# Septal Secretion of Protein A in *Staphylococcus aureus* Requires SecA and Lipoteichoic Acid Synthesis

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## Abstract

Surface proteins of *Staphylococcus aureus* are secreted across septal membranes for assembly into the bacterial cross-wall. This localized secretion requires the YSIRK/GXXS motif signal peptide, however the mechanisms supporting precursor trafficking are not known. We show here that the signal peptide of staphylococcal protein A (SpA) is cleaved at the YSIRK/GXXS motif. A signal peptide mutant defective for cleavage can be crosslinked to SecA, SecDF and LtaS. SecA depletion blocks precursor targeting to septal membranes, whereas deletion of *secDF* diminishes SpA secretion into the cross-wall. Depletion of LtaS blocks lipoteichoic acid synthesis and promotes precursor trafficking to peripheral membranes. We propose a model whereby SecA directs SpA precursors to lipoteichoic acid-rich septal membranes for YSIRK/GXXS motif cleavage and secretion into the cross-wall.

## Introduction

Surface proteins of *Staphylococcus aureus* and other gram-positive cocci enter the secretory pathway via their N-terminal signal peptides (Uhlén et al., 1984, DeDent et al., 2008). Once translocated across the membrane, surface proteins are covalently linked to cell wall peptidoglycan via sortase A-catalyzed cleavage at the LPXTG motif of C-terminal sorting signals (Schneewind et al., 1992, Schneewind et al., 1995, Mazmanian et al., 1999). Some, but not all surface proteins are secreted at septal membranes and incorporated into cross-wall peptidoglycan (Cole and Hahn, 1962, Carlsson et al., 2006, DeDent et al., 2008). Following division and separation of spherical daughter cells, cross-wall anchored surface proteins are displayed over large segments of the bacterial surface (DeDent et al., 2007). Cross-wall

trafficking of surface proteins requires a signal peptide with YSIRK/GXXS motif (Carlsson et al., 2006, DeDent et al., 2008). The YSIRK/GXXS motif is positioned N-terminal of the hydrophobic core, common to all signal peptide precursors traveling the Sec pathway (Emr et al., 1978, Emr et al., 1981, von Heijne, 1986).

Gram-positive bacteria rely on cell wall-anchored surface proteins for adherence to host tissues, evasion from host immune responses and acquisition of host-specific nutrients (Foster et al., 2014). Surface proteins with YSIRK/GXXS signal peptides are produced with high abundance and fulfill essential virulence functions during infection. For example, staphylococcal protein A (SpA) is well known for its attribute of binding to host immunoglobulin and disrupting adaptive immune responses (Forsgren and Sjöquist, 1966, Kim et al., 2016). SpA is synthesized as a precursor with an N-terminal YSIRK/GXXS signal peptide and a C-terminal LPXTG motif sorting signal (Abrahmsén et al., 1985, Schneewind et al., 1992). After initiation into the secretion pathway, the signal peptide is cleaved by signal peptidase (Abrahmsén et al., 1985, Schallenberger et al., 2012). Sortase A recognizes the LPXTG motif of the sorting signal, cleaves the polypeptide between the threonine (T) and the glycine (G) of the LPXTG motif and forms an acyl-enzyme intermediate with the C-terminal threonine (Mazmanian et al., 1999, Ton-That et al., 1999). The acyl-enzyme is relieved by the nucleophilic attack of the amino-group of the pentaglycine crossbridge within lipid II, the precursor for peptidoglycan synthesis (Ton-That et al., 2000, Perry et al., 2002). The product of this reaction, surface protein linked to lipid II, is then incorporated into peptidoglycan via the transglycosylation and transpeptidation reactions of cell wall synthesis (Ton-That et al., 1997, Ton-That and Schneewind, 1999).

Newly synthesized SpA is secreted into the cross-wall compartment, bounded by septal membranes of burgeoning cells during division (DeDent et al., 2007). Upon completion of peptidoglycan synthesis within the cross-wall, its peptidoglycan layer is split (Frankel et al., 2011). The adjacent cells separate and assume a spherical shape, resulting in SpA display on the bacterial surface (DeDent et al., 2007). Staphylococci divide perpendicular to previous cell division planes (Tzagoloff and Novick, 1977). By incorporating secreted polypeptides into newly synthesized cross-walls, staphylococci distribute SpA and other sortase A-anchored products over the bacterial surface (DeDent et al., 2008). However, not all sortase-anchored products traffic to septal membranes. Those that are secreted at polar membranes are also anchored to peptidoglycan but are not distributed over the bacterial surface (DeDent et al., 2008). In *S. aureus* strain Newman, thirteen different sortase-anchored surface proteins and four additional proteins are endowed with YSIRK/GXXS signal peptides for septal secretion: lipase (Lip), glycerol-ester hydrolase (Geh), murein hydrolase LytN and the cell size determinant Ebh (Yu and Götz, 2012, Frankel et al., 2011, Cheng et al., 2014).

The mechanisms supporting YSIRK/GXXS precursor secretion at septal membranes are not known. Here we show that the signal peptide of SpA is cleaved at the YSIRK/GXXS motif. Amino acid substitutions in the signal peptide that affect cleavage also impair SpA secretion. When used as a bait for the crosslinking of membrane proteins, purification of the SpA Ser$^{18}$Leu (S18L) precursor identified SecA, SecDF and LtaS. We studied the contribution of these factors towards protein A secretion into the cross-wall compartment.

## Results

**SpA signal peptide variants defective for septal secretion**

To facilitate the analysis of signal peptide mutants, we generated $SpA_{ED}$, a variant of protein A that is truncated for its C-terminal immunoglobulin binding domains, region X (Xr) and the LPXTG sorting signal (*Figure 1a*). *S. aureus* WY110 (Δ*spa* Δ*sbi*, p$SpA_{ED}$) cultures expressing $spa_{ED}$ were fractionated into culture supernatant (S) and bacterial pellet (P) and analyzed by immunoblotting. $SpA_{ED}$ was found in the extracellular medium; its precursor species was detected in the bacterial pellet (*Figure 1bc*). Site-directed mutagenesis was used to generate short deletions and amino acid substitutions in the signal peptide of $SpA_{ED}$ (*Figure 1b*). Deletion of the YISRK motif (ΔYSIRK) diminished the abundance of the $SpA_{ED/\Delta YSIRK}$ precursor and its processing (*Figure 1b*). Single amino acid substitutions at two positions in the YSIRK motif (I9S and R10A) resulted in precursor accumulation (*Figure 1b*). Further, the R10A variant exhibited diminished secretion and accumulated a precursor species that migrated faster on SDS-PAGE than the full-length precursor (*Figure 1bc*). Amino acid substitution at lysine 11 (K11A) of the YSIRK motif had no effect on $SpA_{ED/K11A}$ precursor processing and secretion (*Figure 1bc*). Deletion of GIAS (ΔGIAS) or of the two variable residues in the GXXS motif (ΔIA) caused precursor accumulation and blocked precursor processing (*Figure 1bc*). Substitution of glycine 15 (G15L) reduced the abundance of $SpA_{ED/G15L}$ and led to the accumulation of a unique precursor species that migrated faster on SDS-PAGE than full-length precursor (*Figure 1b*). Substitution of serine 18 (S18L) caused accumulation of full-length and processed precursors as well as reduced secretion (*Figure 1bc*).

*S. aureus* WY110 cultures were fractionated into culture supernatant (S), cell wall extract (W), membranes (M) and cytoplasm (C). The $SpA_{ED}$ precursor was found in the

cytoplasm and membrane, whereas mature product was secreted into the culture supernatant (S) (*Figure 2a*). Precursors of the $SpA_{ED/\Delta IA}$ and $SpA_{ED/R10A}$ variants accumulated mostly in the cytoplasm, whereas the $SpA_{ED/S18L}$ precursor was located predominantly in the membrane (*Figure 2ab*). Pulse-labeling experiments revealed that wild-type $SpA_{ED}$ precursor was processed within 60 seconds into mature, secreted product (*Figure 2c*). In contrast, processing of the $SpA_{ED/\Delta IA}$, $SpA_{ED/R10A}$ and $SpA_{ED/S18L}$ precursors was delayed (*Figure 2c*). To test whether signal peptide variations affect trafficking of full-length SpA, mutations encoding the ΔIA, R10A and S18L variants were introduced into wild-type *spa*. Wild-type and mutant staphylococci were treated with trypsin to remove all surface proteins from the bacterial surface and incubated for 20 min to allow for cell wall deposition of newly synthesized SpA. To localize SpA, bacteria were viewed by fluorescence microscopy after labeling with SpA-specific monoclonal antibody and Alexa Fluor 647-conjugated secondary IgG (red) and with BODIPY FL-vancomycin (green), which binds to cell wall peptidoglycan. As expected, wild-type SpA was assembled in the cross-wall compartment, whereas $SpA_{SP\text{-}SasF}$, which is secreted via a canonical signal peptide, was deposited into peripheral segments of the cell wall envelope (DeDent et al., 2008)(*Figure 2d*). $SpA_{\Delta IA}$, $SpA_{R10A}$ and $SpA_{S18L}$ exhibited defects in surface display, consistent with their observed defects in precursor processing and secretion (*Figure 2d*). Residual amounts of cross-wall localization were observed for $SpA_{R10A}$ and $SpA_{S18L}$, whereas $SpA_{\Delta IA}$ was not detected in the cross-wall compartment (*Figure 2e*). Together these data indicate that some features of the YSIRK/GXXS motif, specifically $Arg^{10}$, $Ser^{18}$ and the GXXS motif, are crucial for septal secretion of SpA in *S. aureus* (*Figure 2e*).

**Processing of SpA signal peptide variants**

Wild-type $SpA_{ED}$, $SpA_{ED/\Delta IA}$, $SpA_{ED/R10A}$ and $SpA_{ED/S18L}$ were purified from staphylococcal membranes via affinity chromatography, analyzed by Coomassie-stained SDS-PAGE and identified by Edman degradation (*Figure 3a*). For wild-type $SpA_{ED}$, full-length precursor ($SpA_{ED}$-1, starting at $Met^{1}$), as well as two precursors with faster mobility on SDS-PAGE ($SpA_{ED}$-2 and $SpA_{ED}$-3) and mature product ($SpA_{ED}$-4), i.e. SpsB signal peptidase-cleaved $SpA_{ED}$ starting at $Ala^{37}$, were identified (*Figure 3ab*). Edman degradation revealed that $SpA_{ED}$-2 is a product of proteolytic cleavage within the YSIRK/GXXS motif (N-terminus $Gly^{13}$). $SpA_{ED}$-3 is a product of further cleavage, as Edman degradation identified its N-terminal amino acid 10 residues downstream (N-terminus $Thr^{23}$) (*Figure 3ab*). Purified $SpA_{ED}$-1 precursor as well as its $SpA_{ED}$-2, $SpA_{ED}$-3 and $SpA_{ED}$-4 cleavage products were analyzed by MALDI-TOF-MS, confirming the predicted mass of the precursor and its cleaved species (*Table 1*). SDS-PAGE and Edman analysis of the $SpA_{ED/S18L}$ sample revealed the same four species as wild-type $SpA_{ED}$, albeit that the abundance of $SpA_{ED/S18L}$-1 and $SpA_{ED/S18L}$-4 were increased over those of of $SpA_{ED/S18L}$-2 and $SpA_{ED/S18L}$-3 (*Figure 3ab*). Analysis of the $SpA_{ED/R10A}$ sample also identified four species, including $SpA_{ED/\ R10A}$-1 precursor, $SpA_{ED/\ R10A}$-3 cleavage product (N-terminus $Thr^{23}$) and $SpA_{ED/\ R10A}$-4 mature product (N-terminus $Ala^{37}$), whereas $SpA_{ED/\ R10A}$-2 represented a variant cleavage product (N-terminus $Ala^{10}$) (*Figure 3ab*). The $SpA_{ED/\Delta IA}$ sample yielded the same precursor and cleavage species as $SpA_{ED}$ and $SpA_{ED/S18L}$, however the abundance of $SpA_{ED/\Delta IA}$-1 was increased over that of $SpA_{ED/\Delta IA}$-2, $SpA_{ED/\Delta IA}$-3 and $SpA_{ED/\Delta IA}$-4 (*Figure 3ab*). Taken together, these data indicate that the SpA precursor ($SpA_{ED}$-1) is cleaved between $Leu^{12}$ and $Gly^{13}$, which are positioned between the two motifs (underlined) of the YSIRKL/GVGIAS sequence. The R10A

substitution alters the cleavage site and diminishes precursor cleavage, whereas the S18L substitution and ΔIA deletion diminish precursor cleavage without altering the cleavage site between the YSIRK/GXXS motifs. Precursor cleavage between $Gly^{22}$ and $Thr^{23}$ was observed for all SpA variants, suggesting that it represents a proteolytic event unrelated to the function of the YSIRK/GXXS motif in targeting SpA to septal membranes.

**Identification of proteins cross-linked to a mutant SpA precursor**

We used a biochemical approach to identify staphylococcal proteins that interact with SpA precursor in septal membranes. The $SpA_{S18L}$ precursor accumulates in septal membranes (*Figure 2bc*), and $SpA_{ED/S18L}$ precursor can be purified from these membranes (*Figure 3ab*). After crosslinking with formaldehyde, $SpA_{ED/S18L}$ precursor and associated species were isolated via affinity chromatography, heat-treated to resolve crosslinks and analyzed by Coomassie-stained SDS-PAGE and immunoblotting with anti-SpA (*Figure 4ab*). As compared to $SpA_{ED/SP\text{-}SasF}$, which does not traffic to septal membranes and was not crosslinked to other proteins, several proteins were specifically crosslinked to $SpA_{ED/S18L}$ and were identified by mass spectrometry (*Figure 4ab* and *Table S1*). Most crosslinked proteins are components of the peptidoglycan (PBP2, MurE2, MurG, FemA, FemB, FemX), wall teichoic acid (TagB, TagF) and lipoteichoic acid synthesis pathways (LtaS) that are known to be localized to septal membranes (Pinho and Errington, 2005, Mann et al., 2013, Reichmann et al., 2014). We also identified EzrA, a cell division machinery component (Steele et al., 2011). SecA and SecDF, members of the bacterial protein secretory pathway (Oliver and Beckwith, 1981, Gardel et al., 1987, Pogliano and Beckwith, 1994), and LtaS, lipoteichoic acid synthase (Gründling and Schneewind, 2007), were

selected for further study. Immunoblotting confirmed SecA crosslinking to the $SpA_{ED/S18L}$ precursor (*Figure 4c*).

**SecA depletion in *S. aureus***

In *Escherichia coli*, *secA* is an essential gene (Oliver and Beckwith, 1981). SecA functions as an ATPase that moves many, but not all, precursor proteins across the SecYEG translocon (Tsirigotaki et al., 2017). To study the contribution of *secA* towards the septal secretion of SpA, we generated an inducible allele, $P_{spac}$-*secA*, in *S. aureus* WY223 (*Figure 5a*). When induced with isopropyl β-D-1-thiogalactoside (IPTG), *S. aureus* WY223 ($P_{spac}$-*secA*) forms colonies on agar and replicates in liquid media culture in a manner similar to wild-type *S. aureus* (*Figure 5bc*). However, in the absence of IPTG, *S. aureus* WY223 cannot form colonies or replicate in broth culture (*Figure 5bc*). Following dilution of bacteria from IPTG-containing media into broth without inducer, *S. aureus* WY223 replicates for 3 hours at a rate similar to wild-type (*Figure 5b*). Upon further dilution and incubation, *S. aureus* WY223 eventually exhibits growth retardation and arrest (6-hour time point). When analyzed by immunoblotting with SecA-specific antibody, SecA was depleted in *S. aureus* WY223 ($P_{spac}$-*secA*) cultures 3 hours following dilution into inducer free medium. After 6 hours of incubation, SecA could no longer be detected (*Figure 5d*).

**SecA depletion blocks SpA secretion**

After dilution into media with and without inducer, wild-type (*S. aureus* RN4220) and $P_{spac}$-*secA* (*S. aureus* WY223) were subjected to pulse labeling with [$^{35}$S]methionine and protein A

184 precursor processing was analyzed by immunoprecipitation. In wild-type, SpA precursors are
185 processed within 60 seconds; similar rates of processing were observed when the $P_{spac}$-*secA*
186 mutant was grown with IPTG inducer (*Figure 6a*). In the absence of IPTG, SpA precursor
187 processing was slowed to about 5 min, indicating that SecA depletion inhibits precursor
188 translocation (*Figure 6a*). When analyzed by fluorescence microcopy in trypsin-treated
189 staphylococci incubated for 20 min without protease, wild-type *S. aureus* deposited protein A
190 into the cross wall (*Figure 6b, yellow arrow*). Cross wall localization was diminished in $P_{spac}$-*secA*
191 mutant bacteria grown without IPTG inducer and restored to wild-type levels when bacteria
192 were grown in the presence of inducer (*Figure 6c*). Six hours after dilution into broth without
193 IPTG inducer, *S. aureus* WY223 ($P_{spac}$-*secA*) cells were grossly enlarged and surrounded by a thin
194 layer of peptidoglycan with aberrant cross-wall formation (*Figure 6d*, *blue arrow*); at this time
195 point, SpA could not be detected in the bacterial envelope. As a control, growth of *S. aureus*
196 WY223 in the presence of IPTG did not affect cell size and SpA deposition into the cell wall
197 (*Figure 6d*).

198 We wondered whether SecA depletion affects the secretion of other staphylococcal
199 proteins. Glycerol-ester hydrolase (Geh) is synthesized as a precursor with YSIRK/GXXS signal
200 peptide motif (Lee and Iandolo, 1986). Following secretion at septal membranes into the cross-
201 wall compartment, Geh is subsequently released into the extracellular medium (Yu and Götz,
202 2012). When analyzed by immunoblotting of proteins in the extracellular medium, depletion of
203 SecA in *S. aureus* WY223 ($P_{spac}$-*secA*) caused a reduction in secreted Geh, as compared to wild-
204 type staphylococci or *S. aureus* WY223 grown in the presence of IPTG (*Figure 6e*). Staphylooccal
205 nuclease (Nuc), a secreted protein that contribute to the pathogenesis of human and animal

infections, is synthesized as a precursor with a canonical signal peptide (Phonimdaeng et al., 1990, Shortle, 1983). The abundance of secreted Nuc was also diminished in SecA-depleted cultures of *S. aureus* WY223 (*Figure 6e*). As a control, production of sortase A in staphylococcal membranes was not affected by the depletion of SecA. Taken together, these data indicate that SecA is essential for *S. aureus* growth and for the secretion of precursors with canonical and YSIRK/GXXS signal peptides.

**Localization of SecA and SpA precursors in staphylococci**

To localize SecA within *S. aureus*, we generated a translational hybrid between *secA* and the structural gene for super-folder green fluorescent protein (*gfp*) (Pedelacq et al., 2006) under transcriptional control of the $P_{tet}$ promoter in *S. aureus* WY230 ($P_{spac}$-*secA*, $P_{tet}$-*secA:sfGFP*, *Figure 7a*). Expression of *secA:sfGFP* in the $P_{spac}$-*secA* variant restored bacterial growth in the absence of IPTG inducer, indicating that *secA-gfp* is functional (*Figure 7b*). Growth restoration occurred in the presence and in the absence of anhydrotetracycline (ATc), suggesting that *secA:sfGFP* must be expressed even in the absence of the $P_{tet}$ inducer (*Figure 7b*). Immunoblotting staphylococcal cell extracts 3 and 6 hours following dilution into media lacking IPTG revealed that *S. aureus* WY230 indeed produced small amounts SecA:sfGFP in the absence of ATc (*Figure 7c*). In the presence of ATc inducer, the abundance of SecA-GFP was increased (*Figure 7c*). As expected, wild-type SecA was depleted when *S. aureus* WY230 was cultured for 3 or 6 hours without the IPTG inducer (*Figure 7b*). However, under SecA depleting conditions, even small amounts of SecA-sfGFP in *S. aureus* WY230 (-ATc) restored precursor processing of pulse-labeled SpA (*Figure 7d*). SpA precursor processing was accelerated to levels faster than

wild-type when *S. aureus* WY230 cultures were grown in the presence of ATc (*Figure 7d*). Fluorescence microscopy of *S. aureus* WY230 stained with the membrane dye FM4-64 (red) revealed SecA-sfGFP localization to plasma membranes (*Figure 7e*). In dividing cells, SecA-sfGFP was found on septal (*yellow arrow*) and on polar membranes (*orange arrow*, *Figure 7e*). ATc-induced overexpression of SecA-sfGFP caused accumulation of hybrid protein throughout the cytoplasm (*Figure 7e*). Thus, in *S. aureus* WY230, SecA-sfGFP is associated throughout the plasma membranes and not restricted to the septal membrane.

The envelope of trypsin-treated, paraformaldehyde-fixed *S. aureus* WY223 ($P_{spac}$-*secA*) was permeabilized with murein hydrolase and with detergent to detect intracellular precursors via microscopy with fluorescent antibody (Harry et al., 1995, Pinho and Errington, 2003). In *S. aureus* WY223 producing wild-type levels of SecA ($P_{spac}$-*secA* + IPTG), SpA precursors were localized to septal membranes (*Figure 8a*). In other images, SpA precursors appeared as two puncta or ring deposits at septal membranes, reminiscent of FtsZ and of the division rings that are known to accumulate at this site (*Figure 8ab*)(Lutkenhaus, 1993). In contrast, under SecA depleting conditions (-IPTG), SpA precursors in *S. aureus* WY223 were associated with polar membranes and were not localized to septal membranes (*Figure 8a*). These results suggest that in staphylococci with a functional secretion pathway, SpA precursor are localized to the vicinity of septal division rings. However, in cells lacking functional secretion machines, SpA precursors are located throughout the cytoplasm and cannot traffic to septal membranes.

**SecDF contributes to SpA secretion**

The s*ecDF* gene is not essential for protein secretion and *S. aureus* growth, however *secDF* mutants exhibit diminished secretion of many precursors secreted via canonical and YSIRK-GXXS signal peptides (Quiblier et al., 2011, Quiblier et al., 2013). SecDF is a member of the resistance nodulation and cell division (RND) membrane protein family with 12-transmembrane spanning segments. SecDF functions as a membrane-integrated chaperone. Sustained by the proton motive force, SecDF catalyzes ATP-independent translocation and folding of proteins on the *trans*-side of the plasma membrane (Tsukazaki et al., 2011). *S. aureus* expresses two additional RND proteins, here designated Rnd2 (SAOUHSC_02525) and Rnd3 (SAOUHSC_02866) (Quiblier et al., 2011). The *rnd2* gene is located downstream of *femX*, whose product tethers glycine from glycyl-tRNA to the ε-amino group of lysine in lipid II peptidoglycan precursor [$C_{55}$-$(PO_4)_2$-MurNAc(L-Ala-D-iGln-L-Lys-Da-Ala-D-Ala)-GlcNac](Rohrer et al., 1999). Rnd2 product interacts with FemB, PBP1 and PBP2 (Quiblier et al., 2011). As SecDF, FemB and PBP2 were each found crosslinked to $SpA_{ED/S18L}$ precursors (*Table S1*), we asked whether *secDF*, *rnd2* and *rnd3* contribute to septal secretion of SpA. Compared to wild-type *S. aureus*, the Δ*secDF* mutant (*S. aureus* WY418) accumulated $SpA_{ED}$ precursor in bacterial cells and secreted reduced amounts of mature $SpA_{ED}$ into the extracellular medium (*Figure 9ab*). *S. aureus rnd2* (WY416) and *rnd3* (WY400) mutants exhibited wild-type levels of $SpA_{ED}$ secretion (*Figure 9ab*). A variant lacking all three genes, Δ*secDF* Δ*rnd23*, accumulated precursors at a level similar to the Δ*secDF* mutant (*Figure 9ab*). When analyzed for other secreted proteins, the Δ*secDF* mutant secreted diminished amounts of Geh and failed to secrete Coa, whose precursor is secreted via a canonical signal peptide (Phonimdaeng et al., 1990), while the Δ*rnd2* and Δ*rnd3* variants displayed wild-type phenotypes (*Figure 9ab*). Thus, SecDF, but not Rnd2 and Rnd3, contributes

271 to protein secretion in *S. aureus*. Immunofluorescence microscopy experiments revealed that
272 septal secretion of SpA was diminished in the Δ*secDF* and Δ*secDF* Δ*rnd23* mutants (*Figure 9c*).
273 Unlike SecA-depleted cells, where SpA precursors failed to associate with septal membranes,
274 Δ*secDF* and Δ*secDF* Δ*rnd23* mutants exhibited puncta of SpA precursors and rings of low
275 fluorescent intensity at septal membranes (*Figure 9c*). The diminished abundance of SpA
276 precursors at septal membranes was restored to wild-type levels by plasmid-borne expression
277 of *secDF* in Δ*secDF* (pSecDF) and in Δ*secDF* Δ*rnd23* (pSecDF) staphylococci (*Figure 9c*). These
278 data suggest that SecDF aids in the translocation of SpA across staphylococcal membranes but
279 is not required precursor targeting to septal membranes.
280
281 **LtaS is required for septal localization of SpA**
282 LtaS-mediated synthesis of lipoteichoic acid, a polyglycerol-phosphate polymer decorated with
283 esterified D-alanyl and GlcNAc residues, is essential for *S. aureus* growth and cell division
284 (Gründling and Schneewind, 2007). Earlier work generated *S. aureus* $P_{spac}$-*ltaS*, a strain with
285 IPTG-inducible expression of lipoteichoic acid synthase. In the absence of IPTG inducer, LtaS is
286 depleted in *S. aureus* ANG499 ($P_{spac}$-*ltaS*), providing an experimental system to study the effects
287 of LTA synthesis on septal secretion of SpA (Gründling and Schneewind, 2007). Surface proteins
288 were removed with trypsin and staphylococci were incubated for 20 min to localize deposition
289 of newly synthesized SpA (*Figure 10bc*). LtaS depletion (-IPTG) resulted in SpA deposition into
290 polar peptidoglycan, whereas under LtaS inducing conditions (+IPTG) SpA was localized in the
291 cross-wall (*Figure 10bc*). We asked whether SpA precursors are mislocalized to polar
292 membranes under conditions of LtaS depletion. Microscopic analysis of trypsin-treated,

lysostaphin- and detergent-permeabilized staphylococci revealed SpA targeting to septal membranes in wild-type (*S. aureus* RN4220) and in IPTG-induced *S. aureus* ANG499 ($P_{spac}$-*ltaS*)(*Figure 10d*). In contrast, without IPTG inducer, *S. aureus* ANG499 mislocalized SpA precursors to polar membranes (*Figure 10d*). Protein secretion and cell wall anchoring of SpA were analyzed by immunoblotting in *S. aureus* cultures separated into culture supernatant (S) and bacterial sediment (P, pellet) samples. These experiments revealed that LtaS depletion in *S. aureus* ANG499 ($P_{spac}$-*ltaS*, -IPTG) diminished the abundance of cell wall anchored SpA without affecting the secretion of Geh and Nuc (*Figure 10e*). Consistent with the immunoblotting results, LtaS depletion diminished the overall surface distribution of SpA (*Figure 10f*), in agreement with the hypothesis that cross-wall targeting via the YSIRK/GXXS signal peptide, but not polar secretion, is responsible for efficient surface distribution of proteins in staphylococci (Carlsson et al., 2006, DeDent et al., 2008). Together these data indicate that LtaS depletion and a block in lipoteichoic acid synthesis abolished SpA precursor trafficking to septal membranes without affecting its secretion at polar membranes.

## Discussion

Cell wall-anchored surface proteins with YSIRK/GXXS motif signal peptides have been identified in streptococcal and staphylococcal species (Tettelin et al., 2005, Rosenstein and Götz, 2000). Although sortase-anchored surface proteins are found in many different gram-positive bacteria, the signal peptides of surface proteins in rod-shaped bacteria of the genus *Actinomyces, Bacillus*, *Clostridium*, and *Listeria* do not contain the YSIRK/GXXS motif. Common features of staphylococci and streptococci are their spherical or ovoid cell shapes and cell wall synthesis

315 programs at septal membranes; in staphylococci this compartment is designated as the cross-
316 wall (Giesbrecht et al., 1976, Touhami et al., 2004, Monteiro et al., 2015). Earlier work
317 demonstrated that the YSIRK/GXXS motif of the SpA precursor is dispensable for sortase-
318 catalyzed cell wall anchoring (Bae and Schneewind, 2003). However, precursors with
319 YSIRK/GXXS motif signal peptides are targeted for secretion at septal membranes and sortase-
320 mediated deposition into the cross wall compartment (Carlsson et al., 2006, DeDent et al.,
321 2008). After completion of cross-wall synthesis, peptidoglycan splitting and cell separation, the
322 anchored products of *spa* and of other genes with YSIRK/GXXS motif signal peptides are
323 distributed over the bacterial surface (Cole and Hahn, 1962, DeDent et al., 2007). In contrast,
324 surface proteins with canonical signal peptides are deposited by sortase into polar
325 peptidoglycan but cannot be distributed over bacterial surfaces (Carlsson et al., 2006, DeDent
326 et al., 2008).

327 Although it is clear that YSIRK/GXXS signal peptides are necessary and sufficient for
328 septal secretion of proteins, the mechanisms supporting such trafficking were heretofore not
329 known. We show that the YSIRK/GXXS signal peptide of SpA is cleaved between $Leu^{11}$ and $Gly^{12}$,
330 separating the YSIRK sequence from the GXXS motif and from the remainder of the signal
331 peptide. Amino acid substitutions and deletions that affect precursor cleavage and secretion
332 map to three of the four residues (underlined) that are strictly conserved in the YSIRK/GXXS
333 motif: $Ile^{9}$, $Arg^{10}$ and $Ser^{18}$. Substitution of the fourth residue, $Gly^{15}$, with Leu resulted in
334 diminished abundance of precursor substrate and secreted product and in accumulation of a
335 cleaved precursor species (*Figure 1b*). On the basis of these observations, we are compelled to
336 speculate that YSIRK/GXXS motif cleavage may represent a mechanism for precursor

translocation at septal membranes. For example, the YSIRK/GXXS motif may inhibit a key function of the adjacent hydrophobic core within signal peptides: promoting the membrane translocation of precursors. Such inhibitory mechanism could be relieved by a YSIRK/GXXS protease that localizes to the septal membrane. Other mechanisms of proteolytic control for YSIRK/GXXS mediated signal peptide function can also be thought of. Importantly, the discovery of two sequential proteolytic events, YSIRK/GXXS motif cleavage and the signal peptidase-mediated cut provide new experimental opportunities for the testing of predictive models. SpA precursors were also cut between $Gly^{22}$ and $Thr^{23}$, a site that is located within the hydrophobic core of the signal peptide. Mass spectrometry analysis of the *S. aureus* COL secretome also identified signal peptide fragments that had been generated by cleavage in the hydrophobic core, including SpA signal peptides cleaved between $Gly^{22}$ and $Thr^{23}$ (Ravipaty and Reilly, 2010). The significance of signal peptide cleavage in the hydrophobic core is not known, as amino acid substitutions preventing such proteolysis have not be studied for their effect on protein secretion or membrane integrity. We presume that cleavage at $Gly^{22}$/$Thr^{23}$ may not be related to septal secretion. Cleavage at the hydrophobic core may enable staphylococci to remove the products of processed signal peptides from the membrane. For example, products of degradative proteolysis have been observed during processing of SpA LPXTG motif sorting signal, which is cleaved between Thr (T) and Gly (G) and within the hydrophobic core (Navarre and Schneewind, 1994).

Precursors with amino acid substitutions or deletions in the YSIRK/GXXS motif are thought to accumulate in septal membranes, however these variants typically exhibit diminished secretion and cell wall anchoring in the cross wall (DeDent et al., 2008, Yu and Götz,

2012). Our observations corroborate these findings and suggest that not all features of the YSIRK/GXXS motif are required for precursor targeting to septal membranes. We took advantage of the $SpA_{ED/S18L}$ precursor and used affinity chromatography to purify crosslinked proteins. Several crosslinked proteins were already known to be located in septal membranes (PBP2, EzrA, LtaS), consistent with $SpA_{ED/S18L}$ precursor accumulation in this compartment. Among the crosslinked proteins are two components of the secretion machinery, SecA and SecDF, as well as LtaS, which catalyzes the synthesis of lipoteichoic acid in septal membranes (Tsirigotaki et al., 2017, Percy and Gründling, 2014).

The subcellular localization of the Sec apparatus has been examined in streptococci, enterococci and in *Bacillus subtilis*. A spiral pattern of Sec translocase has been reported for *B. subtilis* (Campo et al., 2004). In *S. pyogenes*, contradictory results have been reported for immunogold-labelling and electron microscopy experiments: SecA was localized to a single microdomain and also found distributed throughout the plasma membrane (Carlsson et al., 2006, Rosch and Caparon, 2004). In *S. pneumoniae*, SecA localization changed during cell cycle progression. In early divisional cells, SecA was predominantly localized to septal membranes, whereas during later stages of division SecA was hemispherically distributed within the region between septa and at the future equators of dividing cells (Tsui et al., 2011). *Streptococcus agalactiae* SecA was localized to septal membranes, whereas SecA was detected as a single microdomain in *Streptococcus mutans* and *Enterococcus faecalis* (Brega et al., 2013, Hu et al., 2008, Kline et al., 2009). We show here that *S. aureus* SecA is localized to the plasma membrane and is not spatially restricted to septal membranes or microdomains. This

distribution is consistent with our proposed role of SecA, promoting precursor translocation at polar and septal membranes.

When studied for its contribution to septal secretion, SecDF chaperone allows large amounts of protein A to be deposited into the cross-wall peptidoglycan and promotes secretion of YSIRK/GXXS motif precursors (SpA and Geh). Nevertheless, *secDF* is not essential for septal targeting or secretion of SpA precursors. In contrast, cells depleted for SecA, accumulate SpA precursors that cannot traffic to septal membranes in the cytoplasm. Finally, LtaS-depleted staphylococci are unable to synthesize lipoteichoic acid and cannot direct precursors to the septal area; instead, SpA is directed to polar membranes. We have incorporated these observations into a model whereby septal accumulation of LtaS and of lipoteichoic acids functions as a determinant for SecA-mediated targeting of SpA precursors. Following precursor cleavage at the YSIRK/GXXS motif, truncated SpA (SpA-2) is moved across the membrane, aided by the proton-motif force and by the chaperone activity of SecDF. Once translocated, SpA is cleaved by signal peptidase to generate SpA-4 and by sortase at the LPXTG motif of its C-terminal sorting signal (Navarre and Schneewind, 1994, Ton-That et al., 1999). The resulting sortase-acyl intermediate is then incorporated into cross wall peptidoglycan for distribution on the bacterial surface (Schneewind et al., 1995).

## Materials and methods

### Bacterial strains and growth conditions

*E. coli* strains were grown in Luria-Bertani broth (LB) or LB agar. *S. aureus* strains were grown in tryptic soy broth (TSB) or tryptic soy agar (TSA). Ampicillin (100 µg/ml) was used for plasmid

selection in *E. coli*. Chloramphenicol was used for selection of pOS1 derivatives (10 µg/ml) and pCL55 derivatives (5 µg/ml) in *S. aureus* (Lee et al., 1991). Erythromycin (Erm 10 µg/ml) was used for selection of *ermB* marked *bursa aurealis* transposon mutants in *S. aureus* WY110 (Δ*spa* Δ*sbi*) and 10 µg/ml Erm plus 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was used for pMutin–HA-5'secA selection in *S. aureus*. Protein expression from P*spac* promoter was induced with 1 mM IPTG. Anydrotetracycline (ATc, 200 ng/ml) was used to induce expression from the tetracycline-inducible promoter in pCL55-$P_{tet}$ constructs.

**Plasmids and strains**

To avoid mutations in the *spa* gene, all cloning procedures were performed at 30°C. All the pOS1-derivative and pCL55-derivative plasmids were constructed in *E. coli* DC10B (Monk et al., 2012) and transformed to *S. aureus* strains by electroporation (Schneewind and Missiakas, 2014). All the plasmids or strains constructed were confirmed by sequencing (*Table S2*). Primers used in this study are listed in *Table S3*. To avoid cross reaction in SpA immunoblot and purification, *S. aureus* WY110 (Δ*spa* Δ*sbi)* was generated by transducing *the sbi::ermB* allele from the Phoenix library (Bae et al., 2004) into *S. aureus* SEJ1, i.e. *S. aureus* RN4220 carrying Δ*spa.* Phage transduction was performed as described previously (Schneewind and Missiakas, 2014). To construct $pSpA_{ED}$, primers 10 and 69 were used to amplify the *spa* promoter and $spa_{ED}$ coding sequence encoding IgBDs E and D (-40 bp upstream of the transcription start site of *spa* to 459 bp of *spa* coding sequence) from chromosomal DNA of *S. aureus* RN4220. The PCR product was digested with EcoRI and BamHI, and ligated with plasmid pOS1 (Schneewind et al., 1993). To generate mutations and deletions within SpA signal peptide sequence, quick-change

mutagenesis was performed as follows: primers pairs (*Table S3*) that contain desired mutation or deletion were used to PCR amplify $pSpA_{ED}$. The PCR products were digested with DpnI and transformed to *E. coli* DC10B. Plasmid variants confirmed by DNA sequencing were transformed to *S. aureus* WY110. To construct pCL55-SpA and its derivatives, primer pairs 175 and 177 were used to PCR amplify the *spa* promoter and full-length *spa* coding sequence. PCR products were digested with BamHI and KpnI and ligated into pCL55 cut with the same enzymes (Lee et al., 1991). The resulting plasmid pCL55-SpA was used as template for PCR mutagenesis of its signal peptide mutant derivatives via quick-change mutagenesis as described above. To construct pCL55-$SpA_{SP\text{-}SasF}$, primer pairs 175 and 21 were used to amplify the promoter sequence of *spa*. Primers 22 and 23 were used to amplify coding sequence for the signal peptide sequence of *sasF.* Last, primers 24 and 177 were used amplify *spa* coding sequence for the E and D IgBDs. All three DNA fragments were ligated via SOE (splicing by overlap extension) PCR, digested with BamHI and KpnI, and then ligated into pCL55 cut with the same restriction enzymes. pCL55-derivaties were transformed to *S. aureus* WY110 and integrated into the chromosome at the *geh* locus (Lee et al., 1991). The integration was confirmed by PCR. To construct the *secA* depletion strain *S. aureus* WY223 ($P_{spac}$-*secA*), primers 189 and 190 were used to amplify the ribosome binding site and the first 656 bp of the *secA* gene. The PCR product was digested with HindIII and KpnI and ligated with pMutin–HA (Bacillus Genetic Stock Center, Columbus, OH). The resulting plasmid pMutin–HA-5'secA was transformed into RN4220 and integrated at the *secA* locus in the chromosome. Clones were selected on TSA supplemented with 10 µg/ml erythromycin and 1 mM IPTG. To construct pCL55-$P_{tet}$-*secA:sfGFP*, primers 180 and 181 were used to amplify *secA* full-length coding sequence together with its ribosome binding site.

446 Primers 182 and 183 were used to amplify *sfGFP* gene from pCX-sfGFP (Yu and Götz, 2012). The
447 two DNA fragments were joined together by SOE. The resulting *secA:sfGFP* hybrid, which
448 contains the 'Gly-Gly-Ala-Ala-Gly-Ala' between SecA and sfGFP, was digested with AvrII-BglII
449 and ligated with pCL55-$P_{tet}$ (Gründling and Schneewind, 2007). pCL55-$P_{tet}$-*secA:sfGFP* was
450 transformed to into *S. aureus* WY223 ($P_{spac}$-*secA*) and integrated into the chromosome at the
451 *geh* locus, thereby generating *S. aureus* WY230 ($P_{spac}$-*secA,* $P_{tet}$-*secA:sfGFP*). Plasmid pKOR1
452 based allelic replacement strategy (Bae and Schneewind, 2006) was used to generate the
453 Δ*secDF* (*S. aureus* WY418) and Δ*rnd2* (*S. aureus* WY416) knock-out mutants. *S. aureus* WY412, a
454 mutant with Δ*secDF* Δ*rnd23* mutations, was generated by transducing *the rnd3::ermB* allele
455 from the Phoenix library into *S. aureus* carrying Δ*secDF* and Δ*rnd2* mutations. To construct the
456 complementation plasmid pSecDF, the *secDF* ORF and 274 bp upstream sequence were PCR
457 amplified with primers 315 and 316, digested with EcoRI and BamHI, and ligated into pOS1 cut
458 with the same enzymes. The resulting plasmid, pSecDF, was transformed into *S. aureus* strains
459 WY418 (Δ*secDF*) and WY412 (Δ*secDF* Δ*rnd23*).
460
461 **Cell fractionation and immunoblotting**
462 Bacterial overnight cultures were diluted 1: 100 into fresh TSB and grown to $OD_{600}$ 0.8. One ml
463 culture was centrifuged at 18,000 ×*g* for 5 min in an Eppendorf tube. The culture supernatant
464 (S) was transferred to another tube and proteins were precipitated with 10 % trichloroacetic
465 acid (TCA) on ice for 30 min. The bacterial sediment (P, pellet) was suspended in 1 ml Tris-
466 buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl]and incubated with 20 µg/ml lysostaphin at 37°C
467 for 30 min. After cell lysis, proteins from the cell pellet were precipitated with 10% TCA. To

468 localize proteins in different cellular compartments, cell fractionation was performed as
469 follows: 1 ml culture ($OD_{600}$=0.8) was centrifuged at 18,000 ×*g* for 5 min in an Eppendorf tube.
470 The supernatant was transferred to a new tube and proteins were precipitated with 10% TCA
471 (S, supernatant). The pellet was suspended in 1 ml TSM [50 mM Tris-HCl (pH 7.5), 0.5 M
472 sucrose, 10 mM $MgCl_2$] and incubated with 20 µg/ml lysostaphin for 10 min at 37°C. After
473 centrifugation at 18,000 ×*g* for 5 min, the supernatant (cell wall fraction) was transferred to
474 another tube. The protoplast pellet was suspended in 1 ml Tris-buffer and subjected to freeze-
475 thaw cycle for 3 times in dry ice/ethanol bath and warm water. Membranes were in the cell
476 lysate were sedimented by ultracentrifugation 150,000 ×*g* for 40 min. Supernatant was
477 transferred to another tube (cytosolic fraction) whereas the pellet (membrane fraction) was
478 suspended in 1 ml Tris-buffer and precipitated with 10% TCA. After TCA precipitation on ice for
479 30 min, proteins were sedimented at 18,000 ×*g* for 10 min, washed with ice-cold acetone, air-
480 dried and solubilized in 100 µl 1×SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 2 % SDS, 10 %
481 glycerol, 5 % 2-mercaptoethanol, 0.01 % bromophenol blue]. For immunoblotting, protein
482 samples were separated on 10%, 12% or 15% SDS-PAGE and transferred to polyvinylidene
483 difluoride (PVDF) membranes. Membranes were blocked with 5% milk for 45 min. As needed,
484 50 µl human IgG (Sigma) was added to 10 ml block-solution to block SpA cross-reaction. Primary
485 antibodies were affinity-purified rabbit polyclonal antibodies against $SpA_{KKAA}$ (1:10,000
486 dilution), rabbit serum of anti-SrtA (1:20,000 dilution), rabbit serum of anti-SecA (1:10,000
487 dilution), rabbit serum of anti-Geh (1:10,000 dilution), polyclonal antibodies of anti-Coa
488 (1:5,000 dilution), rabbit serum of anti-Nuc (1:5,000 dilution), and anti-GFP rabbit serum
489 (1:10,000 dilution) (Invitrogen). Membranes were incubated with primary antibodies for 1 h,

washed three times for 5 min with TBST [50 mM Tris(pH 7.5), 150 mM NaCl, 0.1% Tween 20], incubated with secondary anti-rabbit IgG linked with HRP for 1 h, washed and developed using enhanced chemiluminescence substrates. The intensity of immunoblot signals was analyzed and measured with Image J software (Schneider et al., 2012). Statistical analysis was performed using GraphPad Prism software. One-way ANOVA (Dunnett's multiple comparisons test) was used to compare the mean for each variant with the mean for $SpA_{ED}$ wild-type (*Figure 1c, 2b*).

**Pulse-labeling**

Staphylococcal cultures were grown to mid-log phase ($OD_{600}$ 0.8) in TSB and bacteria sedimented by centrifugation at 18,000 ×*g* for 5 min. Bacterial pellets were washed twice and suspended in 1 ml minimal medium 4. $^{35}$S-methionine/cysteine (100 µl =100 µCi Perkin Elmer) was added to bacterial suspensions, vortexed and incubated for 60 seconds at 37°C. 250 µl was removed and immediately mixed with 250 µl ice-cold 10% TCA to quench all metabolic activity (time 0'). Chase solution (50 µl of 2 mg/ml methionine, 2 mg/ml cysteine and 10 mg/ml casamino acids) was added to the remainder of bacterial suspension and incubated for 1, 5 and 20 min. At each time point, 250 µl bacterial suspension was removed and mixed with 250 µl ice-cold 10% TCA. TCA precipitated cells were washed with acetone, dried and suspended in 1 ml 0.5 M Tris-HCl (pH 7.0) containing 20 µg/ml lysostaphin. After lysostaphin treatment at 37°C for 1 h, cell lysate was precipitated with 7% TCA, washed with acetone, dried, suspended in 50 µl 4% SDS, 0.5 M Tris-HCl (pH 7.5) and allowed to incubate for 30 min prior to boiling. Subsequently, samples were incubated for 1 h with rabbit polyclonal anti-$SpA_{KKAA}$ antibody (Kim et al., 2010) that was 1:1000 diluted in 1 ml RIPA buffer (0.1% SDS, 0.5% deoxycholic acid, 1%

512 Triton X-100, 50 mM Tris-HCl pH 8.0, 150 mM NaCl). Protein A sepharose (50 µl of 50% slurry,
513 Sigma) was added to each sample and incubated for 1 h followed by five washes with 1 ml RIPA
514 buffer. Proteins bound to the beads were solubilized by boiling in 15 µl 2×SDS sample buffer for
515 10 min and separated on 10% (SpA) or 15% SDS-PAGE ($SpA_{ED}$). Gels were dried on Whatman 3
516 M paper and autoradiographed on X-ray film for 48 hours or longer.
517
518 **Purification of $SpA_{ED/S18L}$, Edman degradation and MALDI-TOF mass spectrometry**
519 Overnight bacterial cultures of *S. aureus* WY110 ($pSpA_{ED}$ or its derivatives) were diluted 1: 100
520 into 4 liters of TSB and grown to $OD_{600}$ 2. Staphylococci were sedimented by centrifugation at
521 8,000 ×*g* for 10 min. Bacteria were suspended in 30 ml of Tris-buffer, 0.5 (vol/vol) 0.1 mm
522 sterilized glass beads were added and peptidoglycan was broken with 15×1 min pulses in a
523 bead-beating instrument (MP Biomedicals). Samples were centrifuged at 7000 ×*g* for 10 min to
524 sediment glass beads. The supernatant was transferred to another tube and centrifuged at
525 33,000 ×*g* for 1 hour at 4 °C. The membrane sediment was suspended in 30 ml RIPA buffer and
526 incubated for 1 hour with rotation. RIPA extract was centrifuged at centrifuged at 33,000 ×*g* for
527 1 hour at 4 °C. The supernatant was removed and subjected to affinity chromatography. Two ml
528 50% suspension of IgG sepharose (GE Healthcare) was loaded onto column. Column bed was
529 washed with 7 ml 0.1 M glycine (pH 3.0), twice with 14 ml 50 mM Tris-HCl (pH 7 .5) and once
530 with 10 ml RIPA buffer. RIPA membrane extracts were loaded onto the column followed with
531 two washes with 14 ml RIPA buffer and once with 10 ml 50 mM Tris-HCl (pH 7 .5). Proteins were
532 eluted by adding four times 1 ml 0.1 M glycine (pH 3.0) to the column and immediately
533 neutralizing the eluate with 25 µl of 1.5 M Tris (pH 8.8). For Edman degradation, the purified

534 $SpA_{ED}$ precursors were 10-fold concentrated via Amicon® Ultra-0.5 ml Centrifugal Filters (10 kD
535 cut off). Proteins were separated by 15% SDS-PAGE, electro-transferred to PVDF and stained by
536 Coomassie-Brilliant Blue. Bands of interest were excised and subjected to Edman degradation
537 (Alphalyse, Inc, CA, USA). For MALDI-TOF mass spectrometry analysis, 1 µl of $SpA_{ED}$ samples
538 were mixed with 1 µl of 10 mg/ml sinapic acid, dried on the Bruker MTP 384 massive plate, and
539 examined in a Bruker Autoflex Speed MALDI-TOF mass spectrometry in the linear positive-ion
540 mode using peptide standards for calibration.
541
542 **Crosslinking of $SpA_{ED}$ precursor**
543 Overnight cultures of *S. aureus* WY110 (Δ*spa* Δ*sbi,* $pSpA_{ED/S18L}$) and *S. aureus* WY110 (Δ*spa* Δ*sbi,*
544 $pSpA_{ED/SP\text{-}SasF}$) were each diluted 1: 100 into 4 L TSB and grown to $OD_{600}$ 2. Formaldehyde (0.9%,
545 methanol free) was added to the bacterial culture and incubated for 20 min with shaking.
546 Cross-linking was quenched by adding 400 ml ice-cold 0.125 M glycine and rotating the sample
547 for 10 min. Staphylococci were sedimented by centrifugation at 8,000 ×*g* for 10 min. Bacteria
548 were suspended in 30 ml of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and washed twice in the
549 same buffer. Sterilized 0.1 mm glass beads 0.5 (vol/vol) were added and peptidoglycan broken
550 with 15×1 min pulses in a bead-beating instrument (MP Biomedicals). Samples were
551 centrifuged at 7000 ×*g* for 10 min to sediment glass beads. The supernatant was transferred to
552 another tube and centrifuged at 33,000 ×*g* for 1 hour at 4 °C. The membrane sediment was
553 suspended in 30 ml 50 mM Tris-HCl (pH 7 .5), 2% n-dodecyl β-D-maltoside (DDM) and incubated
554 at 4 °C overnight. Samples were subjected to ultracentrifugation at 150,000 ×*g* for 40 min. The
555 supernatant was subjected to affinity chromatography on IgG sepharose affinity purification as

556 described above. Eluate was concentrated via Amicon® Ultra-0.5 ml 10 kD Centrifugal Filters
557 and mixed with equal volume of 2×SDS sample buffer. To reverse the cross-linking, samples
558 were either boiled at 90°C for 20 min or, as a control, incubated at 60°C (no reversal of cross-
559 linking). Proteins in all samples were separated on 12% SDS-PAGE and bands of interest excised
560 as indicated in Figure 4 and subjected to protein identification and semi-quantitative analysis at
561 the Harvard University Taplin Mass Spectrometry Facility (*Table S1*).
562
563 **SecA depletion and SecA-sfGFP induction**
564 Overnight cultures of *S. aureus* RN4220 (WT), *S. aureus* WY223 ($P_{spac}$-*secA*) and *S. aureus* WY230
565 ($P_{spac}$-*secA,* $P_{tet}$-*secA:sfGFP*) were grown in TSB and 1 mM IPTG. Overnight cultures were washed
566 twice with an equal volume of TSB and diluted 1:100 into fresh TSB with or without 1 mM IPTG
567 and with or without 200 ng/ml ATc. After 3 h growth at 37°C, cultures were diluted into fresh
568 TSB with or without IPTG or ATc and subjected to further growth at 37°C. Growth was
569 monitored by sampling cultures at timed intervals and measuring optical density. One ml
570 bacterial culture was removed after 3 hours (prior to the second 1:100 dilution) and after 6
571 hours (3 hours after the second 1:100 dilution). Samples were processed for protein secretion
572 and immunoblotting assays or analyzed by fluorescence microscopy. A similar procedure was
573 performed for LtaS depletion using *S. aureus* ANG499 (P*spac-ltaS*). Samples from the LtaS
574 depletion experiments were analyzed after 3 hours growth with or without 1 mM IPTG.
575
576 **Fluorescence microscopy**

To observe SpA targeting to the cross-wall, 2 ml mid-log phase *S. aureus* culture ($OD_{600}$ 0.8) were centrifuged at 18,000 ×*g* for 5 min, supernatant removed, bacteria washed once in 2 ml PBS and suspended in 1 ml PBS containing 0.5 mg/ml trypsin (Sigma). After incubation at 37°C for 1 hour, staphylococci were washed twice with PBS, suspended in fresh TSB containing 2.5 mg/ml soybean trypsin inhibitor (Sigma) and incubated at 37°C for 20 min with rotation. 250 µl of the cell suspension was removed and immediately mixed with fixation solution (2.5% paraformaldehyde and 0.006% glutaraldehyde in PBS). The cells were fixed for 20 min at room temperature, washed three times with PBS and applied to poly-L-lysine coated 8-well glass slides (MP Biomedicals) for 5 min. Excess and non-adherent cells were washed away with PBS. Mobilized cells were blocked with 3% BSA in PBS for 45 min and incubated with SpA-specific mouse hybridoma monoclonal antibody 5A10 (Kim et al., 2010) (diluted 1:4,000 in 3% BSA) for 1 hour. Cells were washed 8 times with PBS and further incubated in dark with Alexa Fluor 647 conjugated anti-mouse IgG (1:500 in 3% BSA) (Invitrogen) for 1 hour. Cells were washed 10 times with PBS and incubated with 1 µg/ml BODIPY-FL vancomycin (ThermoFisher) for 10 min in the dark followed by washing 5 times with PBS. A drop of SlowFade® Gold reagent (Molecular Probes) was applied to samples before sealing coverslips with nail polish. Fluorescent images were visualized and captured on a Leica SP5 Tandem Scanner Spectral 2-Photon Confocal microscope with 100×oil objective. Identical settings and exposure times were applied to all samples.

To image SpA display on the staphylococcal surface, 1 ml of mid-log phase *S. aureus* cultures were centrifuged at 18,000 ×*g* for 5 min and supernatant removed. Bacteria were washed once in 2 ml PBS and suspended in 1 ml PBS and mixed with fixation solution. Cells

were fixed for 20 min at room temperature, washed three times with PBS and applied to poly-L-lysine coated 8-well glass slides (MP Biomedicals) for 5 min, stained with vancomycin and αSpA antibodies and analyzed by fluorescence microscopy.

To localize intracellular SpA, 2 ml of mid-log phase staphylococcal cultures were centrifuged at 18,000 ×*g* for 5 min and supernatant removed. Bacteria were washed once in 2 ml PBS and suspended in 1 ml PBS, 0.5 mg/ml trypsin (Sigma). After incubation at 37°C for 1 hour, staphylococcal cells were washed twice with PBS and fixed with fixation solution. The cells were fixed for 15 min at room temperature and 30 min on ice, washed three times with PBS and suspended in 1 ml GTE buffer [50 mM glucose, 20 mM Tris-HCl (pH 7.5), 10 mM EDTA]. Lysostaphin (10 µg/ml) was added and 50 µl cell suspensions were immediately applied to poly-L-lysine coated 8-well glass slides and incubated for 1 min. Non-adherent cells were removed and PBS, 0.2% Triton X-100 was applied to samples for 10 seconds. Excessive liquid was aspirated and slides were air-dried. Dried slides were immediately dipped in methanol at -20°C for 5 min, and in acetone at -20°C for 30 s and then allowed to dry completely. Afterwards, the cells on the slides were re-hydrated with PBS for 5 min, blocked with 3% BSA, stained with the membrane dye Nile red (Sigma) and rabbit antibodies specific for SpA followed by Alexa-Fluor-488 conjugated goat-anti-rabbit-IgG and analyzed by fluorescence microscopy as described above.

To visualize the sub-cellular localization of SecA-sfGFP, samples from 3 h growth cultures were removed as described above. Bacteria were sedimented by centrifugation and washed twice in PBS. Cells were stained with 1 µg/µl FM4-64FX (Molecular Probes) for 10 min in the dark and were then fixed with fixation solution for 20 min. After washing twice with PBS,

cells were applied to poly-L-lysine coated glass slides, SlowFade® Gold reagent added, coverslips sealed and samples imaged by fluorescence microscopy.

All the images were analyzed in Image J software (Schneider et al., 2012). To quantify the frequency of SpA cross-wall localization at 20 min regeneration after trypsin digestion, numbers of diplococci and numbers of cross-wall localized SpA were counted manually using the cell counter tool in Image J. Diplococci were defined as two daughter cells that had divided and formed a cross-wall but had not yet separated. Cross-wall localized SpA signals were defined as lines at the cross-wall of diplococci. Diplococci were counted in vancomycin stained images and cross-wall localized SpA was counted in merged images. The frequency was determined by dividing cross-wall localized SpA by the number of diplococci. An example of the counting method is displayed in the *Source file to Figure 2e*. At least two random images were acquired per sample for each experiment. Three or more independent experiments were performed and data from more than 200 diplococci were analyzed for statistically significant differences using one-way ANOVA with Dunnett's multiple comparison test comparing staphylococci expressing wild-type and *spa* variants (Figure 2e). In *Figure 6c* and *Figure 10c* the Tukey's multiple comparison test was used to analyze differences between multiple groups.

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644

## 645 Figure legends

646 **Figure 1.** Mutagenesis of the signal peptide of staphylococcal protein A (SpA). (a) Schematic
647 illustrating the primary structure of SpA and of $SpA_{ED}$ with the immunoglobulin binding domains
648 (IgBDs, designated E, A, B, C and D), region X (Xr), LysM domain and LPXTG sorting signal.
649 Cleavage sites for signal peptidase and sortase A are indicated. The amino acid sequence of the
650 SpA signal peptide is displayed. YSIRK/GXXS motif residues are printed in red. (b) The structural
651 genes for $SpA_{ED}$ and its variants were cloned into pOS1, expressed from the *spa* promoter in *S.*
652 *aureus* WY110 (Δ*spa* Δ*sbi*) and secretion of $SpA_{ED}$ was analyzed by immunoblotting with SpA-
653 specific antibody in the culture supernatant (S) and lysostaphin-digested bacterial pellets (P). (c)
654 Percent secretion of wild-type $SpA_{ED}$ and its variants was quantified from triplicate experiments
655 as the intensity of immunoblotting signals in the supernatant (S) divided by the sum signals in
656 (S+P) fractions ×100. Statistical significance was analyzed with one-way ANOVA comparing each
657 variant with wild-type and *p* values were recorded: WT vs. ΔGIAS, $p$=0.031; WT vs. ΔIA,
658 $p$=0.0032; WT vs. R10A, $p$=0.0116; WT vs. S18L, $p$=0.0172. * denotes $p$<0.05, ** denotes
659 $p$<0.01.

660

661 **Figure 2.** SpA signal peptide variants defective in precursor processing and septal secretion. (a)
662 *S. aureus* cultures were fractionated into cytoplasm (C), membrane (M), cell wall (W) and
663 culture supernatant (S) and analyzed by immunoblotting with αSpA to reveal the subcellular

location of wild-type $SpA_{ED}$ precursor and secreted product of the $SpA_{ED/\Delta IA}$, $SpA_{ED/S18L}$, and $SpA_{ED/R10A}$ variants. Immunoblotting with αSrtA and αL6 was used to establish fractionation and loading controls. (b) Quantification of immunoblot signal intensity in (a) using Image J. Precursor abundance (%) the bacterial cytoplasm (C) and membrane (M) was quantified from triplicate experiments as the intensity of immunoblotting signals divided by the sum signals in all four fractions (C+M+W+S)×100. Statistical significance was analyzed with one-way ANOVA comparing each variant with wild-type and *p* values were recorded: for [C/(C+M+W+S)]×100, WT vs. ΔIA, $p<0.0001$; WT vs. S18L, $p=0.0042$; WT vs. R10A, $p<0.0001$; for [M/(C+M+W+S)]×100, WT vs. ΔIA, $p=0.0056$; WT vs. S18L, $p<0.0001$; WT vs. R10A, $p=0.0405$. **** denotes $p<0.0001$, ** denotes $p<0.01$, * denotes $p<0.05$. (c) *S. aureus* cultures were pulse-labeled for 60 seconds with [$^{35}$S]methionine and labeling quenched by adding an excess of non-radioactive methionine (chase). At timed intervals during the pulse (0') or 1 (1'), 5 (5'), and 20 (20') minutes after the pulse (chase), culture aliquots were precipitated with trichloroacetic acid (TCA), lysostaphin-treated, immunoprecipitated with αSpA and analyzed by autoradiography. (d) *S. aureus* WY110 (Δ*spa* Δ*sbi*) harboring chromosomal pCL55-insertions of wild-type *spa* (SpA), $spa_{\Delta IA}$ ($SpA_{\Delta IA}$) $spa_{S18L}$ ($SpA_{S18L}$), $spa_{R10A}$ ($SpA_{R10A}$) $spa_{SP\text{-}SasF}$ ($SpA_{SP\text{-}SasF}$) or pCL55 alone (Vector) were treated with trypsin to remove SpA. Bacteria were incubated for 20 min to allow for secretion and cell wall deposition of newly synthesized SpA. Samples were incubated with BODIPY-FL vancomycin (Vancomycin) (green) to stain the bacterial cell wall and with SpA-specific monoclonal antibody and Alexa fluor 647-labeled secondary IgG (red) to reveal SpA. (e) SpA-positive staphylococci in images derived from samples in (d) were analyzed for SpA deposition at the cross wall of diplococci (n=200). Data from three independent experiments were used to derive the mean

686 (±SEM) and were analyzed for significant differences with one-way ANOVA for comparisons
687 between wild-type and mutant SpA. *p* values were recorded: SpA vs. $SpA_{\Delta IA}$, $p<0.0001$; SpA vs.
688 $SpA_{S18L}$, $p=0.0006$; SpA vs. $SpA_{R10A}$, $p=0.0004$; SpA vs. $SpA_{SP\text{-}SasF}$, $p<0.0001$; SpA vs. Vector,
689 $p<0.0001$. **** denotes $p<0.0001$, *** denotes $p<0.001$.
690
691 **Figure 3.** Proteolytic cleavage of the SpA signal peptide. (a) Wild-type $SpA_{ED}$ or $SpA_{ED/\Delta IA}$,
692 $SpA_{ED/S18L}$, and $SpA_{ED/R10A}$ variants precursors and cleavage products were purified from
693 detergent-solubilized staphylococcal membranes using affinity chromatography on IgG-
694 sepharose and analyzed on Coomassie-Blue stained SDS-PAGE. Full length precursors (1) and
695 their cleavage products (2-4) were analyzed by Edman degradation and MALDI-TOF mass
696 spectrometry. See Table 1 for mass spectrometry data. (b) Schematic illustrating the proteolytic
697 cleavage sites for each of the four precursors $SpA_{ED}$, $SpA_{ED/\Delta IA}$, $SpA_{ED/S18L}$ and $SpA_{ED/R10A}$.
698
699 **Figure 4.** Crosslinking of staphylococcal proteins to $SpA_{ED/S18L}$ or $SpA_{ED/SP\text{-}SasF}$. (a) Bacteria from *S.*
700 *aureus* WY110 ($pSpA_{ED/S18L}$) and *S. aureus* WY110 ($pSpA_{ED/\ SP\text{-}SasF}$) cultures were crosslinked with
701 0.9% formaldehyde, membrane proteins detergent-solubilized and $SpA_{ED/S18L}$ as well as $SpA_{ED/SP\text{-}}$
702 $_{SasF}$. precursors purified by affinity chromatography on IgG-sepharose. Eluate was treated for 20
703 min at 90°C to reverse cross-linking or kept at 60°C (cross-linked control) and analyzed on
704 Coomassie-stained SDS-PAGE. Bands were excised as indicated and individual proteins
705 identified via ESI-MS analyses of tryptic peptides and data comparison with *in silico* trypsin-
706 cleaved translation products derived from the genome sequence of *S. aureus*. Immunoblotting

of 90°C samples to validate crosslinking of $SpA_{ED/S18L}$ staphylococcal proteins (b) and to SecA (c). See Table S1 for a summary of proteins crosslinked to $SpA_{ED/S18L}$.

**Figure 5.** Depletion of SecA in *S. aureus*. (a) Diagram of the *secA* gene locus in *S. aureus* RN4220 (wild-type parent, WT) and its $P_{spac}$-*secA* variant. (b) Bacteria from overnight cultures of wild-type *S. aureus* and *S. aureus* $P_{spac}$-*secA* grown in TSB with 1 mM IPTG were washed and suspended in fresh TSB with or without 1 mM IPTG. Subsequent growth was monitored as increased absorbance at 600 nm ($A_{600}$). After three hours, cultures were diluted 1:100 into fresh TSB with or without 1 mM IPTG and incubated for additional growth measurements. (c) *S. aureus* $P_{spac}$-*secA* was streaked on tryptic soy agar with or without 1 mM IPTG supplement and incubated for 16 hours at 37°C for growth. (d) Culture samples retrieved after 3 and 6 hours in (b) were analyzed by immunoblotting with antibodies against SecA (αSecA).

**Figure 6.** SecA depletion diminishes septal secretion of SpA in *S. aureus*. (a) SpA precursor processing of [$^{35}$S]methionine pulse-labeled *S. aureus* RN4220 (WT) or *S. aureus* $P_{spac}$-*secA* grown in the presence or absence of 1 mM IPTG. Bacteria were pulse-labeled for 60 sec with radioactive methionine and then incubated with an excess of non-radioactive methionine. During the pulse (0') or 1 (1'), 5 (5') and 20 (20') min after the addition of excess unlabeled methionine, culture aliquots were withdrawn, precipitated with TCA, digested with lysostaphin, and subjected to SDS-PAGE and autoradiography of immunoprecipitated SpA. Wild-type *S. aureus* (WT) and its $P_{spac}$-*secA* variant were grown for 3 (b) and 6 hours (d) in the presence or absence of 1 mM IPTG (see Figure 5) and treated with trypsin to remove SpA from the bacterial

729 surface. Bacteria were incubated for 20 min to allow for secretion and cell wall deposition of
730 newly synthesized SpA. Samples were incubated with BODIPY-FL vancomycin (green) to stain
731 the bacterial cell wall and with SpA-specific monoclonal antibody and Alexa Fluor 647-labeled
732 secondary IgG (red) to reveal SpA. As a control for SpA-specific staining, the *S. aureus* Δ*spa*
733 variant grown in the absence of IPTG was analyzed by fluorescence microscopy. (c) SpA-positive
734 staphylococci in images derived from samples in (b) were analyzed for SpA deposition at the
735 cross wall of diplococci (n=200). Data from three independent experiments were used to derive
736 the mean (±SEM), were analyzed for significant differences with one-way ANOVA and *p* values
737 were recorded: RN4220-IPTG vs. RN4220+IPTG, non-significant (ns); RN4220-IPTG vs. WY223-
738 IPTG, $p$=0.0003; WY223-IPTG vs. WY223+IPTG, $p$<0.0001, RN4220+IPTG vs. WY223+IPTG, ns. (e)
739 SecA depletion diminishes secretion of staphylococcal proteins. Protein samples from the
740 extracellular medium and bacterial pellet of *S. aureus* RN4220 (WT) and *S. aureus* $P_{spac}$-*secA*
741 cultures grown for 3 hours in the presence or absence of 1 mM IPTG were analyzed by
742 immunoblotting with antibodies against glycerol-ester hydrolase (αGeh), nuclease (αNuc) and
743 sortase A (αSrtA).
744
745 **Figure 7.** SecA localization in staphylococci. (a) Diagram of the *secA* gene locus and of the
746 pCL55-mediated *att* insertion site for *secA-sfGFP* in the staphylococcal genome. (b) Bacteria
747 from overnight cultures of *S. aureus* RN4220 (WT), *S. aureus* $P_{spac}$-*secA* and *S. aureus* $P_{spac}$-
748 *secA*/$P_{tet}$-*secA*:*sfGFP* grown in TSB with 1 mM IPTG were washed and suspended in fresh TSB
749 without IPTG and with or without 1 mM anhydro-tetracycline (ATc); growth was monitored as
750 increased absorbance at 600 nm ($A_{600}$). After three hours, cultures were diluted 1:100 into fresh

751 TSB without IPTG and with or without 1 mM ATc and incubated for further growth
752 measurements. (c) Culture samples retrieved after 3 and 6 hours from the experiment detailed
753 in (b) were analyzed by immunoblotting with rabbit antibodies against SecA (αSecA) and sfGFP
754 (αGFP). (d) [$^{35}$S]methionine-labeled *S. aureus* cultures incubated for three hours as described in
755 (b) were analyzed during the 60 sec pulse with radioactive methionine (0) and 1, 5 and 20 min
756 after the addition of excess unlabeled methionine via SDS-PAGE and autoradiography of
757 immunoprecipitated SpA. (e) Fluorescence microscopy of bacteria from *S. aureus* cultures
758 incubated for three hours as described in (b). Bacteria were stained with the membrane dye
759 FM4-64 (red) and analyzed for SecA-sfGFP fluorescence (green).
760
761 **Figure 8.** Intracellular trafficking of SpA in the presence and absence of SecA. (a) *S. aureus* $P_{spac}$-
762 s*ecA* cells were grown in the presence (+SecA) or absence of 1 mM IPTG (-SecA) and, alongside
763 *S. aureus* Δ*spa* control cells, were trypsin treated to remove extracellular surface proteins and
764 fixed with para-formaldehyde. Samples were then treated with lysostaphin (+Lysostaphin) or
765 left untreated (-Lysostaphin), incubated with detergent and SpA-specific rabbit antibodies and
766 Alexa Fluor 488-labeled goat-anti-rabbit-IgG (green) and with Nile red to reveal bacterial
767 membranes. Bright-field microscopy (BF) images were acquired to reveal the contours of all
768 bacterial cells. Scale bar, 1 µm. (b) Additional samples (#1, #2 and #3) of *S. aureus* $P_{spac}$-s*ecA*
769 cells were grown in the presence of 1 mM IPTG (+SecA), trypsin treated, fixed with
770 formaldehyde, lysostaphin treated, incubated with detergent and with SpA-specific antibody
771 (green) and Nile red.
772

773 **Figure 9.** SecDF is required for SpA trafficking to septal membranes. (a) *S. aureus* cultures were
774 centrifuged to sediment the bacteria into a pellet (P) and separate them from the extracellular
775 medium (S, supernatant). Following lysostaphin digestion of bacteria, proteins in both fractions
776 were precipitated with TCA and analyzed by immunoblotting with αSpA. (b) *S. aureus* cultures
777 were fractionated as described in (a) and subjected to immunoblotting with antibodies specific
778 for glycerol-ester hydrolase (αGeh), coagulase (αCoa) and sortase A (αSrtA). (c) Fluorescence
779 microscopy of bacteria from cultures of *S. aureus* RN4220 (WT, wild-type), WY418 (Δ*secDF*),
780 WY416 (Δ*rnd2*), WY400 (Δ*rnd3*) and WY412 (Δ*secDF* Δ*rnd23*) mutants with and without
781 expression plasmid for wild-type *secDF* (pSecDF) as well as *S. aureus* SEJ1 (Δ*spa*) *as* control.
782 Bacteria were trypsin treated to remove extracellular surface proteins and fixed with para-
783 formaldehyde. Samples were treated with lysostaphin, incubated with detergent and SpA-
784 specific rabbit antibodies and Alexa Fluor 488-labeled goat-anti-rabbit-IgG (green) and with Nile
785 red to reveal bacterial membranes. BF identifies the bright-field microscopy view of
786 fluorescence microscopy images. Scale bar, 1 µm.
787
788 **Figure 10.** Localization of SpA secretion in LtaS-depleted *S. aureus*. (a) Schematic to illustrate
789 the *ltaS* locus in *S. aureus* RN4220 and ANG499. (b) Fluorescence microscopy with BODIPY-FL-
790 vancomycin (green) and αSpA (red) stained samples 20 min after trypsin removal of surface
791 proteins from the staphylococcal envelope to detect newly synthesized SpA. Scale bar, 2 µm. (c)
792 SpA-positive staphylococci in images derived from samples in (b) were analyzed for SpA
793 deposition at the cross wall of diplococci (n=200). Data from three independent experiments
794 were used to derive the mean (±SEM) and were analyzed for significant differences with one-

way ANOVA for comparisons between *S. aureus* RN4220 (WT) and ANG499 grown with (+LtaS) and without IPTG (-LtaS). *p* values were recorded: RN4220-IPTG vs. ANG499-IPTG, $p < 0.0001$; ANG499-IPTG vs. ANG499+IPTG, $p < 0.0001$. (d) Fluorescence microscopy to localize intracellular SpA in *S. aureus* strains RN4220 (WT) and ANG499 ($P_{spac}$-*ltaS*) grown with and without IPTG induction for 3 hours. Bacteria were trypsin treated to remove extracellular surface proteins and fixed with para-formaldehyde. Samples were then treated with lysostaphin, incubated with detergent and SpA-specific rabbit antibodies and Alexa Fluor 488-labeled goat-anti-rabbit-IgG (green) and with Nile red to reveal bacterial membranes. BF identifies the bright-field microscopy view of fluorescence microscopy images. Scale bar, 2 µm. (e) The culture supernatant (S) and bacterial pellet (P) samples of *S. aureus* RN4220 and ANG499 grown for three hours in the presence or absence of IPTG were immunoblotted with antibodies specific for SpA (αSpA), glycerol-ester hydrolase (αGeh), coagulase (αCoa), nuclease (αNuc) and sortase A (αSrtA). (f) Fluorescence microscopy of staphylococci to measure surface display of protein A in bacteria stained with BODIPY-FL-vancomycin (Van-FL) (green) and αSpA (red) without trypsin treatment. Scale bar, 2 µm.

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**Table 1. MALDI-TOF-MS ion signals of purified $SpA_{ED}$ species and their variants**

| Protein | Observed *m/z* | [a]Calculated *m/z* | Δobs.- calc. |
|---|---|---|---|
| $SpA_{ED}$-1 | 16776.47 | 16777.7 | 1.23 |
| $SpA_{ED}$-2 | 15273.92 | 15273.78 | 0.14 |
| $SpA_{ED}$-3 | 14418.92 | 14418.78 | 0.14 |
| $SpA_{ED}$-4 | 13152.40 | 13152.32 | 0.08 |
| $SpA_{ED/S18L}$-1 | 16803.02 | 16803.78 | 0.76 |
| $SpA_{ED/S18L}$-2 | 15298.89 | 15299.86 | 0.97 |
| $SpA_{ED/S18L}$-3 | 14417.89 | 14418.78 | 0.89 |
| $SpA_{ED/S18L}$-4 | 13152.22 | 13152.32 | 0.10 |
| $SpA_{ED/\Delta IA}$-1 | 16592.95 | 16593.46 | 0.51 |
| $SpA_{ED/\Delta IA}$-2 | 15088.71 | 15089.54 | 0.83 |
| $SpA_{ED/\Delta IA}$-3 | 14417.86 | 14418.78 | 0.92 |
| $SpA_{ED/\Delta IA}$-4 | 13151.55 | 13152.32 | 0.77 |
| $SpA_{ED/R10A}$-1 | 16692.99 | 16692.59 | 0.40 |
| $SpA_{ED/R10A}$-2 | 15584.97 | 15586.2 | 1.23 |
| $SpA_{ED/R10A}$-3 | 14418.46 | 14418.78 | 0.32 |
| $SpA_{ED/R10A}$-4 | 13152.07 | 13152.32 | 0.25 |

[a]Based on average mass calculated with the online ExPASy tool.

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**Table S1. List of ESI-MS identified tryptic peptides crosslinked to $SpA_{ED/S18L}$**

| Number of peptides | Gene Locus or Symbol | UniProt reference |
|---|---|---|
| 26 | *secA* | (O06446_SECA_STAA8) |
| 21 | *alaS* | (Q2FXV9_SYA_STAA8) |
| 18 | *murG* | (Q2FYL5_MURG_STAA8) |
| 15 | *polA* | (Q2FXN9_Q2FXN9_STAA8) |
| 15 | SAOUHSC_01854 | (Q2G245_Q2G245_STAA8) |
| 15 | *femA* | (Q2FYR2_FEMA_STAA8) |
| 11 | *clpC* | (Q2G0P5_CLPC_STAA8) |
| 10 | SAOUHSC_01810 | (Q2FXM5_Q2FXM5_STAA8) |
| 10 | *ezrA* | (Q2FXK8_EZRA_STAA8) |
| 9 | *pbp2* | (Q2FYI0_Q2FYI0_STAA8) |
| 9 | *murE2* | (Q2FWZ9_Q2FWZ9_STAA8) |
| 9 | UDP-N-acetylglucosamine pyrophosphorylase | (Q2FW81_URTF_STAA8) |
| 9 | *femB* | (Q2FYR1_FEMB_STAA8) |
| 9 | *tagB* | (Q2G1C2_Q2G1C2_STAA8) |
| 8 | *tagF* | (Q2G1C1_Q2G1C1_STAA8) |
| 7 | *ltaS* | (Q2G093_LTAS_STAA8) |
| 7 | *femX* | (Q2FVZ4_FEMX_STAA8) |
| 6 | 1-acyl-sn-glycerol-3-phosphate acyltransferases domain protein | (Q2FXJ7_Q2FXJ7_STAA8) |
| 6 | *purL* | (Q2FZJ0_PURL_STAA8) |
| 6 | Conserved hypothetical phage | (Q2FY82_Q2FY82_STAA8) |

| | protein | |
|---|---|---|
| 6 | SAOUHSC_02447 | (Q2G2D7_Q2G2D7_STAA8) |
| 6 | SAOUHSC_01347 | (Q2FYS9_Q2FYS9_STAA8) |
| 6 | SAOUHSC_01180 | (Q2G264_Q2G264_STAA8) |
| 5 | *clpB* | (Q2FZS8_Q2FZS8_STAA8) |
| 5 | *dltD* | (Q2FZW3_Q2FZW3_STAA8) |
| 5 | *cshA* | (Q2FWH5_Y2316_STAA8) |
| 5 | *prfC* | (Q2FZP4_RF3_STAA8) |
| 5 | *parE* | (Q2FYS5_PARE_STAA8) |
| 5 | *parC* | (Q2FYS4_PARC_STAA8) |
| 5 | *betA* | (Q2FV11_BETA_STAA8) |
| 5 | SAOUHSC_02417 | (Q2FW86_Q2FW86_STAA8) |
| 5 | SAOUHSC_02274 | (Q2FWL5_Q2FWL5_STAA8) |
| 5 | SAOUHSC_01960 | (Q2FXA5_Q2FXA5_STAA8) |
| 5 | SAOUHSC_01908 | (Q2G1W5_Q2G1W5_STAA8) |
| 5 | SAOUHSC_01613 | (Q2FY52_Q2FY52_STAA8) |
| 5 | SAOUHSC_01026 | (Q2FZH8_Q2FZH8_STAA8) |
| 5 | SAOUHSC_00309 | (Q2G146_Q2G146_STAA8) |
| 5 | SAOUHSC_00113 | (Q2G1K9_Q2G1K9_STAA8) |
| 4 | *saeS* | (Q2G2U1_SAES_STAA8) |
| 4 | *rplK* | (P0A0F4_RL11_STAA8) |
| 4 | *rplE* | (Q2FW18_RL5_STAA8) |
| 4 | *murC* | (Q2FXJ0_MURC_STAA8) |
| 4 | *metN2* | (Q2FZZ2_METN2_STAA8) |
| 4 | *lip2* | (Q2G155_LIP2_STAA8) |
| 4 | SAOUHSC_02859 | (Q2FV77_Q2FV77_STAA8) |
| 4 | SAOUHSC_02582 | (Q2FVV9_FDHL_STAA8) |
| 4 | SAOUHSC_02363 | (Q2FWD6_ALD1_STAA8) |
| 4 | SAOUHSC_01884 | (Q2FXG3_Q2FXG3_STAA8) |

| | | |
|---|---|---|
| 4 | SAOUHSC_01855 | (Q2G247_Y1855_STAA8) |
| 4 | SAOUHSC_01723 | (Q2FXV8_Q2FXV8_STAA8) |
| 4 | SAOUHSC_01679 | (Q2FXZ6_Q2FXZ6_STAA8) |
| 4 | SAOUHSC_01460 | (Q2FYI6_Q2FYI6_STAA8) |
| 4 | SAOUHSC_01321 | (Q2FYV3_Q2FYV3_STAA8) |
| 4 | SAOUHSC_00893 | (Q2FZU7_Q2FZU7_STAA8) |
| 4 | SAOUHSC_00637 | (Q2G2L2_Q2G2L2_STAA8) |
| 4 | SAOUHSC_00531 | (Q2G0M9_Q2G0M9_STAA8) |
| 3 | *tgt* | (Q2FXT6_TGT_STAA8) |
| 3 | *queA* | (Q2FXT5_QUEA_STAA8) |
| 3 | *prs* | (Q2G0S2_Q2G0S2_STAA8) |
| 3 | *pcrA* | (Q53727_PCRA_STAA8) |
| 3 | *hemE* | (Q2FXA3_DCUP_STAA8) |
| 3 | *gyrA* | (Q2G2Q0_GYRA_STAA8) |
| 3 | *gpsA* | (Q2FYG1_GPDA_STAA8) |
| 3 | *glmU* | (Q2G0S3_GLMU_STAA8) |
| 3 | SAOUHSC_02684 | (Q2FVL8_Q2FVL8_STAA8) |
| 3 | SAOUHSC_02627 | (Q2G2W3_Q2G2W3_STAA8) |
| 3 | SAOUHSC_02317 | (Q2FWH4_Q2FWH4_STAA8) |
| 3 | SAOUHSC_02134 | (Q2G234_Q2G234_STAA8) |
| 3 | SAOUHSC_01973 | (Q2G2T1_Q2G2T1_STAA8) |
| 3 | SAOUHSC_01728 | (Q2FXV3_Q2FXV3_STAA8) |
| 3 | SAOUHSC_01673 | (Q2FY01_Q2FY01_STAA8) |
| 3 | SAOUHSC_01612 | (Q2FY53_Q2FY53_STAA8) |
| 3 | SAOUHSC_01584 | (Q2FY81_Q2FY81_STAA8) |
| 3 | SAOUHSC_01499 | (Q2FYF3_Q2FYF3_STAA8) |
| 3 | SAOUHSC_01490 | (Q2FYG2_Q2FYG2_STAA8) |
| 3 | SAOUHSC_01071 | (Q2FZF9_Q2FZF9_STAA8) |
| 3 | SAOUHSC_00875 | (Q2FZW0_Q2FZW0_STAA8) |

| | | |
|---|---|---|
| 3 | SAOUHSC_00756 | (Q2G065_Q2G065_STAA8) |
| 3 | SAOUHSC_00731 | (Q2G089_Q2G089_STAA8) |
| 3 | SAOUHSC_00584 | (Q2G0I1_Q2G0I1_STAA8) |
| 3 | SAOUHSC_00480 | (Q2G0R6_Q2G0R6_STAA8) |
| 3 | SAOUHSC_00467 | (Q2G0S7_Q2G0S7_STAA8) |
| 3 | SAOUHSC_00139 | (Q2G1I3_Q2G1I3_STAA8) |
| 2 | *pknB* | (Q2FZ64_Q2FZ64_STAA8) |
| 2 | *tsaD* | (Q2FWL2_TSAD_STAA8) |
| 2 | *topB* | (Q2FW03_TOP3_STAA8) |
| 2 | *topA* | (Q2FZ32_TOP1_STAA8) |
| 2 | *rsmH* | (P60393_RSMH_STAA8) |
| 2 | *rsmA* | (Q2G0T0_RSMA_STAA8) |
| 2 | *rpsG* | (P48940_RS7_STAA8) |
| 2 | *rpsC* | (Q2FW12_RS3_STAA8) |
| 2 | *rplM* | (Q2FW38_RL13_STAA8) |
| 2 | *rplI* | (Q2G2T3_RL9_STAA8) |
| 2 | *rplF* | (Q2FW21_RL6_STAA8) |
| 2 | *potA* | (Q2G2A7_POTA_STAA8) |
| 2 | *mutS* | (Q2FYZ9_MUTS_STAA8) |
| 2 | *murE* | (Q2FZP6_MURE_STAA8) |
| 2 | *lysA* | (Q2FYN4_Q2FYN4_STAA8) |
| 2 | *gcvT* | (Q2FY33_GCST_STAA8) |
| 2 | *fmt* | (Q2FZ68_FMT_STAA8) |
| 2 | *ebpS* | (Q2FYF1_EBPS_STAA8) |
| 2 | *dnaJ* | (Q2FXZ3_DNAJ_STAA8) |
| 2 | *atpG* | (Q2FWE9_Q2FWE9_STAA8) |
| 2 | *aroC* | (Q2FYG9_AROC_STAA8) |
| 2 | *alr1* | (Q9ZAH5_ALR1_STAA8) |
| 2 | *ald2* | (Q2FXL7_DHA2_STAA8) |

| | | |
|---|---|---|
| 2 | SAOUHSC_02980 | (Q2G220_Q2G220_STAA8) |
| 2 | SAOUHSC_02899 | (Q2FV40_Q2FV40_STAA8) |
| 2 | SAOUHSC_02875 | (Q2FV62_Q2FV62_STAA8) |
| 2 | SAOUHSC_02382 | (Q2FWB6_Q2FWB6_STAA8) |
| 2 | SAOUHSC_02145 | (Q2FWX6_Q2FWX6_STAA8) |
| 2 | SAOUHSC_02133 | (Q2G235_Q2G235_STAA8) |
| 2 | SAOUHSC_01998 | (Q2G281_Q2G281_STAA8) |
| 2 | SAOUHSC_01816 | (Q2FXL9_Y1816_STAA8) |
| 2 | SAOUHSC_01794 | (Q2FXP2_Q2FXP2_STAA8) |
| 2 | SAOUHSC_01791 | (Q2FXP5_Q2FXP5_STAA8) |
| 2 | SAOUHSC_01766 | (Q2FXR9_Q2FXR9_STAA8) |
| 2 | SAOUHSC_01660 | (Q2FY14_Q2FY14_STAA8) |
| 2 | SAOUHSC_01615 | (Q2FY50_Q2FY50_STAA8) |
| 2 | SAOUHSC_01606 | (Q2FY59_Q2FY59_STAA8) |
| 2 | SAOUHSC_01486 | (Q2FYG6_Q2FYG6_STAA8) |
| 2 | SAOUHSC_01249 | (Q2G2Q2_Q2G2Q2_STAA8) |
| 2 | SAOUHSC_01199 | (Q2FZ53_Q2FZ53_STAA8) |
| 2 | SAOUHSC_01184 | (Q2FZ67_Q2FZ67_STAA8) |
| 2 | SAOUHSC_01054 | (Q2G2G7_Y1054_STAA8) |
| 2 | SAOUHSC_01014 | (Q2FZI9_Q2FZI9_STAA8) |
| 2 | SAOUHSC_00834 | (Q2G000_Q2G000_STAA8) |
| 2 | SAOUHSC_00794 | (Q2G033_Q2G033_STAA8) |
| 2 | SAOUHSC_00508 | (Q2G242_Q2G242_STAA8) |
| 2 | SAOUHSC_00442 | (Q2G0T5_Q2G0T5_STAA8) |
| 1 | yajC | (Q2FXT7_Q2FXT7_STAA8) |
| 1 | *secDF* | (Q2FXT8_Q2FXT8_STAA8) |
| 1 | *scaH* | (Q2G222_Y2979_STAA8) |
| 1 | *murI* | (Q2FZC6_MURI_STAA8) |
| 1 | *msrR* | (Q7BHL7_MSRR_STAA8) |

| | | |
|---|---|---|
| 1 | *msrB* | (P0A088_MSRB_STAA8) |
| 1 | *xerD* | (Q2FY74_Q2FY74_STAA8) |
| 1 | *uvrC* | (Q2FZD0_UVRC_STAA8) |
| 1 | *uvrA* | (Q2G046_Q2G046_STAA8) |
| 1 | *ugtP* | (Q2FZP7_UGTP_STAA8) |
| 1 | *tagX* | (O05154_TAGX_STAA8) |
| 1 | *srrB* | (Q2FY80_SRRB_STAA8) |
| 1 | *sarZ* | (Q2FVN3_SARZ_STAA8) |
| 1 | *sarS* | (Q2G1N7_SARS_STAA8) |
| 1 | *sarR* | (Q9F0R1_SARR_STAA8) |
| 1 | *sarA* | (Q2G2U9_SARA_STAA8) |
| 1 | *gdpp* | (Q2G2T6_Q2G2T6_STAA8) |
| 1 | *ruvB* | (Q2FXT4_RUVB_STAA8) |
| 1 | *rpsL* | (P0A0H0_RS12_STAA8) |
| 1 | *rpsD* | (Q2FXK6_RS4_STAA8) |
| 1 | *rpmI* | (Q2FXQ0_RL35_STAA8) |
| 1 | *rplW* | (Q2FW08_RL23_STAA8) |
| 1 | *rplP* | (Q2FW13_RL16_STAA8) |
| 1 | *rplN* | (Q2FW16_RL14_STAA8) |
| 1 | *rot* | (Q9RFJ6_ROT_STAA8) |
| 1 | *rnr* | (Q2G024_Q2G024_STAA8) |
| 1 | *recG* | (O50581_RECG_STAA8) |
| 1 | *pyrF* | (Q2FZ71_PYRF_STAA8) |
| 1 | *putP* | (Q2FWY7_PUTP_STAA8) |
| 1 | *nusG* | (Q2G0P2_NUSG_STAA8) |
| 1 | *mutS2* | (Q2FZD3_MUTS2_STAA8) |
| 1 | *mqo* | (Q2FVQ5_Q2FVQ5_STAA8) |
| 1 | *moaA* | (P69848_MOAA_STAA8) |
| 1 | *infC* | (Q2FXP9_IF3_STAA8) |

| | | |
|---|---|---|
| 1 | *hslO* | (Q2G0Q9_HSLO_STAA8) |
| 1 | *hemH* | (Q2FXA4_Q2FXA4_STAA8) |
| 1 | *guaC* | (Q2FYU4_GUAC_STAA8) |
| 1 | *gmk* | (Q2G1U0_KGUA_STAA8) |
| 1 | *ftsY* | (Q2FZ48_Q2FZ48_STAA8) |
| 1 | *dltC* | (Q2FZW4_DLTC_STAA8) |
| 1 | *dinG* | (Q2FYH5_DING_STAA8) |
| 1 | *cvfB* | (Q2FYP3_CVFB_STAA8) |
| 1 | *copA* | (Q2FV64_COPA_STAA8) |
| 1 | *cinA* | (Q2FZ10_Q2FZ10_STAA8) |
| 1 | *bioA* | (Q2FVJ6_BIOA_STAA8) |
| 1 | *atpF* | (Q2G2F8_ATPF_STAA8) |
| 1 | *addA* | (Q2FZT5_ADDA_STAA8) |
| 1 | SAOUHSC_02525 (RND2) | (Q2FVZ5_Q2FVZ5_STAA8) |
| 1 | SAOUHSC_03016 | (Q2FUT5_Q2FUT5_STAA8) |
| 1 | SAOUHSC_02971 | (Q2FUX4_Q2FUX4_STAA8) |
| 1 | SAOUHSC_02956 | (Q2FUY9_Q2FUY9_STAA8) |
| 1 | SAOUHSC_02947 | (Q2FUZ8_Q2FUZ8_STAA8) |
| 1 | SAOUHSC_02791 | (Q2FVC2_Q2FVC2_STAA8) |
| 1 | SAOUHSC_02760 | (Q2FVF4_Q2FVF4_STAA8) |
| 1 | SAOUHSC_02727 | (Q2FVI3_Q2FVI3_STAA8) |
| 1 | SAOUHSC_02723 | (Q2FVI7_Q2FVI7_STAA8) |
| 1 | SAOUHSC_02690 | (Q2G1U8_Q2G1U8_STAA8) |
| 1 | SAOUHSC_02681 | (Q2FVM1_Q2FVM1_STAA8) |
| 1 | SAOUHSC_02668 | (Q2FVN4_Q2FVN4_STAA8) |
| 1 | SAOUHSC_02660 | (Q2FVP2_Q2FVP2_STAA8) |
| 1 | SAOUHSC_02649 | (Q2FVQ3_Q2FVQ3_STAA8) |
| 1 | SAOUHSC_02648 | (Q2FVQ4_Q2FVQ4_STAA8) |
| 1 | SAOUHSC_02629 | (Q2G2W1_Q2G2W1_STAA8) |

| | | |
|---|---|---|
| 1 | SAOUHSC_02614 | (Q2FVS7_Q2FVS7_STAA8) |
| 1 | SAOUHSC_02601 | (Q2FVU1_Q2FVU1_STAA8) |
| 1 | SAOUHSC_02583 | (Q2FVV8_Q2FVV8_STAA8) |
| 1 | SAOUHSC_02555 | (Q2FVW8_Q2FVW8_STAA8) |
| 1 | SAOUHSC_02554 | (Q2FVW9_Q2FVW9_STAA8) |
| 1 | SAOUHSC_02553 | (Q2FVX0_Q2FVX0_STAA8) |
| 1 | SAOUHSC_02544 | (Q2FVX8_Q2FVX8_STAA8) |
| 1 | SAOUHSC_02406 | (Q2FW93_Q2FW93_STAA8) |
| 1 | SAOUHSC_02381 | (Q2FWB7_Q2FWB7_STAA8) |
| 1 | SAOUHSC_02374 | (Q2FWC4_Q2FWC4_STAA8) |
| 1 | SAOUHSC_02357 | (Q2FWE2_Q2FWE2_STAA8) |
| 1 | SAOUHSC_02352 | (Q2G2F5_Q2G2F5_STAA8) |
| 1 | SAOUHSC_02197 | (Q2FWT3_Q2FWT3_STAA8) |
| 1 | SAOUHSC_02161 | (Q2FWW1_Q2FWW1_STAA8) |
| 1 | SAOUHSC_02098 | (Q2FX09_Q2FX09_STAA8) |
| 1 | SAOUHSC_01987 | (Q2FX90_Q2FX90_STAA8) |
| 1 | SAOUHSC_01979 | (Q2FX98_Q2FX98_STAA8) |
| 1 | SAOUHSC_01978 | (Q2FX99_Y1978_STAA8) |
| 1 | SAOUHSC_01977 | (Q2FXA0_Y1977_STAA8) |
| 1 | SAOUHSC_01969 | (Q2G2T0_Q2G2T0_STAA8) |
| 1 | SAOUHSC_01966 | (Q2G2F2_Q2G2F2_STAA8) |
| 1 | SAOUHSC_01915 | (Q2G2V2_Q2G2V2_STAA8) |
| 1 | SAOUHSC_01877 | (Q2FXH0_Q2FXH0_STAA8) |
| 1 | SAOUHSC_01869 | (Q2FXH8_Q2FXH8_STAA8) |
| 1 | SAOUHSC_01867 | (Q2FXI0_Q2FXI0_STAA8) |
| 1 | SAOUHSC_01846 | (Q2G294_Q2G294_STAA8) |
| 1 | SAOUHSC_01825 | (Q2FXL0_Q2FXL0_STAA8) |
| 1 | SAOUHSC_01812 | (Q2FXM3_Q2FXM3_STAA8) |
| 1 | SAOUHSC_01803 | (Q2FXN2_Q2FXN2_STAA8) |

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|---|---|---|
| 1 | SAOUHSC_01801 | (Q2FXN4_Q2FXN4_STAA8) |
| 1 | SAOUHSC_01744 | (Q2FXT9_Q2FXT9_STAA8) |
| 1 | SAOUHSC_01734 | (Q2FXU8_Q2FXU8_STAA8) |
| 1 | SAOUHSC_01732 | (Q2FXV0_Q2FXV0_STAA8) |
| 1 | SAOUHSC_01700 | (Q2FXY0_Q2FXY0_STAA8) |
| 1 | SAOUHSC_01664 | (Q2FY10_PDRP_STAA8) |
| 1 | SAOUHSC_01659 | (Q2FY15_Q2FY15_STAA8) |
| 1 | SAOUHSC_01652 | (Q2FY21_Q2FY21_STAA8) |
| 1 | SAOUHSC_01610 | (Q2FY55_Y1610_STAA8) |
| 1 | SAOUHSC_01587 | (Q2FY78_Q2FY78_STAA8) |
| 1 | SAOUHSC_01488 | (Q2FYG4_Q2FYG4_STAA8) |
| 1 | SAOUHSC_01487 | (Q2FYG5_Q2FYG5_STAA8) |
| 1 | SAOUHSC_01480 | (Q2FYH2_Q2FYH2_STAA8) |
| 1 | SAOUHSC_01455 | (Q2FYJ0_Q2FYJ0_STAA8) |
| 1 | SAOUHSC_01436 | (Q2FYK4_Y1436_STAA8) |
| 1 | SAOUHSC_01284 | (Q2FYY8_Q2FYY8_STAA8) |
| 1 | SAOUHSC_01279 | (Q2FYZ3_Q2FYZ3_STAA8) |
| 1 | SAOUHSC_01267 | (Q2FZ04_Q2FZ04_STAA8) |
| 1 | SAOUHSC_01258 | (Q2FZ13_Q2FZ13_STAA8) |
| 1 | SAOUHSC_01214 | (Q2FZ39_Q2FZ39_STAA8) |
| 1 | SAOUHSC_01198 | (Q2FZ54_Q2FZ54_STAA8) |
| 1 | SAOUHSC_01179 | (Q2G266_Q2G266_STAA8) |
| 1 | SAOUHSC_01031 | (Q2FZH3_Q2FZH3_STAA8) |
| 1 | SAOUHSC_01016 | (Q2FZI7_Q2FZI7_STAA8) |
| 1 | SAOUHSC_00989 | (Q2FZL1_Q2FZL1_STAA8) |
| 1 | SAOUHSC_00982 | (Q2FZL8_Q2FZL8_STAA8) |
| 1 | SAOUHSC_00974 | (Q2FZM6_Q2FZM6_STAA8) |
| 1 | SAOUHSC_00951 | (Q2FZP9_Y951_STAA8) |
| 1 | SAOUHSC_00946 | (Q2FZQ4_Q2FZQ4_STAA8) |

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|---|---|---|
| 1 | SAOUHSC_00925 | (Q2FZR5_Q2FZR5_STAA8) |
| 1 | SAOUHSC_00909 | (Q2FZT1_Q2FZT1_STAA8) |
| 1 | SAOUHSC_00897 | (Q2FZU3_Q2FZU3_STAA8) |
| 1 | SAOUHSC_00873 | (Q2FZW2_Q2FZW2_STAA8) |
| 1 | SAOUHSC_00855 | (Q2FZX9_2NPD_STAA8) |
| 1 | SAOUHSC_00847 | (Q2FZY7_Q2FZY7_STAA8) |
| 1 | SAOUHSC_00792 | (Q2G035_Y792_STAA8) |
| 1 | SAOUHSC_00730 | (Q2G090_Q2G090_STAA8) |
| 1 | SAOUHSC_00727 | (Q2G094_Q2G094_STAA8) |
| 1 | SAOUHSC_00711 | (Q2G2T9_Q2G2T9_STAA8) |
| 1 | SAOUHSC_00707 | (Q2G238_Q2G238_STAA8) |
| 1 | SAOUHSC_00640 | (Q2G2L3_Q2G2L3_STAA8) |
| 1 | SAOUHSC_00639 | (Q2G2K9_Q2G2K9_STAA8) |
| 1 | SAOUHSC_00547 | (Q2G0L3_Q2G0L3_STAA8) |
| 1 | SAOUHSC_00483 | (Q2G0R3_Q2G0R3_STAA8) |
| 1 | SAOUHSC_00444 | (Q2G0T4_Y444_STAA8) |
| 1 | SAOUHSC_00413 | (Q2G0W1_Y413_STAA8) |
| 1 | SAOUHSC_00398 | (Q2G0X5_Q2G0X5_STAA8) |
| 1 | SAOUHSC_00333 | (Q2G1V4_Q2G1V4_STAA8) |
| 1 | SAOUHSC_00307 | (Q2G148_Q2G148_STAA8) |
| 1 | SAOUHSC_00269 | (Q2G178_Q2G178_STAA8) |
| 1 | SAOUHSC_00268 | (Q2G179_Q2G179_STAA8) |
| 1 | SAOUHSC_00261 | (Q2G185_Q2G185_STAA8) |
| 1 | SAOUHSC_00236 | (Q2G1A9_Q2G1A9_STAA8) |
| 1 | SAOUHSC_00196 | (Q2G1C9_Q2G1C9_STAA8) |
| 1 | SAOUHSC_00126 | (Q2G1J6_Q2G1J6_STAA8) |
| 1 | SAOUHSC_00039 | (Q2G1R3_Q2G1R3_STAA8) |

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**Table S2. Strains and plasmids used in this study**

| Strain or plasmid | Description | Reference or source |
|---|---|---|
| *E. coli* DC10B | Cloning strain | (Monk et al., 2012) |
| *E. coli* DH5α | Cloning strain | (Hanahan, 1983) |
| *S. aureus* RN4220 | *S. aureus* laboratory strain | (Kreiswirth et al., 1983) |
| *S. aureus* SEJ1 | Δ*spa* in RN4220 | (Gründling and Schneewind, 2007) |
| *S. aureus* WY110 | Δ*spa* Δ*sbi, sbi*::*ermB* in *S. aureus* SEJ1 | This work |
| *S. aureus* WY223 | $P_{spac}$-*secA* in *S. aureus* RN4220 | This work |
| *S. aureus* WY230 | $P_{tet}$-*secA:sfGFP* in *S. aureus* WY223 | This work |
| *S. aureus* ANG499 | $P_{spac}$-*ltaS* in *S. aureus* RN4220 | (Gründling and Schneewind, 2007) |
| *S. aureus* WY418 | Δ*secDF* in *S. aureus* RN4220 | This work |
| *S. aureus* WY416 | Δ*rnd2* in *S. aureus* RN4220 | This work |
| *S. aureus* WY400 | Δ*rnd3* in *S. aureus* RN4220 | This work |
| *S. aureus* WY412 | Δ*secDF* Δ*rnd2*Δ*rnd3* in *S. aureus* RN4220 | This work |
| pOS1 | *E. coli/S. aureus* shuttle vector | (Schneewind et al., 1993) |
| $pSpA_{ED}$ | *spa* promoter, signal peptide and IgBDs E and D in pOS1 | This work |
| $pSpA_{ED/R10A}$ | R10A variant of $pSpA_{ED}$ | This work |
| $pSpA_{ED/S18L}$ | S18L variant of of $pSpA_{ED}$ | This work |
| $pSpA_{ED/\Delta IA}$ | ΔIA variant of $pSpA_{ED}$ | This work |
| pCL55 | *S. aureus* integration vector | (Lee et al., 1991) |
| pCL55-SpA | Full length *spa* with its native promoter cloned in pCL55 | This work |
| pCL55-$SpA_{R10A}$ | R10A variant of pCL55-SpA | This work |
| pCL55-$SpA_{S18L}$ | S18L variant of pCL55-SpA | This work |
| pCL55-$SpA_{\Delta IA}$ | ΔIA variant of pCL55-SpA | This work |
| pCL55-$SpA_{SP\text{-}SasF}$ | SpA signal peptide replaced by SasF signal peptide in pCL55-SpA | This work |
| pCL55-$P_{tet}$ | pCL55 with anhydro-tetracycline inducible | (Gründling and |

| | | |
|---|---|---|
| | promoter | Schneewind, 2007) |
| pCL55-$P_{tet}$-*secA:sfGFP* | SecA-sfGFP hybrid cloned into pCL55-$P_{tet}$ | This work |
| pMutin–HA | Single copy integration vector | Bacillus Genetic Stock Center |
| pMutin–HA-5'secA | *secA* promoter and 656 bp in pMutin-HA | This work |
| pKOR1-*secDF* | allelic replacement vector for secDF1 deletion | This work |
| pKOR1-*rnd2* | allelic replacement vector for *secDF2* deletion | This work |
| pSecDF | *secDF* ORF and 274 bp upstream in pOS1 | This work |

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**Table S3. Oligonucleotide primers used in this study**

| Primer | [a]Direction | Sequence | Plasmid |
|---|---|---|---|
| 10 | F | GCGTAGTATTGCAATACATAATTCGTTA | $pSpA_{ED}$ |
| 69 | R | TTTTGGATCCTTACATTTTCGGTGCTTGAGATTCGTT | $pSpA_{ED}$ |
| 38 | R | TGCAATACCTACACCTAGAATGTTTTTCTTTTTCA | $pSpA_{ED/\Delta YSIRK}$ |
| 39 | F | GAAAAAGAAAAACATTCTAGGTGTAGGTATTGCATC | $pSpA_{ED/\Delta YSIRK}$ |
| 49 | R | TGTACCTAAAGTTACTACACCTAGTTTACGA | $pSpA_{ED/\Delta GIAS}$ |
| 50 | F | TCGTAAACTAGGTGTAGTAACTTTAGGTACA | $pSpA_{ED/\Delta GIAS}$ |
| 51 | R | TGTACCTAAAGTTACTGCAATTACACCTAGT | $pSpA_{ED/\Delta G15\Delta S18}$ |
| 52 | F | ACTAGGTGTAATTGCAGTAACTTTAGGTACA | $pSpA_{ED/\Delta G15\Delta S18}$ |
| 53 | R | ACCTAAAGTTACAGAACCTACACCTAGTTTAC | $pSpA_{ED/\Delta IA}$ |
| 54 | F | GTAAACTAGGTGTAGGTTCTGTAACTTTAGGT | $pSpA_{ED/\Delta IA}$ |
| 55 | R | AGTTACAGATGCAATTAATACACCTAGTTTACGA | $pSpA_{ED/G15L}$ |
| 56 | F | TCGTAAACTAGGTGTATTAATTGCATCTGTAACT | $pSpA_{ED/G15L}$ |
| 57 | R | TGTACCTAAAGTTACTAATGCAATACCTACACCT | $pSpA_{ED/S18L}$ |
| 58 | F | AGGTGTAGGTATTGCATTAGTAACTTTAGGTACA | $pSpA_{ED/S18L}$ |
| 70 | R | AGTAATGTACCTAAAGTTACCAATGCAATTAATACACCTAGT | $pSpA_{ED/G15L/S18L}$ |
| 71 | F | ACTAGGTGTATTAATTGCATTGGTAACTTTAGGTACATTACT | $pSpA_{ED/G15L/S18L}$ |
| 72 | R | AGTAATGTACCTAAAGTTACAGATGCAATTACACCTAGTTTACGA | $pSpA_{ED/\Delta G15}$ |
| 73 | F | TCGTAAACTAGGTGTAATTGCATCTGTAACTTTAGGTACATTACT | $pSpA_{ED/\Delta G15}$ |
| 74 | R | ACCTAAAGTTACTGCAATACCTACACCTAGTTTACGAATTGA | $pSpA_{ED/\Delta S18}$ |
| 75 | F | TCAATTCGTAAACTAGGTGTAGGTATTGCAGTAACTTTAGGT | $pSpA_{ED/\Delta S18}$ |
| 84 | R | ATACCTACACCTAGTTTAGCAATTGAATAAATGTTT | $pSpA_{ED/R10A}$ |
| 85 | F | AAACATTTATTCAATTGCTAAACTAGGTGTAGGTAT | $pSpA_{ED/R10A}$ |
| 92 | R | TAGTTTACGAATTGAAGCAATGTTTTTCTTTTTCA | $pSpA_{ED/Y7A}$ |
| 93 | F | TGAAAAAGAAAAACATTGCTTCAATTCGTAAACTA | $pSpA_{ED/Y7A}$ |
| 94 | R | TACACCTAGTTTACGAGATGAATAAATGTTTTTCT | $pSpA_{ED/I9S}$ |
| 95 | F | AGAAAAACATTTATTCATCTCGTAAACTAGGTGTA | $pSpA_{ED/I9S}$ |
| 96 | R | TGCAATACCTACACCTAGAGCACGAATTGAATAAATGT | $pSpA_{ED/K11A}$ |
| 97 | F | ACATTTATTCAATTCGTGCTCTAGGTGTAGGTATTGCA | $pSpA_{ED/K11A}$ |
| 175 | F | GCGGGATCCTAGTATTGCAATACATAATTCGTTA | pCL55-SpA |
| 177 | R | GCGGGTACCTTATAGTTCGCGACGACGTCCAGCT | pCL55-SpA |
| 21 | R | ATTAATACCCCCTGTATGTATTTGT | $pCL55\text{-}SpA_{SP\text{-}SasF}$ |
| 22 | F | TACAGGGGGTATTAATATGGCTAAATATCGAGGGAAAC | $pCL55\text{-}SpA_{SP\text{-}SasF}$ |

| | | | |
|---|---|---|---|
| 23 | R | TGTTGAGCTTCATCGTGTTGCGCAGCTTGGGCATCGTACGGCAAGA | pCL55-$SpA_{SP\text{-}SasF}$ |
| 24 | F | GCGCAACACGATGAAGCTCAACAA | pCL55-$SpA_{SP\text{-}SasF}$ |
| 189 | F | CCCAAGCTTTAGCTAAAGGAGCGAACGAAATGGGA | pMUTIN-HA-5'*secA* |
| 190 | R | GCGGGTACCTGAGTCAACCTCATCAATGATTGCA | pMUTIN-HA-5'*secA* |
| 180 | F | CTCCCTAGGTAAAGGAGCGAACGAAATGGGAT | pCL55-$P_{tet\text{-}}$ *secA:sfGFP* |
| 181 | R | TGCAGCTCCTGCGGCGCCTCCTTTTCCATGGCAATTTTTGA | pCL55-$P_{tet\text{-}}$ *secA:sfGFP* |
| 182 | F | AGGAGGCGCCGCAGGAGCTGCATCAAAAGGTGAAGAATT | pCL55-$P_{tet\text{-}}$ *secA:sfGFP* |
| 183 | R | CTCAGATCTTTATTTATATAATTCATCCATACCA | pCL55-$P_{tet\text{-}}$ *secA:sfGFP* |
| 295 | F | GGGGACAAGTTTGTACAAAAAAGCAGGCTAATATTGTCATTGTATCCCGCTTCT | pKOR1-*secDF* |
| 313 | R | ACATACGTAAATATCGAACGATGAAAAGATTTTAGT | pKOR1-*secDF* |
| 314 | F | TCATCGTTCGATATTTACGTATGTATTTAGAATACT | pKOR1-*secDF* |
| 298 | R | GGGGACCACTTTGTACAAGAAAGCTGGGTTGAACATACAGAGCAGTTTATGCCT | pKOR1-*secDF* |
| 305 | F | GGGGACAAGTTTGTACAAAAAAGCAGGCTACATACTCCACAGATATTTTAGA | pKOR1-*rnd2* |
| 306 | R | TGAATATAGATAATATAAAAGCCATAAAAGCGGT | pKOR1-*rnd2* |
| 307 | F | TGGCTTTTATATTATCTATATTCAAAAATATTTTACT | pKOR1-*rnd2* |
| 308 | R | GGGGACCACTTTGTACAAGAAAGCTGGGTTCGATCCTGATGTTGAAGTTGAT | pKOR1-*rnd2* |
| 315 | F | GCGGAATTCTGAGAAGTGGTATTAAAAAGGATGA | pSecDF |
| 316 | R | GCGGGATCCTTAAACTAAAATCTTTTCATCGTTCGA | pSecDF |

[a]PCR primer direction for forward (F) or reverse (R) amplification of template DNA.

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