE) library (Bae *et al*, 2004) (Supplementary Table 1). Mutations were transduced with bacteriophage φ85 into SEJ1 or SEJ2 and grown in TSB supplemented with 10 μg ml⁻¹ erythromycin. Following plasmid transformation, *E. coli* DH5α transformants were selected in the presence of 100 μg ml⁻¹ ampicillin. Following transformation of relevant *S. aureus* strains, transformants were selected by growth on 10 μg ml⁻¹ chloramphenicol.

Phage transduction, plasmids and mutagenesis

φ85 transductants were confirmed by inverse PCR and DNA sequencing as described (Bae *et al*, 2004). For plasmid complementation studies, the open reading frame of surface protein genes, for example, *clfA* or *sasF*, and 1000 bp of upstream sequence was PCR amplified from *S. aureus* Newman chromosomal DNA with primers AAAGAATTCGTTGTAATGCATTTTCTATAAGATAGAAGCTA AAAGGAG and AAAGCTAGCAATAATTAAGTGTGATGAATTAATT ATTAATTATATTCAAACAA for *clfA* or AAAGAATTCATGAAACGATGCTCAATATAGCAGAC and AAAGCTAGCTTCATTATAACGTTAAA ATGATGGACAATCTATTCA for *sasF*. Resulting PCR products were digested with the restriction enzymes *NheI* and *BamHI* and ligated into pOS1 (Schneewind *et al*, 1993) cut with the same enzymes. Restriction sites flanking coding sequence for N-terminal signal peptides were cloned by the QuikChange method (Stratagene). Specifically, an *XhoI* site was inserted 30 nucleotides downstream of the predicted cleavage site with primers GTTACGCAATCTGATAGC CTCGAGGCAAGTAACGAAAGCAAAAG and CTTTGTCTTTCGTTAC TTGCTCGAGGCTATCAGATTGCGTAAC (*clfA*) or GGATACGAAAT TTCAAACTCGAGATATTATCTAAGCAAG and CTTGCTTAGATAAT ATCTCGAGTTTGAATTTTCAGTATCC (*sasF*). An *AvrII* site was generated following mutation of nucleotides 9 bp upstream from the annotated open reading frame start site with primers TAGTT AATATAAATGACTTTTACCTAGGGGAATAAATGAATATGAAGA

AA and TTTCTTCATATTCATTTTATTCCTAGGTAAAAAGTCATT TTATATTAACCTA (*clfA*) or TAATTCCTTTGAAGGAAGTAAGTGACCT AGGAGTATGTTGATG GCTAAATATCGA and TCGATATTTAGCCAT-CAACATACTCTAGGTCACCTTACTTCCTCAAAGAATTA (*sasF*). Following confirmation by restriction digest and DNA sequencing analysis, the resulting plasmids, pClfA or pSasF, or empty vector were transformed by electroporation into the *S. aureus* strains *Δspa*, *clfA::ermB* or *Δspa*, *sasF::ermB*, respectively. For construction of signal peptide exchange hybrid proteins, pClfA or pSasF was digested with the restriction enzymes *AvrII* and *XhoI*. The resulting fragments were ligated together (*clfA* signal peptide insert ligated into the *sasF* vector fragment and *sasF* signal peptide insert ligated into the *clfA* vector fragment), generating pSP_{ClfA}-SasF and pSP_{SasF}-ClfA, respectively. Plasmid signal peptide exchanges were screened by colony PCR and confirmed by DNA sequencing analysis and pSP_{ClfA}-SasF and pSP_{SasF}-ClfA were transformed into *S. aureus*. The plasmid pClfA_{YSIRKscramble} was cloned from pClfA using QuikChange mutagenesis (Stratagene) with the primers AAAAAAGAAAAA TTAACACGCATCAAAATCGATTGGGTGGCTGGCGTGCTTGTAGGT and ACCTACAAGCACGCCAGCCACCCGAATCGATTTTGATGCGTGTT TAATTTTTCTTTTTT. The integration plasmids pCL55-SP_{ClfA}-SasF and pCL55-ClfA_{YSIRKscramble} were generated by subcloning the SP_{ClfA}-SasF or ClfA_{YSIRKscramble} fragment into *SmaI*-digested pCL55 by blunt end ligation following digestion with *EcoRI* and *NheI* and generation of blunt ends with Klenow enzyme. pCL55-SasF was generated following PCR amplification of SasF using the same primers described for pSasF. The resulting PCR product was ligated together with *SmaI*-digested pCL55. pCL55-SP_{ClfA}-SasF, pClfA_{YSIRKscramble} and pCL55-SasF were transformed by electroporation into the *S. aureus* strains.

Immunofluorescence microscopy

Overnight cultures of *S. aureus* cells were diluted 1:100 into fresh growth media and grown to mid-log phase (OD₆₆₀ of ~0.6) and equivalent of 4–10 cell divisions as surface proteins are regulated by *agr* (Novick, 2003). The culture (6 ml) was centrifuged for 5 min at 8000g. Bacterial cell pellets were washed twice with 10 ml phosphate-buffered saline (PBS, 10 mM sodium phosphate, pH 7.0). Cells were suspended in 500 µl of PBS, sonicated for 15 s and fixed with 2.5% paraformaldehyde, 0.006% glutaraldehyde in 30 mM PBS (pH 7.4) for 20 min at room temperature. Cells were washed three times with 1 ml PBS, suspended in a final volume of 250 µl of 3% bovine serum albumin (BSA) in PBS as a blocking reagent for nonspecific antibody binding, and incubated for 1 h at room temperature or overnight at 4°C with shaking. Primary antibodies were adsorbed prior to incubation with fixed staphylococci by pre-incubation of immune sera with acetone precipitates of transduced φN₃ library mutant staphylococci lacking the gene to which the serum was raised. Following blocking, staphylococci were sedimented by centrifugation at 8000g for 3 min and suspended in 250 µl of adsorbed primary antibody (1:500 in 3% BSA-PBS) and incubated for 1 h at room temperature with rotation. Primary incubation was followed by incubation with Alexafluor 647-IgG secondary, and BODIPY-vancomycin (Invitrogen Molecular Probes). Cells were washed five times with 1 ml PBS containing

0.025% Tween 20 (PBS-T) followed by 1 h incubation in the dark with 250 µl of 1:250 goat anti-rabbit Alexafluor 647-conjugated secondary antibody in 3% BSA-PBS. Staphylococci were sedimented and incubated with 200 µl of 1 µg ml⁻¹ BODIPY-vancomycin conjugate for 5 min. Cells were again washed five times with 1 ml PBS and suspended in 100 µl. Cell suspensions were applied to L-poly-lysine-coated coverslips for 3 min followed by three washes with 300 µl PBS. Slides were mounted with *N*-propylgallate, sealed, viewed and images were collected with a Leica SP5 AOBs spectral two-photon confocal microscope (DeDent *et al*, 2007). Captured images and Z-stacks were processed with ImageJ software and plugins (Abramoff *et al*, 2004).

Double labelling and trypsin treatment experiments

To prevent cross-reactivity of secondary antibodies for labelling of two surface proteins, primary antibodies were either labelled using the Zenon Alexafluor rabbit IgG labelling kit (conjugated Fab fragments; Invitrogen) or directly conjugated to Alexafluor 488 or 647 (Invitrogen Molecular Probes). Prior to conjugation, ClfA- or SasF-specific rabbit serum was incubated with acetone-precipitated cells of each mutant, supernatant was applied to a protein A column (Pierce), eluted and dialysed overnight with PBS. Following incubation of cells with 3% BSA-PBS, cells were incubated for 1 h at room temperature with both αClfA-Alexafluor 488 and αSasF-Alexafluor 647 (1:25 in 3% BSA-PBS). Cells were washed and prepared for microscopy as described. For trypsin treatment, staphylococci were treated for 1 h with 0.2 mg ml⁻¹ trypsin in PBS at 37°C. Cells were washed twice with 10 ml PBS and suspended in 1 ml TSB containing 1 mM phenylmethylsulphonyl fluoride (PMSF) (Figure 6) or soyabean trypsin inhibitor (Figure 8; Supplementary Figure 5) and grown at 37°C. Aliquots of cells were removed at 0, 20 or 40 min following addition of PMSF and growth at 37°C. Cells were sonicated for 15 s, fixed, labelled with primary-conjugated antibodies, and slides were prepared as described. Images were collected with a Leica SP5 AOBs spectral two-photon confocal microscope and z-series scans were sampled in 120-nm increments.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Dr Vytas Bindokas, Director of the University of Chicago BSD Light Microscopy Core Facility, for assistance with microscopy experiments, Juliane Bubeck-Wardenburg, Gabriella Garufi, Luciano Marraffini, Alice Cheng, Hwan Keun Kim and Brandon Wojcik for critical reading of the paper, and members of our laboratory for discussion. This study was supported by a grant from the National Institute of Allergy and Infectious Diseases, Infectious Disease Branch (AI38897) to OS. AD acknowledges support from the Molecular Cell Biology Training Grant T32GM007183 at the University of Chicago.

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