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

3

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9 ## Abstract

10 Surface proteins of *Staphylococcus aureus* are secreted across septal membranes for assembly
11 into the bacterial cross-wall. This localized secretion requires the YSIRK/GXXS motif signal
12 peptide, however the mechanisms supporting precursor trafficking are not known. We show
13 here that the signal peptide of staphylococcal protein A (SpA) is cleaved at the YSIRK/GXXS
14 motif. A signal peptide mutant defective for cleavage can be crosslinked to SecA, SecDF and
15 LtaS. SecA depletion blocks precursor targeting to septal membranes, whereas deletion of
16 *secDF* diminishes SpA secretion into the cross-wall. Depletion of LtaS blocks lipoteichoic acid
17 synthesis and promotes precursor trafficking to peripheral membranes. We propose a model
18 whereby SecA directs SpA precursors to lipoteichoic acid-rich septal membranes for YSIRK/GXXS
19 motif cleavage and secretion into the cross-wall.
20

21 ## Introduction

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

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31 trafficking of surface proteins requires a signal peptide with YSIRK/GXXS motif (Carlsson et al.,
32 2006, DeDent et al., 2008). The YSIRK/GXXS motif is positioned N-terminal of the hydrophobic
33 core, common to all signal peptide precursors traveling the Sec pathway (Emr et al., 1978, Emr
34 et al., 1981, von Heijne, 1986).

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

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52 Newly synthesized SpA is secreted into the cross-wall compartment, bounded by septal
53 membranes of burgeoning cells during division (DeDent et al., 2007). Upon completion of
54 peptidoglycan synthesis within the cross-wall, its peptidoglycan layer is split (Frankel et al.,
55 2011). The adjacent cells separate and assume a spherical shape, resulting in SpA display on the
56 bacterial surface (DeDent et al., 2007). Staphylococci divide perpendicular to previous cell
57 division planes (Tzagoloff and Novick, 1977). By incorporating secreted polypeptides into newly
58 synthesized cross-walls, staphylococci distribute SpA and other sortase A-anchored products
59 over the bacterial surface (DeDent et al., 2008). However, not all sortase-anchored products
60 traffic to septal membranes. Those that are secreted at polar membranes are also anchored to
61 peptidoglycan but are not distributed over the bacterial surface (DeDent et al., 2008). In *S.*
62 *aureus* strain Newman, thirteen different sortase-anchored surface proteins and four additional
63 proteins are endowed with YSIRK/GXXS signal peptides for septal secretion: lipase (Lip),
64 glycerol-ester hydrolase (Geh), murein hydrolase LytN and the cell size determinant Ebh (Yu and
65 Götz, 2012, Frankel et al., 2011, Cheng et al., 2014).

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

72

73 ## Results

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

75 To facilitate the analysis of signal peptide mutants, we generated SpA_{ED}, a variant of protein A
76 that is truncated for its C-terminal immunoglobulin binding domains, region X (Xr) and the
77 LPXTG sorting signal (*Figure 1a*). *S. aureus* WY110 (Δ*spa* Δ*sbi*, $pSpA_{ED}$) cultures expressing spa_{ED}
78 were fractionated into culture supernatant (S) and bacterial pellet (P) and analyzed by
79 immunoblotting. SpA_{ED} was found in the extracellular medium; its precursor species was
80 detected in the bacterial pellet (*Figure 1bc*). Site-directed mutagenesis was used to generate
81 short deletions and amino acid substitutions in the signal peptide of SpA_{ED} (*Figure 1b*). Deletion
82 of the YISRK motif (ΔYSIRK) diminished the abundance of the $SpA_{ED/\Delta YSIRK}$ precursor and its
83 processing (*Figure 1b*). Single amino acid substitutions at two positions in the YSIRK motif (I9S
84 and R10A) resulted in precursor accumulation (*Figure 1b*). Further, the R10A variant exhibited
85 diminished secretion and accumulated a precursor species that migrated faster on SDS-PAGE
86 than the full-length precursor (*Figure 1bc*). Amino acid substitution at lysine 11 (K11A) of the
87 YSIRK motif had no effect on $SpA_{ED/K11A}$ precursor processing and secretion (*Figure 1bc*).
88 Deletion of GIAS (ΔGIAS) or of the two variable residues in the GXXS motif (ΔIA) caused
89 precursor accumulation and blocked precursor processing (*Figure 1bc*). Substitution of glycine
90 15 (G15L) reduced the abundance of $SpA_{ED/G15L}$ and led to the accumulation of a unique
91 precursor species that migrated faster on SDS-PAGE than full-length precursor (*Figure 1b*).
92 Substitution of serine 18 (S18L) caused accumulation of full-length and processed precursors as
93 well as reduced secretion (*Figure 1bc*).

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

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96 cytoplasm and membrane, whereas mature product was secreted into the culture supernatant
97 (S) (*Figure 2a*). Precursors of the $SpA_{ED/\Delta IA}$ and $SpA_{ED/R10A}$ variants accumulated mostly in the
98 cytoplasm, whereas the $SpA_{ED/S18L}$ precursor was located predominantly in the membrane
99 (*Figure 2ab*). Pulse-labeling experiments revealed that wild-type SpA_{ED} precursor was processed
100 within 60 seconds into mature, secreted product (*Figure 2c*). In contrast, processing of the
101 $SpA_{ED/\Delta IA}$, $SpA_{ED/R10A}$ and $SpA_{ED/S18L}$ precursors was delayed (*Figure 2c*). To test whether signal
102 peptide variations affect trafficking of full-length SpA, mutations encoding the ΔIA, R10A and
103 S18L variants were introduced into wild-type *spa*. Wild-type and mutant staphylococci were
104 treated with trypsin to remove all surface proteins from the bacterial surface and incubated for
105 20 min to allow for cell wall deposition of newly synthesized SpA. To localize SpA, bacteria were
106 viewed by fluorescence microscopy after labeling with SpA-specific monoclonal antibody and
107 Alexa Fluor 647-conjugated secondary IgG (red) and with BODIPY FL-vancomycin (green), which
108 binds to cell wall peptidoglycan. As expected, wild-type SpA was assembled in the cross-wall
109 compartment, whereas $SpA_{SP\text{-}SasF}$, which is secreted via a canonical signal peptide, was
110 deposited into peripheral segments of the cell wall envelope (DeDent et al., 2008)(*Figure 2d*).
111 $SpA_{\Delta IA}$, SpA_{R10A} and SpA_{S18L} exhibited defects in surface display, consistent with their observed
112 defects in precursor processing and secretion (*Figure 2d*). Residual amounts of cross-wall
113 localization were observed for SpA_{R10A} and SpA_{S18L}, whereas $SpA_{\Delta IA}$ was not detected in the
114 cross-wall compartment (*Figure 2e*). Together these data indicate that some features of the
115 YSIRK/GXXS motif, specifically Arg^{10}, Ser^{18} and the GXXS motif, are crucial for septal secretion of
116 SpA in *S. aureus* (*Figure 2e*).
117

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118 **Processing of SpA signal peptide variants**

119 Wild-type SpA$_{ED}$, SpA$_{ED/\Delta IA}$, SpA$_{ED/R10A}$ and SpA$_{ED/S18L}$ were purified from staphylococcal
120 membranes via affinity chromatography, analyzed by Coomassie-stained SDS-PAGE and
121 identified by Edman degradation (*Figure 3a*). For wild-type SpA$_{ED}$, full-length precursor (SpA$_{ED}$-
122 1, starting at Met^{1}), as well as two precursors with faster mobility on SDS-PAGE (SpA$_{ED}$-2 and
123 SpA$_{ED}$-3) and mature product (SpA$_{ED}$-4), i.e. SpsB signal peptidase-cleaved SpA$_{ED}$ starting at
124 Ala^{37}, were identified (*Figure 3ab*). Edman degradation revealed that SpA$_{ED}$-2 is a product of
125 proteolytic cleavage within the YSIRK/GXXS motif (N-terminus Gly^{13}). SpA$_{ED}$-3 is a product of
126 further cleavage, as Edman degradation identified its N-terminal amino acid 10 residues
127 downstream (N-terminus Thr^{23}) (*Figure 3ab*). Purified SpA$_{ED}$-1 precursor as well as its SpA$_{ED}$-2,
128 SpA$_{ED}$-3 and SpA$_{ED}$-4 cleavage products were analyzed by MALDI-TOF-MS, confirming the
129 predicted mass of the precursor and its cleaved species (*Table 1*). SDS-PAGE and Edman analysis
130 of the SpA$_{ED/S18L}$ sample revealed the same four species as wild-type SpA$_{ED}$, albeit that the
131 abundance of SpA$_{ED/S18L}$-1 and SpA$_{ED/S18L}$-4 were increased over those of of SpA$_{ED/S18L}$-2 and
132 SpA$_{ED/S18L}$-3 (*Figure 3ab*). Analysis of the SpA$_{ED/R10A}$ sample also identified four species, including
133 SpA$_{ED/ R10A}$-1 precursor, SpA$_{ED/ R10A}$-3 cleavage product (N-terminus Thr^{23}) and SpA$_{ED/ R10A}$-4
134 mature product (N-terminus Ala^{37}), whereas SpA$_{ED/ R10A}$-2 represented a variant cleavage
135 product (N-terminus Ala^{10}) (*Figure 3ab*). The SpA$_{ED/\Delta IA}$ sample yielded the same precursor and
136 cleavage species as SpA$_{ED}$ and SpA$_{ED/S18L}$, however the abundance of SpA$_{ED/\Delta IA}$-1 was increased
137 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
138 indicate that the SpA precursor (SpA$_{ED}$-1) is cleaved between Leu^{12} and Gly^{13}, which are
139 positioned between the two motifs (underlined) of the <u>YSIRK</u>L/GV<u>GIAS</u> sequence. The R10A

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140 substitution alters the cleavage site and diminishes precursor cleavage, whereas the S18L
141 substitution and ΔIA deletion diminish precursor cleavage without altering the cleavage site
142 between the YSIRK/GXXS motifs. Precursor cleavage between Gly^{22} and Thr^{23} was observed for
143 all SpA variants, suggesting that it represents a proteolytic event unrelated to the function of
144 the YSIRK/GXXS motif in targeting SpA to septal membranes.
145

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

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

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162 selected for further study. Immunoblotting confirmed SecA crosslinking to the $SpA_{ED/S18L}$

163 precursor (*Figure 4c*).

164

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

166 In *Escherichia coli*, *secA* is an essential gene (Oliver and Beckwith, 1981). SecA functions as an

167 ATPase that moves many, but not all, precursor proteins across the SecYEG translocon

168 (Tsirigotaki et al., 2017). To study the contribution of *secA* towards the septal secretion of SpA,

169 we generated an inducible allele, P_{spac}-*secA*, in *S. aureus* WY223 (*Figure 5a*). When induced with

170 isopropyl β-D-1-thiogalactoside (IPTG), *S. aureus* WY223 (P_{spac}-*secA*) forms colonies on agar and

171 replicates in liquid media culture in a manner similar to wild-type *S. aureus* (*Figure 5bc*).

172 However, in the absence of IPTG, *S. aureus* WY223 cannot form colonies or replicate in broth

173 culture (*Figure 5bc*). Following dilution of bacteria from IPTG-containing media into broth

174 without inducer, *S. aureus* WY223 replicates for 3 hours at a rate similar to wild-type (*Figure*

175 *5b*). Upon further dilution and incubation, *S. aureus* WY223 eventually exhibits growth

176 retardation and arrest (6-hour time point). When analyzed by immunoblotting with SecA-

177 specific antibody, SecA was depleted in *S. aureus* WY223 (P_{spac}-*secA*) cultures 3 hours following

178 dilution into inducer free medium. After 6 hours of incubation, SecA could no longer be

179 detected (*Figure 5d*).

180

181 **SecA depletion blocks SpA secretion**

182 After dilution into media with and without inducer, wild-type (*S. aureus* RN4220) and P_{spac}-*secA*

183 (*S. aureus* WY223) were subjected to pulse labeling with $[^{35}S]$methionine and protein A

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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

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206 infections, is synthesized as a precursor with a canonical signal peptide (Phonimdaeng et al.,
207 1990, Shortle, 1983). The abundance of secreted Nuc was also diminished in SecA-depleted
208 cultures of *S. aureus* WY223 (*Figure 6e*). As a control, production of sortase A in staphylococcal
209 membranes was not affected by the depletion of SecA. Taken together, these data indicate that
210 SecA is essential for *S. aureus* growth and for the secretion of precursors with canonical and
211 YSIRK/GXXS signal peptides.
212
213 **Localization of SecA and SpA precursors in staphylococci**
214 To localize SecA within *S. aureus*, we generated a translational hybrid between *secA* and the
215 structural gene for super-folder green fluorescent protein (*gfp*) (Pedelacq et al., 2006) under
216 transcriptional control of the P_{tet} promoter in *S. aureus* WY230 (P_{spac}-*secA*, P_{tet}-*secA:sfGFP*,
217 *Figure 7a*). Expression of *secA:sfGFP* in the P_{spac}-*secA* variant restored bacterial growth in the
218 absence of IPTG inducer, indicating that *secA-gfp* is functional (*Figure 7b*). Growth restoration
219 occurred in the presence and in the absence of anhydrotetracycline (ATc), suggesting that
220 *secA:sfGFP* must be expressed even in the absence of the P_{tet} inducer (*Figure 7b*).
221 Immunoblotting staphylococcal cell extracts 3 and 6 hours following dilution into media lacking
222 IPTG revealed that *S. aureus* WY230 indeed produced small amounts SecA:sfGFP in the absence
223 of ATc (*Figure 7c*). In the presence of ATc inducer, the abundance of SecA-GFP was increased
224 (*Figure 7c*). As expected, wild-type SecA was depleted when *S. aureus* WY230 was cultured for 3
225 or 6 hours without the IPTG inducer (*Figure 7b*). However, under SecA depleting conditions,
226 even small amounts of SecA-sfGFP in *S. aureus* WY230 (-ATc) restored precursor processing of
227 pulse-labeled SpA (*Figure 7d*). SpA precursor processing was accelerated to levels faster than

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

235 The envelope of trypsin-treated, paraformaldehyde-fixed *S. aureus* WY223 (P_{spac}-*secA*)
236 was permeabilized with murein hydrolase and with detergent to detect intracellular precursors
237 via microscopy with fluorescent antibody (Harry et al., 1995, Pinho and Errington, 2003). In *S.*
238 *aureus* WY223 producing wild-type levels of SecA (P_{spac}-*secA* + IPTG), SpA precursors were
239 localized to septal membranes (*Figure 8a*). In other images, SpA precursors appeared as two
240 puncta or ring deposits at septal membranes, reminiscent of FtsZ and of the division rings that
241 are known to accumulate at this site (*Figure 8ab*)(Lutkenhaus, 1993). In contrast, under SecA
242 depleting conditions (-IPTG), SpA precursors in *S. aureus* WY223 were associated with polar
243 membranes and were not localized to septal membranes (*Figure 8a*). These results suggest that
244 in staphylococci with a functional secretion pathway, SpA precursor are localized to the vicinity
245 of septal division rings. However, in cells lacking functional secretion machines, SpA precursors
246 are located throughout the cytoplasm and cannot traffic to septal membranes.
247

248 **SecDF contributes to SpA secretion**

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249 The *secDF* gene is not essential for protein secretion and *S. aureus* growth, however *secDF*
250 mutants exhibit diminished secretion of many precursors secreted via canonical and YSIRK-
251 GXXS signal peptides (Quiblier et al., 2011, Quiblier et al., 2013). SecDF is a member of the
252 resistance nodulation and cell division (RND) membrane protein family with 12-transmembrane
253 spanning segments. SecDF functions as a membrane-integrated chaperone. Sustained by the
254 proton motive force, SecDF catalyzes ATP-independent translocation and folding of proteins on
255 the *trans*-side of the plasma membrane (Tsukazaki et al., 2011). *S. aureus* expresses two
256 additional RND proteins, here designated Rnd2 (SAOUHSC_02525) and Rnd3 (SAOUHSC_02866)
257 (Quiblier et al., 2011). The *rnd2* gene is located downstream of *femX*, whose product tethers
258 glycine from glycyl-tRNA to the ε-amino group of lysine in lipid II peptidoglycan precursor [C_{55}-
259 $(PO_4)_2$-MurNAc(L-Ala-D-iGln-L-Lys-Da-Ala-D-Ala)-GlcNac](Rohrer et al., 1999). Rnd2 product
260 interacts with FemB, PBP1 and PBP2 (Quiblier et al., 2011). As SecDF, FemB and PBP2 were each
261 found crosslinked to $SpA_{ED/S18L}$ precursors (*Table S1*), we asked whether *secDF*, *rnd2* and *rnd3*
262 contribute to septal secretion of SpA. Compared to wild-type *S. aureus*, the Δ*secDF* mutant (*S.*
263 *aureus* WY418) accumulated SpA_{ED} precursor in bacterial cells and secreted reduced amounts
264 of mature SpA_{ED} into the extracellular medium (*Figure 9ab*). *S. aureus rnd2* (WY416) and *rnd3*
265 (WY400) mutants exhibited wild-type levels of SpA_{ED} secretion (*Figure 9ab*). A variant lacking all
266 three genes, Δ*secDF* Δ*rnd23*, accumulated precursors at a level similar to the Δ*secDF* mutant
267 (*Figure 9ab*). When analyzed for other secreted proteins, the Δ*secDF* mutant secreted
268 diminished amounts of Geh and failed to secrete Coa, whose precursor is secreted via a
269 canonical signal peptide (Phonimdaeng et al., 1990), while the Δ*rnd2* and Δ*rnd3* variants
270 displayed wild-type phenotypes (*Figure 9ab*). Thus, SecDF, but not Rnd2 and Rnd3, contributes

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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,

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293 lysostaphin- and detergent-permeabilized staphylococci revealed SpA targeting to septal
294 membranes in wild-type (*S. aureus* RN4220) and in IPTG-induced *S. aureus* ANG499 (P_{spac}-
295 *ltaS*)(*Figure 10d*). In contrast, without IPTG inducer, *S. aureus* ANG499 mislocalized SpA
296 precursors to polar membranes (*Figure 10d*). Protein secretion and cell wall anchoring of SpA
297 were analyzed by immunoblotting in *S. aureus* cultures separated into culture supernatant (S)
298 and bacterial sediment (P, pellet) samples. These experiments revealed that LtaS depletion in *S.*
299 *aureus* ANG499 (P_{spac}-*ltaS*, -IPTG) diminished the abundance of cell wall anchored SpA without
300 affecting the secretion of Geh and Nuc (*Figure 10e*). Consistent with the immunoblotting
301 results, LtaS depletion diminished the overall surface distribution of SpA (*Figure 10f*), in
302 agreement with the hypothesis that cross-wall targeting via the YSIRK/GXXS signal peptide, but
303 not polar secretion, is responsible for efficient surface distribution of proteins in staphylococci
304 (Carlsson et al., 2006, DeDent et al., 2008). Together these data indicate that LtaS depletion
305 and a block in lipoteichoic acid synthesis abolished SpA precursor trafficking to septal
306 membranes without affecting its secretion at polar membranes.
307

308 ## Discussion

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

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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

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

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

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359 2012). Our observations corroborate these findings and suggest that not all features of the
360 YSIRK/GXXS motif are required for precursor targeting to septal membranes. We took
361 advantage of the $SpA_{ED/S18L}$ precursor and used affinity chromatography to purify crosslinked
362 proteins. Several crosslinked proteins were already known to be located in septal membranes
363 (PBP2, EzrA, LtaS), consistent with $SpA_{ED/S18L}$ precursor accumulation in this compartment.
364 Among the crosslinked proteins are two components of the secretion machinery, SecA and
365 SecDF, as well as LtaS, which catalyzes the synthesis of lipoteichoic acid in septal membranes
366 (Tsirigotaki et al., 2017, Percy and Gründling, 2014).

367 The subcellular localization of the Sec apparatus has been examined in streptococci,
368 enterococci and in *Bacillus subtilis*. A spiral pattern of Sec translocase has been reported for *B.*
369 *subtilis* (Campo et al., 2004). In *S. pyogenes*, contradictory results have been reported for
370 immunogold-labelling and electron microscopy experiments: SecA was localized to a single
371 microdomain and also found distributed throughout the plasma membrane (Carlsson et al.,
372 2006, Rosch and Caparon, 2004). In *S. pneumoniae*, SecA localization changed during cell cycle
373 progression. In early divisional cells, SecA was predominantly localized to septal membranes,
374 whereas during later stages of division SecA was hemispherically distributed within the region
375 between septa and at the future equators of dividing cells (Tsui et al., 2011). *Streptococcus*
376 *agalactiae* SecA was localized to septal membranes, whereas SecA was detected as a single
377 microdomain in *Streptococcus mutans* and *Enterococcus faecalis* (Brega et al., 2013, Hu et al.,
378 2008, Kline et al., 2009). We show here that *S. aureus* SecA is localized to the plasma
379 membrane and is not spatially restricted to septal membranes or microdomains. This

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

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

397

398 Materials and methods

399 Bacterial strains and growth conditions

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

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402 selection in *E. coli*. Chloramphenicol was used for selection of pOS1 derivatives (10 μg/ml) and
403 pCL55 derivatives (5 μg/ml) in *S. aureus* (Lee et al., 1991). Erythromycin (Erm 10 μg/ml) was
404 used for selection of *ermB* marked *bursa aurealis* transposon mutants in *S. aureus* WY110 (Δ*spa*
405 Δ*sbi*) and 10 μg/ml Erm plus 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was used for
406 pMutin–HA-5'secA selection in *S. aureus*. Protein expression from P*spac* promoter was induced
407 with 1 mM IPTG. Anydrotetracycline (ATc, 200 ng/ml) was used to induce expression from the
408 tetracycline-inducible promoter in pCL55-P_{tet} constructs.
409

410 **Plasmids and strains**

411 To avoid mutations in the *spa* gene, all cloning procedures were performed at 30°C. All the
412 pOS1-derivative and pCL55-derivative plasmids were constructed in *E. coli* DC10B (Monk et al.,
413 2012) and transformed to *S. aureus* strains by electroporation (Schneewind and Missiakas,
414 2014). All the plasmids or strains constructed were confirmed by sequencing (*Table S2*). Primers
415 used in this study are listed in *Table S3*. To avoid cross reaction in SpA immunoblot and
416 purification, *S. aureus* WY110 (Δ*spa* Δ*sbi)* was generated by transducing *the sbi::ermB* allele
417 from the Phoenix library (Bae et al., 2004) into *S. aureus* SEJ1, i.e. *S. aureus* RN4220 carrying
418 Δ*spa.* Phage transduction was performed as described previously (Schneewind and Missiakas,
419 2014). To construct $pSpA_{ED}$, primers 10 and 69 were used to amplify the *spa* promoter and
420 spa_{ED} coding sequence encoding IgBDs E and D (-40 bp upstream of the transcription start site
421 of *spa* to 459 bp of *spa* coding sequence) from chromosomal DNA of *S. aureus* RN4220. The PCR
422 product was digested with EcoRI and BamHI, and ligated with plasmid pOS1 (Schneewind et al.,
423 1993). To generate mutations and deletions within SpA signal peptide sequence, quick-change

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424 mutagenesis was performed as follows: primers pairs (*Table S3*) that contain desired mutation
425 or deletion were used to PCR amplify pSpA$_{ED}$. The PCR products were digested with DpnI and
426 transformed to *E. coli* DC10B. Plasmid variants confirmed by DNA sequencing were transformed
427 to *S. aureus* WY110. To construct pCL55-SpA and its derivatives, primer pairs 175 and 177 were
428 used to PCR amplify the *spa* promoter and full-length *spa* coding sequence. PCR products were
429 digested with BamHI and KpnI and ligated into pCL55 cut with the same enzymes (Lee et al.,
430 1991). The resulting plasmid pCL55-SpA was used as template for PCR mutagenesis of its signal
431 peptide mutant derivatives via quick-change mutagenesis as described above. To construct
432 pCL55-SpA$_{SP\text{-}SasF}$, primer pairs 175 and 21 were used to amplify the promoter sequence of *spa*.
433 Primers 22 and 23 were used to amplify coding sequence for the signal peptide sequence of
434 *sasF.* Last, primers 24 and 177 were used amplify *spa* coding sequence for the E and D IgBDs. All
435 three DNA fragments were ligated via SOE (splicing by overlap extension) PCR, digested with
436 BamHI and KpnI, and then ligated into pCL55 cut with the same restriction enzymes. pCL55-
437 derivaties were transformed to *S. aureus* WY110 and integrated into the chromosome at the
438 *geh* locus (Lee et al., 1991). The integration was confirmed by PCR. To construct the *secA*
439 depletion strain *S. aureus* WY223 (P$_{spac}$-*secA*), primers 189 and 190 were used to amplify the
440 ribosome binding site and the first 656 bp of the *secA* gene. The PCR product was digested with
441 HindIII and KpnI and ligated with pMutin–HA (Bacillus Genetic Stock Center, Columbus, OH).
442 The resulting plasmid pMutin–HA-5'secA was transformed into RN4220 and integrated at the
443 *secA* locus in the chromosome. Clones were selected on TSA supplemented with 10 µg/ml
444 erythromycin and 1 mM IPTG. To construct pCL55-P$_{tet}$-*secA:sfGFP*, primers 180 and 181 were
445 used to amplify *secA* full-length coding sequence together with its ribosome binding site.

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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

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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,

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490 washed three times for 5 min with TBST [50 mM Tris(pH 7.5), 150 mM NaCl, 0.1% Tween 20],
491 incubated with secondary anti-rabbit IgG linked with HRP for 1 h, washed and developed using
492 enhanced chemiluminescence substrates. The intensity of immunoblot signals was analyzed
493 and measured with Image J software (Schneider et al., 2012). Statistical analysis was performed
494 using GraphPad Prism software. One-way ANOVA (Dunnett's multiple comparisons test) was
495 used to compare the mean for each variant with the mean for SpA_{ED} wild-type (*Figure 1c, 2b*).
496

497 **Pulse-labeling**

498 Staphylococcal cultures were grown to mid-log phase (OD_{600} 0.8) in TSB and bacteria
499 sedimented by centrifugation at 18,000 ×*g* for 5 min. Bacterial pellets were washed twice and
500 suspended in 1 ml minimal medium 4. ^{35}S-methionine/cysteine (100 µl =100 µCi Perkin Elmer)
501 was added to bacterial suspensions, vortexed and incubated for 60 seconds at 37°C. 250 µl was
502 removed and immediately mixed with 250 µl ice-cold 10% TCA to quench all metabolic activity
503 (time 0'). Chase solution (50 µl of 2 mg/ml methionine, 2 mg/ml cysteine and 10 mg/ml
504 casamino acids) was added to the remainder of bacterial suspension and incubated for 1, 5 and
505 20 min. At each time point, 250 µl bacterial suspension was removed and mixed with 250 µl ice-
506 cold 10% TCA. TCA precipitated cells were washed with acetone, dried and suspended in 1 ml
507 0.5 M Tris-HCl (pH 7.0) containing 20 µg/ml lysostaphin. After lysostaphin treatment at 37°C for
508 1 h, cell lysate was precipitated with 7% TCA, washed with acetone, dried, suspended in 50 µl
509 4% SDS, 0.5 M Tris-HCl (pH 7.5) and allowed to incubate for 30 min prior to boiling.
510 Subsequently, samples were incubated for 1 h with rabbit polyclonal anti-SpA_{KKAA} antibody (Kim
511 et al., 2010) that was 1:1000 diluted in 1 ml RIPA buffer (0.1% SDS, 0.5% deoxycholic acid, 1%

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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

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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

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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**

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

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

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

602 To localize intracellular SpA, 2 ml of mid-log phase staphylococcal cultures were
603 centrifuged at 18,000 ×*g* for 5 min and supernatant removed. Bacteria were washed once in 2
604 ml PBS and suspended in 1 ml PBS, 0.5 mg/ml trypsin (Sigma). After incubation at 37°C for 1
605 hour, staphylococcal cells were washed twice with PBS and fixed with fixation solution. The
606 cells were fixed for 15 min at room temperature and 30 min on ice, washed three times with
607 PBS and suspended in 1 ml GTE buffer [50 mM glucose, 20 mM Tris-HCl (pH 7.5), 10 mM EDTA].
608 Lysostaphin (10 µg/ml) was added and 50 µl cell suspensions were immediately applied to poly-
609 L-lysine coated 8-well glass slides and incubated for 1 min. Non-adherent cells were removed
610 and PBS, 0.2% Triton X-100 was applied to samples for 10 seconds. Excessive liquid was
611 aspirated and slides were air-dried. Dried slides were immediately dipped in methanol at -20°C
612 for 5 min, and in acetone at -20°C for 30 s and then allowed to dry completely. Afterwards, the
613 cells on the slides were re-hydrated with PBS for 5 min, blocked with 3% BSA, stained with the
614 membrane dye Nile red (Sigma) and rabbit antibodies specific for SpA followed by Alexa-Fluor-
615 488 conjugated goat-anti-rabbit-IgG and analyzed by fluorescence microscopy as described
616 above.

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

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621 cells were applied to poly-L-lysine coated glass slides, SlowFade® Gold reagent added,
622 coverslips sealed and samples imaged by fluorescence microscopy.
623 All the images were analyzed in Image J software (Schneider et al., 2012). To quantify
624 the frequency of SpA cross-wall localization at 20 min regeneration after trypsin digestion,
625 numbers of diplococci and numbers of cross-wall localized SpA were counted manually using
626 the cell counter tool in Image J. Diplococci were defined as two daughter cells that had divided
627 and formed a cross-wall but had not yet separated. Cross-wall localized SpA signals were
628 defined as lines at the cross-wall of diplococci. Diplococci were counted in vancomycin stained
629 images and cross-wall localized SpA was counted in merged images. The frequency was
630 determined by dividing cross-wall localized SpA by the number of diplococci. An example of the
631 counting method is displayed in the *Source file to Figure 2e*. At least two random images were
632 acquired per sample for each experiment. Three or more independent experiments were
633 performed and data from more than 200 diplococci were analyzed for statistically significant
634 differences using one-way ANOVA with Dunnett's multiple comparison test comparing
635 staphylococci expressing wild-type and *spa* variants (Figure 2e). In *Figure 6c* and *Figure 10c* the
636 Tukey's multiple comparison test was used to analyze differences between multiple groups.
637

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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 $\times$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

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664 location of wild-type SpA_{ED} precursor and secreted product of the $SpA_{ED/\Delta IA}$, $SpA_{ED/S18L}$, and
665 $SpA_{ED/R10A}$ variants. Immunoblotting with αSrtA and αL6 was used to establish fractionation and
666 loading controls. (b) Quantification of immunoblot signal intensity in (a) using Image J.
667 Precursor abundance (%) the bacterial cytoplasm (C) and membrane (M) was quantified from
668 triplicate experiments as the intensity of immunoblotting signals divided by the sum signals in
669 all four fractions (C+M+W+S)×100. Statistical significance was analyzed with one-way ANOVA
670 comparing each variant with wild-type and p values were recorded: for [C/(C+M+W+S)]×100,
671 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,
672 WT vs. ΔIA, $p=0.0056$; WT vs. S18L, $p<0.0001$; WT vs. R10A, $p=0.0405$. **** denotes $p<0.0001$,
673 ** denotes $p<0.01$, * denotes $p<0.05$. (c) *S. aureus* cultures were pulse-labeled for 60 seconds
674 with $[^{35}S]$methionine and labeling quenched by adding an excess of non-radioactive methionine
675 (chase). At timed intervals during the pulse (0') or 1 (1'), 5 (5'), and 20 (20') minutes after the
676 pulse (chase), culture aliquots were precipitated with trichloroacetic acid (TCA), lysostaphin-
677 treated, immunoprecipitated with αSpA and analyzed by autoradiography. (d) *S. aureus* WY110
678 (Δ*spa* Δ*sbi*) harboring chromosomal pCL55-insertions of wild-type *spa* (SpA), $spa_{\Delta IA}$ ($SpA_{\Delta IA}$)
679 spa_{S18L} (SpA_{S18L}), spa_{R10A} (SpA_{R10A}) $spa_{SP\text{-}SasF}$ ($SpA_{SP\text{-}SasF}$) or pCL55 alone (Vector) were treated
680 with trypsin to remove SpA. Bacteria were incubated for 20 min to allow for secretion and cell
681 wall deposition of newly synthesized SpA. Samples were incubated with BODIPY-FL vancomycin
682 (Vancomycin) (green) to stain the bacterial cell wall and with SpA-specific monoclonal antibody
683 and Alexa fluor 647-labeled secondary IgG (red) to reveal SpA. (e) SpA-positive staphylococci in
684 images derived from samples in (d) were analyzed for SpA deposition at the cross wall of
685 diplococci (n=200). Data from three independent experiments were used to derive the mean

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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 $SpA_{ED/SP\text{-}SasF}$
702 . 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

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707 of 90°C samples to validate crosslinking of $SpA_{ED/S18L}$ staphylococcal proteins (b) and to SecA (c).
708 See Table S1 for a summary of proteins crosslinked to $SpA_{ED/S18L}$.
709
710 **Figure 5.** Depletion of SecA in *S. aureus*. (a) Diagram of the *secA* gene locus in *S. aureus* RN4220
711 (wild-type parent, WT) and its P_{spac}-*secA* variant. (b) Bacteria from overnight cultures of wild-
712 type *S. aureus* and *S. aureus* P_{spac}-*secA* grown in TSB with 1 mM IPTG were washed and
713 suspended in fresh TSB with or without 1 mM IPTG. Subsequent growth was monitored as
714 increased absorbance at 600 nm (A_{600}). After three hours, cultures were diluted 1:100 into fresh
715 TSB with or without 1 mM IPTG and incubated for additional growth measurements. (c) *S.*
716 *aureus* P_{spac}-*secA* was streaked on tryptic soy agar with or without 1 mM IPTG supplement and
717 incubated for 16 hours at 37°C for growth. (d) Culture samples retrieved after 3 and 6 hours in
718 (b) were analyzed by immunoblotting with antibodies against SecA (αSecA).
719
720 **Figure 6.** SecA depletion diminishes septal secretion of SpA in *S. aureus*. (a) SpA precursor
721 processing of [^{35}S]methionine pulse-labeled *S. aureus* RN4220 (WT) or *S. aureus* P_{spac}-*secA*
722 grown in the presence or absence of 1 mM IPTG. Bacteria were pulse-labeled for 60 sec with
723 radioactive methionine and then incubated with an excess of non-radioactive methionine.
724 During the pulse (0') or 1 (1'), 5 (5') and 20 (20') min after the addition of excess unlabeled
725 methionine, culture aliquots were withdrawn, precipitated with TCA, digested with lysostaphin,
726 and subjected to SDS-PAGE and autoradiography of immunoprecipitated SpA. Wild-type *S.*
727 *aureus* (WT) and its P_{spac}-*secA* variant were grown for 3 (b) and 6 hours (d) in the presence or
728 absence of 1 mM IPTG (see Figure 5) and treated with trypsin to remove SpA from the bacterial

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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

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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 *secA* 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}-*secA*
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

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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-

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

810

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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)

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	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)

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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)

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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)

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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)

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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)

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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)

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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

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	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	$pCL55\text{-}SpA_{SP\text{-}SasF}$

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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.

1016

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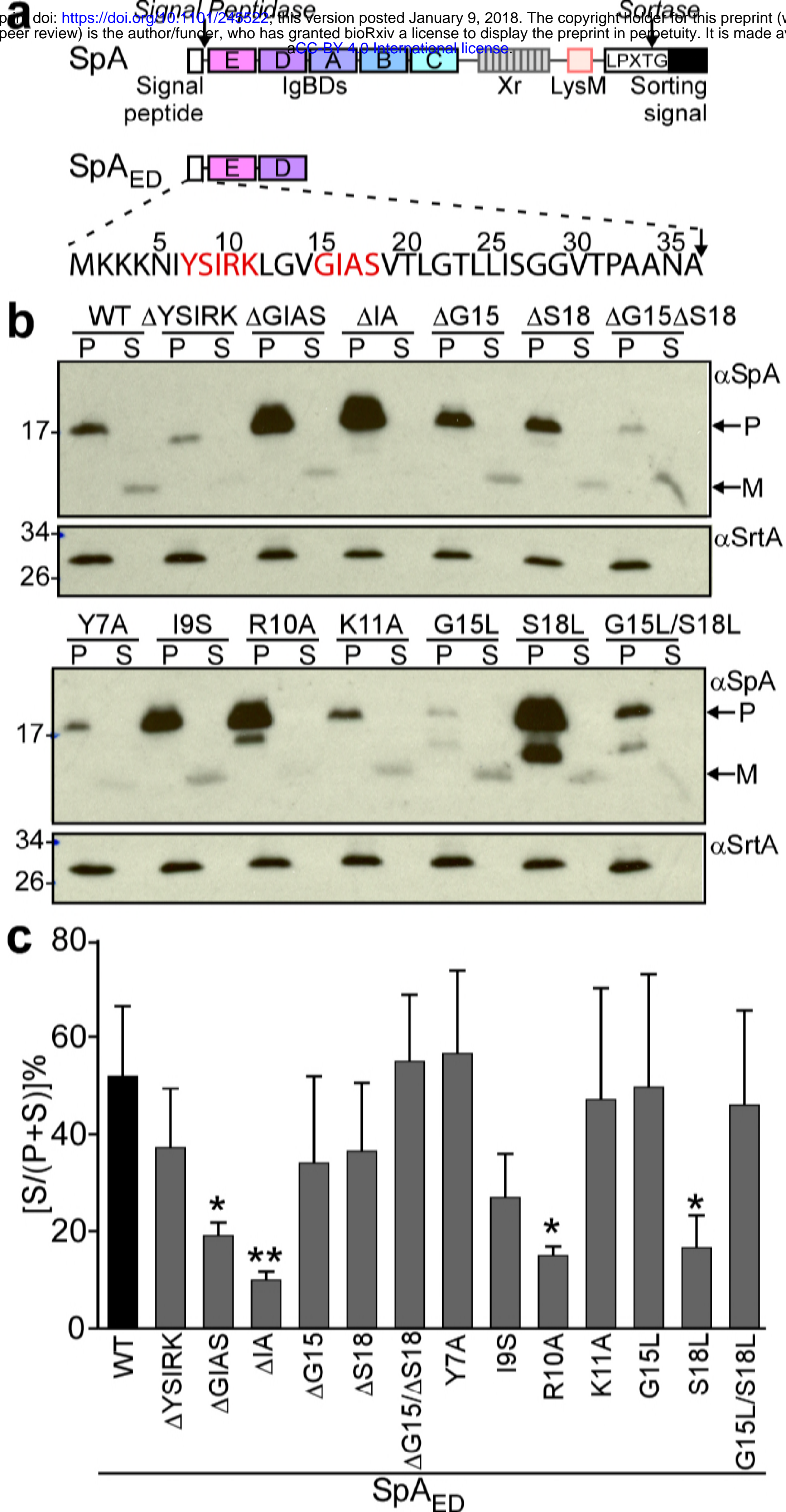

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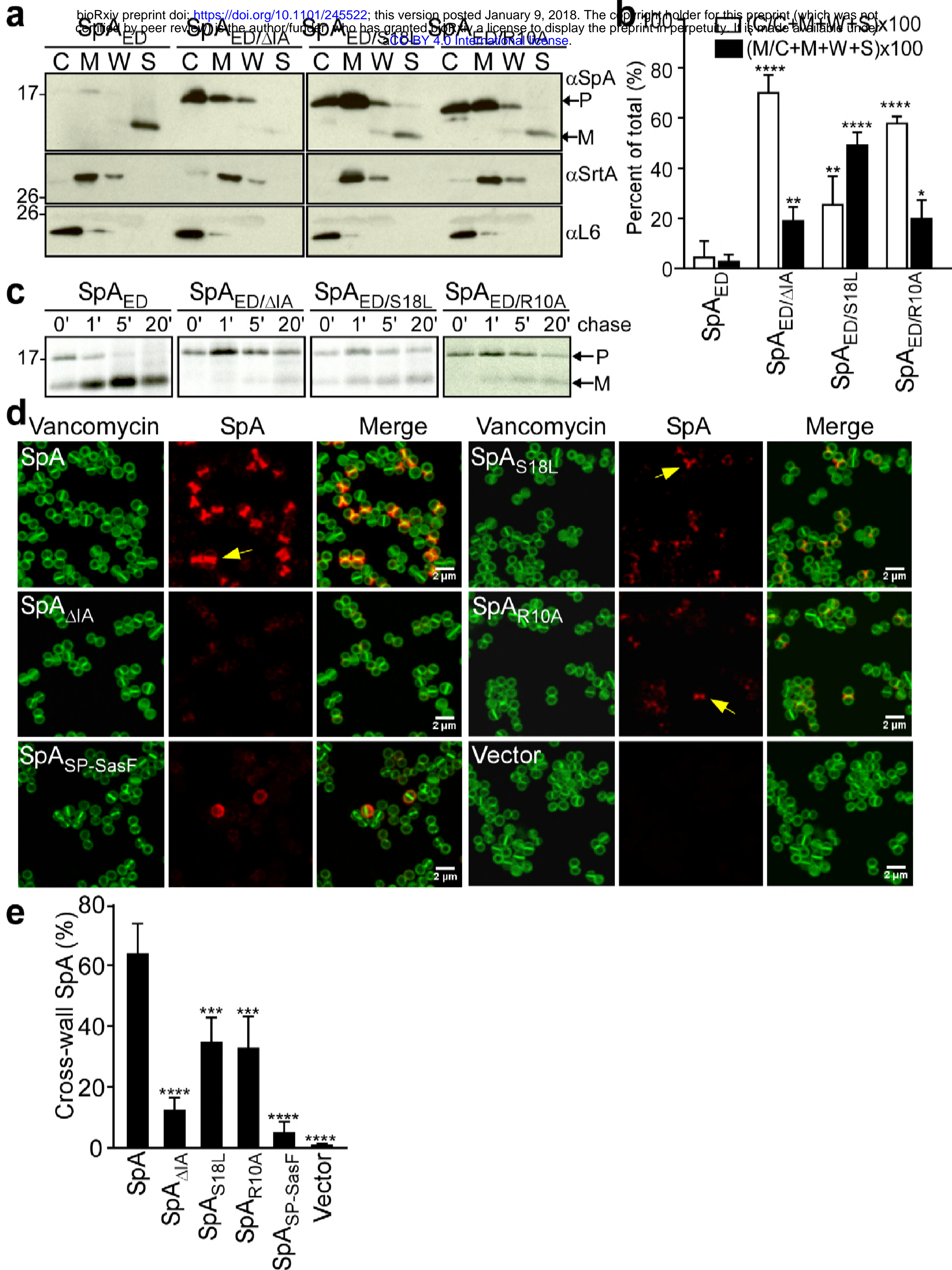

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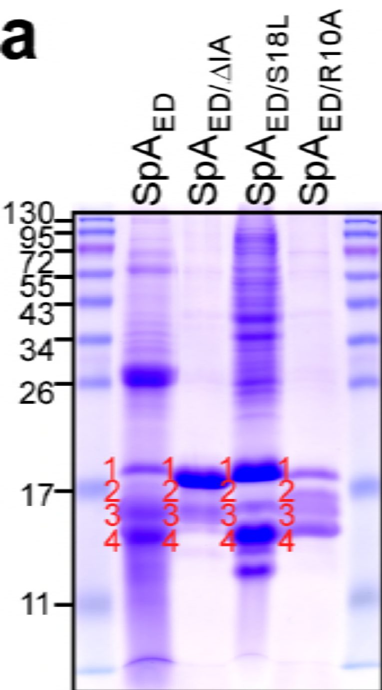

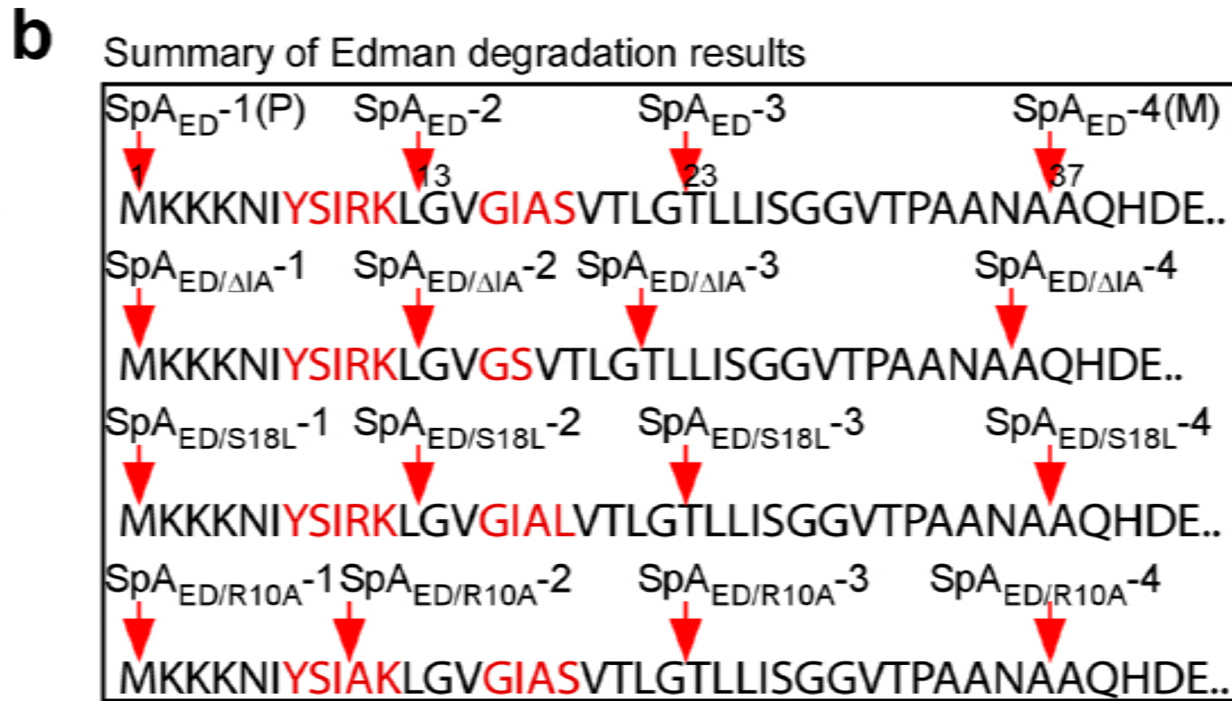

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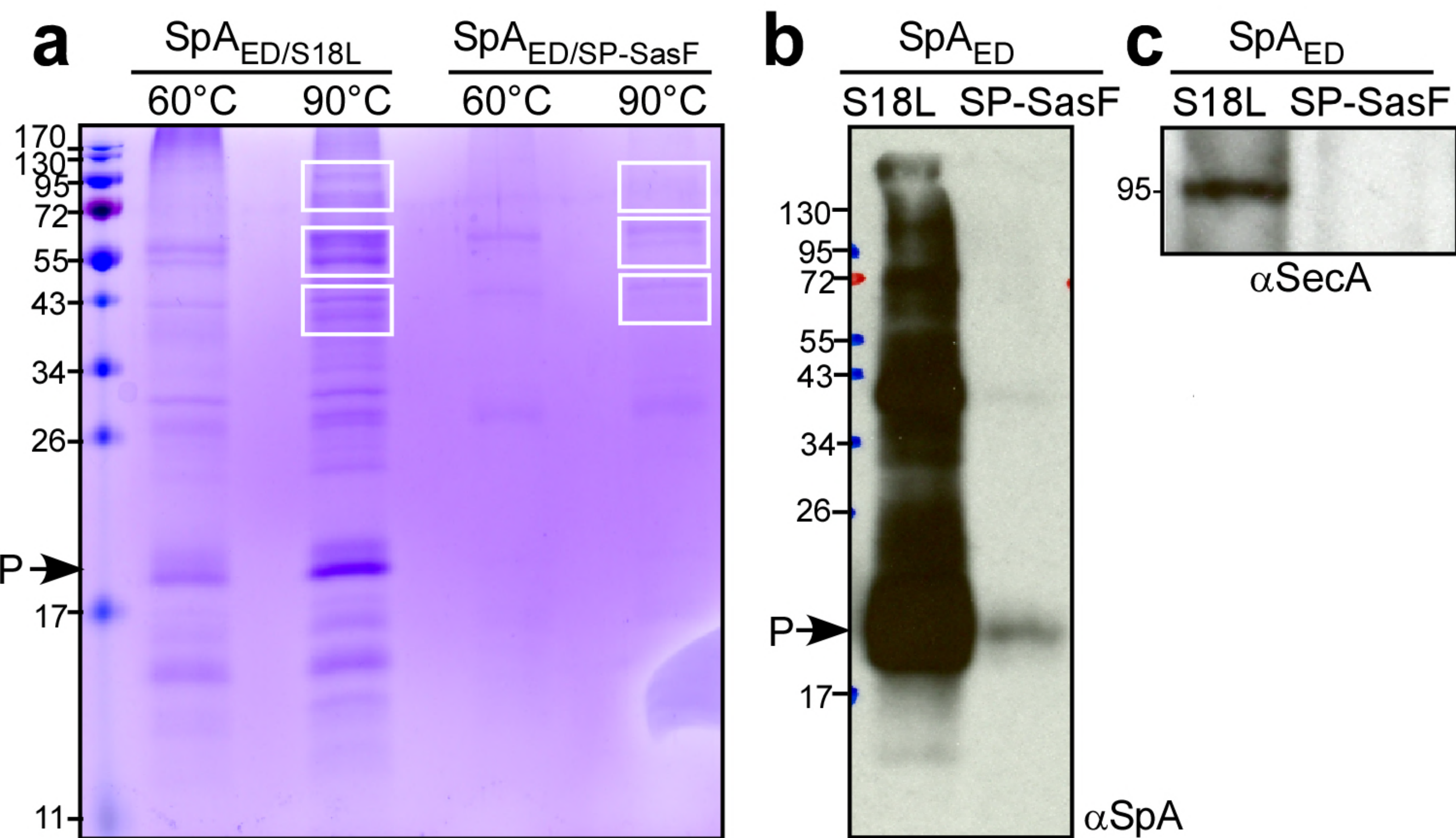

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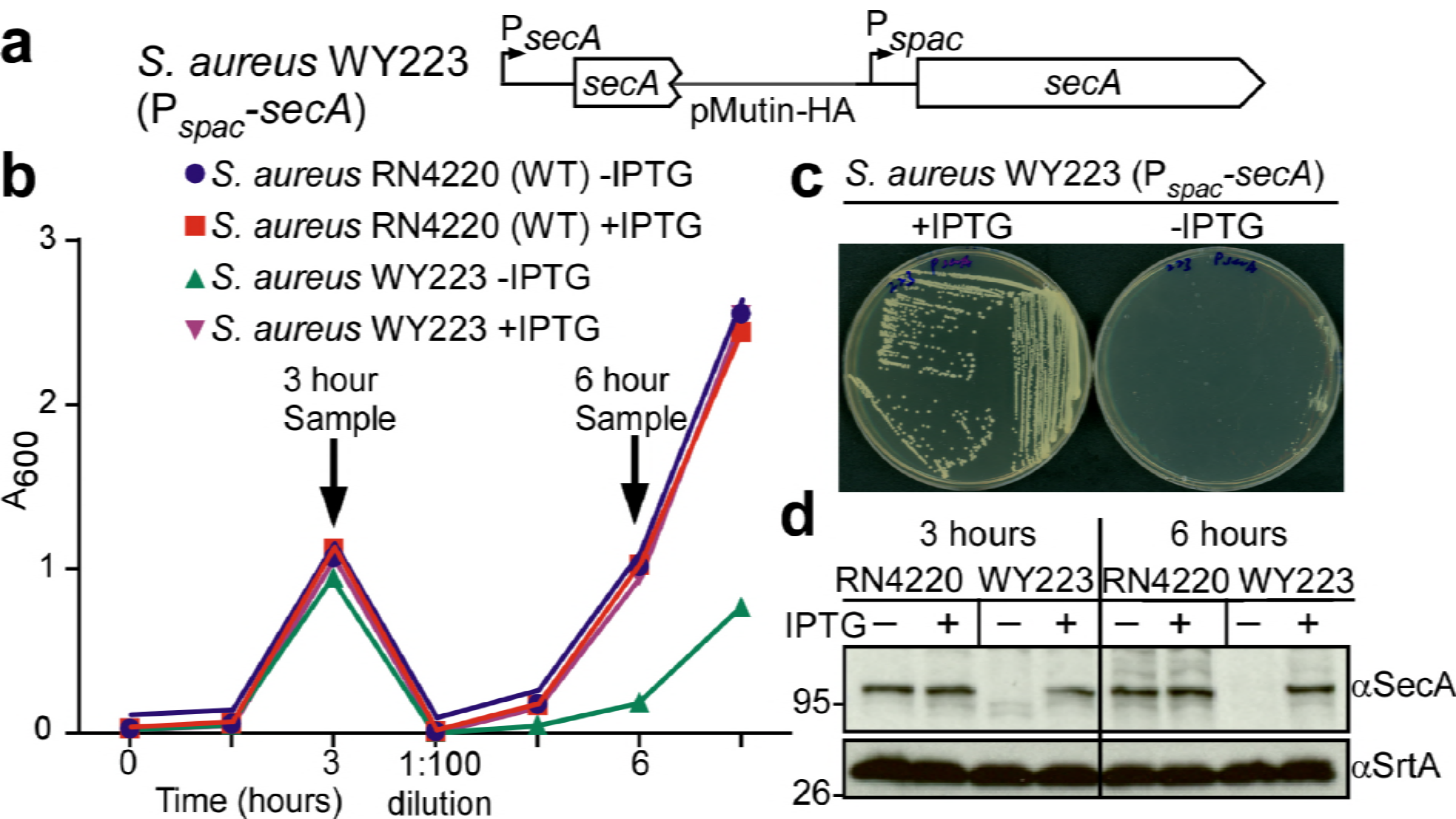

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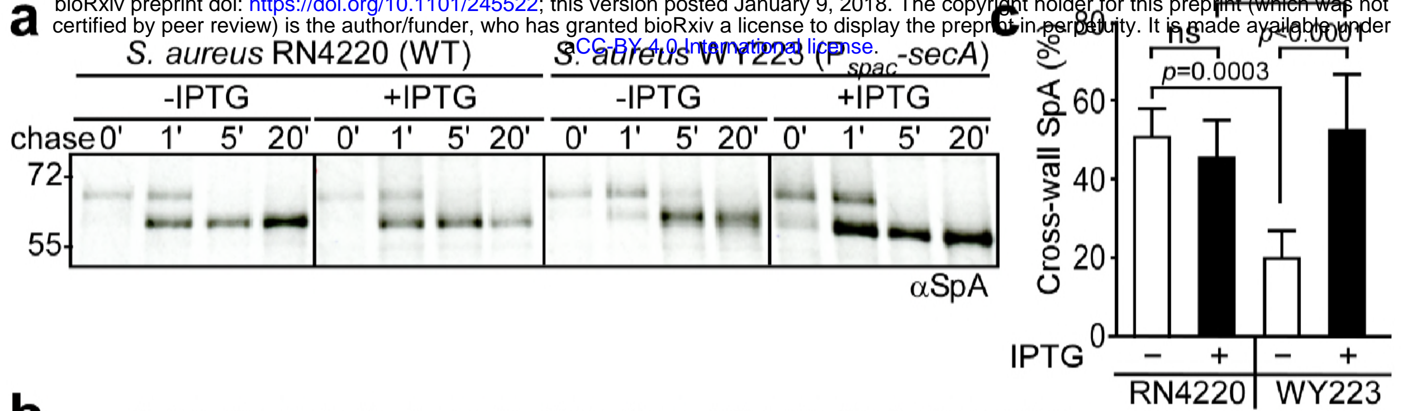

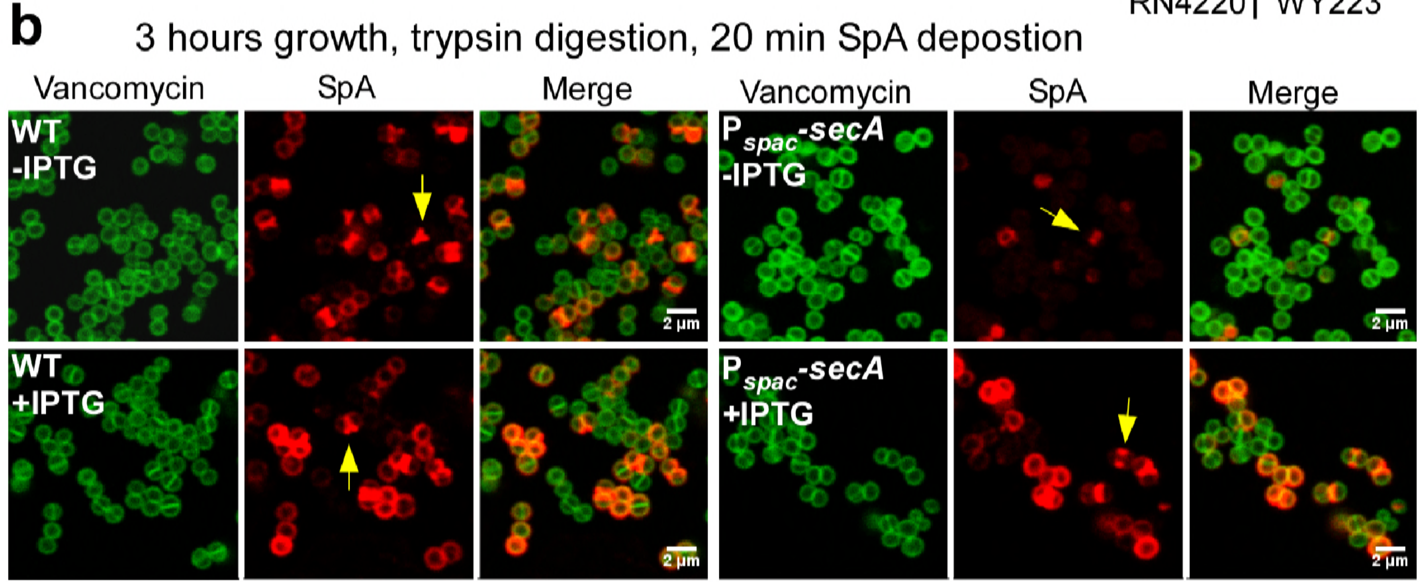

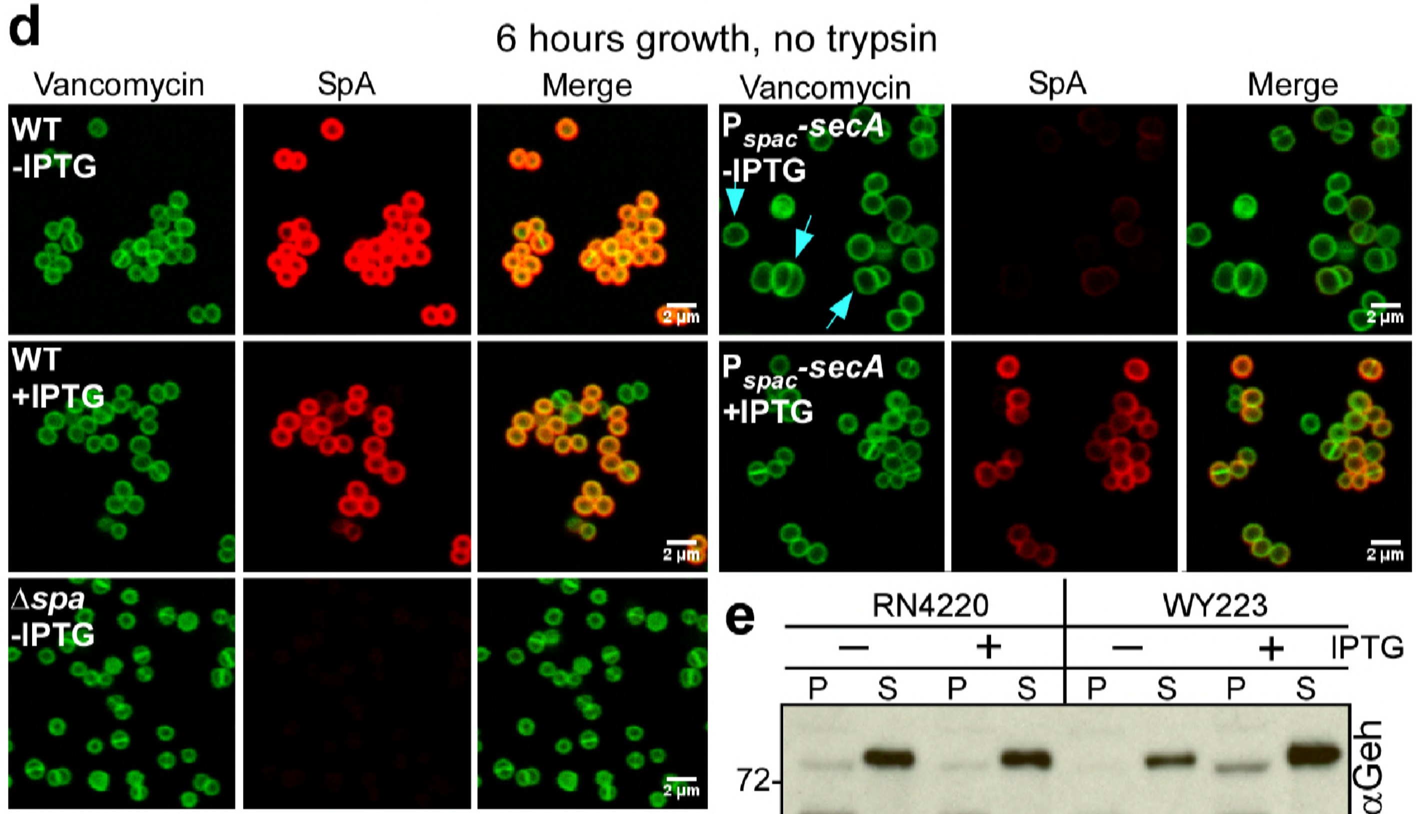

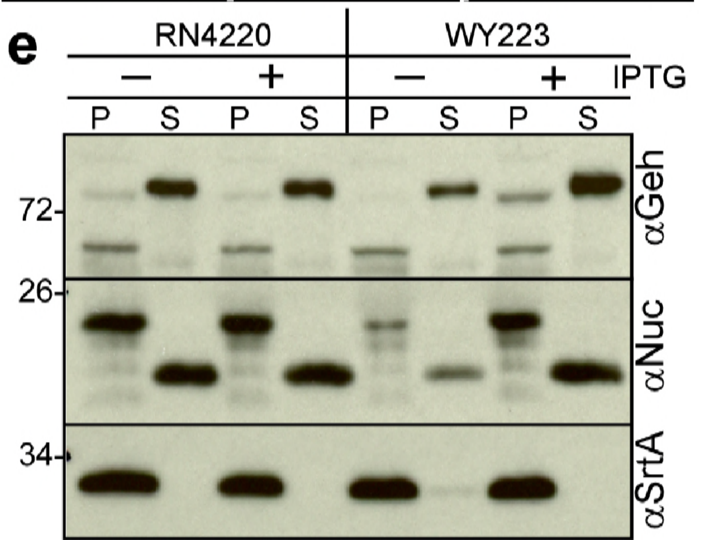

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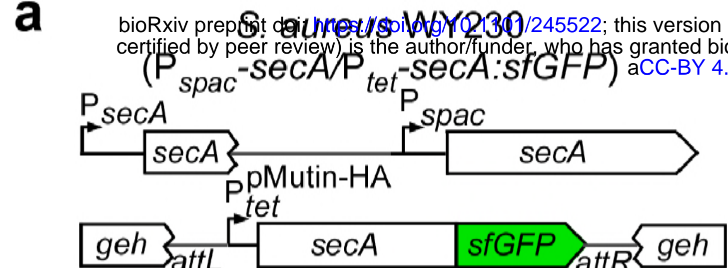
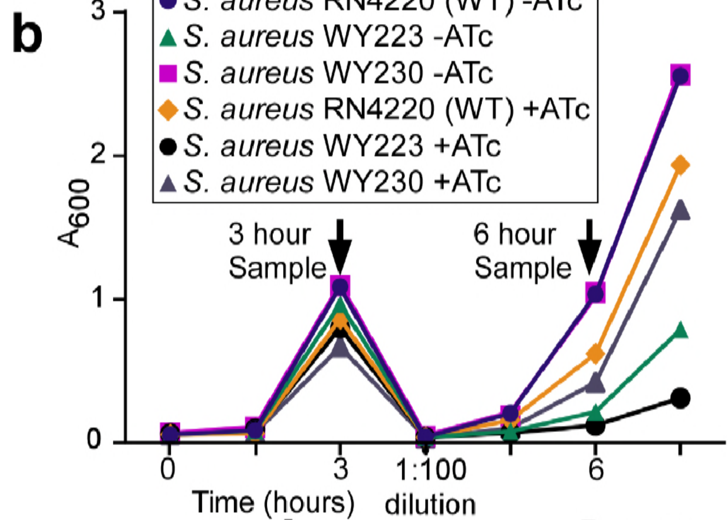
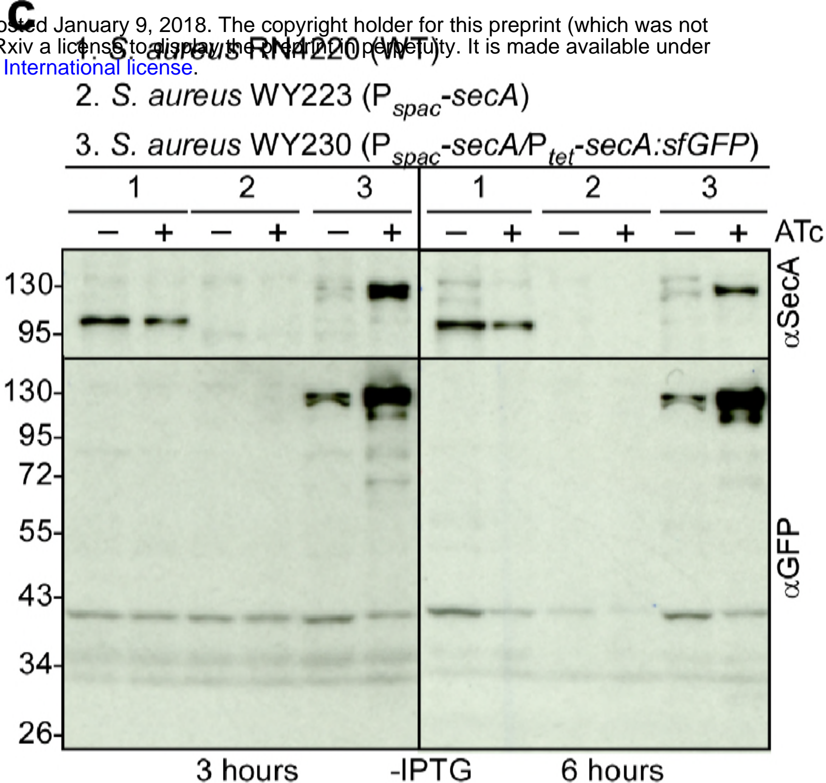
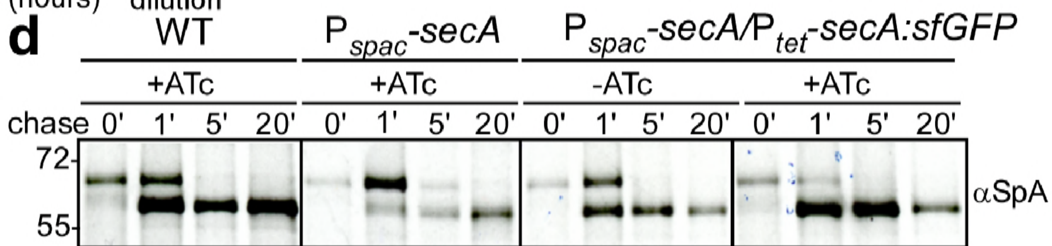
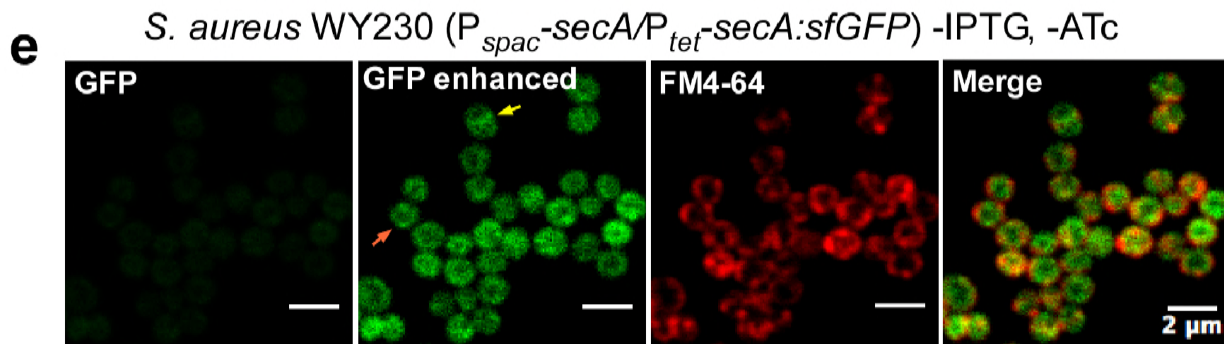




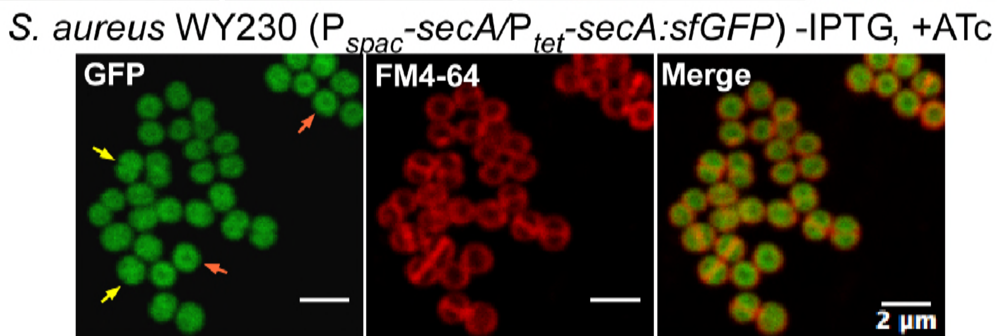




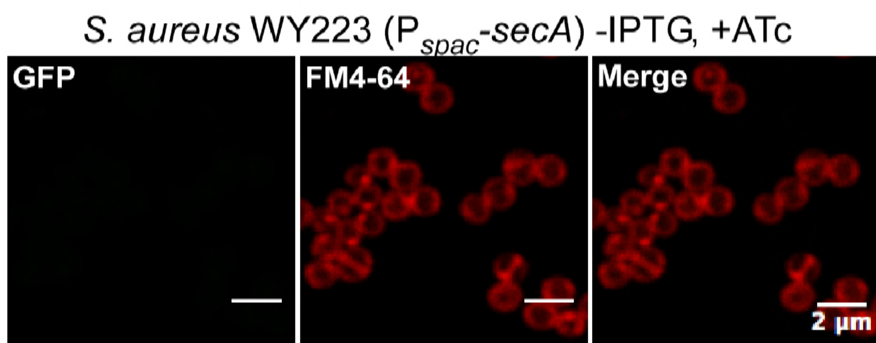
S. aureus WY230 (Pspac-secA/Ptet-secA:sfGFP) -IPTG, -ATc



S. aureus WY230 (Pspac-secA/Ptet-secA:sfGFP) -IPTG, +ATc



S. aureus WY223 (Pspac-secA) -IPTG, +ATc



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a BF SpA Nile Red Merge

+SecA

-SecA

Δ*spa*

+Lysostaphin

+SecA, -Lysostaphin

b BF SpA Nile Red Merge

#1 +SecA

#2 +SecA

#3 +SecA

+Lysostaphin

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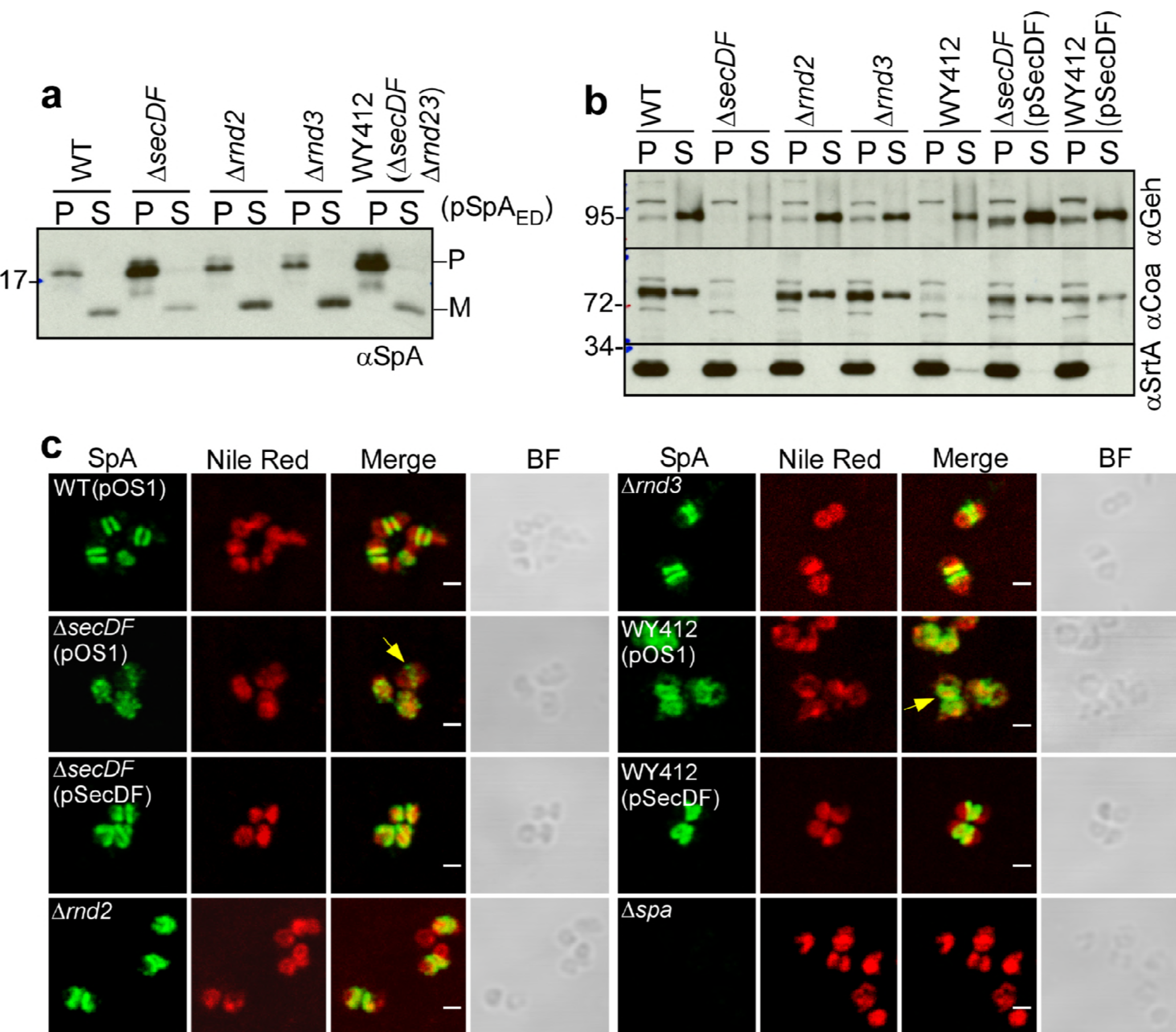

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