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Published in:
Nature Microbiology

DOI:
[10.1038/nmicrobiol.2016.183](https://doi.org/10.1038/nmicrobiol.2016.183)

Publication date:
2016

Document Version
Peer reviewed version

[Link to publication in Discovery Research Portal](#)

Citation for published version (APA):

Cao, Z., Kneuper, H., Casabona, M. G., Chalmers, J. D., & Palmer, T. (2016). The type VII secretion system of *Staphylococcus aureus* secretes a nuclease toxin that targets competitor bacteria. *Nature Microbiology*, 2, 1-11. [16183]. <https://doi.org/10.1038/nmicrobiol.2016.183>

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1 **The Ess/Type VII secretion system of *Staphylococcus***
2 ***aureus* secretes a nuclease toxin that targets competitor**
3 **bacteria**

4

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11

12

13 **Summary**

14 The type VII protein secretion system (T7SS) plays a critical role in the virulence of human
15 pathogens including *Mycobacterium tuberculosis* and *Staphylococcus aureus*. Here we report
16 that the *S. aureus* T7SS secretes a large nuclease toxin, EsaD. The toxic activity of EsaD is
17 neutralised during its biosynthesis through complex formation with an antitoxin, EsaG, which
18 binds to its C-terminal nuclease domain. The secretion of EsaD is dependent upon a further
19 accessory protein, EsaE, that does not interact with the nuclease domain, but instead binds
20 to the EsaD N-terminal region. EsaE has a dual cytoplasmic/membrane localization and
21 membrane-bound EsaE interacts with the T7SS secretion ATPase, EssC, implicating EsaE in
22 targeting the EsaDG complex to the secretion apparatus. EsaD and EsaE are co-secreted
23 whereas EsaG is found only in the cytoplasm and may be stripped off during the secretion
24 process. Strain variants of *S. aureus* that lack *esaD* encode at least two copies of EsaG-like
25 proteins most likely to protect themselves from the toxic activity of EsaD secreted by *esaD*⁺
26 strains. In support of this, a strain overproducing EsaD elicits significant growth inhibition
27 against a sensitive strain. We conclude that T7SSs may play unexpected and key roles in
28 bacterial competitiveness.

29

30 Protein secretion systems are used by bacteria to interact with and manipulate their
31 environments, and play critical roles in the secretion of virulence factors. Gram-negative
32 bacteria produce numerous secretion systems that transport substrates across the cell
33 envelope¹. Many Gram-positive bacteria also produce a specialised protein secretion
34 machinery termed the Type VII secretion system (T7SS). T7SSs are found in representatives
35 of the Actinobacteria and Firmicutes phyla. The system was first described in the
36 Actinobacterial pathogens *Mycobacterium tuberculosis* and *M. bovis* where T7SS ESX-1 was
37 shown to be essential for virulence and to secrete two small proteins ESAT-6 and CFP-10,
38 subsequently renamed EsxA and EsxB²⁻⁴. In addition to secreting EsxA/B proteins,
39 Mycobacterial T7SSs can also secrete much larger proteins of the PE/PPE family⁵, which are
40 highly abundant in the genomes of some species⁶.

41 A distantly related T7SS, termed T7b⁷, is also found in Firmicutes such as *Bacillus subtilis*^{8,9}
42 and *Staphylococcus aureus*¹⁰. T7SSs share two common types of components: a membrane-
43 bound hexameric ATPase of the FtsK/SpoIIIE protein family^{11,12} and at least one EsxA/EsxB-
44 related protein¹¹. EsxA and EsxB are members of the WXG100 superfamily that form dimeric
45 helical hairpins^{13,14}, and in Firmicutes EsxA is exported as a folded homo-dimer^{14,15}. The *S.*
46 *aureus* Ess system comprises six core components^{10,16} (Figs 1A; S1). In addition to EssC,
47 three further membrane proteins EsaA, EssA, EssB are essential for T7 secretion activity^{16,17}
48 along with the secreted protein EsxA¹⁰ and the predicted cytoplasmic protein EsaB¹⁶. All
49 except one strain of *S. aureus* examined to date encode the six core T7 components but there
50 is strain variability in the repertoire of T7 substrate proteins¹⁸. Studies using strains with the
51 NCTC8325 T7S gene cluster organisation, including Newman, USA300 and RN6390 have
52 identified EsxB, EsxC and EsxD as secreted substrates^{10,16,19,20}. These three proteins are
53 small (~100-130 aa), and their precise functions remain to be elucidated.
54 The T7SS has previously been shown to contribute to virulence in mouse infection
55 models^{10,16,21} and to facilitate release of intracellular *S. aureus* from epithelial cells²². Here we
56 identify a further function for the *S. aureus* T7SS in bacterial competition. We show that EsaD
57 is a T7 nuclease substrate that interacts with two Ess accessory proteins, EsaG, an antitoxin,
58 and EsaE, a putative chaperone, during its biosynthesis. Strains of *S. aureus* that do not
59 encode the EsaD substrate harbour *esaG* homologues, most likely to protect themselves from
60 killing by EsaD-producing strains. In support of this we demonstrate EsaD-dependent growth
61 inhibition of *S. aureus*. Our findings confirm that the Gram-positive T7SS has anti-bacterial
62 activity in addition to anti-eukaryotic function.

63

64 **Results**

65 **EsaD is not required for T7SS activity.** EsaD is encoded within the T7SS gene cluster (Fig
66 1a) and has been reported to be a membrane-bound T7SS accessory factor²³, however its
67 absence from some *S. aureus* strains indicates it is unlikely to be a critical component of the
68 secretion machinery¹⁸. We constructed an in-frame *esaD* deletion and asked whether it was

69 required for secretion of the core component EsxA and the substrate protein EsxC. Fig 1b
70 shows that EsxA was still secreted by the *esaD* strain, but very little EsxC was detected. To
71 circumvent this we overproduced EsxC from a plasmid (Fig 1c), and in this case could clearly
72 detect EsxC in culture supernatants of the *esaD* strain. We conclude that EsaD is not essential
73 for T7-dependent secretion.

74 **EsaD is a predicted nuclease that is secreted by the T7SS.** EsaD encoded by strain
75 NCTC8325 is predicted to be a 614 residue protein, and sequence analysis suggests that the
76 C-terminal ~170 aa comprise a nuclease domain (Fig S2). In accord with EsaD having toxic
77 activity, we were unable to clone *esaD* unless we introduced a H528A codon substitution at
78 the predicted nuclease active site. When HA-tagged EsaD(H528A) was produced in either *S.*
79 *aureus* RN6390 or COL strains, tagged protein was detected in the supernatant (Fig 1d, e).
80 The lack of EsaD in supernatants of the cognate *essC* strains strongly suggests that EsaD is
81 a T7SS secreted substrate.

82 EsaD has previously been reported as a membrane protein²³. However, topology prediction
83 programmes (e.g. TMHMM) do not predict transmembrane regions. To explore the location of
84 cellular EsaD we fractionated cells producing EsaD(H528A)-HA and Fig 1f shows the tagged
85 protein (migrating as a double band) was clearly detected only in the cytoplasmic fraction. We
86 conclude EsaD is not a membrane protein.

87 **EsaD has toxic activity that is neutralised by EsaG.** To confirm that EsaD is a toxin, it was
88 essential to clone the wild-type gene. We could readily clone *esaD* if the downstream gene,
89 *esaG*, was included, but not if this sequence was omitted. We were eventually able to obtain
90 a clone of *esaD* in pT7.5²⁴. Expression of genes from this vector is under control of the T7
91 promoter which is not recognised by *E. coli* RNA polymerase. Even so, the clone we obtained,
92 which gave very small colonies, harboured a V584Y substitution. We reasoned that this might
93 serve to lower the stability/toxicity of EsaD. When this construct was introduced into *E. coli*
94 BL21(DE3) (that encodes an inducible copy of the phage T7 RNA polymerase), growth ceased
95 when the inducer, IPTG was added (Fig 2a) but cells continued to grow if *esaG* was co-

96 expressed with wild type *esaD*. We conclude that EsaD has toxic activity that is modulated by
97 EsaG.

98 Examination of *E. coli* cells by microscopy showed that production of EsaD(V584Y) resulted
99 in cell elongation (Fig S3a,b), a hallmark of the SOS response induced by DNA damage^{25,26},
100 consistent with EsaD exhibiting DNase activity. We confirmed EsaD-induced DNA damage
101 using TUNEL, which labels the ends of fragmented DNA^{27,28}. Fluorescence microscopy
102 showed TUNEL staining in a subset of cells producing EsaD(V584Y) (Fig S3c) that could also
103 be detected by flow cytometry (red dots in FACS plot in Fig S3d). Production of EsaD was
104 also associated with an increase in side scatter, consistent with changes in cellular
105 morphology seen by microscopy. We conclude that EsaD results in DNA damage when
106 produced in the cytoplasm of *E. coli*.

107 **EsaG interacts with the nuclease domain of EsaD.** The presence of *esaG* counteracts the
108 toxic activity of EsaD suggesting that *esaG* is an antitoxic gene. Antitoxins may be proteins or
109 RNA²⁹. Inspection of *esaG* indicates that it is a probable protein-coding gene, producing a
110 protein of the uncharacterised DUF600 family. To investigate whether EsaG interacts with
111 EsaD we co-produced EsaD(H528A)-His with EsaG-HA in *S. aureus* and purified tagged EsaD
112 from cell lysates. Fig 2c shows that EsaG co-purifies with EsaD indicating the proteins form a
113 complex.

114 EsaD-EsaG interaction was also confirmed by bacterial two hybrid assay (Fig 2b). EsaG (163
115 aa) is significantly smaller than EsaD, suggesting it may interact with only part of EsaD. We
116 genetically separated EsaD into predicted nuclease domain (EsaD₄₂₁₋₆₁₄, harbouring the
117 H528A codon substitution) and N-terminal region (EsaD₁₋₄₂₀). Fig 2b shows that EsaG
118 interacts specifically with the predicted nuclease domain. This was confirmed biochemically
119 by co-purification of EsaG-HA with His-tagged EsaD₄₂₁₋₆₁₄ (Fig 2d). We conclude that EsaG is
120 a proteinaceous antitoxin that blocks EsaD activity by direct interaction.

121 To investigate directly whether EsaD has DNase activity, we overproduced His-tagged wild
122 type or H528A variants of the nuclease domain in *E. coli* in the presence of EsaG and purified
123 His-tagged EsaD in the presence of 8M urea to unfold the protein and detach bound EsaG

124 (Fig 2e). After refolding and eluting from the Ni-resin, the EsaD nuclease domains were
125 incubated with plasmid DNA in the presence of Mg²⁺ or Zn²⁺ ions. Fig 2f shows that wild type
126 EsaD specifically degraded plasmid DNA in the presence of Mg²⁺, and could also degrade
127 genomic and linear DNA (Fig S5). The H528A variant showed some DNase activity, but
128 appeared to be much less potent than EsaD, as expected. We conclude that EsaD is a Mg²⁺-
129 dependent DNase.

130 Fractionation of cells overproducing tagged EsaG shows that it is found exclusively in the
131 cytoplasm (Fig S6). However, as the T7SS can export protein complexes¹⁵, we tested whether
132 it could be co-secreted if overproduced with EsaD. Fig S7 shows that in the presence of
133 secreted EsaD(H528A), EsaG localised only to the cell fraction and does not appear to be co-
134 secreted with its partner protein. We then asked whether EsaG was required for secretion of
135 EsaD. We were not able to delete *esaG* from *S. aureus* unless we also deleted *esaD*,
136 consistent with its antitoxic role. Production of HA-tagged EsaD(H528A) in the *esaDG* mutant
137 strain resulted in no detectable EsaD in the supernatant and very little in the cells (Fig S8).
138 We conclude that EsaG is required for the stability/secretion of EsaD. EsaG was not, however,
139 required for the secretion of either EsxA or EsxC (Fig S9) and is therefore not a general T7S
140 accessory factor.

141 **EsaE also interacts with EsaD and is required for its secretion or stability.** Studies of
142 Mycobacterial T7SS have revealed that PE/PPE substrate proteins interact with specific
143 chaperones that facilitate their secretion³⁰⁻³². To investigate whether any additional soluble
144 proteins encoded at the *S. aureus* T7SS locus interact with EsaD, we used bacterial two hybrid
145 analysis. Fig 3a shows no evidence for EsaD interaction with any known secretion substrates
146 (EsxB, EsxC or EsxD) or soluble machinery components (EsaB or EsxA). Interaction was
147 detected between EsaD and EsaE, which was also confirmed biochemically as EsaE-HA co-
148 purified with EsaD(H528A)-His when the two proteins were co-produced in *S. aureus* (Fig 3b).
149 To determine whether EsaE interacted with the nuclease domain of EsaD or elsewhere on the
150 protein, we screened EsaE interaction with EsaD₁₋₄₂₀ or EsaD₄₂₁₋₆₁₄(H528A) (Fig 3c). The
151 results show that EsaE specifically interacts with the non-nuclease region of EsaD. We tried

152 to confirm these genetic observations with co-purification experiments but were not able to
153 stably produce the truncated EsaD₁₋₄₂₀.

154 We constructed an in-frame deletion of *esaE* to determine whether it was required for EsaD
155 secretion. We were readily able to obtain the *esaE* mutant, consistent with EsaE not being an
156 antitoxin but playing some other role in EsaD biosynthesis. Production of HA-tagged
157 EsaD(H528A) in this strain resulted in no detectable EsaD in the supernatant and very little in
158 the cells (Fig S8), suggesting EsaE is required for its stability or secretion. Although EsaE was
159 not required for EsxA secretion, there was no apparent secretion of EsxC in the *esaE* mutant,
160 even if EsxC was overproduced from a plasmid (Fig S9). Thus EsaE is required for efficient
161 secretion of EsaD and at least one further T7SS substrate.

162 His-tagged EsaE was found almost exclusively in the cellular fraction, suggesting it is not, by
163 itself, a T7SS substrate (Fig 3d). We did, however, routinely observe that overproduction of
164 tagged EsaE led to a dramatic increase in the level of extracellular EsxA (Fig 3d), for reasons
165 that are unclear. However, when EsaE-His was co-produced with EsaD(H528A)-HA, notable
166 secretion of His-tagged EsaE could now be detected suggesting that these two proteins are
167 co-exported as a complex.

168 **EsaD/E/G form a ternary complex.** We next assessed whether EsaD could form a ternary
169 complex with EsaE and EsaG. Control experiments (Fig S10) showed no direct interaction
170 between EsaE and EsaG by either two hybrid or co-purification experiments. When
171 EsaD(H528A)-Myc, EsaE-HA and EsaG-His were co-produced in *E. coli* and EsaE-HA
172 immunoprecipitated, EsaG-His and Myc-tagged EsaD were also co-precipitated, consistent
173 with the three proteins forming a ternary complex (Fig 3f,g). Reciprocal experiments where
174 His-tagged EsaG was isolated by Ni-affinity purification resulted in co-purification of EsaE-HA
175 and EsaD-Myc (Fig S12). In this latter experiment, although some full length EsaD-Myc was
176 detected, most of the protein was fragmented to the approximate size of the nuclease domain,
177 consistent with the prior instability of EsaD₁₋₄₂₀ noted previously.

178 **EsaE interacts with the multimeric form of EssC.** Although EsaE is predicted to be soluble,
179 subcellular fractionation showed a proportion of His-tagged EsaE localised to the membrane

180 and was stable to carbonate washing (Fig 4a). As EsaE has a dual cytoplasmic-membrane
181 location, we wondered whether it may play a role in targeting EsaD/EsaG to the membrane-
182 bound secretion machinery. We undertook formaldehyde crosslinking experiments in whole
183 cells of *S. aureus* producing EsaE-His, isolated membranes and blotted for EsaE. Fig 4b
184 shows several EsaE-His crosslinks, including a particularly strong crosslink migrating above
185 250 kD. It has previously been shown that the *S. aureus* T7 ATPase, EssC, forms a high
186 molecular weight multimer^{17,33}. To ascertain whether this high molecular weight crosslink also
187 contained EssC, we repeated the crosslinking experiments in the wild type and *essC* mutant
188 strains. Fig 4c shows that the EsaE-His-containing crosslink migrated with an apparently
189 identical mass as the EssC-containing crosslink and moreover, no such crosslink was
190 detected when *essC* was deleted. We conclude that the membrane-bound form of EsaE
191 interacts with the multimeric form of EssC.

192 These results suggest a model for the biosynthesis and secretion of EsaD (Fig 4d), whereby
193 the interaction of EsaG and EsaE with their respective binding domains on EsaD is essential
194 for maintaining EsaD in a catalytically-inactive, secretion-competent conformation. Our
195 findings support the idea that targeting of the protein complex to the T7 secretion machinery
196 is by virtue of the interaction between EsaE and the assembled EssC multimer and that EsaG
197 is stripped from the complex at some point during secretion and remains in the cytoplasm.
198 Thus EsaD is released from the cell in a form that is immediately active, and the EsaG
199 immunity protein remains in the producing cell where it may potentially serve further protective
200 functions.

201 **Secreted EsaD inhibits the growth of sensitive strains of *S. aureus*.** We next addressed
202 potential roles for EsaD. Gram-negative bacteria utilise a subset of their protein secretion
203 systems to target toxins at bacterial competitors as well as at eukaryotic cells³⁴⁻³⁸. It was noted
204 previously that although approximately 50% of *S. aureus* strains do not carry *esaD*, they
205 encode at least two homologues of *esaG* close to their T7SS gene clusters¹⁸ (Fig 5a)
206 suggesting they may produce EsaG-type proteins as a protective mechanism to prevent killing
207 by EsaD-producing strains. To probe this, we assessed whether EsaG homologues from non

208 *esaD*-containing strains MRSA252, ST398 and EMRSA15 could interact with EsaD(H528A)
209 using two hybrid analysis. At least one EsaG homologue from each strain was able to interact
210 with EsaD (Fig 5b), supporting the idea that orphan EsaG proteins serve to protect *S. aureus*
211 from EsaD nuclease toxins.

212 A common feature of bacterial toxins, particularly those involved in interspecies competition,
213 is that they are polymorphic^{39,40}. Comparison of EsaD sequences across *esaD*-encoding
214 *S. aureus* strains shows extensive sequence variability within the nuclease domain but away
215 from the predicted catalytic site (centred around H528; Fig S13). A likely explanation is that
216 substitutions in this region of EsaD alter affinity for a cognate EsaG antitoxin and render
217 normally resistant strains susceptible to attack by EsaD sequence variants. In this context it is
218 interesting to note a cluster of EsaG homologues are encoded directly downstream of *esaDG*
219 in *S. aureus* *esaD*-containing strains that is highly variable in number¹⁸. For example
220 NCTC8325 encodes five of these (Fig 5a), whereas COL encodes eleven. These genes are
221 not co-transcribed with *esaDG* in strain RN6390 (Fig S14), however analysis of RNA-seq data
222 from¹⁸ indicated that transcripts from these genes are present under laboratory growth
223 conditions.

224 We could readily delete the cluster of *esaG*-like genes from RN6390 indicating they are not
225 required to neutralise EsaD, consistent with our prior conclusion that EsaG itself is the cognate
226 EsaD antitoxin. Loss of this *esaG*-like gene cluster, or indeed absence of all EsaG-encoding
227 proteins (including EsaG) also did not affect the secretion of EsxA or EsxC (Fig S9) confirming
228 these proteins are not essential components of the T7SS machinery. However, two hybrid
229 assay shows that they are able to interact with EsaD (Fig 5c), but that the interaction is not as
230 strong as that seen for EsaD-EsaG, raising the possibility that they serve to protect RN6390
231 from EsaD sequence variants produced by other strains of *S. aureus*.

232 Together, the results presented above strongly suggest that *S. aureus* uses its T7SS to
233 secrete a nuclease toxin that targets rival bacteria. To confirm this, we used *S. aureus* strain
234 COL, which shows the highest level of T7S activity in laboratory growth media¹⁶ producing
235 plasmid-encoded EsaDG as attacker, and incubated it with variants of strain RN6390. Fig 5d

236 shows that there is an approximate two-log decrease in recovered prey cells when they are
237 co-cultured with a T7SS⁺ strain of COL compared with a T7SS mutant strain, demonstrating
238 there is T7-dependent growth inhibition. Importantly, this is completely dependent upon the
239 toxic activity of EsaD as COL producing the H528A variant of EsaD no longer exhibited
240 detectable growth inhibition (Fig 5d). Finally, as expected, EsaG offered some protection
241 against the inhibitory effect of secreted EsaD as the RN6390 wild type tended to be less
242 susceptible to EsaD-dependent growth inhibition than a strain lacking EsaG homologues, and
243 the protective effect was enhanced by overproduction of plasmid-encoded EsaG in the prey
244 cells. We conclude that *S. aureus* can use its T7SS to target bacterial competitors.

245

246 **Discussion**

247 It is well established that T7SSs play critical roles in mammalian infection and virulence. Here
248 we demonstrate an important novel role for the *S. aureus* T7SS in the secretion of a nuclease
249 toxin, EsaD, involved in interspecies competition. Thus, akin to the Gram-negative Type VI
250 secretion system^{34,41}, the T7SS appears to target eukaryotes and rival bacteria.

251 EsaD is the largest known substrate of the T7b system and the only one for which a function
252 has been identified. We have shown that two T7 accessory factors are essential for the
253 biogenesis of EsaD - EsaG that binds to and neutralises the toxic activity of the nuclease
254 domain and EsaE that interacts with, and potentially stabilises, the N-terminal region. In this
255 context it is interesting to note that large substrates of the Mycobacterial T7a system such as
256 the PE/PPE proteins interact with specific chaperones of the EspG family that keep them in a
257 secretion-competent state³² and deliver them to the cognate secretion machinery³¹. Although
258 there is no detectable sequence similarity between EspG proteins and EsaE, it is possible that
259 the proteins have analogous functions in the two distantly related secretion machineries.

260 The presence of T7SSs in non-pathogenic organisms such as *Streptomyces coelicolor*⁴² and
261 *Bacillus subtilis*^{8,9} has previously been noted. Here we offer a likely explanation for the

262 presence of this secretion system in environmental strains. We have demonstrated that
263 secreted EsaD inhibits the growth of sensitive strains of *S. aureus*, indicating this nuclease
264 toxin is used to target competitor bacteria. In support, strains of *S. aureus* lacking *esaD* encode
265 at least two copies of the EsaG-like antitoxin, presumably as a protective mechanism. It is
266 interesting to note that an EsaD homologue has been reported in *B. subtilis*²³ that likely has
267 toxic activity⁴³, inferring that modulating bacterial competition is a conserved role for T7SSs.

268 It is currently not known how EsaD accesses the cytoplasm of target cells. There is no
269 evidence that T7SSs form large extracellular needle-like structures that could deliver toxins
270 directly into target cells, like to those seen for the Gram-negative Type III, IV and VI secretion
271 systems¹. Instead we suggest that EsaD is released into the environment where it binds to
272 receptors on sensitive cells in a similar manner as bacteriocins and contact-dependent growth
273 inhibition (CDI) toxins, as the first step in a cell entry pathway^{44,45}. Further work will be required
274 to dissect out the mechanism by which EsaD interacts with and traverses the cell envelope of
275 *S. aureus*.

276 *S. aureus* is an important pathogen in polymicrobial human infections, for example infections
277 of the skin and lung. Whether EsaD-mediated interspecies competition is critical for the
278 establishment of virulence in animal and human infections and whether there are additional
279 antibacterial toxins secreted by the *S. aureus* T7SS remain to be established.

280

281 **Methods**

282 **Bacterial strains and growth conditions:** Strains and plasmids used in this study are listed
283 in Tables S1 and S2. *S. aureus* strains were grown in TSB medium at 37°C under vigorous
284 agitation. Where required, chloramphenicol (Cm) at a final concentration of 10 µg/ml was
285 added for plasmid selection. Anhydrotetracycline (ATC) was used as a selection during allelic
286 gene replacement using the pIMAY system (1 µg/ml⁴⁶) or for induction of target gene
287 expression from the pRAB11 plasmid⁴⁷; the concentrations used in each experiment are listed

288 in the appropriate figure legends). *Escherichia coli* was grown aerobically in Lysogeny broth
289 (LB) at 37°C. If required, cultures were supplemented with ampicillin (Amp, 100 µg/ml),
290 Kanamycin (Kan, 50µg/ml) or Cm (15 µg/ml) for plasmid selection. Induction of plasmid-
291 encoded gene expression was achieved by addition of isopropyl-β-D-galactopyranoside
292 (IPTG), as indicated in the text. Light microscopy was carried out using a Zeiss
293 light/fluorescence microscope with a 100x oil objective and images captured using an AXIO
294 camera (Zeiss). The light microscopy images in Fig S3a were performed twice using different
295 biological samples, representative images are shown. Bacterial two-hybrid analyses were
296 performed as described⁴⁸; quantitative assessment of protein interactions was undertaken by
297 plating onto MacConkey medium⁴⁹ containing 0.4% maltose as carbon source, and quantified
298 by β-galactosidase assays (according to the method of⁵⁰) on strains grown to exponential
299 phase at 30°C and permeabilized with toluene. For all of the bacterial two hybrid experiments
300 reported (Figs 2b, 3a, 3c, 5b, 5c, S10a) each interaction pair was scored on MacConkey
301 maltose on at least four different occasions and β-galactosidase assays were performed at
302 least twice, and representative results are presented. RT-PCR was undertaken on RNA
303 prepared from *S. aureus* strain RN6390 grown aerobically in TSB to an OD₆₀₀ of 2, as
304 described previously¹⁶ using primer pairs listed in Table S3, and was performed twice (on the
305 same biological sample – results presented in Fig S14b are representative).

306 **Strain and plasmid construction:** All oligonucleotide primers used in this study and cloning
307 strategies to generate the strains and plasmids are outlined in Table S3. In-frame deletions of
308 *S. aureus* genes were performed by allelic exchange using pIMAY⁴⁶. For each gene, the
309 upstream and downstream regions including at least the first three and last three codons were
310 amplified from RN6390 genomic DNA using primers listed in Table S3. Clones were selected
311 in *E. coli*, verified by DNA sequencing and introduced into *S. aureus* RN6390 strains by
312 electroporation. Chromosomal deletions were verified by amplification of the genomic region
313 from isolated genomic DNA (GeneElute Bacterial Genomic DNA Kit, Sigma Aldrich) and DNA
314 sequencing of the amplified products.

315 To construct strains specifying chromosomally-encoded erythromycin resistance, the
316 erythromycin resistance gene, *ermC*, was integrated into the RN6390 genome after base pair
317 14208 as this region was found to be devoid of transcriptional activity in the closely related
318 strain NCTC 8325-4⁵¹. A synthetic construct comprising *ermC* from *Staphylococcus lentus*
319 plasmid pSTE2 under control of the *rpsF* promoter from *Bacillus subtilis* (purchased from
320 Biomatik; sequence given in Fig S16) was cloned into pIMAY and integrated into the
321 chromosome giving RN6390::*ermC*. Integration of the resistance gene was confirmed by
322 sequencing and by testing for growth in the presence of 5 µg/ml erythromycin. Subsequently
323 the *ermC* cassette was transduced from RN6390::*ermC* into other strains using phage φ11 as
324 described⁵². Transduction was confirmed by PCR amplification using oligonucleotides Intctrl1
325 and Intctrl2 (Table S3).

326 **Bacterial competition experiments:** Overnight cultures of the indicated strains were
327 subcultured in TSB (supplemented with 2µM hemin for attacker strains) and antibiotics as
328 required and cultured with shaking at 37°C. Once OD₆₀₀ of 0.5 was reached, induction of
329 EsaD-HA and EsaG-His production in the attacker strains was initiated by the addition of 500
330 ng/ml of ATC. When cells reached OD₆₀₀ of 2, 20ml of attacker strain, and 1ml of prey were
331 separately harvested and resuspended in 1ml of TSB. 100ul of resuspended attacker cells
332 were mixed with the same volume of prey cells (giving a 20:1 ratio) and incubated at 37°C
333 with shaking for 16 h in sterile Eppendorf tubes. Co-cultures were then serially diluted in TSB
334 and plated on selective agar (LB + 5 µg/ml erythromycin as all prey strains carried
335 chromosomally-integrated *ermC* conferring resistance to erythromycin) for colony-forming unit
336 determination. For experiments where plasmid-encoded EsaG-His was produced in prey cells,
337 once strain RN6390 pEsaG-His reached an OD₆₀₀ of 0.5, EsaG-His production was initiated
338 by the addition of 250 ng/ml of ATC and cultured until an OD₆₀₀ of 2 was reached after which
339 they were used as prey as described above.

340 **TUNEL assay for DNA fragmentation:** DNA fragmentation was detected in fixed cells of *E.*
341 *coli* BL21(DE3) harbouring pT7.5esaD and pT7.5esaDG using the Deadend fluorometric
342 TUNEL system kit (Promega). This was undertaken on two biological replicates and

343 representative results are shown in FigS3 c and d. Following induction of EsaD production by
344 treating cell cultures with 1mM IPTG for 3 hours, cells were pelleted, washed twice with PBS
345 and fixed with 4% formaldehyde in PBS for 30 minutes on ice. Following a further wash with
346 PBS, cells were permeabilized with 1.5% triton X-100 solution in PBS for 1 hour on ice and
347 stored in 70% ice cold ethanol at -20 °C overnight. The following day cells were spun down,
348 washed and resuspended in equilibration buffer for 1 hour at 37°C. The cells were incubated
349 in the dark for 2 hours with fluorescein 12-dUTP and recombinant terminal deoxynucleotidyl
350 transferase (rTdT), after which the reaction was quenched by addition of 2x SCC buffer, and
351 the cells washed with PBS. Following this fluorescein-labelled cells were spotted onto poly-D-
352 lysine-treated slides and analysed by fluorescence microscopy using a Zeiss fluorescence
353 microscope with a 100x oil objective and images captured using an AXIO camera (Zeiss), or
354 detected and quantitated directly by flow cytometry (Flow cytometry Facility, Dundee
355 University). Negative control samples were treated identically except that no rTdT was added.

356 **Purification of the EsaD nuclease domain:** *E. coli* strain M15[prep4] harbouring pQE70-
357 EsaG-EsaG-EsaD₄₂₁₋₆₁₄-His or pQE70-EsaG-EsaG-EsaD₄₂₁₋₆₁₄(H528A)-His was cultured to
358 OD₆₀₀ of 0.5 at 37°C, after which 1mM IPTG was added to each culture. An aliquot of the cells
359 were harvested after 4 hours induction and resuspended in lysis buffer and the remainder of
360 the cells were pelleted and frozen at -80°C for 30 mins. The nuclease domain of EsaD was
361 subsequently purified following a protocol based on that described at
362 http://openwetware.org/wiki/Knight:Purification_of_His-
363 [tagged_proteins/Denaturing_with_refolding](http://openwetware.org/wiki/Knight:Purification_of_His-tagged_proteins/Denaturing_with_refolding) with some modifications, and was undertaken
364 once for each variant (Fig 2e). Briefly, the thawed pellets were resuspended in lysis buffer (8 M
365 Urea, 100 mM NaH₂PO₄, 10mM Tris·HCl, 10mM imidazole, 1 mM PMSF, pH 8.0) and
366 sonicated for 2 mins on ice. The cell lysate was centrifuged at 13,200 × g for 30 min at 4 °C.
367 Nickel affinity resin (Biorad) was equilibrated with lysis buffer and added to the cleared lysate
368 and gently mixed on a rotary shaker for 2 h at 4 °C. The resin was washed five times with
369 denaturing wash buffer (8 M Urea, 100 mM NaH₂PO₄, 150mM NaCl, 20mM imidazole, 1 mM
370 PMSF, pH 8.0) followed by five washes with native wash buffer (50 mM NaH₂PO₄, 500mM

371 NaCl, 20mM imidazole, 1 mM PMSF, pH 8.0). Finally, the bound protein was eluted into 200
372 μ l of elution buffer (50 mM NaH_2PO_4 , 500mM NaCl, 250mM imidazole, 1 mM PMSF, pH 8.0).
373 For nuclease assays, 800 ng of plasmid pT18 was incubated with 0.4 μ g of purified EsaD₄₂₁₋
374 ₆₁₄-His, purified EsaD₄₂₁₋₆₁₄(H528A)-His or an equivalent volume of elution buffer at 37°C for 1
375 hour in a final volume of 20 μ l the presence of either 50mM MgCl_2 or ZnCl_2 as indicated, after
376 which the DNA was analysed by agarose gel electrophoresis. These assays were each
377 performed three times and representative results are presented in Figs 2f and S5.

378 **Cell fractionation, crosslinking and western blotting:** For the isolation of cell and
379 supernatant fractions to assess secretion activity, *S. aureus* strains were subcultured at 1/100
380 from an overnight grown pre-culture into fresh TSB medium. At $\text{OD}_{600\text{nm}}$ of 2, cells were
381 harvested and the supernatant samples precipitated with trichloroacetic acid in the presence
382 of deoxycholate, as described previously¹⁶. Harvested cell samples were washed once with
383 PBS buffer, normalized to an OD_{600} of 2 in PBS and lysed by addition of 50 μ g/ml lysostaphin
384 with incubation at 37°C for 30 min. All samples were mixed with an equal volume of LDS buffer
385 and boiled for 10 min prior to analysis. For the secretion experiments shown in Fig 1b, 1c, 1e,
386 S9a, S9b and S14 representative images are shown from at least two biological replicates,
387 and in Figs 1d, 3d, 3e, S7 and S8 representative images are shown from at least six biological
388 replicates. The fractionation of cells to give cell wall, membrane and cytoplasmic fractions was
389 undertaken as described by¹⁶. Carbonate-washing of membranes was undertaken according
390 to⁵³. All fractionation experiments (Figs 1f, 3a and S6) were undertaken at least twice on
391 separate biological samples, with representative results presented.

392 Formaldehyde crosslinking of cells was undertaken as described previously¹⁷ and crosslinking
393 experiments were performed twice on separate biological samples – the results presented in
394 Fig 3b and c are representative results. Western blotting was performed according to standard
395 protocols using the following antibody dilutions: α -EsxA¹⁶ 1:2500, α -EsxC¹⁶ 1:2000, α -EssC¹⁶
396 1:10000, α -TrxA⁵⁴ 1:25000, α -SrtA (Abcam, catalogue number ab13959) 1:3000, α -HA (HRP-
397 conjugate, Sigma catalogue number H6533) 1:10000, α -His (HRP-conjugate, Abcam

398 catalogue number ab184607) 1:10000, α -Myc (HRP-conjugate, Invitrogen catalogue number
399 R951-25) 1:5000, and goat anti Rabbit IgG HRP conjugate (Bio-Rad, catalogue number 170-
400 6515) 1:10000.

401 **Protein purification by nickel affinity isolation or immunoprecipitation:** Cells of *E. coli* or
402 *S. aureus*, grown as described in the figure legends, were harvested and resuspended in
403 either ice cold (i) resuspension buffer (200 mM NaCl, 20 mM HEPES, pH 7.2) for Ni affinity
404 purification or (ii) phosphate-buffered saline (PBS) for immunoprecipitation. Samples were
405 then supplemented with a few flakes of DNase I, 1 mM PMSF and either lysozyme (for *E. coli*)
406 or lysostaphin (for *S. aureus*). The samples were incubated at 37°C for 0.5-1 hour with gentle
407 mixing on a rotating wheel after which cells were lysed by French press (for *E. coli*) or
408 sonication (*S. aureus*). Unbroken cells and cellular debris was pelleted by centrifugation at
409 17,000 *g* and 4°C for 30 min and the supernatant was retained as the cell lysate. All protein
410 purification experiments shown in Figs 2c, 2d, 3b, 3f, 3g, S4, S10b, S11 and S12 were
411 performed at least twice with different biological replicates.

412 For Ni-affinity purification, 100 μ l Ni-NTA resin (Biorad, catalogue number 156-0131) was
413 equilibrated by washing twice in 1 ml ice cold wash buffer (200 mM NaCl, 15 mM imidazole,
414 20 mM HEPES, pH 7.2). Cell lysate was diluted to 5 μ g/ μ l protein in ice cold wash buffer (200
415 mM NaCl, 15 mM imidazole, 20 mM HEPES, pH 7.2) in a final volume of 1.2 ml, added to the
416 equilibrated Ni-NTA resin and gently agitated for 1 hour at 4°C. The Ni-NTA resin was pelleted
417 by centrifugation, washed four times with 1 ml ice cold wash buffer and finally resuspended
418 in 100 μ l elution buffer (200 mM NaCl, 300 mM imidazole, 20 mM HEPES, pH 7.2), mixing at
419 4°C for 1 hour. The Ni-NTA resin was pelleted, the supernatant carefully removed and retained
420 as the eluted fraction.

421 For immunoprecipitation, 40-100 μ l of Anti-HA Agarose bead suspension (Sigma, catalogue
422 number A2095) were pelleted, washed twice with PBS and mixed with cell lysate which was
423 diluted to 5 μ g/ μ l in 200 μ l PBS. The suspension was incubated with agitation for at least 1
424 hour at 4°C after which the beads were pelleted and washed four times with 1 ml ice cold wash

425 PBS. After the final wash the supernatant was aspirated to leave ~30 µl of PBS above the
426 beads for final resuspension.

427

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429

430 **Acknowledgements**

431 This study was supported by the UK Biotechnology and Biological Sciences Research Council
432 [grant BB/H007571/1] the Medical Research Council [grants G117/519 and MR/M011224/1]
433 the Wellcome Trust [through Early Postdoctoral Training Fellowship for Clinician Scientists
434 WT099084MA to JDC, Investigator Award 110183/Z/15/Z to TP and Institutional Strategic
435 Support Fund 105606/Z/14/Z to the University of Dundee], Tenovus Scotland [through project
436 grant T14/10] and a China Scholarship Council PhD studentship (to ZC). We thank Dr Grant
437 Buchanan for constructing some of the bacterial two hybrid clones used in this study, Dr James
438 Cargill for advice with synthetic construct design and co-purification experiments, Marilia
439 Costa for her assistance with microscopy, Dr Ewan Murray and Professor Paul Williams
440 (University of Nottingham) for supplying us with phage $\phi 11$, Professor Jan-Maarten van Dijk
441 (University of Groningen) for the kind gift of anti-TrxA antiserum, Professor Ralph Bertram
442 (University of Tübingen) for pRAB11 and Professor Frank Sargent and Dr Sarah Coulthurst
443 for helpful discussion and advice.

444

445 **Author contributions** - ZC, MGC, HK, JDC and TP designed experiments, ZC, MGC and HK
446 carried out experimental work, ZC, MGC, HK, JDC and TP undertook data analysis, TP wrote
447 the paper.

448

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608

609 **Main text figure legends**

610 **Fig 1. EsaD is a substrate of the T7SS.** a. The *ess* locus – genes coding for core components
611 of the secretion machinery are in green, secreted components yellow and proteins
612 investigated as part of this study in white. b. and c. EsaD is not required for secretion of EsxA
613 and EsxC – (b) the RN6390 wild-type or isogenic deletion strains, as indicated, were cultured
614 in TSB medium to OD₆₀₀ of 2 or (c) the indicated strains harbouring pRAB11 (empty) or
615 pRAB11-EsxC were cultured in TSB medium to OD₆₀₀ of 0.5, then supplemented with ATC
616 (50ng/ml; to induce plasmid-encoded gene expression) until OD₆₀₀ of 2. Cells were pelleted
617 and the supernatant (sn) was retained as the secreted protein fraction. Samples of the
618 supernatant and whole cells (an equivalent of 200µl of culture supernatant and 10µl of cells
619 adjusted to OD1) were separated on 12 % bis-Tris gels and immunoblotted with the indicated
620 antisera (with TrxA serving as a cytoplasmic control). Note that the samples were run on the
621 same gel but intervening lanes have been spliced out (unspliced version is shown in Fig S9).
622 (d) and (e). EsaD is secreted in an *essC*-dependent manner. The indicated *S. aureus* strains
623 harbouring pRAB11 (empty) or pRAB11-EsaD(H528A)-HA were treated as described in (c)
624 except that 250ng/ml ATC was used to induce EsaD(H528A)-HA production and an equivalent
625 of 250µl of supernatant and 10µl of cells adjusted to OD₆₀₀ of 1 were loaded f. Cells of the wild
626 type *S. aureus* strain, RN6390, harbouring pRAB11 or pRAB11-EsaD(H528A)-HA from (d)
627 were fractionated into cytoplasmic (cyt) and membrane (m) fractions. Samples of each fraction
628 (20µl aliquot of cyt, and 2mg of membrane) were separated on 12% bis-Tris gels and
629 immunoblotted using either anti-HA, anti-EssB (membrane protein control) or anti-TrxA
630 (cytoplasmic control) antisera.

631

632 **Fig 2. EsaDG form a nuclease toxin-antitoxin pair.** a. EsaD is toxic to *E. coli*. *E. coli*
633 BL21(DE3) harbouring pT7.5 (empty vector), pT7.5-*esaD*(V584Y) or pT7.5-*esaDG* was
634 cultured to OD₆₀₀ 0.5, supplemented with 1 mM IPTG (time zero) and OD₆₀₀ measured at 1 hr
635 intervals ($n=3$ biological replicates, error bars are \pm SD). b. – d. EsaG interacts with the

636 nuclease domain of EsaD. b. Interactions between pT25-EsaG and EsaD variants fused to
637 pT18 assessed by β -galactosidase activity assay in *E. coli* BTH101. BTH101 harbouring pT25
638 and pT18 was the negative control. Error bars are \pm SD ($n=3$ biological replicates). Student's
639 *t*-test gives *p* values < 0.00001 for EsaD/EsaG and EsaD₄₂₁₋₆₁₄/EsaG relative to the negative
640 control. Inset shows the same strain/plasmid combinations on MacConkey maltose plates. c.
641 and d. Top two panels: *S. aureus* RN6390 carrying c. pRAB11 (empty vector), pRAB11-EsaG-
642 HA or pRAB11-EsaD(H528A)-His-EsaG-HA, or e. pRAB11 (empty), pRAB11-EsaG-HA,
643 pRAB11-EsaD₄₂₁₋₆₁₄(H528A)-His or pRAB11-EsaD₄₂₁₋₆₁₄(H528A)-His-EsaG-HA was cultured
644 to OD₆₀₀ of 0.5, then supplemented with ATC (500ng/ml). Cells were harvested at OD₆₀₀ 3,
645 lysed and histidine-tagged EsaD purified. Cell lysate (load) and eluted fractions (20 μ l of each)
646 were analysed by western blot with anti-His and anti-HA antisera. Bottom two panels show
647 repeat experiments of: c. the EsaD(H528A)-His-EsaG-HA co-purification or d. the EsaD₄₂₁₋
648 ₆₁₄(H528A)-His-EsaG-HA co-purification. Samples of load (10 μ l), flow through (20 μ l), final
649 wash (30 μ l) and elution fraction (30 μ l) were analysed using the same antisera. Coomassie-
650 stained samples of the load and elute fractions are shown in Fig S4. e. *E. coli* M15[prep4]
651 harbouring pQE70 alone (empty) pQE70-EsaG-EsaG-EsaD₄₂₁₋₆₁₄-His or pQE70-EsaG-EsaG-
652 EsaD₄₂₁₋₆₁₄(H528A)-His were cultured to OD₆₀₀ of 0.5 and supplemented with 1mM IPTG. An
653 aliquot was harvested after 4 hours' induction and resuspended in lysis buffer. His-tagged
654 EsaD nuclease domain (wild type or H528A variant) was purified in the presence of 8M urea,
655 refolded and eluted as described in Methods. 10 μ l of each sample were separated (12% bis-
656 Tris gel) and stained using coomassie instant blue. f. EsaD is a Mg²⁺-dependent DNase.
657 Plasmid DNA was incubated with purified EsaD₄₂₁₋₆₁₄-His, EsaD₄₂₁₋₆₁₄(H528A)-His or buffer
658 alone with either 50mM MgCl₂ or ZnCl₂, after which the DNA was analysed by agarose gel
659 electrophoresis.

660

661

662 **Fig 3. EsaE is co-secreted with EsaD and together with EsaG they form a ternary**
663 **complex.** a – c. EsaE interacts with EsaD. Interactions between a. pT25-EsaD(H528A) and
664 the indicated fusions to pT18 or c. pT25-EsaE and EsaD₁₋₄₂₀ or EsaD₄₂₁₋₆₁₄(H528A) fused to
665 pT18 as well as pT18-EsaE and full length EsaD(H528A) fused to pT25 assessed by β -
666 galactosidase activity assay in BTH101. BTH101 harbouring pT25 and pT18 was the negative
667 control. Error bars are \pm SD ($n=3$ biological replicates). Student's *t*-test gives *p* values <
668 0.00001 for EsaD(H528A)/EsaE, EsaD(H528A)/EsaG, EsaD/EsaE, and EsaD₁₋₄₂₀/EsaE
669 relative to the negative control. Inset shows the same strain and plasmid combinations on
670 MacConkey maltose plates. b. Top two panels - *S. aureus* RN6390 carrying pRAB11 (empty),
671 pRAB11-EsaE-HA or pRAB11-EsaD(H528A)-His-EsaE-HA was cultured to OD₆₀₀ of 0.5
672 supplemented with 500ng/ml ATC and harvested at OD₆₀₀ of 3. Cells were lysed and histidine-
673 tagged EsaD(H528A) was purified. Cell lysate (load) and eluted fractions (20 μ l of each) were
674 analysed by western blot with anti-His and anti-HA antisera. Coomassie-stained samples of
675 these fractions are shown in Fig S4. Bottom two panels show repeats of EsaD(H528A)-His-
676 EsaE-HA co-purification. Samples of load (10 μ l), flow through (20 μ l), final wash (30 μ l) and
677 elution fraction (30 μ l) were analysed using the same antisera. d and e. EsaE is co-secreted
678 with EsaD. *S. aureus* RN6390 harbouring pRAB11 (empty) and either d. pRAB11-EsaE-His
679 or e. pRAB11-EsaD-His-EsaE-HA was cultured to OD₆₀₀ of 0.5 supplemented with 250ng/ml
680 ATC and harvested at OD₆₀₀ of 3. Samples of supernatant and cells (equivalent to 250 μ l
681 supernatant and 10 μ l cells adjusted to OD₆₀₀ of 1) were separated on 12% bis-Tris gels and
682 immunoblotted with the indicated antisera. f. EsaE, EsaD and EsaG form a ternary complex.
683 *E. coli* M15[pRep4] carrying pQE70 (empty) or pQE70- EsaE-HA-EsaD(H528A)-Myc-EsaG-
684 His was cultured to OD₆₀₀ of 0.5, supplemented with 2 mM IPTG for 4 hours, harvested and
685 lysed. HA-tagged EsaE was purified and 10 μ l, 25 μ l and 35 μ l of the elution fractions were
686 analysed by western blot with anti-HA, anti-His and anti-myc antibodies, respectively.
687 Coomassie-stained samples of these fractions are shown in Fig S10. g. Samples of load
688 (10 μ l), flow through (20 μ l), final wash (30 μ l) and elution fraction (30 μ l) from the EsaE-HA-

689 EsaD(H528A)-Myc-EsaG-His co-purification experiment shown in f. were analysed by western
690 blotting with the same antisera.

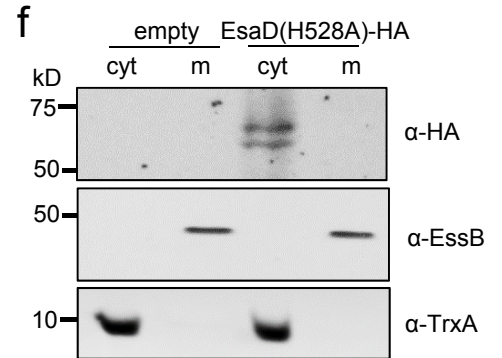
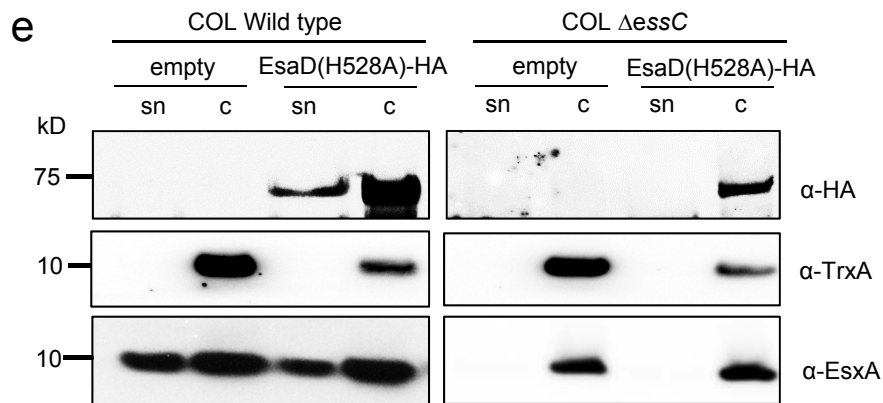
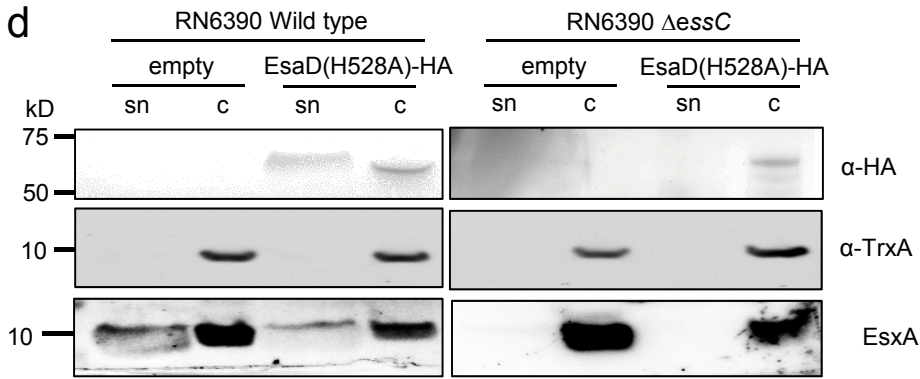
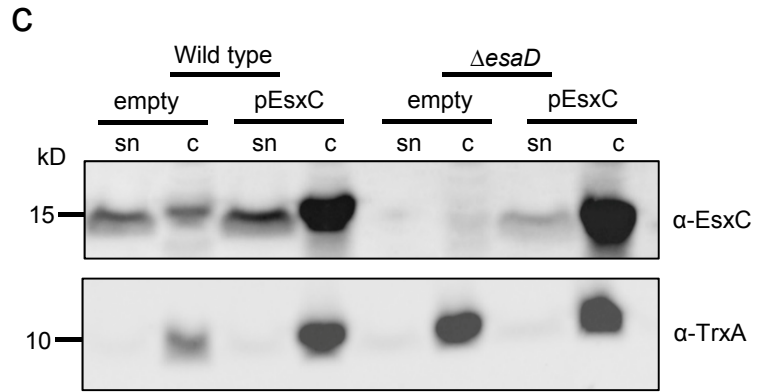
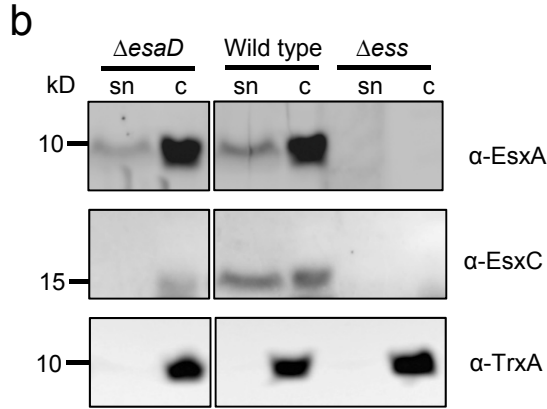
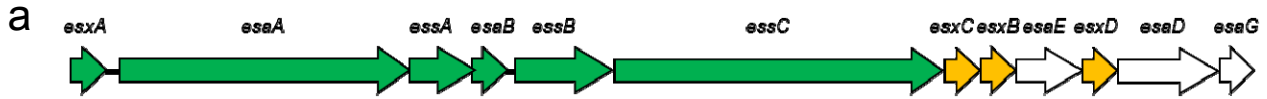
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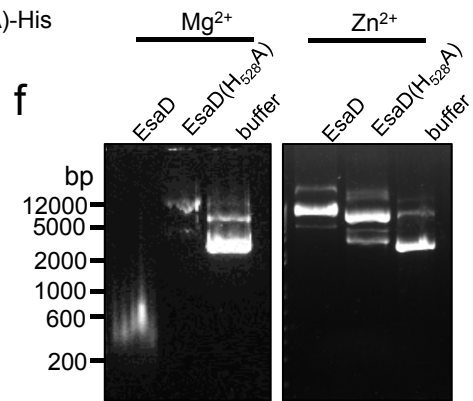
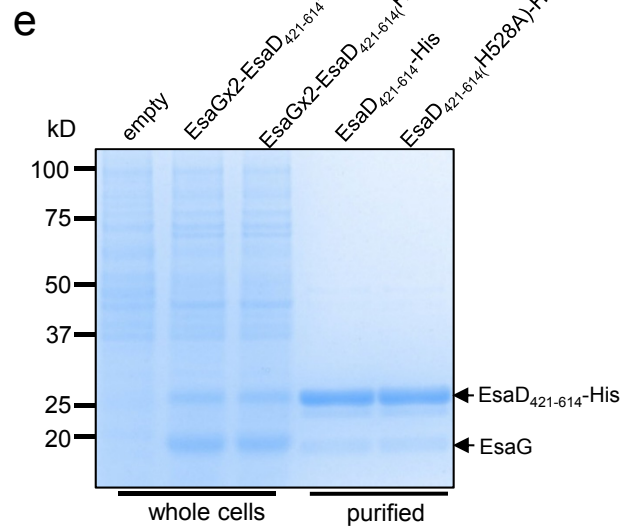
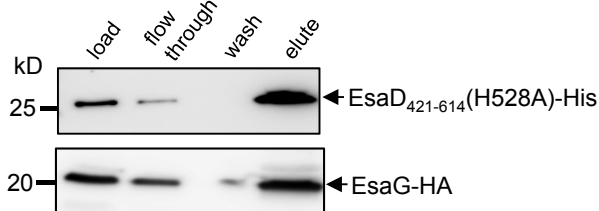
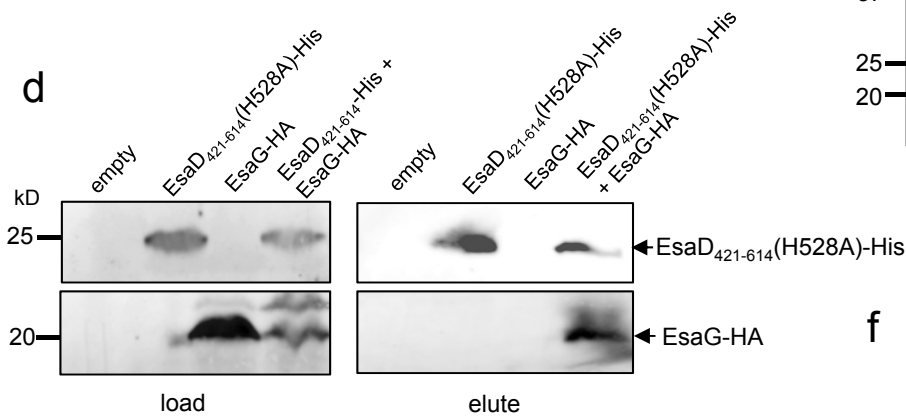
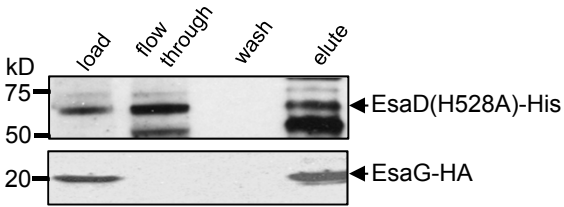
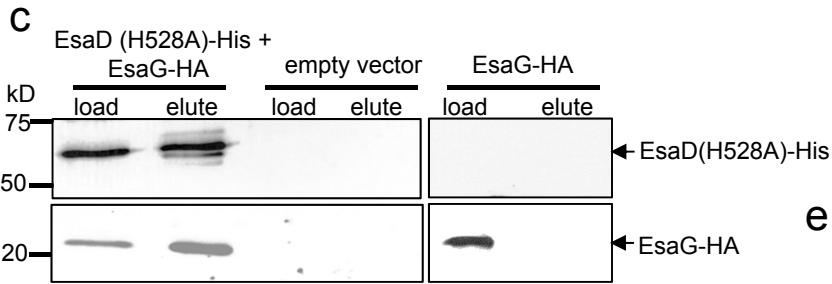
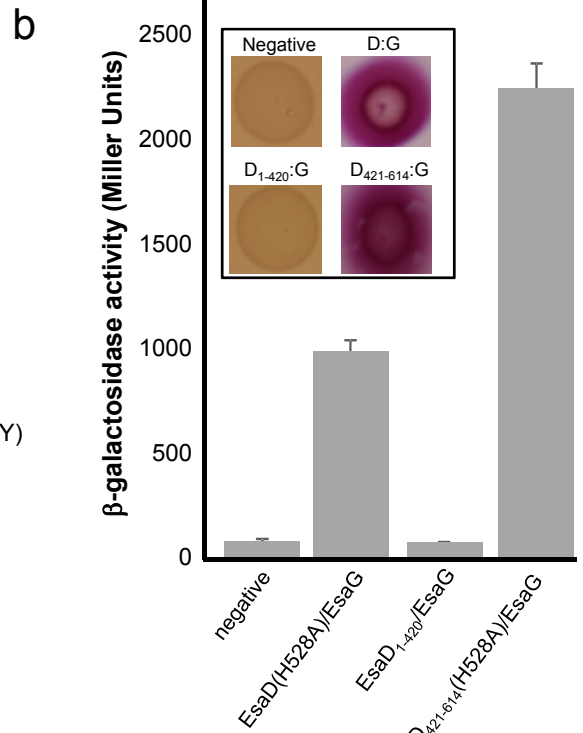
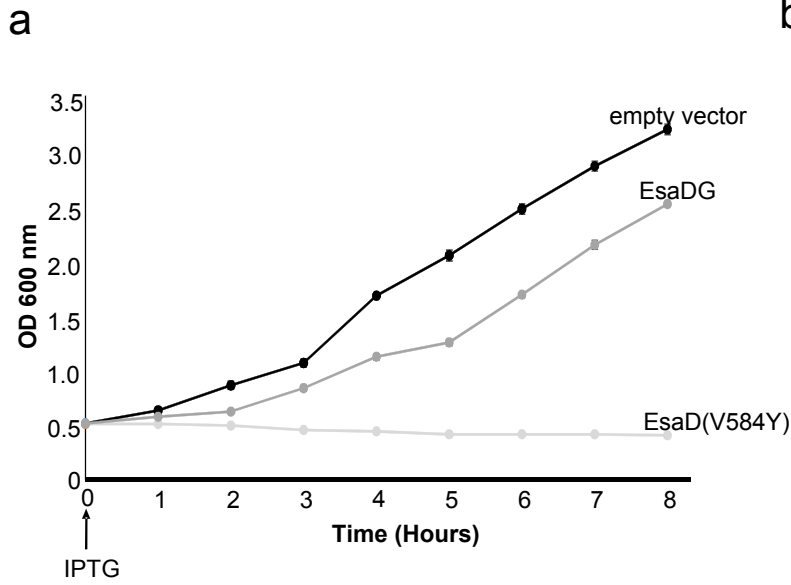
692 **Fig 4. EsaE is a membrane-associated protein that interacts with multimeric EssC.** a. A
693 proportion of EsaE is bound to the membrane. The *S. aureus* wild type strain, RN6390,
694 harbouring pRAB11 (empty) or pRAB11-EsaE-His was cultured in TSB medium to OD₆₀₀ of
695 0.5, supplemented with ATC (250ng/ml) and harvested at OD₆₀₀ of 2. Cells were fractionated
696 into cell wall (cw), cytoplasmic (cyt) and membrane (m) fractions. An aliquot of the membrane
697 fraction was washed with 0.2 M Na₂CO₃ (m+). Samples of each fraction (20µl aliquot of cw and
698 cyt; 2mg of membrane) were separated on 12% bis-Tris gels and immunoblotted using either
699 anti-His or anti-sortase A (SrtA) antisera. b and c. EsaE crosslinks to a multimeric form of
700 EssC. b. Whole cells of the *S. aureus* wild type (RN6390), or c. the wild type and the isogenic
701 *essC* deletion strain, as indicated, harbouring pRAB11 (empty) or pRAB11-EsaE-His were
702 cultured in TSB medium to OD₆₀₀ of 0.5 supplemented with ATC (250ng/ml) and at OD₆₀₀ of
703 2, cells were incubated with paraformaldehyde (PFA) as described under Methods. Following
704 quenching, cells were lysed and membrane fractions prepared, and membrane protein (1mg
705 for samples from the wild type strain, 10mg for samples from the *essC* strain) loaded on b. a
706 bis-Tris gel containing 12% acrylamide or c. SDS-gels containing 5 % acrylamide (bottom
707 panel in part C showing EsaE-His monomer is 12% bis-Tris gel) and analysed by western blot
708 with the indicated antisera. d. Model for EsaD synthesis and secretion. Following synthesis of
709 EsaD (shown in green), **1** EsaE binds to the N terminal region of the protein and **2** EsaG binds
710 to the nuclease domain to prevent activity against the DNA of the producing cell. **3** the ternary
711 complex is targeted to the secretion machinery facilitated by the interaction of EsaE with
712 multimeric EssC. **4** EsaG is released from EsaD during the transport step and **5** the EsaD-
713 EsaE complex is secreted out of the cell via the T7SS.

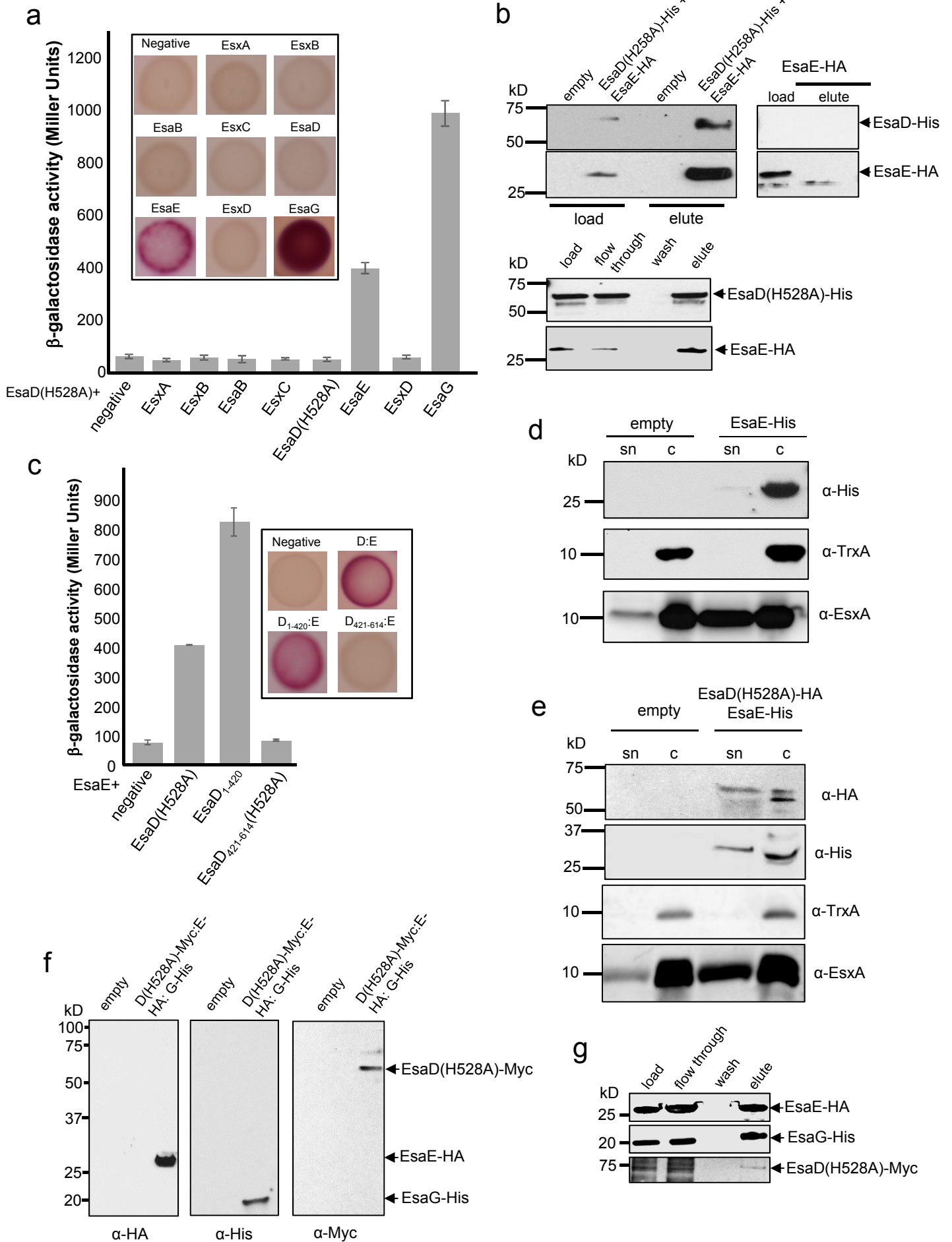
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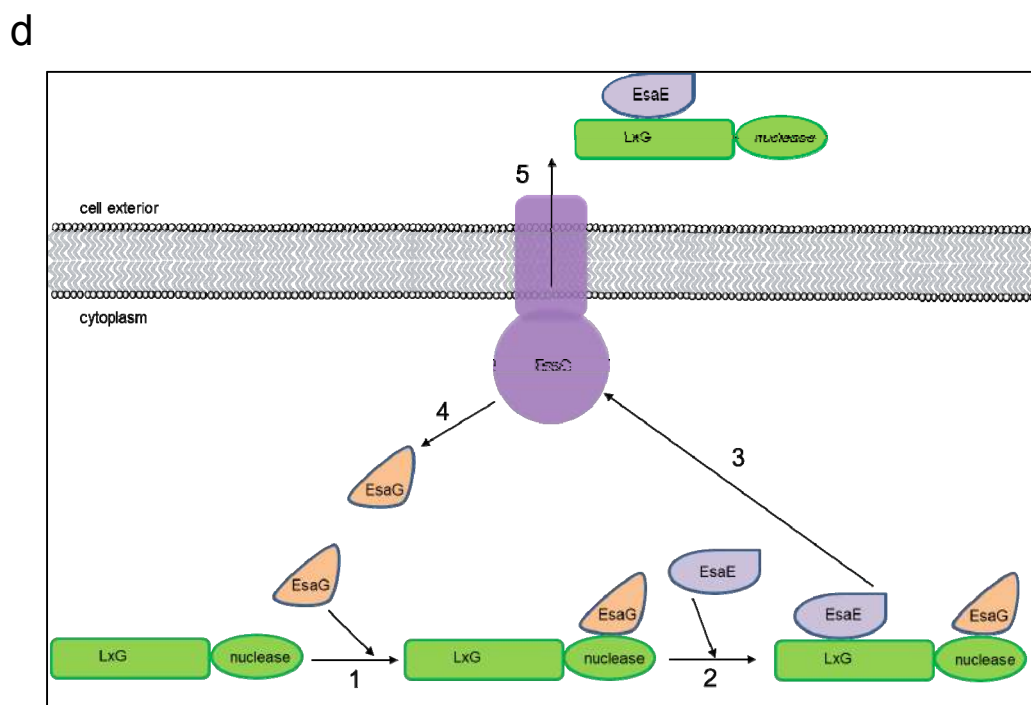
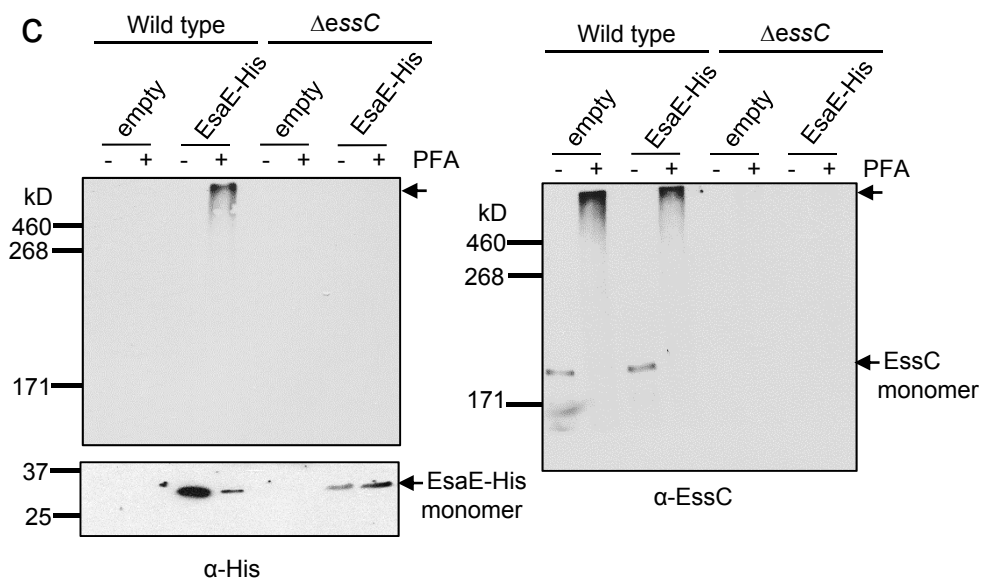
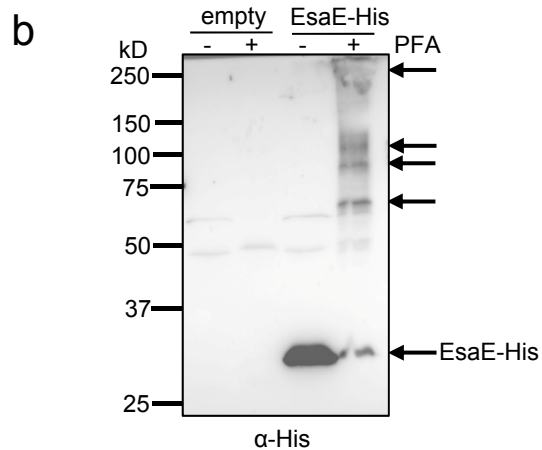
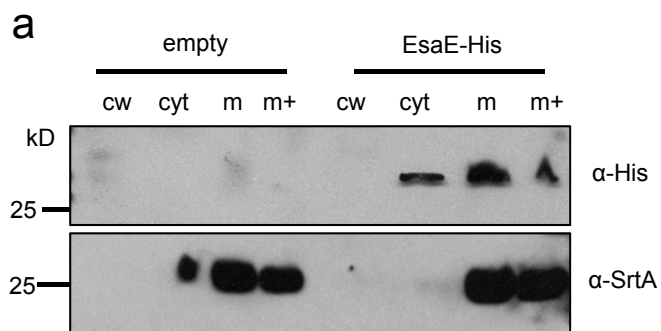
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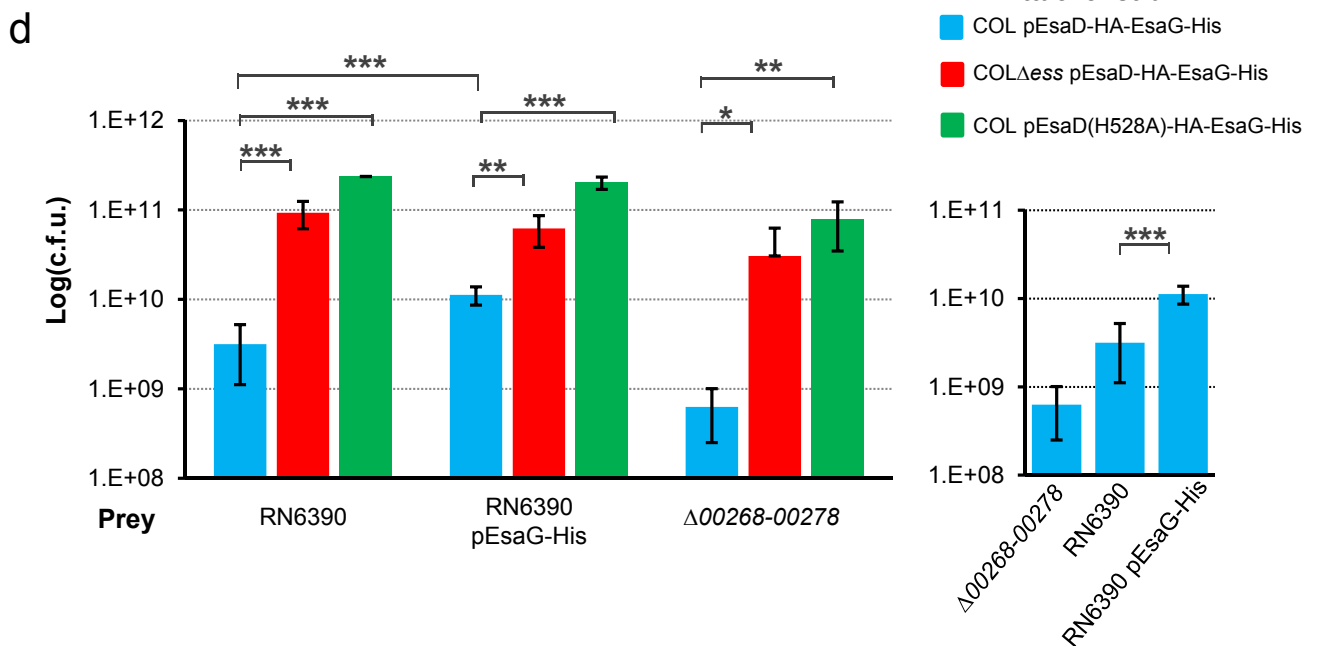
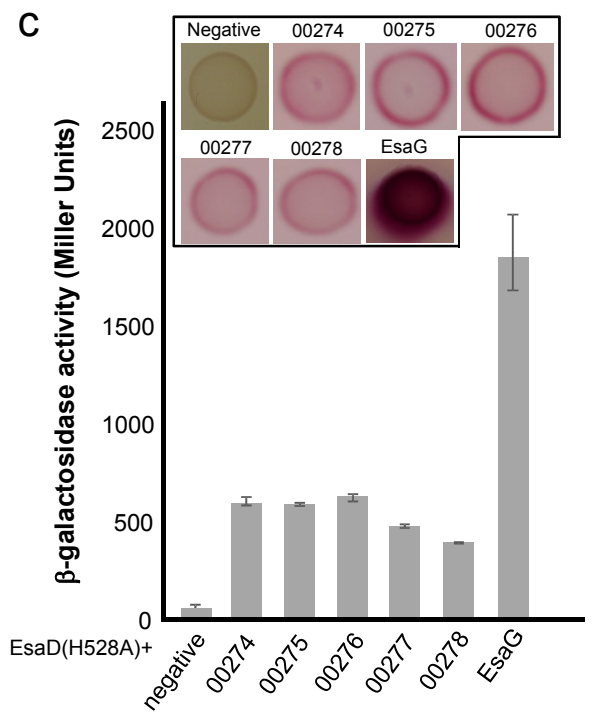
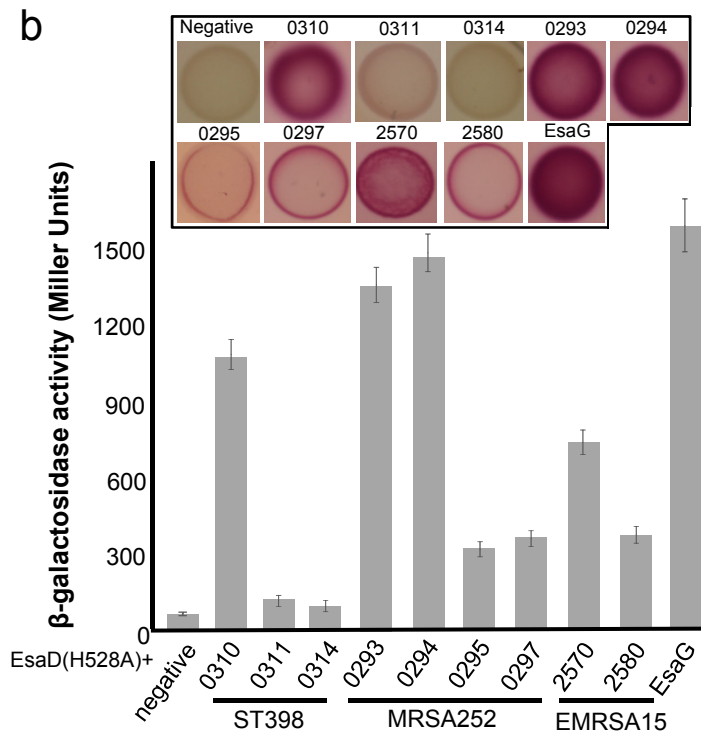
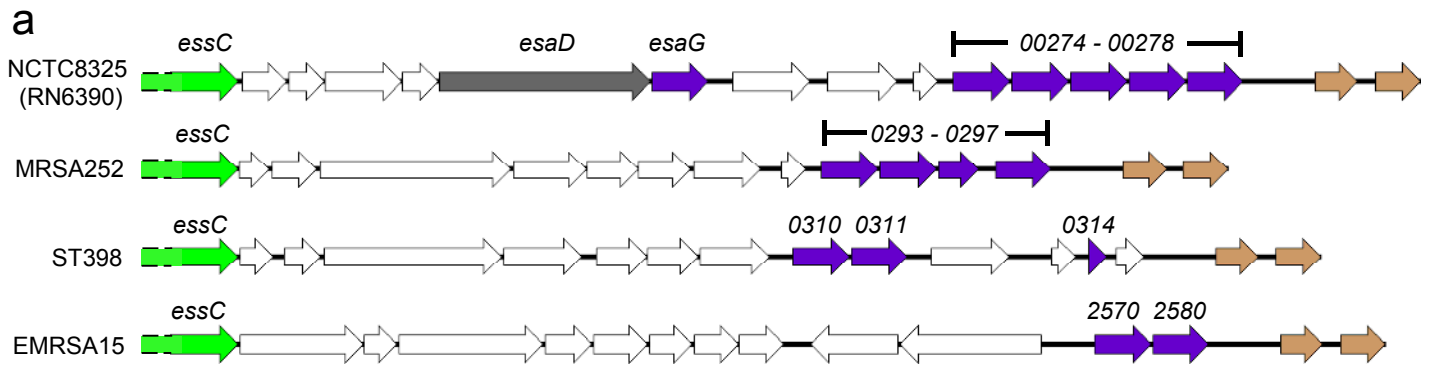
716 **Fig 5. Secreted EsaD kills sensitive strains of *S. aureus*.** a. EsaG homologues are encoded
717 in *S. aureus* strains that lack *esaD*. DUF600-family proteins encoded at the *ess* loci in *S.*
718 *aureus* strains NCTC8325 (parental strain of RN6390), MRSA252, ST398 and EMRSA15.
719 Genes encoding DUF600 proteins are shaded in purple, *essC* is shaded green and *esaD* grey.
720 The two genes shaded in brown are highly conserved across all strains and define the 3'
721 boundary of the *ess* locus¹⁸. b. and c. Interactions between pT25-EsaD(H528A) and DUF600
722 proteins; b. from strains ST398, MRSA252 and EMRSA15, and c. from strain NCTC8325. In
723 each case the DUF600 reading frame was fused to pT18 and interaction with full length EsaD
724 fused to pT25 assessed by β -galactosidase activity assay in *E. coli* BTH101. BTH101
725 harbouring pT25 and pT18 was the negative control. Error bars are \pm SD ($n=3$ biological
726 replicates). Student's *t*-test gives *p* values < 0.00001 relative to the negative control. Insets
727 shows the same strain and plasmid combinations on MacConkey maltose plates. d. *In vitro*
728 growth competition assays between the indicated attacker and prey strains in liquid medium.
729 In each case the attacker strain (COL or COL Δ *ess*) overproduced EsaD-HA or EsaD(H528A)-
730 HA along with EsaG-His as described in Methods, and was incubated with either RN6390,
731 RN6390 Δ 00268-00278 or RN6390 pEsaG-His as prey, as indicated. To the right, the three
732 prey strains incubated with COL pEsaD-HA-EsaG-His as attacker are replotted next to each
733 other to allow a more direct comparison. In all experiments five biological replicates of each
734 attacking strain was used against a single culture of prey. Bars represent the average value
735 of c.f.u. of prey bacteria at the end of the experiment. Asterisks indicate significant differences
736 in c.f.u. * *p* value < 0.05 ; ** *p* value < 0.005 , *** *p* value < 0.0005 . Comparison of RN6390 with
737 RN6390 Δ 00268-00278 survival when COL pEsaD-HA-EsaG-His was used as attacker was
738 not significant ($p = 0.069$). Error bars are \pm SD ($n = 5$ biological replicates).











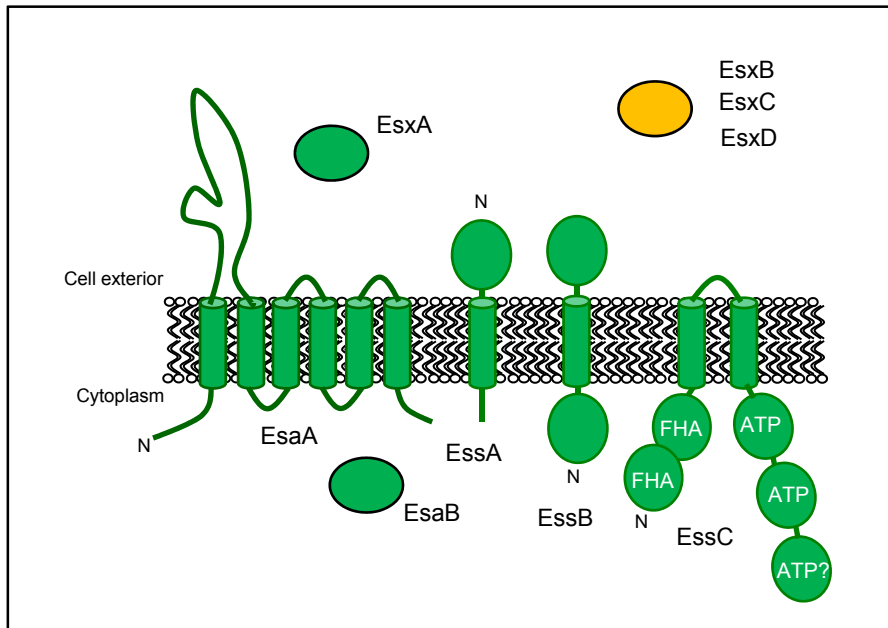
The Ess/Type VII secretion system of *Staphylococcus aureus* secretes a nuclease toxin that targets competitor bacteria

Supplementary information

Supplementary Figures 1-16

Supplementary Tables 1-3

Supplementary References



Supplementary Figure 1. Localization and predicted topologies of Ess components in *S. aureus* strain RN6390. Essential components of the secretion machinery are shown in green and known secreted substrates in yellow. FHA – forkhead associated domain. ATP – P-loop ATP-binding domain.

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Spy_Spd1 29 : A-----ARVRTYPM--SHANTHAKNTVS-SALDPFTANQL-----QLGELDNINRATFSHQ-QDRHETKDVRTKINYDFVWHNYQFFPYGDGSKSSWVMNRGHLVGYQFCGLNDEPRNLVAMTAWLNT----GA
Sau_EssD 441 : EMN-SSKYVESPNYTKV---EFGEHYAF--LRPRLKLANIEMIT--PTGHIYRTDHKGRIKEVYVDNIS-KDGD-----RNSHAQHTVGEEL-RLP-----DDGGHLLIARAFEGGSKD-IDNLVAQSKFINPFFREKGGH
Bsu_YeeF 493 : SSG-RRTPAPHVFPVTV---KYGEHAFARWR-KVLRPNIEIKTI-KEGTYITIDNYGRITSVK-ADLQ-GEAK---RQCYAQTNAQKPD-RKP-----DDGGHLLIATCFKGGSGQ-FDNLVEMNSQINRS---GGK
Sepi 445 : GSN-SVHYHEDPNLTKV---EYGDHYDFRFMKPKRLKANIEMIT--PHGIVYRTDHKGRIKEVYADDIS-LDGG-----RNTYAQHTVGERL-RLP-----DDGGHLLIARAFEGGSKD-IDNLVEQSKYINRSFKEKGD
Ble 534 : AVK-----QEQLRKVEVSGVNHHTRVVDG-KKALRPNISITTI-QNGRMYKTI-SEGRIKSVAN-LQ-GLAK---RNTYAQHKVGGV-LIS-----GDDGGHLLIASIFKGGSD-IDNLVEMNANLNR---SE
Bcl 446 : G-----NRRVSD----DIIRDGSHLGR-DGTLKPNVYQAGEYNYQNKIDELGRITDFENAD--D-KLTK---RGNRLSHKSNTPG-KEP-----GHAGHLLIADRFEGGSD-IDNLVVSQSSSVNL----SK
Bli 509 : SKTKTDIKVDSKTIHKV---KYGDHTFVVKR-KVLRPNIEIKTI-PVGYTYKTIHKGRITNVSGK-LNQ-GAAK---RQKYAQRHAREL-RLK-----TDEGGHLLIASIFEGSGK-LDNLVEMDGNLNR---GE
Bha 393 : GS--DNFAGATRISNRV---EYGDHYDFRVDK-KVLRPNIEEMT--PEGIVYKTI-DHGRITHVEGD--IS-GAAK---RNTYAQHTVGERL-RLP-----GDDGGHLLIASIFKGGSD-IDNLVEMNANLNEY---E
Bthu 304 : GTG-----EGPVKV---NYGEQYAFEKR-KKILKPNVEMTS-KEGTYITIDSQGRVASCESG-LQ-GDGK---RNTYAQRVVEGNI-RLD-----DDGGHLLIATIFKGGSGN-MDNLVEMNSNLNR---GE
Bce 412 : GTG-----EGPVKV---NYGEQYAFEKR-KKILKPNVEMTS-KEGTYITIDSQGRVASCESG-LQ-GDGK---RNTYAQRVVEGNI-RLD-----DDGGHLLIATIFKGGSGN-MDNLVEMNSNLNR---GE
Bpu 466 : KTG-RRLPAPKSPPTVV---SYGDHYVWVKR-KVLRPNVVEST-QCGTYITIDHYGRIVKVAQASD-KYGEVK---RQCYAQSNSQKPI-RLL-----DDGGHLLIATIFKGGSD-IDNLVEMNSQINRS---GGK
Afl 328 : ----SEKASKGIENI---KYGEQYAFINR-KKALKPNIEEMT--KEGTYITIDRGRITSSVEAK--LE-GKAD---RNTYAQRVVEGERL-RLP-----NDEGGHLLIASIFKGGSD-IDNLVEMNATLNR---SE
Cle 549 : KLEDSKLIVGEGKAGVV---EYGEQYDFIGN-KVLRPNVEMID-SNGRMYKTI-DKGRISNVQGN-LQ-GEQV---RNTYAQRVVEGNI-RLP-----TDEGGHLLIGSCFNGSGQ-IDNLVQONSSINRA---GGE
Cpa 210 : GTG-EGESNKAPSPTKV---NYGDHTFQGR-KKALKPNVEMKS-PDGYTYRTDISHGRIVECEGD-IV-GSAE---RNTYAQRVVEGKI-RLP-----DDGGHLLIAGCFRGLKT-IDNLVQONSCINRS---GGK
Sha 416 : GTG-N-TSIKIKEISEV---NYGDHTFQGRGRRLTPNVRVVI-EDGMYKTI-DELGRITDVEADNLI-QEAD---RNLGMQRAVAREL-RLP-----DDGGHLLIGSCFNGSGE-IDNLVAQNSQINRS---GGQ
Strep 448 : -----VSKAGKGSVDNIIRDGSHFDE-VGRLKPNVYKQGEFFENLQIDGLRITDWNAS--E-QLTE-----RNLGRLSHDSSTPG-KLP-----GHAGHLLIAGDRFEGGSE-IDNLVSQLSIVNL----SD

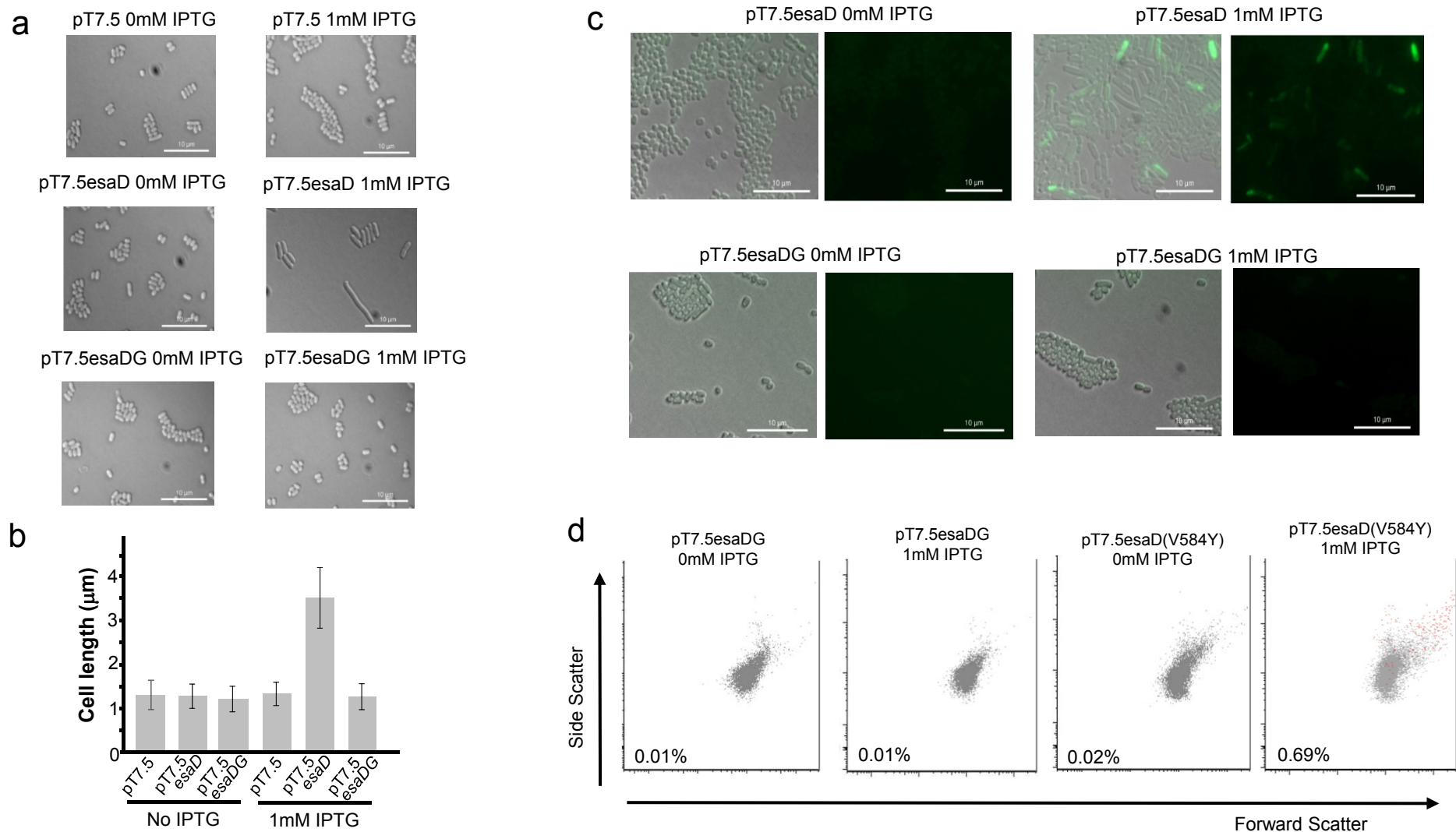
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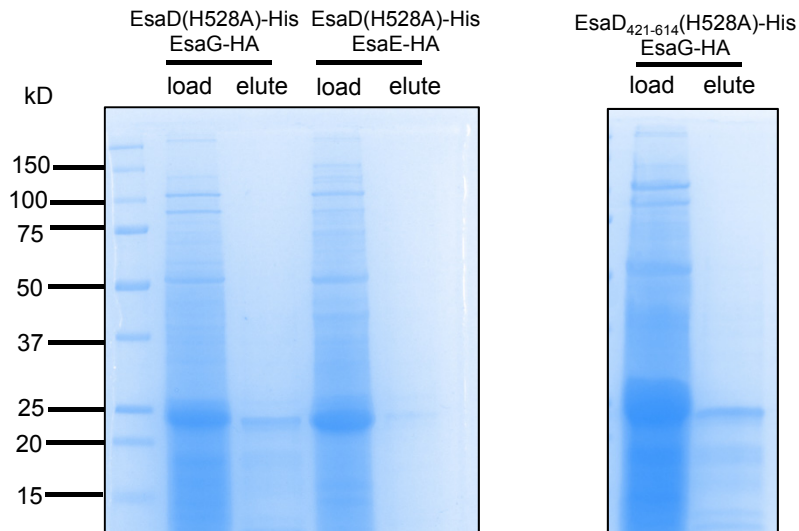
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Sau_EssD 560 : WYN-----LQKELQEFNSGKE-VKNIKMEVVRKSGNSQRETIKRVEMDINGERNI--RRILNK-----
Bsu_YeeF 610 : WYE-----NEQELAKKLSKKPPKAVQIEPVIYSGDSLRESYELVITKIGSRKEI-SVSIKIQPGG-----
Sepi 566 : WYK-----LQKELQKELQKKEGKE-VKNIKMEVVRKSGDSQRETIQKRVEMDINNEKDI--KSIKMI-----
Ble 645 : YKM-----LQNTAKKLEAGK--TVEVKEPIYKSGNSKREDEEPIYKIDGRKES--EILTYTS-----
Bcl 551 : YKK-----LQNTAKKLEEGK--TVEVKEPKVMEGNSLRESSLDEEMDIDGRMRF--TSIGK-----
Bli 624 : WKK-----LQNTAKKLEKGR--TVEVKEIQPVYKGNSSQREASLEIEMKIGKREWE-VVKEDIKPGGK-----
Bha 505 : YRA-----LQRRTEQALKAVPPKDKVRIQPVYKSGDSHRETEKQVEEMKGNNEWI-LDRLEP-----
Bthu 411 : WKK-----LQNEVANAINDGD--KRVKKEIIPKNSGNSKREDSVIRKRGDEDRLKKNFDVPGGKLEDE-----
Bce 519 : WKK-----LQNEVANAINDGD--KRVKKEIIPKNSGNSKREDSVIRKRGDEDRLKKNFDVPGGKLEDE-----
Bpu 583 : WYQ-----NEQELLSLQKVEVPEKSVYIEAVYHSDSLRESAELVYKRIGESTDY-I-YIKNEYGG-----
Afl 437 : YKS-----LQNTAKKLEEGK--TVEVKEPIYKSGDSRFAKEDVEMKIDGKKYE-VTLT-VYAGGK-----
Cle 666 : WYK-----LQKRLADAKKESG--SNNVNTPIYEGCVRESVVRVDMIDGKIS-KKIINP-----
Cpa 326 : WFE-----LQTEVANAQKIEPPKKTIVRIQDPVYSGDSMRDELKRVTHQDNKRAV-TKRILIQPGG-----
Sha 533 : WYK-----LQTEVANAQKIEPPKKTIVRIQDPVYSGDSMRDELKRVTHQDNKRAV-TKRILIQPGG-----
Strep 556 : YKK-----LQNTAKKLEEGK--TVEVKEPKVMEGNSLRESSLDEEMDIDGFFFS--RRIKK-----

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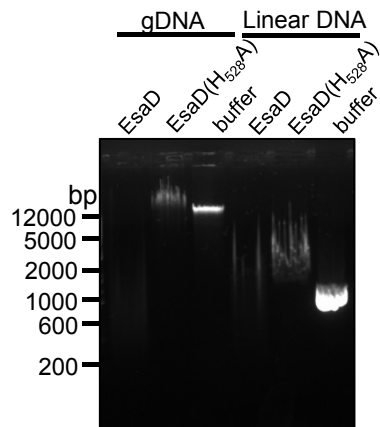
Supplementary Figure 2. EsaD has a predicted nuclease domain at its C-terminus. Alignment of *S. aureus* EsaD with Orthologs identified using BlastP¹⁰. Multiple sequence alignment was generated using Clustal Omega¹¹ with default settings. The *Streptococcus pyogenes* Spd1 protein, for which nuclease activity has been described¹², was included as a reference for the Endonuclease_NS_2 family (PFAM 13930). The position of the catalytically active Histidine 121 in Spd1 (H528 for EsaD) is marked with an asterisk. The position of EsaD V584 that was substituted to a tyrosine in this work is indicated by a red arrow. Abbreviations: Afl, *Anoxybacillus flavithermus*; Bce, *Bacillus cereus*; Bcl, *B. clausii*; Bha, *B. halodurans*; Ble, *B. lentocellum*; Bli, *B. licheniformis*; Bpu, *B. pumilus*; Bsu, *B. subtilis*; Cle, *Clostridium lentocellum*; Cpa, *Cohnella panacarvi*; Sau, *Staphylococcus aureus*; Sepi, *S. epidermidis*; Sha, *Sediminibacillus halophilus*; Spy, *Streptococcus pyogenes*; Strep, *Streptococcus sp.*



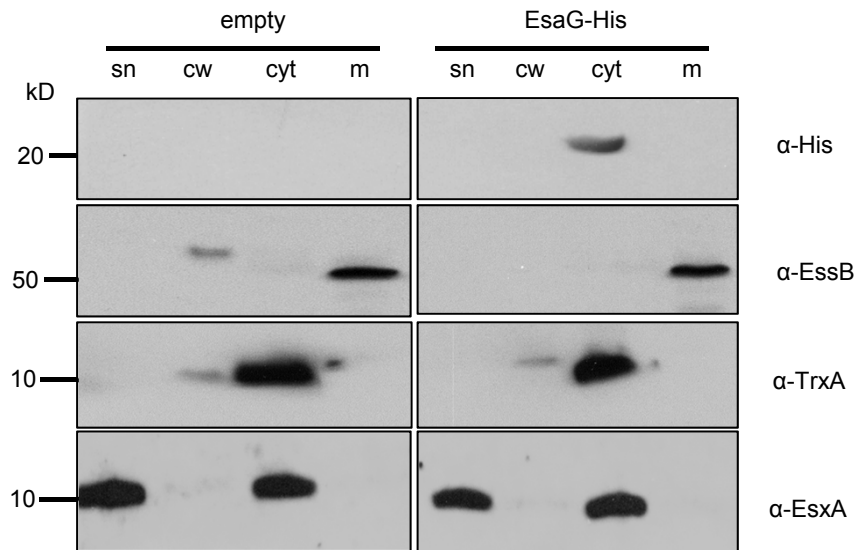
Supplementary Figure 3. Microscopy analysis of *E. coli* cells producing EsaD. *E. coli* strain BL21(DE3) harbouring plasmids pT7.5, pT7.5esaD(V584Y) or pT7.5esaDG was cultured in LB at 37°C to OD₆₀₀ 0.5, an aliquot of cells was removed and the remainder of the sample was supplemented with 1 mM IPTG for a further three hours. **a.** Cells were analysed by light microscopy and **b.** The length of the cells was measured ($n=300$) and the mean length (+/- one standard deviation) is shown. $p < 0.001$ comparing length of cells harbouring pT7.5esaD(V584Y) in the presence and the absence of IPTG induction. or **c.** Cells were treated with the deadend fluorometric TUNEL system kit (Promega) and analysed by fluorescence light microscopy with a FITC filter. **d.** EsaD causes DNA damage. BL21(DE3) harbouring the indicated plasmids were cultured as in **a.**, and cells were harvested prior to IPTG supplementation (0mM IPTG) and after three hour treatment with 1mM IPTG, deoxynucleotidyl transferase dUTP nick end labelled and fluorescence-positive cells quantified by flow cytometry. The percentage of total cells that are scored as fluorescence positive is given in the bottom left-hand corner of each panel.



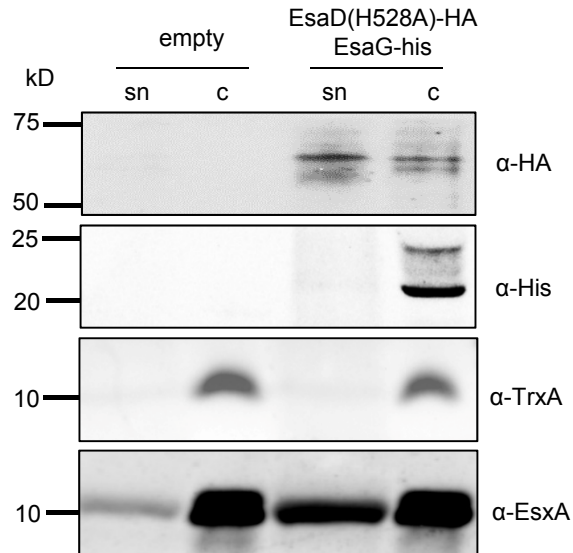
Supplementary Figure 4. Analysis of EsaD(H528A)-His-EsaG-HA, EsaD(H528A)-His-EsaE-HA and EsaD₄₂₁₋₆₁₄(H528A)-His-EsaG-HA co-purification by coomassie staining. The *S. aureus* wild type strain, RN6390, carrying EsaD(H528A)-His-EsaG-HA, pRAB11-EsaD(H528A)-His-EsaE-HA or pRAB11-EsaD(H528A)(421-614)-His-EsaG-HA was cultured in TSB medium to OD₆₀₀ of 0.5 supplemented with ATC (500ng/ml) and harvested at OD₆₀₀ of 3. Cells were lysed and histidine-tagged EsaD(H528A) was purified using nickel affinity beads. The cell lysate (load) and eluted fractions (20µl aliquots of each) were separated by SDS PAGE (12%) and stained with coomassie instant blue. The far left hand lane is the molecular weight marker used in the experiment. Note that these correspond to the same fractions used for western blotting in Fig 2c, Fig 2d and Fig 3b.



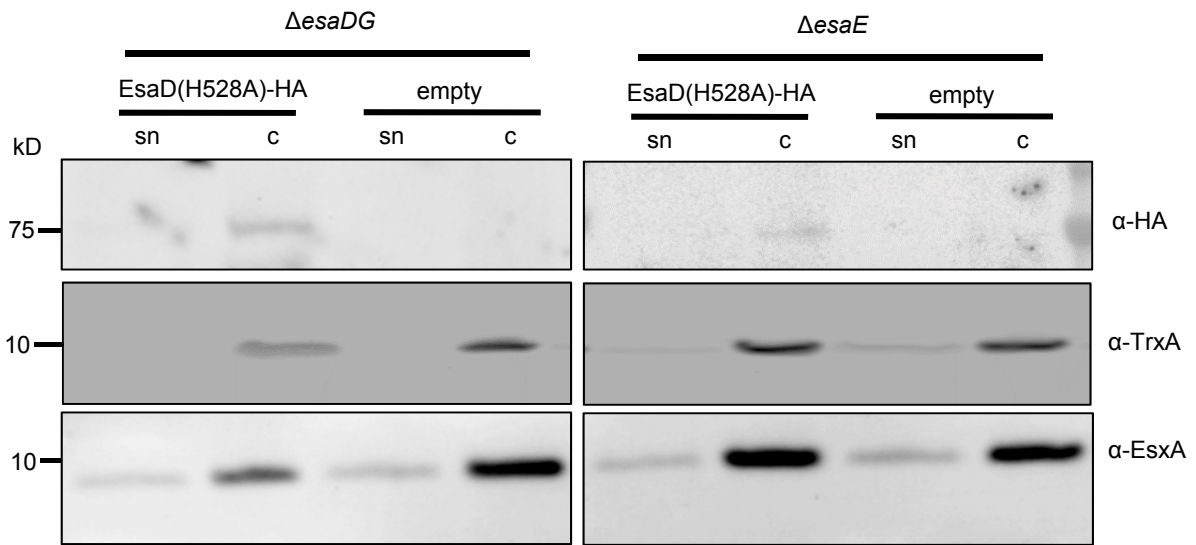
Supplementary Figure 5. EsaD is a Mg²⁺-dependent DNase. 200ng of *S. aureus* gDNA or 400ng of a linear DNA PCR product were incubated with 0.4 µg purified EsaD₄₂₁₋₆₁₄-His, EsaD₄₂₁₋₆₁₄(H528A)-His or an equivalent volume of elution buffer, each supplemented with 50 mM MgCl₂ in a final volume of 20 µl at 37 °C for 20 mins after which the DNA was analysed by 1% agarose gel electrophoresis.



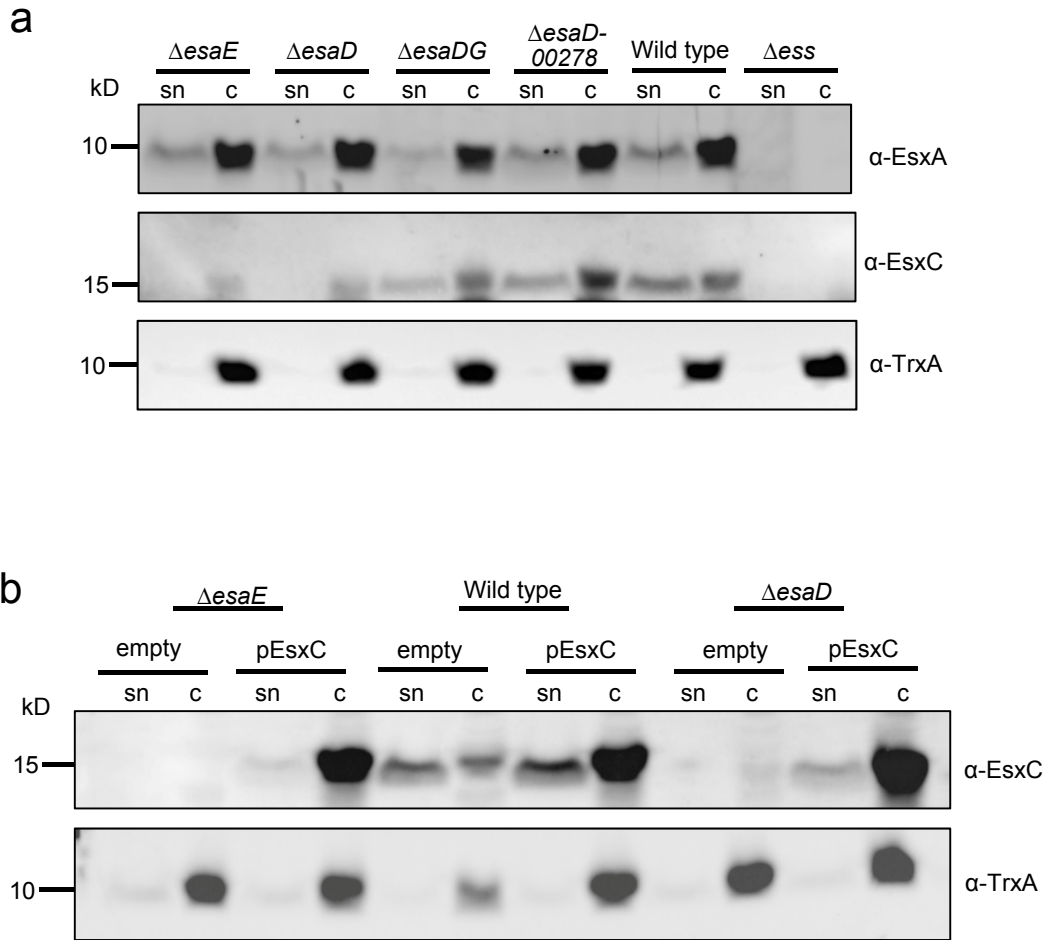
Supplementary Figure 6. EsaG is a cytoplasmic protein. The *S. aureus* wild type strain, RN6390, harbouring pRAB11 (empty) or pRAB11-EsaG-His was cultured in TSB medium to OD₆₀₀ of 0.5, supplemented with ATC (250ng/ml) and harvested at OD₆₀₀ of 2. A sample of the supernatant (sn) was retained as the secreted protein fraction and cells were fractionated into cell wall (cw), cytoplasmic (cyt) and membrane (m) fractions. Samples of each fraction (20µl aliquot of sn, cw, and cyt, 2mg of membrane) were separated on 12% bis-Tris gels and immunoblotted using either anti-His, anti-EssB (membrane protein control), anti-EsxA or anti-TrxA (cytoplasmic control) antisera.



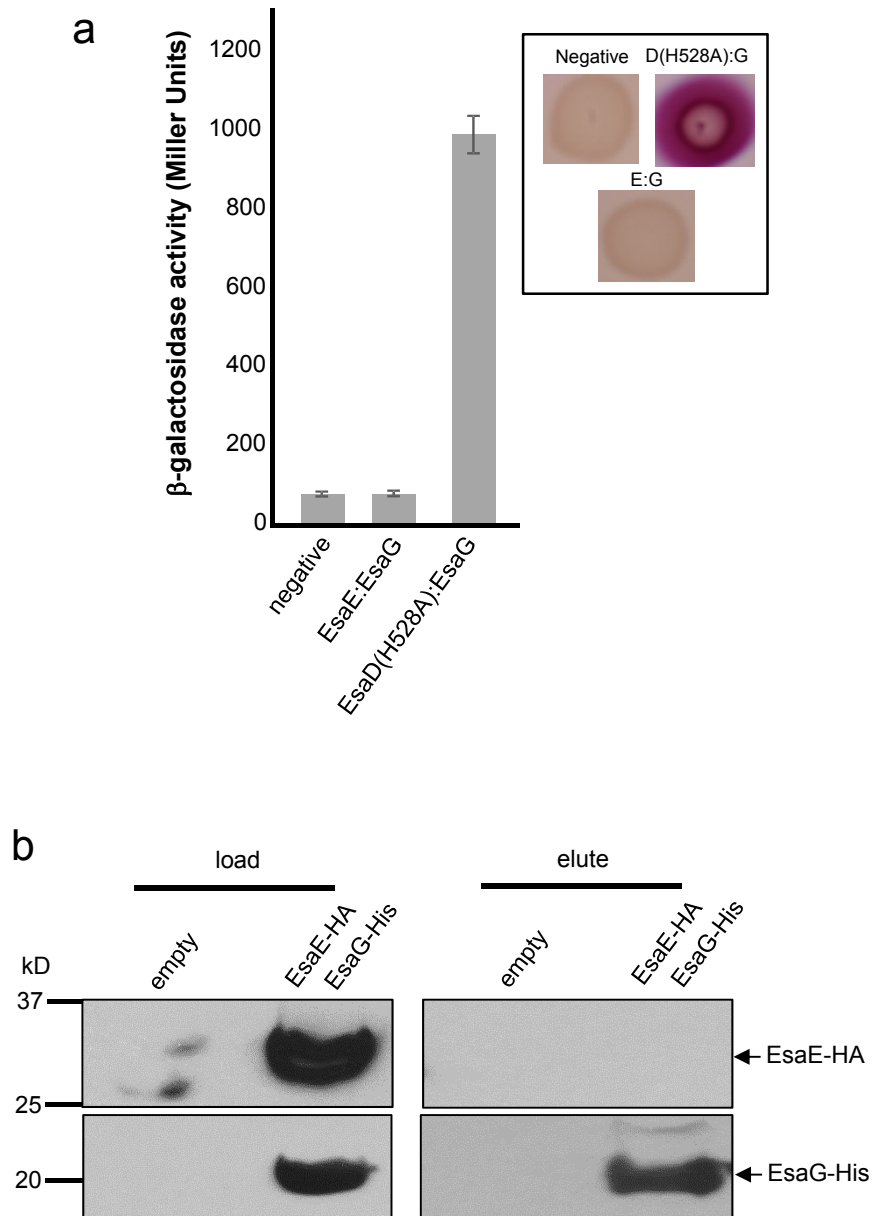
Supplementary Figure 7. EsaG is not co-secreted with EsaD. The *S. aureus* wild type strain, RN6390, harbouring pRAB11 (empty) or pRAB11-EsaD(H528A)-HA-EsaG-His were cultured in TSB medium to OD_{600} of 0.5, supplemented with ATC (250ng/ml) and harvested at OD_{600} of 2. Samples of the supernatant (sn) and cellular (c) fractions (an equivalent of 250 μ l of supernatant and 10 μ l of cells adjusted to OD_{600} of 1) were separated on 12% bis-Tris gels and immunoblotted using the indicated antisera.



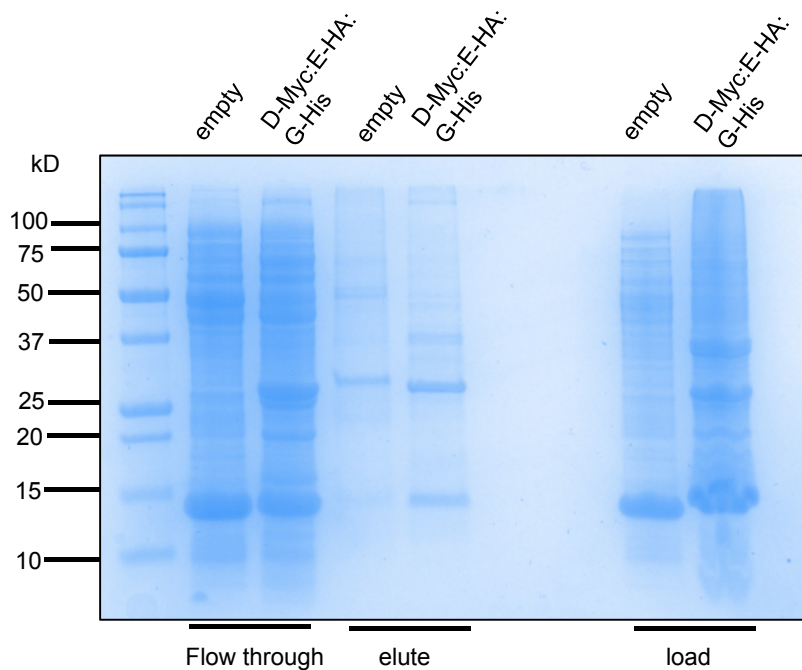
Supplementary Figure 8. EsaD-HA is not detected in the supernatant when *esaE* or *esaG* are deleted. The indicated *S. aureus* strains harbouring pRAB11 (empty) or pRAB11-EsaD(H528A)-HA were cultured in TSB medium until to OD₆₀₀ of 0.5, supplemented with ATC (250ng/ml) and harvested at OD₆₀₀ of 2. Samples of the supernatant (sn) and cellular (c) fractions (an equivalent of 250μl of supernatant and 10μl of cells adjusted to OD₆₀₀ of 1) were separated on 12% bis-Tris gels and immunoblotted using the indicated antisera.



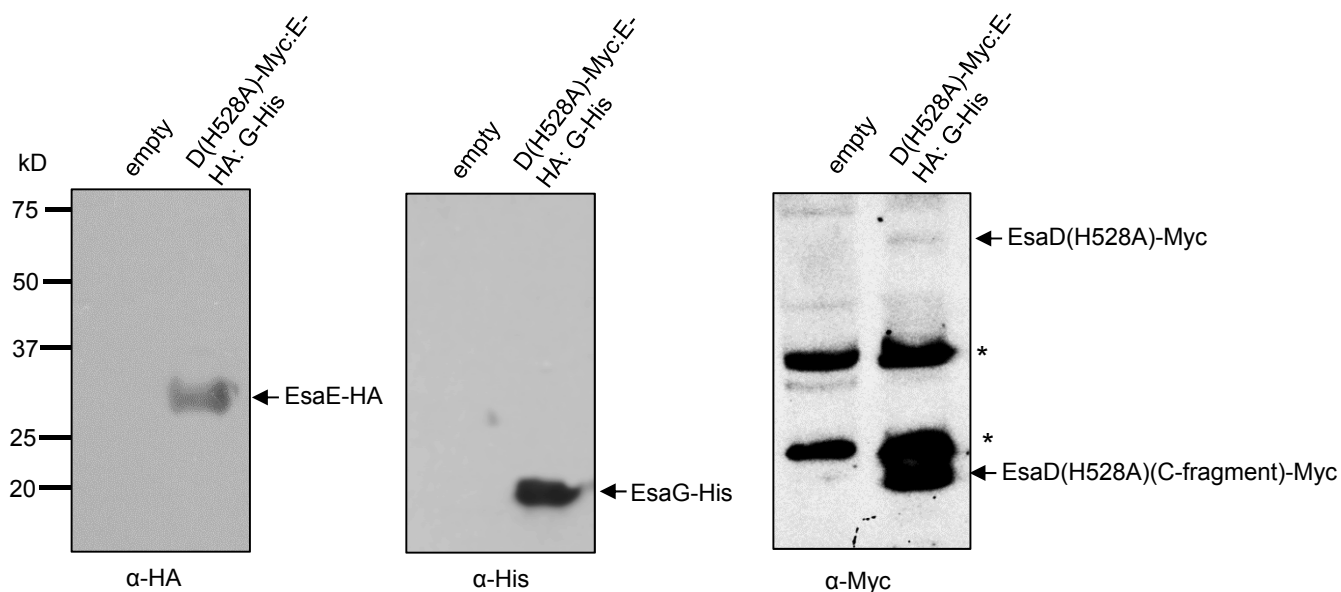
Supplementary Figure 9. EsaD and EsaG proteins are not required for the secretion of EsxA and EsxC. a. The RN6390 wild-type or isogenic deletion strains, as indicated, were cultured in TSB medium or b. The indicated strains harbouring pRAB11 (empty) or pRAB11-EsxC were cultured in TSB medium until an OD₆₀₀ of 0.5 and supplemented with ATC (50ng/ml). a. and b. When cultures reached OD₆₀₀ of 2, cells were spun down and the supernatant (sn) was retained as the secreted protein fraction, while the pellet was retained as the cellular fraction. Samples of the supernatant and cellular fractions (an equivalent of 200μl of supernatant and 10μl of cells adjusted to OD₆₀₀ of 1) were separated on 12 % bis-Tris gels and immunoblotted with the indicated antisera.



Supplementary Figure 10. EsaE and EsaG do not interact with each other. a. Bacterial two-hybrid analysis of strain BTH101 harbouring pT25 and pT18 (negative), pT25-EsaE and pT18-EsaG, or pT25-EsaG and pT18-EsaD(H528A) assessed by β -galactosidase activity assay. Error bars represent the standard deviation ($n=3$ biological replicates). The inset shows the same strain and plasmid combinations scored on MacConkey maltose plates. b. *E. coli* strain M15[pRep4] carrying pQE70 (empty) or pQE70-EsaE-HA-EsaG-His was cultured in LB medium to OD₆₀₀ of 0.5, and then supplemented with 2 mM IPTG for 4 hours, after which cells were harvested and lysed. His-tagged EsaG was purified using Ni-affinity beads, and in each case the elution fractions (10 μ l aliquots of each) were separated by SDS PAGE (12%) and analysed by western blot with anti-HA or anti-His antibodies.



Supplementary Figure 11. Analysis of EsaE-HA-EsaD(H528A)-Myc-EsaG-His co-purification by coomassie staining. *E. coli* M15[pRep4] carrying pQE70 (empty) or pQE70- EsaE-HA-EsaD(H528A)-Myc-EsaG-His was cultured, lysed and EsaE-HA was purified using anti-HA antibody-coupled agarose beads as described in Methods and in the legend to Fig 3. The same samples (10 μ l aliquots of each) as those analysed in Fig 3f were stained using coomassie instant blue following SDS PAGE (12% bis-Tris gel). The far left hand lane is the molecular weight marker used in the experiment and the lanes between the load and elute fractions were left empty.



Supplementary Figure 12. EsaD, EsaE and EsaG form a ternary complex. *E. coli* strain M15[pRep4] carrying pQE70 (empty) or pQE70-EsaE-HA-EsaD(H528A)-Myc-EsaG-His was cultured in LB medium to OD_{600} of 0.5, and then supplemented with 2 mM IPTG for 4 hours, after which cells were harvested and lysed. His-tagged EsaG was purified using Ni-affinity beads, and 20 μ l aliquots the elution fractions (of each) were separated by SDS PAGE (12%) and analysed by western blot with the indicated antisera. Non-specific bands that cross-react with the anti-Myc antibody are indicated with asterisks.

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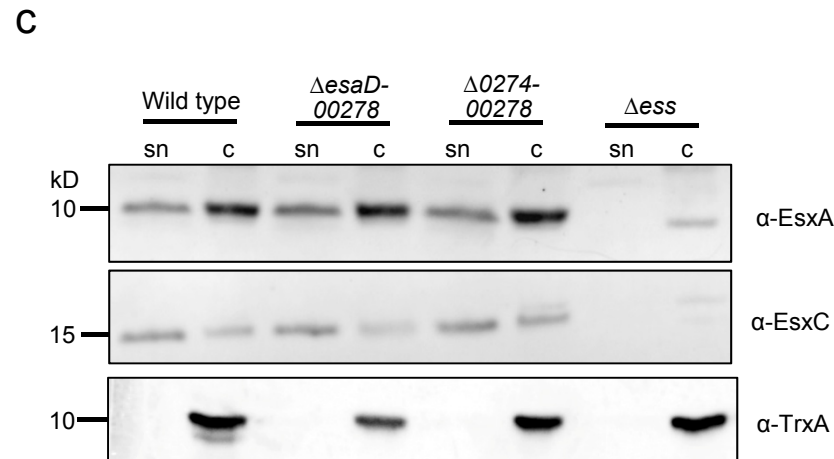
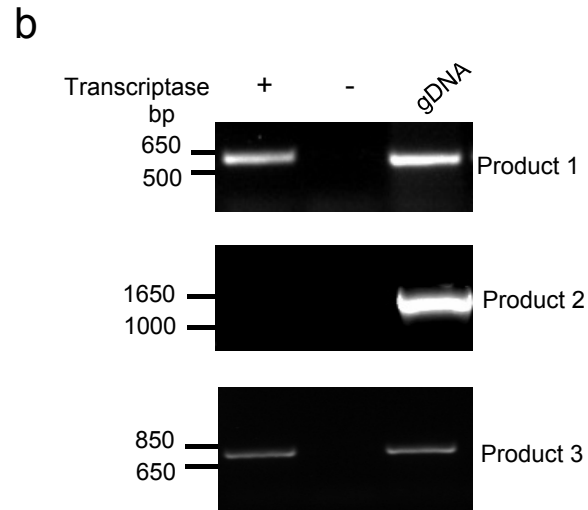
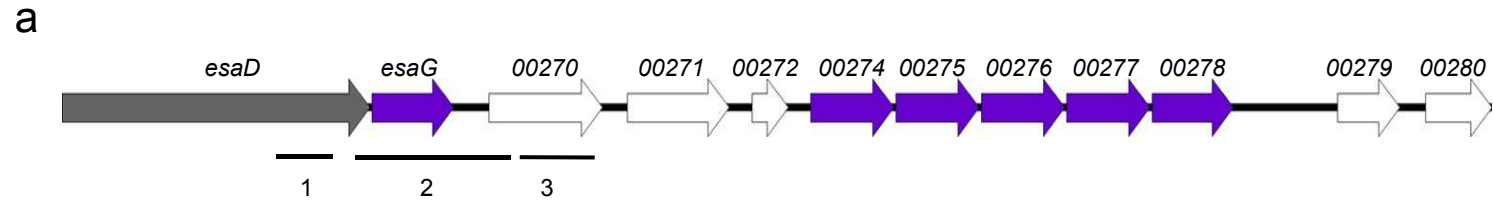
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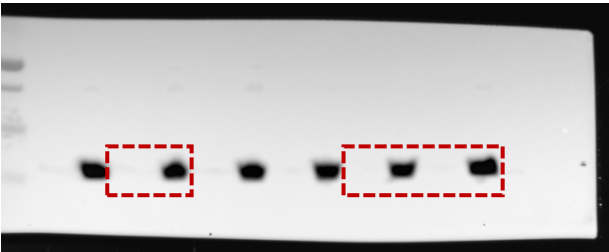
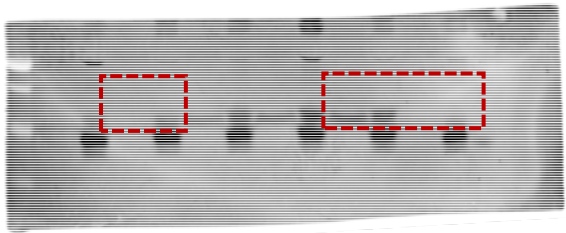
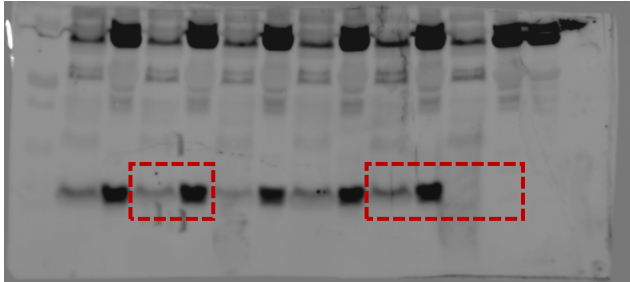
Supplementary Figure 13. Sequence analysis of EsaD proteins shows variability within the C-terminal nuclease domain. EsaD protein sequences from *S. aureus* subsp. *aureus* strains were extracted from the KEGG Genome database (www.genome.jp/kegg/genome.html) and aligned using Clustal Omega on standard settings. Redundant sequences (100% identity) were omitted for clarity.



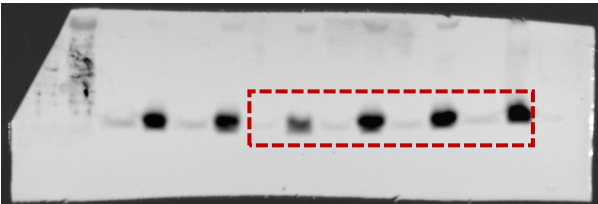
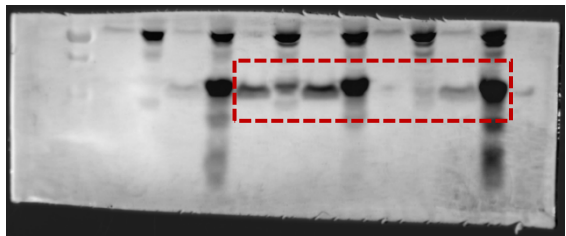
Supplementary Figure 14. Genes encoding additional DUF600 domain proteins are not co-transcribed with *esaG* and are not required for EsxA and EsxC secretion. a. Genetic organisation at the 3' end of the *ess* locus in *S. aureus* strain NCTC8325, with genes encoding DUF600 proteins are shaded in purple. The regions amplified by primer pairs used for RT-PCR analysis (listed in Table S3) are indicated. b. RT-PCR analysis of mRNA isolated from *S. aureus* strain RN6390, using primer pairs. The expected sizes for PCR products 1-3 are 573, 1143 and 684 bp, respectively. c. The RN6390 wild-type or isogenic deletion strains, as indicated, were cultured in TSB medium to OD₆₀₀ of 2, cells (c) were harvested and the supernatant (sn) was retained as the secreted protein fraction. Samples of the supernatant and cellular fractions (an equivalent of 250 μl of supernatant and 10 μl of cells adjusted to OD₆₀₀ of 1) were separated on 12% bis-Tris gels and immunoblotted using either anti-EsxA, anti-EsxC or anti-TrxA antisera.

Supplementary Figure 15. Uncropped images of Figs. 1-4 and Supplementary Figs. 5-10, 12 and 14.

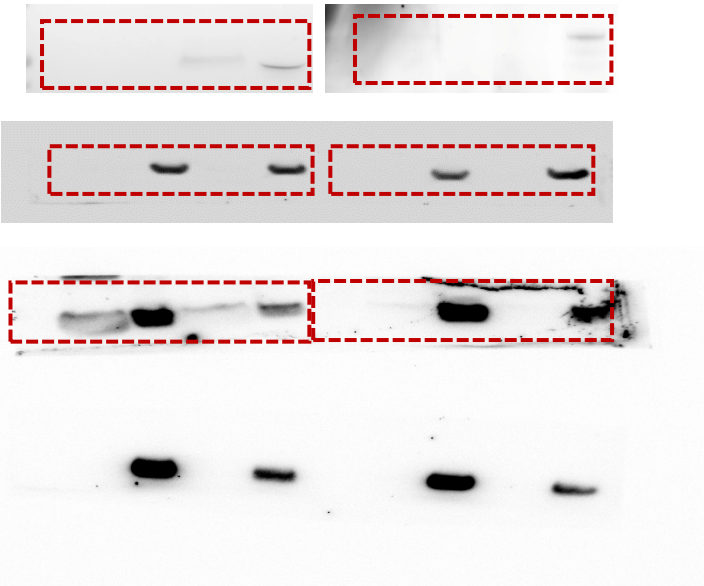
Uncropped Figure 1b



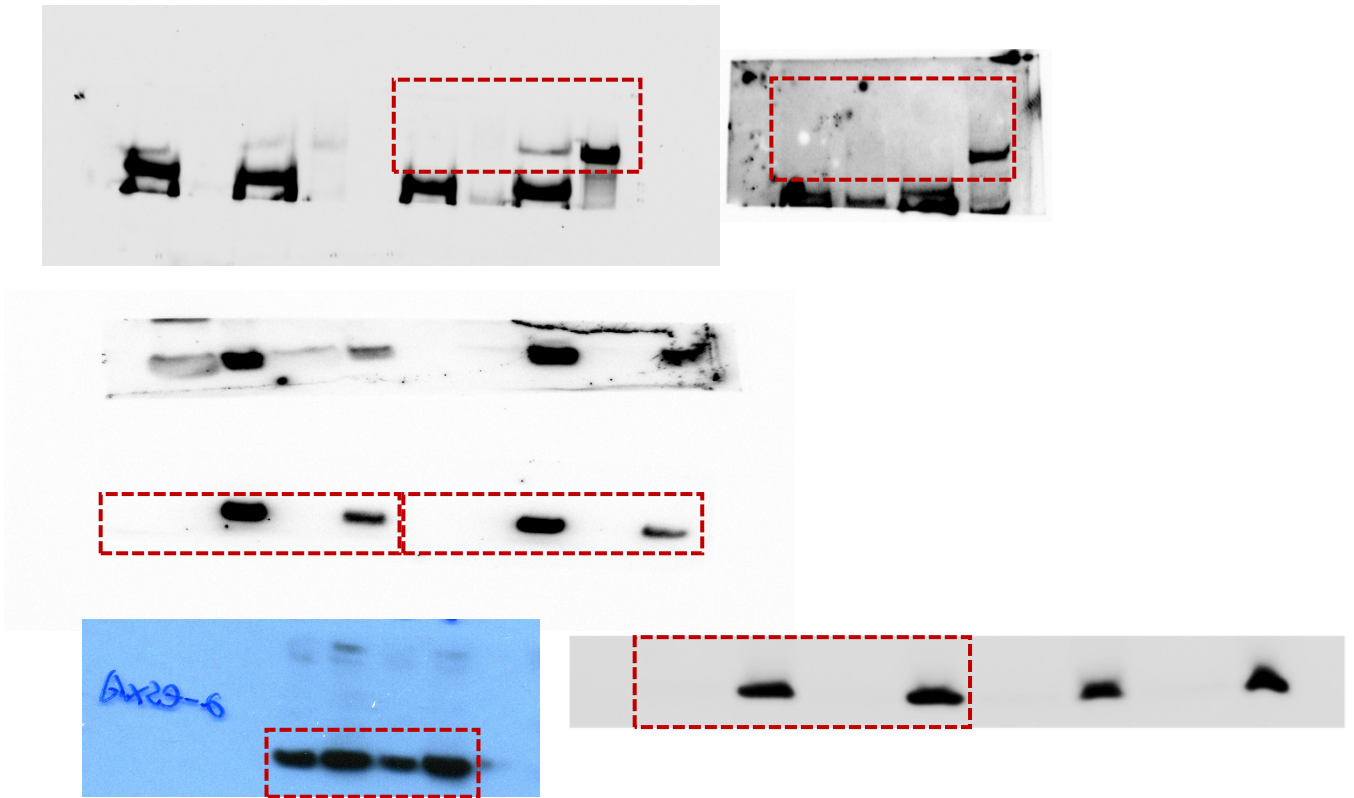
Uncropped Figure 1c



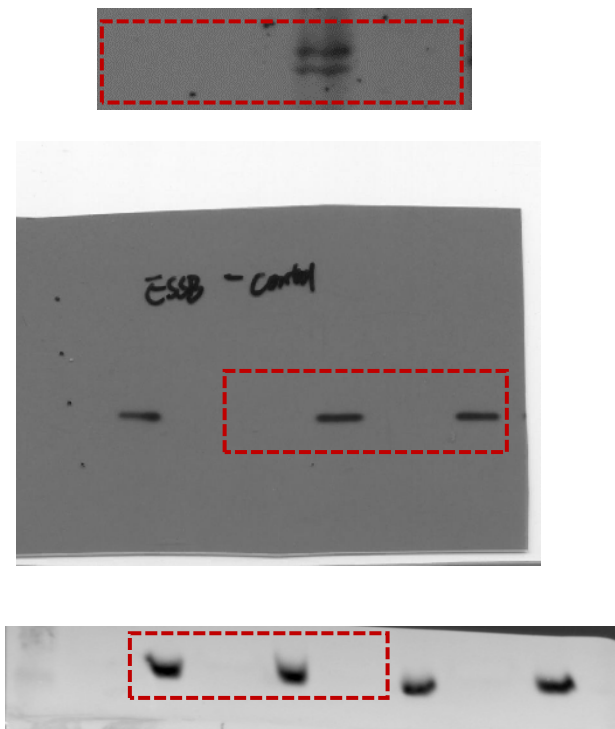
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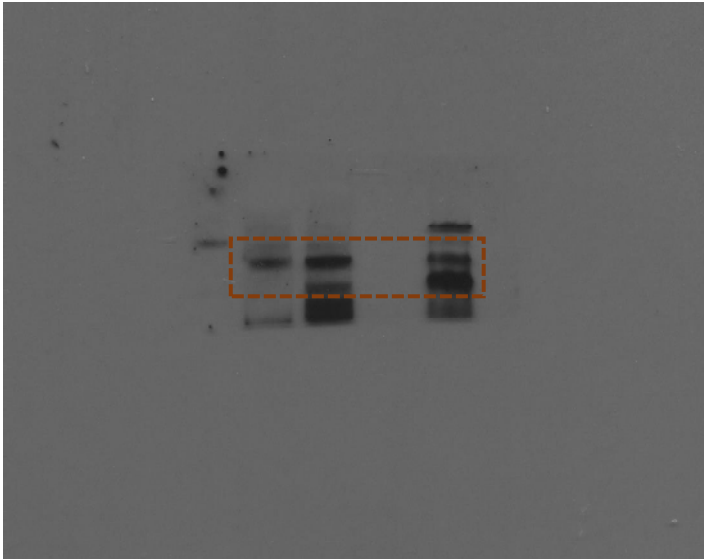
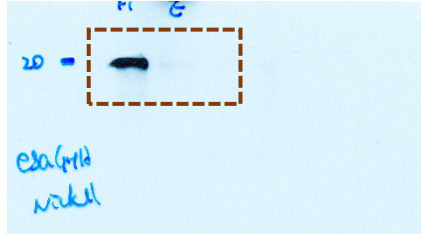
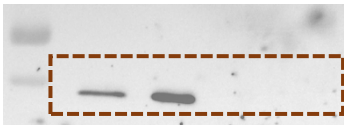
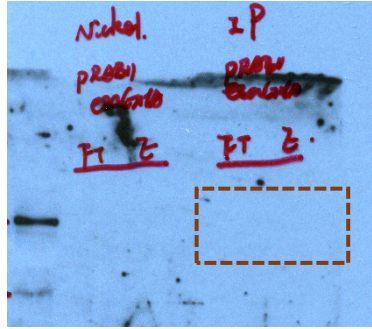
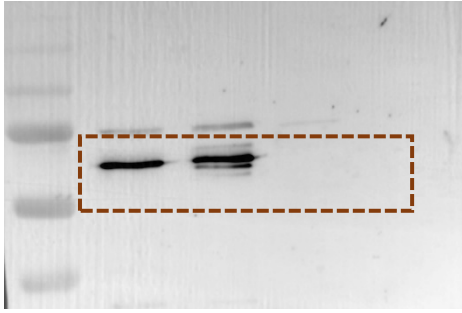
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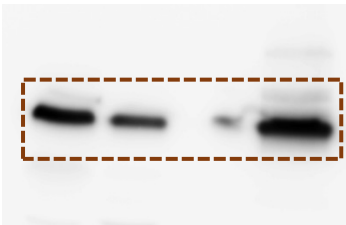
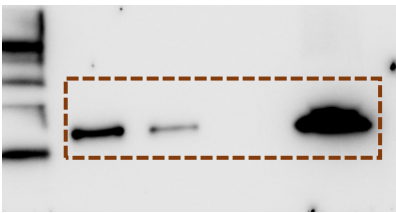
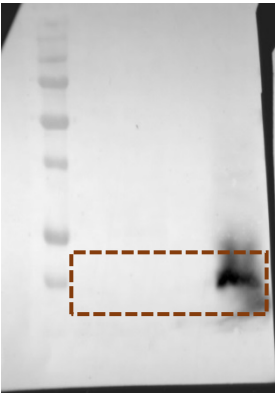
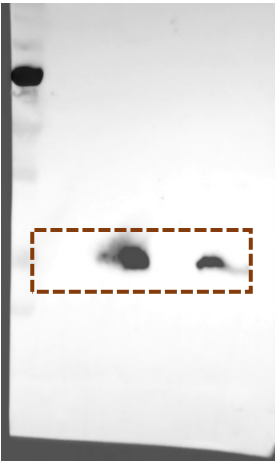
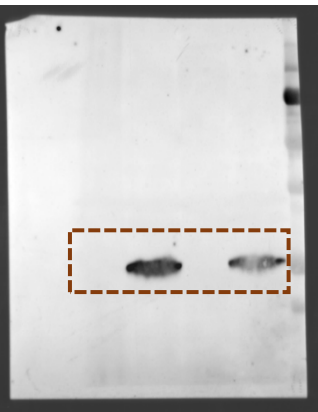
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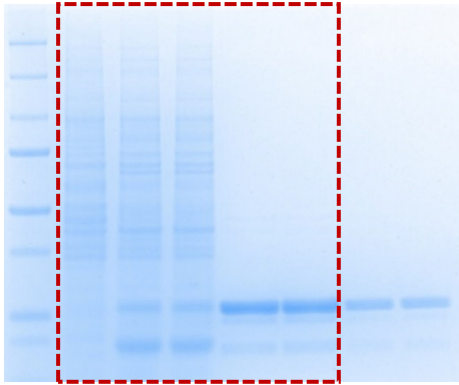
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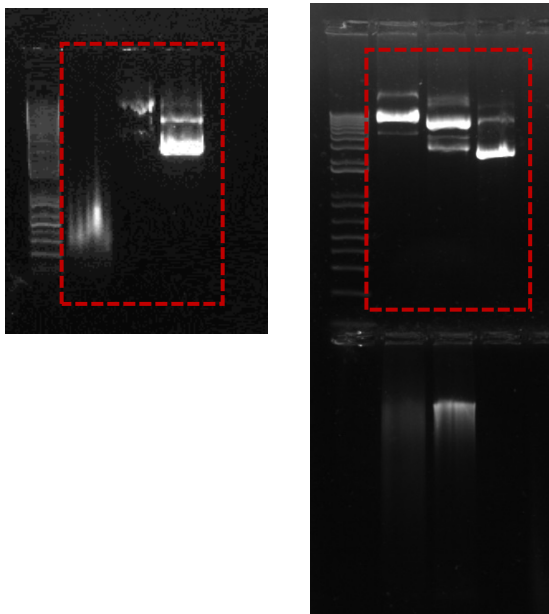
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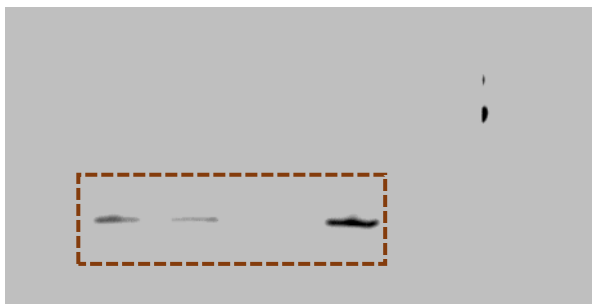
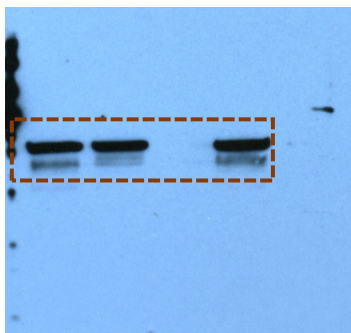
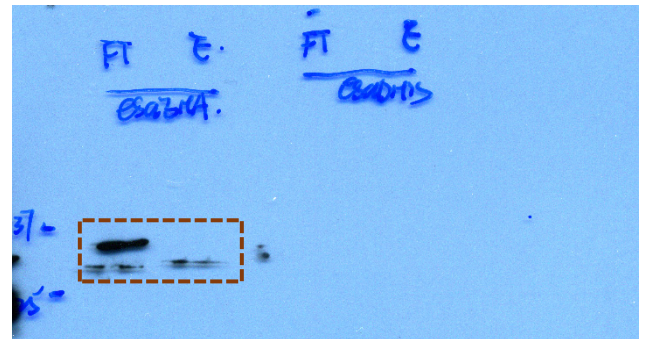
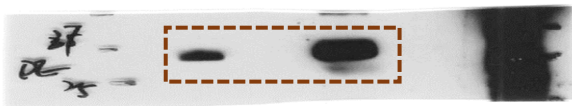
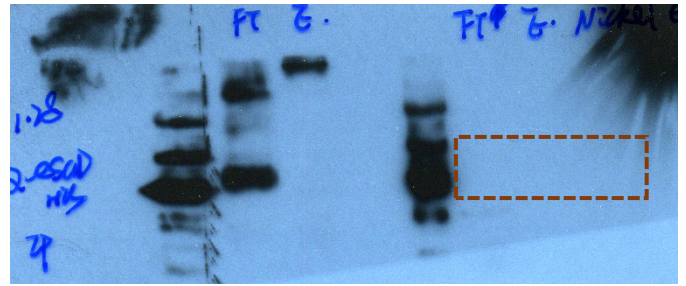
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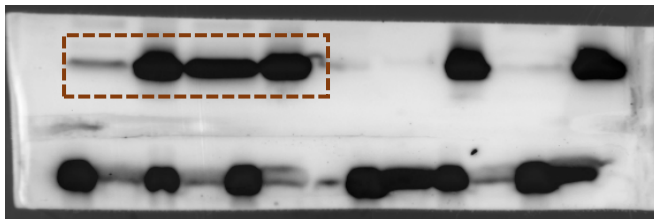
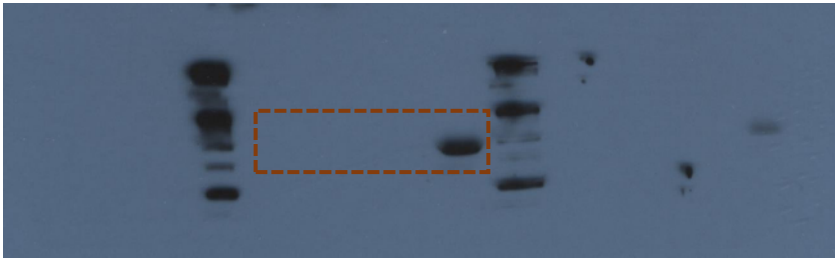
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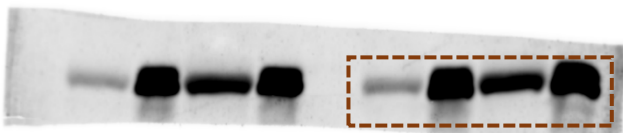
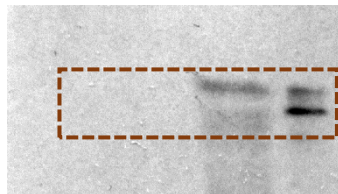
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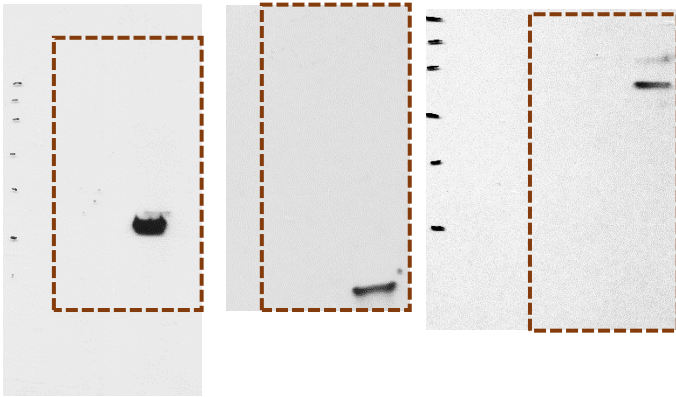
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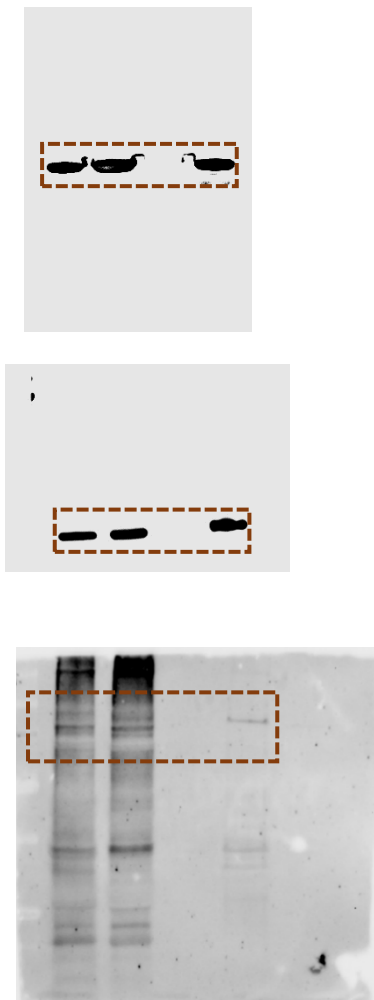
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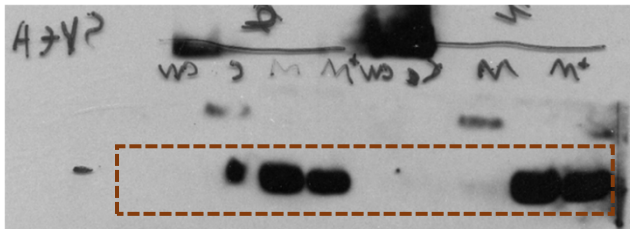
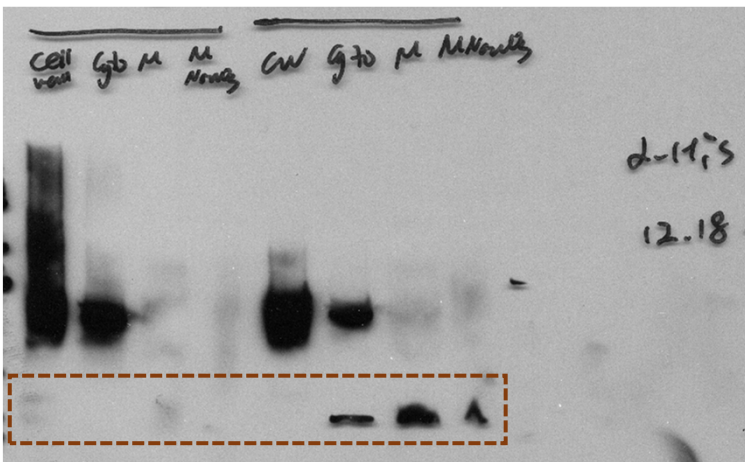
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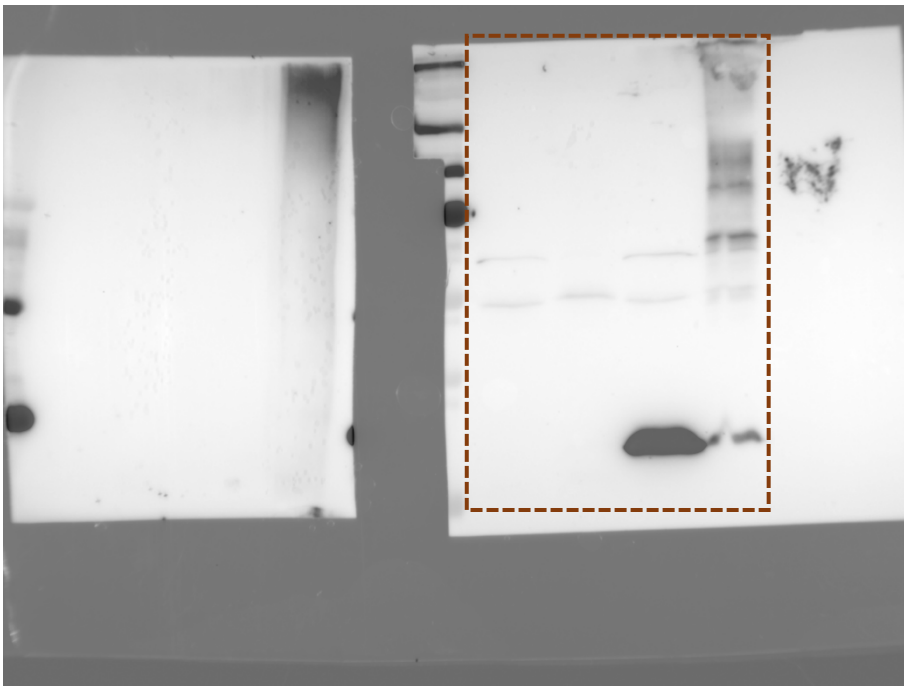
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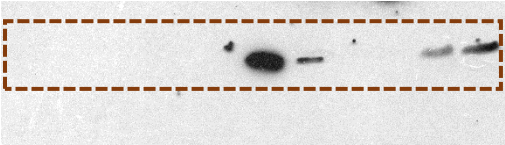
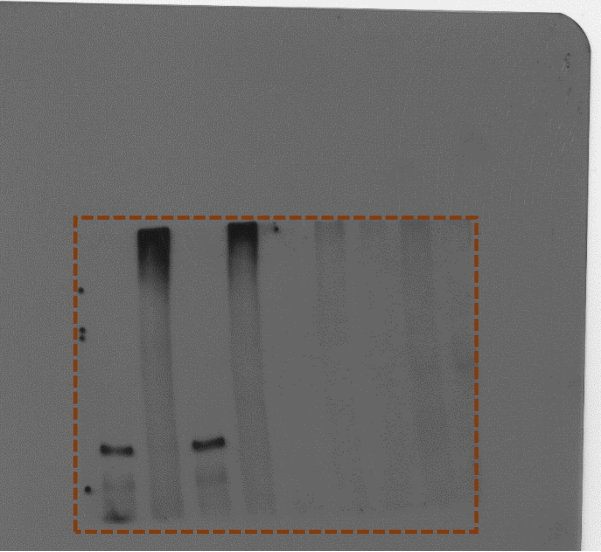
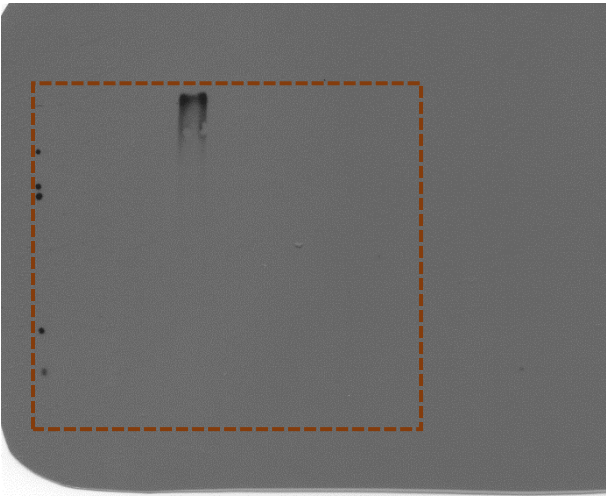
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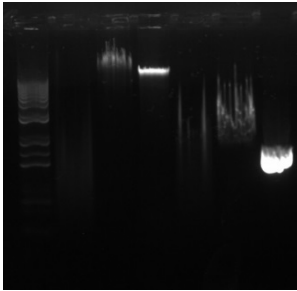
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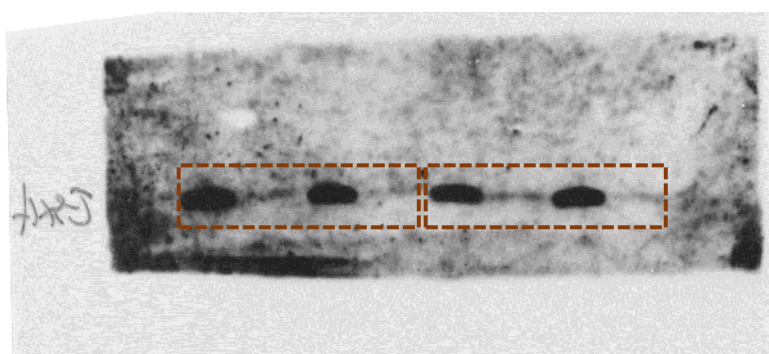
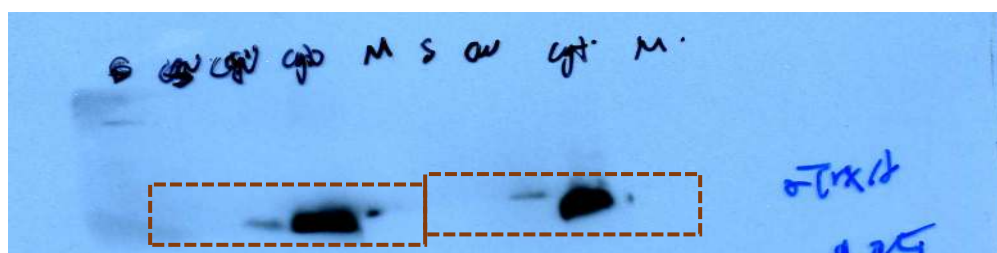
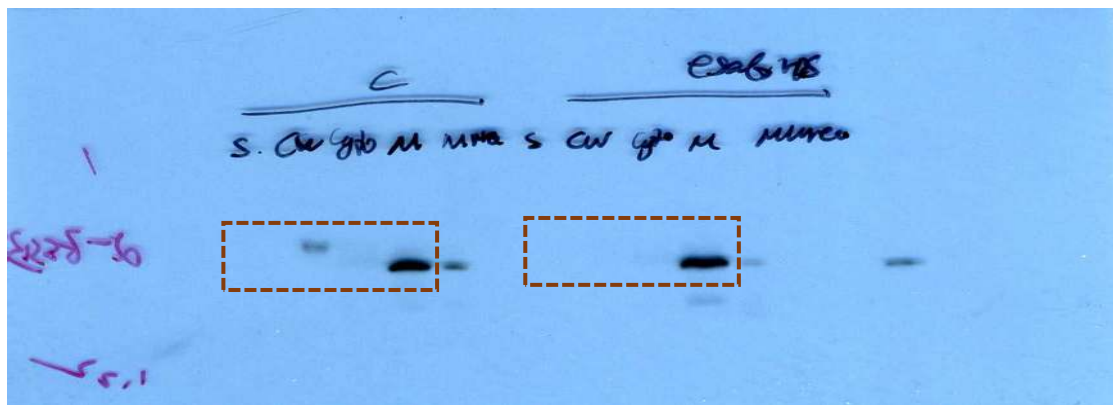
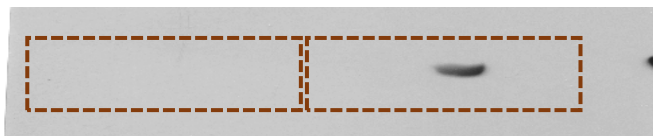
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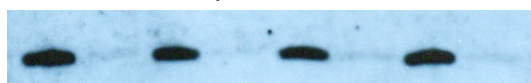
Uncropped Supplementary Figure 5



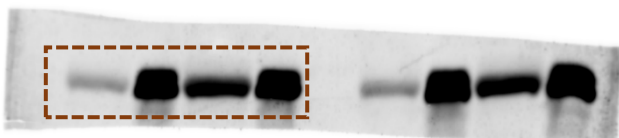
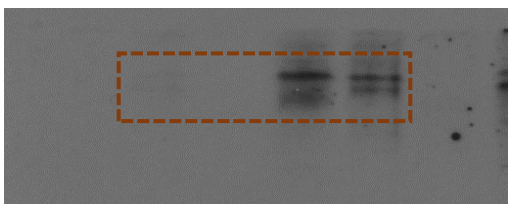
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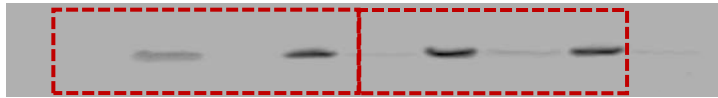
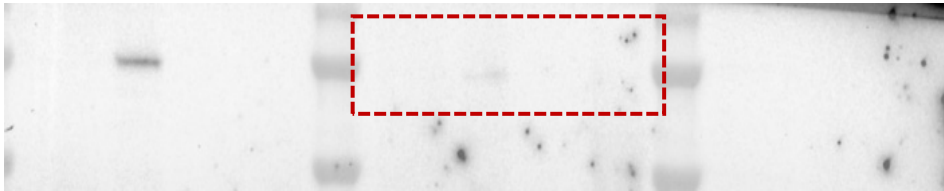
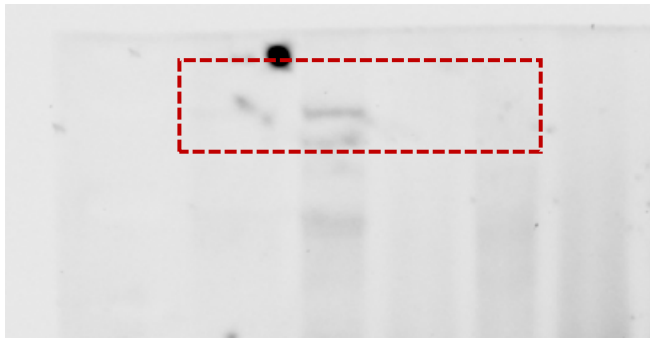
As a shorter exposure



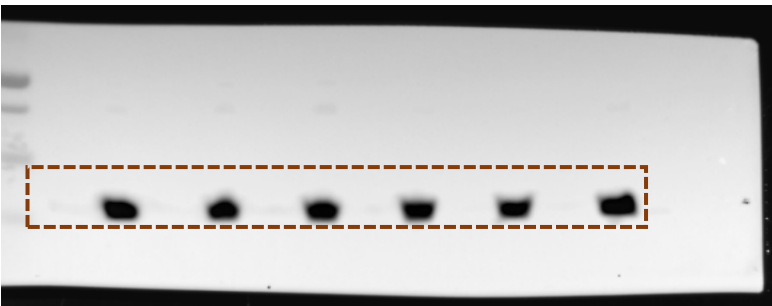
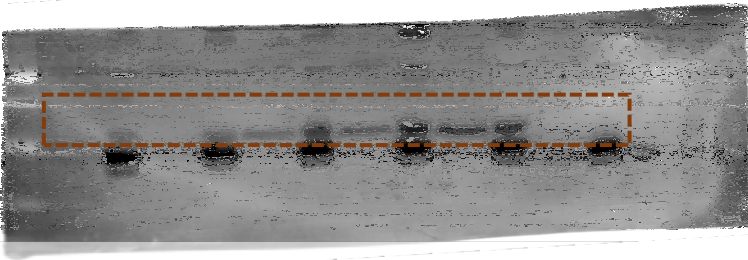
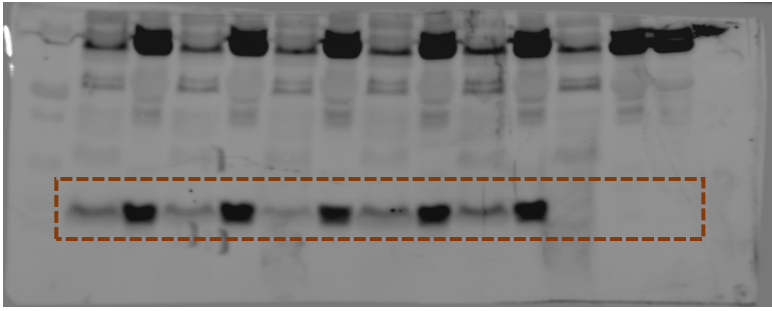
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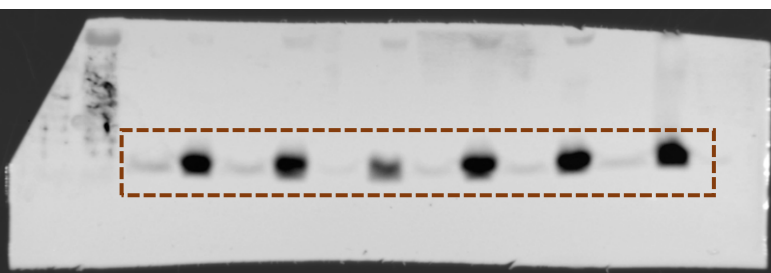
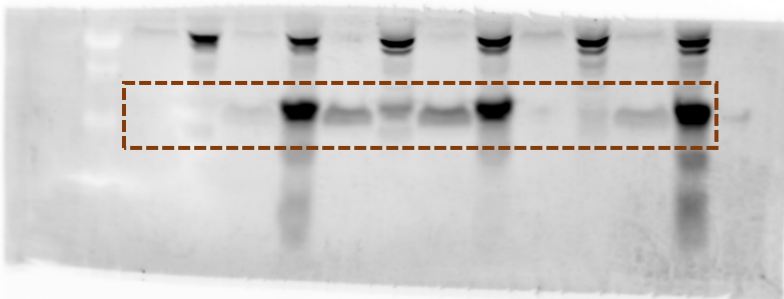
Uncropped Supplementary Figure 8



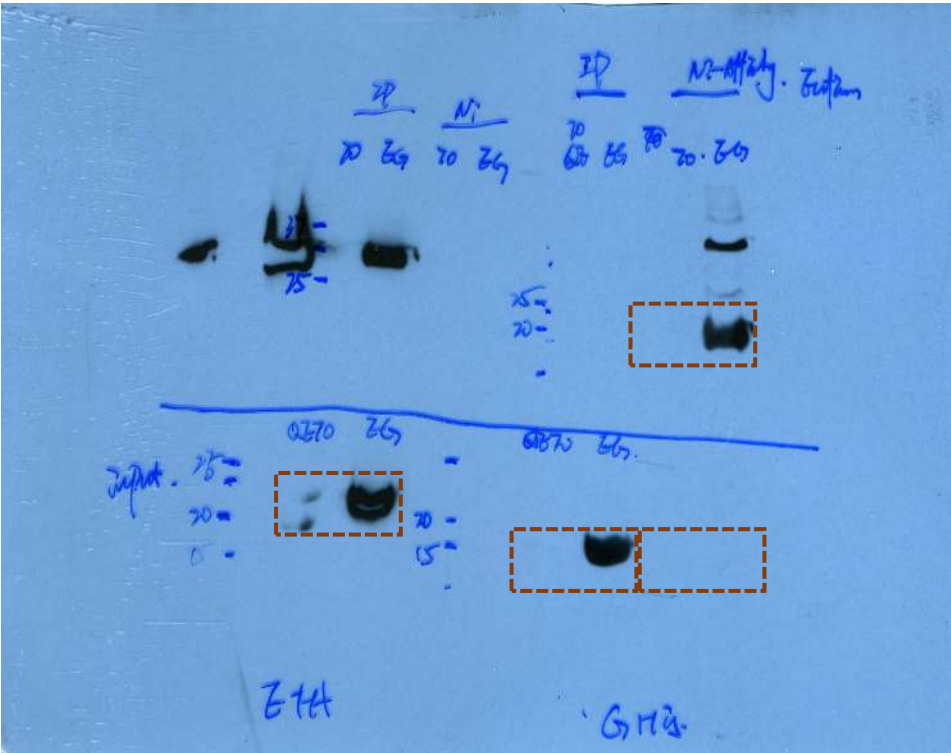
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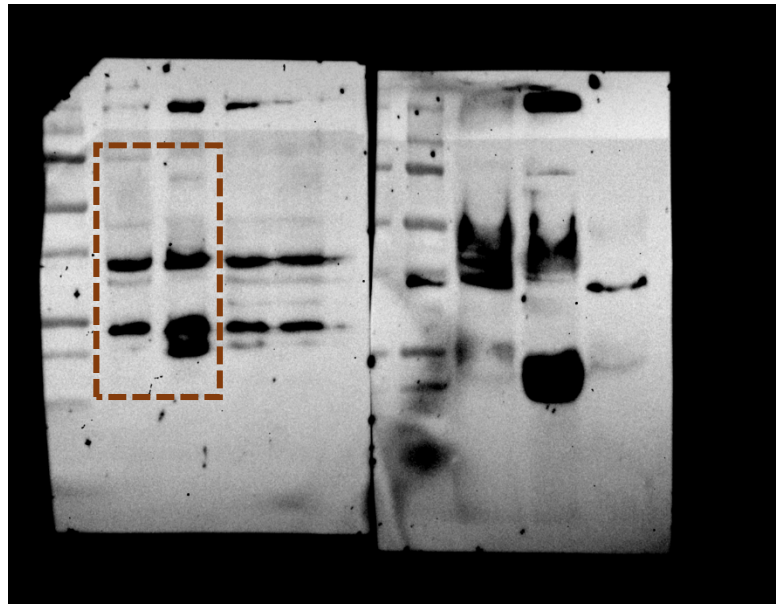
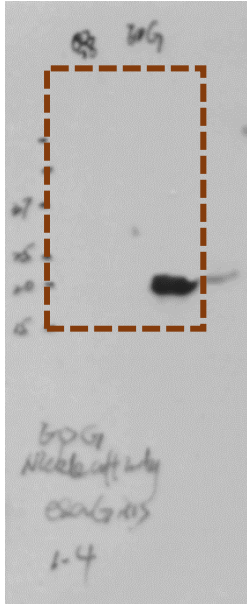
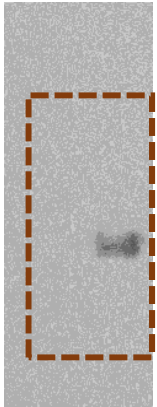
Uncropped Supplementary Figure 9b



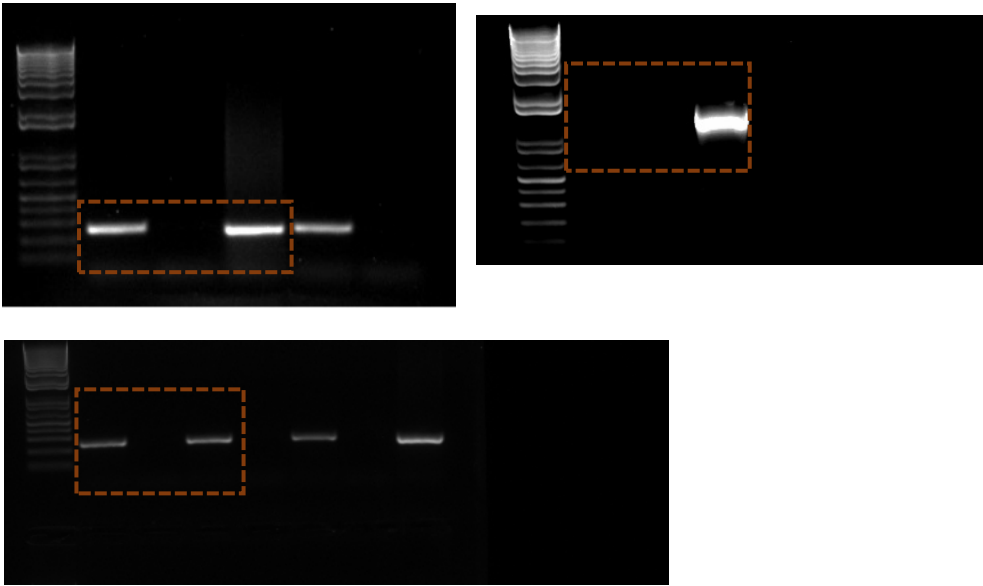
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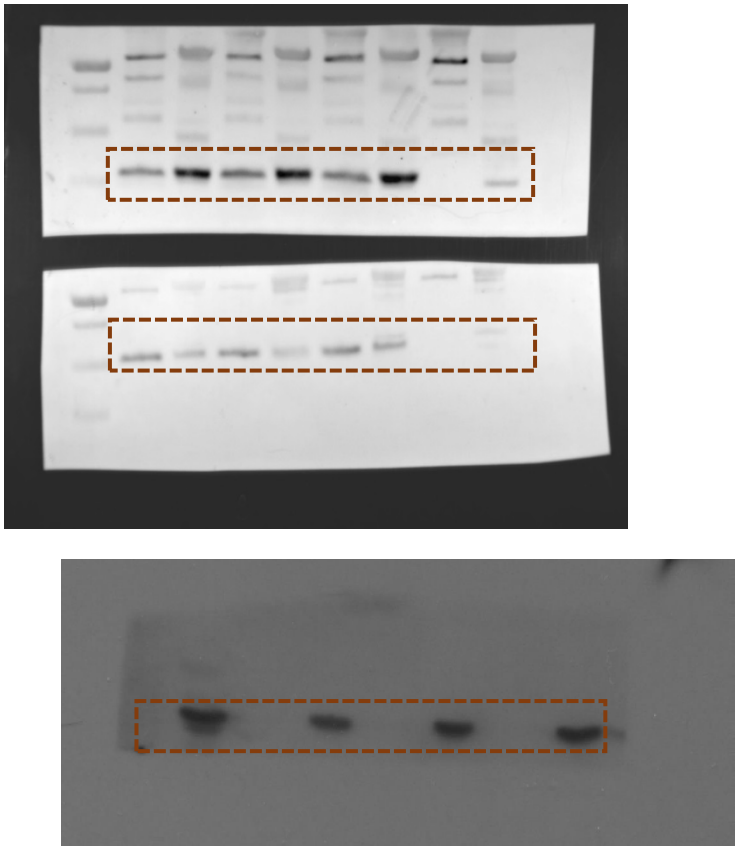
Uncropped Supplementary Figure 12



Uncropped Supplementary Figure 14b



Uncropped Supplementary Figure 14c



Supplementary Figure 16. Synthetic gene sequences used in this study.

Sequence of insert in pQE70 EsaE-HA-EsaD-Myc-EsaG-His

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*ACTCTGAATTCGGTCAGATCGGTCGGTCAGAACTACTACAAATACCGTAAATTCGGTATC
CTGCCGAAACCGAATACGAAATCAACAAAGTTAAAGAAATCGAACAGTACATCAAAGA
ACTGGAAAGATCT*

Restriction sites are underlined, genes (*esaE*, *esaD* and *esaG*) are shown in italics and added tags (HA, Myc) are double underlined. Note that the C-terminal His-tag on *EsaG* is supplied by the vector.

Synthetic pT25-DUF600 constructs

pT25-00274

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CATGCGAATTTGACTTTACAAGAGATGGCAAATTGAATGTATCTTTTGATTATATAGATT
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pT25-00275

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pT25-SAPIG0311

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pT25-SAPIG0314

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pT25-SAR0293

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pT25-SAR0294

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pT25-SAR0295

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pT25-SAR0297

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pT25-SAEMRSA15_02570

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pT25-SAEMRSA15_02580

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Restriction sites are underlined

***ermC* integration synthetic construct**

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GGTGTGGTATTTTCAATTTGGTATTGTGGCTTCGTCTCGAATTC

Restriction sites underlined, *ermC* sequence in italics, *rpsF* promoter sequence from *B. subtilis* and *trxA* rho-independent terminator sequence from *S. aureus* flanking the *ermC* gene are dashed and the whole integration cassette sandwiched between the homology region (622 bp 5', 617 bp 3') for integration.

Synthetic *ess* region from *esxA* to *esxB*, codon optimised for *E. coli*.

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gaagaacgcgaagatattaagcgttcatcagctgatgattgaagatctgaaacagcgcgaaatggatggcccgtttgaaa
agatagcctgtatatttaacgattttaaacccttattgattgcacctatattccggaagatgatgtgaaaaactgattacaaag
gcccggaactggcctgaacattctgtttggtggtcattcataaagaactgattgatgcgatgataaacagattgatgtggcgcg
aaaatgattaaccagtttagcattggcattcgcattagcgcagctttttaaatttcgcttattcagcgcgcaaccggatgataa
gaaaacgaagcgtatattgtggcgaaccaggcgtatcagaaaattcgtggtttaaTAAcaatgaattaataggaggggag
gtatgttATGaacttaacgatattgaaacgatggtgaaagcaaatgaaagatataaaaaacatcgggaagaaattgcgca
tgaattgaagtgcgagcggctatctgcgcaaaagcgaacagataaacgcctggaatttaacctgagctttgcgctggatgat

attgaaagcaccgCGAAAGATgtgcagaccgCGAAAAGCAGCGCGAACAAGATgCGTgaccGTgaaaggCAAAGCGC
CGAACACCCTGTATATTGAAAAACGCAACCTGATGAAACAGAACTGGAAATGCTGGGCGAAGATATTGATAAAAAACAAGA
AAGCTGCAGAAAGCGAAAGAAATTGCGGGCGAAAAGCGAGCGAATATTTAACAAGCGATGAACTAATATTGAGGTG
AAGATATGggcggctataaaggcattaagcggatggcggcaagtgatcaggCGAACAGCTGGCGGGCGAAAACCGC
GAAAGATATTGAAGCGTGCAGAAACAGACCCAGCAGCTGGCGGAATATATTGAAGGCAGCGATTGGGAAGGCCAGTTGCG
AACAAAGTGAAGATGTGCTGCTGATTATGGCGAAATTCAGGAAGAAGTGGTGCAGCCGATGGCGGATCATCAGAAAGCGATT
GATAACCTGAGCCAGAACCTGGCGAAATATGATACCCTGAGCATTAAACAGGGCCTGGATCGCGTGAACCCGTAAAGCTT

Restriction sites underlined, start and stop codons shown in upper case.

Supplementary Table 1. Strains used in this study

Strain	Relevant genotype or description	Source or reference
<i>S. aureus</i> strains		
RN6390	NCTC8325 derivative, <i>rbsU</i> , <i>tcaR</i> , cured of ϕ 11, ϕ 12, ϕ 13	Refence ¹
Δ essC	As RN6390, Δ essC	Reference ²
Δ ess	Complete deletion from <i>esxA</i> – <i>esaG</i>	Reference ²
Δ esaE	As RN6390, Δ esaD	This work
Δ esaD	As RN6390, Δ esaE	This work
Δ esaDG	As RN6390, Δ esaDG	This work
Δ saouhsc00268-00278	As RN6390, Δ esaD-saouhsc00278	This work
Δ saouhsc00274-00278	As RN6390, Δ saouhsc00274-saouhsc00278	This work
RN6390:: <i>ermC</i>	As RN6390, with <i>ermC</i> resistance gene chromosomal insertion	This work
Δ esaDG:: <i>ermC</i>	As Δ esaDG with <i>ermC</i> resistance gene from RN6390:: <i>ermC</i> (phage ϕ 11 transduction)	This work
Δ saouhsc00268-00278:: <i>ermC</i>	As Δ esaD-saouhsc00278 with <i>ermC</i> resistance gene from RN6390:: <i>ermC</i> (phage ϕ 11 transduction)	This work
Δ saouhsc00274-00278:: <i>ermC</i>	As Δ saouhsc00274-saouhsc00278 with <i>ermC</i> resistance gene from RN6390:: <i>ermC</i> (phage ϕ 11 transduction)	This work
COL	MRSA, <i>agr</i>	Reference ³
COL Δ ess	Complete deletion from <i>SacI0271</i> (<i>esxA</i>) - <i>SacI0282</i>	Reference ²
<i>E. coli</i> strains		
JM110	<i>rpsL thr leu thi lacY galK galT ara tonA tsx dam dcm glnV44 Δ(lac-proAB) e14- [F' traD36 proAB⁺ lacI^q lacZΔM15] hsdR17(rK⁻mK⁺)</i>	Stratagene
BL21(DE3)	<i>E. coli</i> B: F ⁻ , <i>dcm</i> , <i>ompT</i> , <i>hsdS</i> (rB ⁻ , mB ⁻), <i>gal</i> , λ DE3	Reference ⁴

M15 [pREP4]	<i>F</i> -, <i>lac</i> , <i>ara</i> , <i>gal</i> , <i>mtl</i> [(<i>Kan</i> ^R , <i>lacI</i>)]	Qiagen
BTH101	<i>F</i> - <i>cya-99</i> , <i>araD139</i> , <i>galE15</i> , <i>galK16</i> , <i>rpsL1</i> (<i>Str</i> ^r), <i>hsdR2</i> , <i>mcrA1</i> , <i>mcrB1</i>	Reference ⁵

Supplementary Table 2. Plasmids used in this study

Plasmid	Relevant genotype or description	Source or reference
pIMAY	<i>E. coli</i> / <i>S. aureus</i> shuttle vector, temperature sensitive, <i>cml</i> ^r	⁶
pIMAY- <i>esaE</i>	pIMAY carrying <i>esaE</i> deletion allele	This work
pIMAY- <i>esaD</i>	pIMAY carrying <i>esaD</i> deletion allele	This work
pIMAY- <i>esaDG</i>	pIMAY carrying <i>esaDG</i> deletion allele	This work
pIMAY-saouhsc00268-00278	pIMAY carrying <i>esaD-saouhsc00278</i> deletion allele	This work
pIMAY-saouhsc00274-00278	pIMAY carrying <i>saouhsc00274-saouhsc00278</i> deletion allele	This work
pRAB11	<i>E. coli</i> / <i>S. aureus</i> shuttle vector, inducible protein expression, <i>amp</i> ^r , <i>cml</i> ^r	Reference ⁷
pRAB11- <i>EsaD</i> (H528A)-HA	pRAB11 producing C-terminally HA-tagged <i>EsaD</i>	This work
pRAB11- <i>EsaE</i> -his	pRAB11 producing C-terminally His-tagged <i>EsaE</i>	This work
pRAB11- <i>EsaG</i> -his	pRAB11 producing C-terminally His-tagged <i>EsaG</i>	This work
pRAB11- <i>EsaD</i> -His- <i>EsaG</i> -HA	pRAB11 producing C-terminally His-tagged <i>EsaD</i> and C-terminally HA-tagged <i>EsaG</i>	This work
pRAB11- <i>EsaD</i> -HA- <i>EsaG</i> -His	pRAB11 producing C-terminally HA-tagged <i>EsaD</i> and C-terminally His-tagged <i>EsaG</i>	This work
pRAB11- <i>EsaD</i> (H528A)-His	pRAB11 producing C-terminally His-tagged H528A substituted <i>EsaD</i>	This work
pRAB11- <i>EsaG</i> -HA	pRAB11 producing C-terminally HA-tagged <i>EsaG</i>	This work
pRAB11- <i>EsaE</i> -HA	pRAB11 producing C-terminally HA-tagged <i>EsaE</i>	This work
pRAB11- <i>EsaD</i> (H528A)-His- <i>EsaE</i> -HA	pRAB11 producing C-terminally His-tagged H528A substituted <i>EsaD</i> and C-terminally HA-tagged <i>EsaE</i>	This work
pRAB11- <i>EsaD</i> (H528A)-HA- <i>EsaE</i> -His	pRAB11 producing C-terminally HA-tagged H528A-substituted <i>EsaD</i> and C-terminally His-tagged <i>EsaE</i>	This work
pRAB11- <i>EsaD</i> ₄₂₁₋₆₁₄ (H528A)-His- <i>EsaG</i> -HA	pRAB11 producing C-terminally His-tagged H528A substituted nuclease domain of <i>EsaD</i> (aa 421-614) and C-terminally HA-tagged <i>EsaG</i>	This work
pRAB11- <i>EsaD</i> ₄₂₁₋₆₁₄ (H528A)-His	pRAB11 producing C-terminally His-tagged H528A substituted nuclease domain of <i>EsaD</i> (aa 421-614)	This work
pRAB11- <i>EsxC</i>	pRAB11 producing native (untagged) <i>EsxC</i>	This work
pQE70	Vector for regulatable protein overproduction in <i>E. coli</i> (T5 promoter). <i>Amp</i> ^r	Qiagen
pQE70- <i>EsaE</i> -HA- <i>EsaD</i> -Myc- <i>EsaG</i> -His ¹	pQE70 producing C-terminally HA-tagged <i>EsaE</i> , C-terminally Myc-tagged <i>EsaD</i> and C-terminally His-tagged <i>EsaG</i>	This work

pQE70-EsaE-HA-EsaD(H528A)-Myc-EsaG-His [†]	pQE70 producing C-terminally HA-tagged EsaE, C-terminally Myc-tagged H528A-substituted EsaD and C-terminally His-tagged EsaG	This work
pQE70-EsaE-HA-EsaG-His	pQE70 producing C-terminally HA-tagged EsaE and C-terminally His-tagged EsaG	This work
pQE70-EsaG-EsaG-EsaD ₄₂₁₋₆₁₄ -His	pQE70 producing tandem copies of EsaG and C-terminally His-tagged EsaD ₄₂₁₋₆₁₄	This work
pQE70-EsaG-EsaG-EsaD ₄₂₁₋₆₁₄ (H528A)-His	pQE70 producing tandem copies of EsaG and C-terminally His-tagged H528A substituted EsaD ₄₂₁₋₆₁₄	This work
pT7.5	Vector for regulatable protein production in <i>E. coli</i> (T7 promoter). Amp ^r	Reference ⁸
pT7.5esaD(V584Y)	pT7.5 producing V584Y substituted EsaD	This work
pT7.5esaDG	pT7.5 producing EsaD and EsaG	This work
pT18	Vector encoding T18 fragment of <i>B. pertussis</i> <i>cyaA</i> ; Amp ^r	Reference ⁹
pT18-EsaE	pT18 carrying <i>esaE</i>	This work
pT18-EsaD(H528A)	pT18 carrying <i>esaD</i> (H528A codon substitution)	This work
pT18-EsaD ₁₋₄₂₀	pT18 carrying <i>esaD</i> (codons 1-420)	This work
pT18-EsaG	pT18 carrying <i>esaG</i>	This work
pT25	Vector encoding T18 fragment of <i>B. pertussis</i> <i>cyaA</i> ; cml ^r	Reference ⁹
pT25-EsxA	pT25 carrying <i>esxA</i>	This work
pT25-EsxB	pT25 carrying <i>esxB</i>	This work
pT25-EsaB	pT25 carrying <i>esaB</i>	This work
pT25-EsxC	pT25 carrying <i>esxC</i>	This work
pT25-EsaE	pT25 carrying <i>esaE</i>	This work
pT25-EsaD(H528A)	pT25 carrying <i>esaD</i> (H528A codon substitution)	This work
pT25-EsaD ₄₂₁₋₆₁₄	pT25 carrying <i>esaD</i> (codons 421-614, with H528A codon substitution)	This work
pT25-EsxD	pT25 carrying <i>esxD</i>	This work
pT25-EsaG	pT25 carrying <i>esaG</i>	This work
pT25-saouhsc00274*	pT25 carrying <i>saouhsc00274</i>	This work
pT25-saouhsc00275*	pT25 carrying <i>saouhsc00275</i>	This work
pT25-saouhsc00276*	pT25 carrying <i>saouhsc00276</i>	This work

pT25-saouhsc00277*	pT25 carrying <i>saouhsc00277</i>	This work
pT25-saouhsc00278*	pT25 carrying <i>saouhsc00278</i>	This work
pT25-SAPIG0310*	pT25 carrying <i>SAPIG0310</i>	This work
pT25-SAPIG0311*	pT25 carrying <i>SAPIG0311</i>	This work
pT25-SAPIG0314*	pT25 carrying <i>SAPIG0314</i>	This work
pT25-SAR0293*	pT25 carrying <i>SAR0293</i>	This work
pT25-SAR0294*	pT25 carrying <i>SAR0294</i>	This work
pT25-SAR0295*	pT25 carrying <i>SAR0295</i>	This work
pT25-SAR0297*	pT25 carrying <i>SAR0297</i>	This work
pT25- SAEMRSA15_02570*	pT25 carrying <i>SAEMRSA15_02570</i>	This work
pT25- SAEMRSA15_02580*	pT25 carrying <i>SAEMRSA15_02580</i>	This work

†Purchased ready-cloned in the pQE70 vector from Biomatik.

*Purchased ready-cloned in the pT25 vector from Genescript.

esaD(421-614aa)-kpnI-for-esxA-RBS	GAAAGGTACCAGGAGGTTTCTACTTATGACACATGGTCCAAAAGATAGTATGGTGAG	esaD(H528A)	KpnI	Construction of pRAB11-EsaD ₄₂₁₋₆₁₄ (H528A)-His ¹
00268-00278A1	CATGGAGCTCGATTGTACAATC	gDNA	SacI	Construction of pIMAY-saouhsc_00268-00278 ³
00268-00278A2	TGTCTGCTACATGTCATGCACCTATCCCTC	gDNA		Construction of pIMAY-saouhsc_00268-00278 ³
00268-00278B1	CATGACATGTAGCAGACATGTTATAAAAGACTGTG	gDNA		Construction of pIMAY-saouhsc_00268-00278 ³
00268-00278B2	GCGCGAGCTCCATCTATTTTCAGTGTTAATTTAC	gDNA	SacI	Construction of pIMAY-saouhsc_00268-00278 ³
00268-00278-out1	TATGTATTTTGCACCATTTAGC			Sequencing primer
00268-00278-out2	CGTTTAAATGTTTGACGCAAGA			Sequencing primer
00274-00278A1	CATGGAGCTCGTATTACATATAGGTGTGGGT	gDNA	SacI	Construction of pIMAY-saouhsc_00274-00278 ³
00274-00278A2	TGTCTGCTACATGTTATCGCCCCTATGTGTTGC	gDNA		Construction of pIMAY-saouhsc_00274-00278 ³
00274-00278B1	GATAACATGTAGCAGACATGTTATAAAAGACTGTG	gDNA		Construction of pIMAY-saouhsc_00274-00278 ³
00274-00278B2	GCGCGGTACCCATCTATTTTCAGTGTTAATTTAC	gDNA	KpnI	Construction of pIMAY-saouhsc_00274-00278 ³
00274-00278-out1	GGAGCGTTTGCTTTTGTGTAA	gDNA		Sequencing primer
0266A1	AGGATCCAGTGCTAATAAGGACAGTG	gDNA	BamHI	Construction of pIMAY-esaE ³
0266A2	CTCTGCTTTATCTTTCATCATGGGTTCCAC	gDNA		Construction of pIMAY-esaE ³
0266B1	ATGAAAGATAAAGCAGAGGAGTAATGACG	gDNA		Construction of pIMAY-esaE ³
0266B2	TTCGGATCCAATTGAGATGCATAATC	gDNA	BamHI	Construction of pIMAY-esaE ³

0268A1	AGAAGGATCCAATGATTGGGACTTTAAAC	gDNA	<i>Bam</i> HI	Construction of pIMAY-esaD ³ and pIMAY-esaDG ³
0268A2	CTTATTTAATTTTGTCATGTCATGCACC	gDNA		Construction of pIMAY-esaD ³ and pIMAY-esaDG ³
0268B1	ATGACAAAATTAATAAGTAGAGGTGCC	gDNA		Construction of pIMAY-esaD ³
0268B2	TGAGGATCCACTTTACGGTATCTATTG	gDNA	<i>Bam</i> HI	Construction of pIMAY-esaD ³
0269A1	GGTTGAATTCGGAGAACACTATGC	gDNA	<i>Eco</i> RI	Construction of pIMAY-esaG ³
0269A2	TTATTCTTCAAATGTCATGTTGGCACCTC	gDNA		Construction of pIMAY-esaD ³
0269B1	ATGACATTTGAAGAATAAACTATCTTAATG	gDNA		Construction of pIMAY-esaD ³ and pIMAY-esaDG ³
0269B2	AAAGGATCCATGTTGCAAATACTGCG	gDNA	<i>Bam</i> HI	Construction of pIMAY-esaG ³ and pIMAY-esaD ³
EsaG-out1	TGGGTCAAACATAAAGCGTGC			Sequencing primer
EsaG-out2	ACGTGAACATTCCGCCAATTAC			Sequencing primer
RT-PCR-primer pair 1- for	ACATGGTCCAAAAGATAGTATGGTGAG	gDNA		RT-PCR
RT-PCR-primer pair 1- rev	GCACCTCTACTTATTTAATATTCTTC	gDNA		RT-PCR
RT-PCR-primer pair 2- for	GAAGAATATTAATAAGTAGAGGTGC	gDNA		RT-PCR
RT-PCR-primer pair 2- rev	GTCATCATCTTCAGTGTTTAATTC	gDNA		RT-PCR
RT-PCR-primer pair 3- for	GATACTCTTCGAAAGCCAGGTGCAC	gDNA		RT-PCR

RT-PCR-primer pair 3-rev	GTCATCATCTTCAGTGTTTAATTC	gDNA		RT-PCR
pT25-EsxA-F	GCGC <u>GGATCCC</u> GCGATGATTA ⁺ AATGAGCCCGGAAG	Synthetic <i>esxA</i> gene sequence [†]	<i>Bam</i> HI	Construction of pT25-EsxA ⁴
pT25-EsxA-R	GCGC <u>GGTACCT</u> TACTGCAGGCCAAAGTTGTTGCTCAGC	Synthetic <i>esxA</i> gene sequence [†]	<i>Kpn</i> I	Construction of pT25-EsxA ⁴
pT25-EsxB-F	GCGC <u>GGATCCC</u> GGGCGGCTATAAAGGCATTAAGC	Synthetic <i>esxB</i> gene sequence [†]	<i>Bam</i> HI	Construction of pT25-EsxB ⁴
pT25-EsxB-R	GCGC <u>GGTACCT</u> TACGGGTTACGCGATCCAGGCCCTG	Synthetic <i>esxB</i> gene sequence [†]	<i>Kpn</i> I	Construction of pT25-EsxB ⁴
pT25-EsaB-F	GCGC <u>GGATCCC</u> AATCAGCACGTA ⁺ AAGTAACATTTG	Synthetic <i>esaB</i> gene sequence [†]	<i>Bam</i> HI	Construction of pT25-EsaB ⁴
pT25-EsaB-R	GCGC <u>GGTACCT</u> TACAGCAGCAGTTTCAGAATATCGCCATCC	Synthetic <i>esaB</i> gene sequence [†]	<i>Kpn</i> I	Construction of pT25-EsaB ⁴
pT25-EsaC-F	GCGC <u>GGATCCC</u> AACTTTAACGATATTGAAACGATG	Synthetic <i>esxC</i> gene sequence [†]	<i>Bam</i> HI	Construction of pT25-EsxC ⁴
pT25-EsaC-R	GCGC <u>GGTACCT</u> TAGTTCATCGCTTTGTTAAAATATTCG	Synthetic <i>esxC</i> gene sequence [†]	<i>Kpn</i> I	Construction of pT25-EsxC ⁴
T25esaD-F	GCGC <u>GGATCCC</u> CACAAAAGATATTGAATATCT	<i>esaD</i> (H528A)	<i>Bam</i> HI	Construction of pT25-EsaD(H528A) ⁴
T25esaD-R	GCGC <u>GGTACC</u> ATTACTTATTTAATATTCTTCTAAT	<i>esaD</i> (H528A)	<i>Kpn</i> I	Construction of pT25-EsaD(H528A) ⁴
T25esaE-F	GCGC <u>GGATCCC</u> AAAGATGTTAAGCGAATAGAT	gDNA	<i>Bam</i> HI	Construction of pT25-EsaE ⁴
T25esaE-R	GCGC <u>GGTACC</u> ATTACTCCTCTGCTTTATTAATAT	gDNA	<i>Kpn</i> I	Construction of pT25-EsaE ⁴
T25esaF-F	GCGC <u>GGATCCC</u> CACGTTGAGTGGAAAAATTAGTG	gDNA	<i>Bam</i> HI	Construction of pT25-EsxD ⁴
T25esaF-R	GCGC <u>GGTACC</u> ATTATCCCTCAATATTATAGT	gDNA	<i>Kpn</i> I	Construction of pT25-EsxD ⁴
T25esaG-F	GCGC <u>GGATCCC</u> CACATTTGAAGAGAAGCTTAG	gDNA	<i>Bam</i> HI	Construction of pT25-EsaG ⁴

T25esaG-R	GCGCGGTACCATTATTCTTCTAGCTCTTTAATA	gDNA	<i>KpnI</i>	Construction of pT25-EsaG ⁴
T25esaD-421-614-F	GCGCGGATCCCACACATGGTCCAAAAGATAG	<i>esaD(H528A)</i>	<i>KpnI</i>	Construction of pT25-EsaD ₄₂₁₋₆₁₄ (H528A) ⁴
T18esaD-F	GCGCGGGCCCCACAAAAGATATTGAATATCT	<i>esaD(H528A)</i>	<i>Apal</i>	Construction of pT18-EsaD(H528A) ⁵
T18esaD-R	GCGCCTCGAGGGCTTATTTAATATTCTTCTAAT	<i>esaD(H528A)</i>	<i>XhoI</i>	Construction of pT18-EsaD(H528A) ⁵
T18esaE-F	GCGCGGGCCCCAAAGATGTTAAGCGAATAGAT	gDNA	<i>Apal</i>	Construction of pT18-EsaE ⁵
T18esaE-R	GCGCCTCGAGGGCTCCTCTGCTTTATTAATAT	gDNA	<i>XhoI</i>	Construction of pT18-EsaE ⁵
T18esaG-F	GCGCGGGCCCCACATTTGAAGAGAAGCTTAG	gDNA	<i>Apal</i>	Construction of pT18-EsaG ⁵
T18esaG-R	GCGCCTCGAGGGTCTTCTAGCTCTTTAATA	gDNA	<i>XhoI</i>	Construction of pT18-EsaG ⁵
T18esaD-1-420-R	GCGCCTCGAGGGCACGTGATTTTGTGGTGTAACAG	gDNA	<i>XhoI</i>	Construction of pT18- <i>esaD</i> ₁₋₄₂₀ ⁵
pQE70- <i>esaE</i> -for	GCGAGCATGCATGAAAGACGTAAACGTATC	Synthetic <i>esaE</i> gene sequence*	<i>SphI</i>	Construction of pQE70-EsaE-HA-EsaG-his
pQE70- <i>esaEG</i> -overlapping-for	GAAATTAACCATGACCAAAAACAAATAATATTAAGAGGAGAAATTA ACCATGACCTTCGAAGAAAACTGTC	Synthetic <i>esaEG</i> gene sequence*		Construction of pQE70-EsaE-HA-EsaG-his
pQE70- <i>esaEG</i> -overlapping-rev	CATATGATGTTCCAGATTATGCATAATGGATCCATTAAGAGGAGA AATTAACCATGACCAAAAACAAATAAT	Synthetic <i>esaEG</i> gene sequence*		Construction of pQE70-EsaE-HA-EsaG-his
EsaG-for- <i>SphI</i>	GCGAGCATGCCCTTCGAAGAAAACTGTCTAAAATC	Synthetic <i>esaG</i> gene sequence*	<i>SphI</i>	Construction of pQE70-EsaG-EsaG-EsaD ₄₂₁₋₆₁₄ -his and pQE70-EsaG-EsaG-EsaD ₄₂₁₋₆₁₄ (H528A)-his ⁶
EsaG-overlapping-for	GTACATCAAAGAA CTGGAATAATAA ATTAAGAGG AGAAATTAACC ATGACCTTCGAAGAAAACTG	Synthetic <i>esaG</i> gene sequence*		Construction of pQE70-EsaG-EsaG-EsaD ₄₂₁₋₆₁₄ -his and pQE70-EsaG-EsaG-EsaD ₄₂₁₋₆₁₄ (H528A)-his ⁶
EsaG-overlapping-rev	CAGTTTTTCTTCGAAGGTCATGGTTAATTTCTCCTCTTTAATTTATTA TTCCAGTTCTTTGATGTAC	Synthetic <i>esaG</i> gene sequence*		Construction of pQE70-EsaG-EsaG-EsaD ₄₂₁₋₆₁₄ -his and

				pQE70-EsaG-EsaG-EsaD421-614(H528A)-his ⁶
EsaG-rev-apal-bamHI	GCGAGGATCCGGGCCCTTATTATTCCAGTTCTTTGATGTACTGTTG	Synthetic <i>esaG</i> gene sequence*	<i>Apal/BamHI</i>	Construction of pQE70-EsaG-EsaG-EsaD421-614-his and pQE70-EsaG-EsaG-EsaD421-614(H528A)-his ⁶
EsaDnu-for-Apal	GCGA GGGCCC ATTAAGAGGAGAAATTAACCATGCACGGTCCG AAAGACTCTATGGTTC	Synthetic <i>esaD</i> gene sequence*	<i>Apal</i>	Construction of pQE70-EsaG-EsaG-EsaD421-614-his and pQE70-EsaG-EsaG-EsaD421-614(H528A)-his ⁶
EsaDnu-rev-BgIII	GCGAAGATCT TTTGTTTCAGGATACGACGGATGTTACG	Synthetic <i>esaD</i> gene sequence*	<i>BgIII</i>	Construction of pQE70-EsaG-EsaG-EsaD421-614-his and pQE70-EsaG-EsaG-EsaD421-614(H528A)-his ⁶
pQE70-esaD-qc-F	ACGACGACGGTGGTGCCTGATCGCGGTATGTTCCGGT	Synthetic <i>esaD</i> gene sequence*		Construction of pQE70-EsaE-HA-EsaD(H528A)-Myc-EsaG-his ⁷
pQE70-esaD-qc-R	ACCGAACATACGCGGATCAGTGCACCACCGTCGTCGT	Synthetic <i>esaD</i> gene sequence*		Construction of pQE70-EsaE-HA-EsaD(H528A)-Myc-EsaG-his ⁷
pQE70-esaG-rev	GCGCAGATCTTTCCAGTTCTTTGATGTACTG	Synthetic <i>esaG</i> gene sequence*	<i>BgIII</i>	Construction of pQE70-EsaE-HA-EsaG-his
pT7.5-esaD--for	AGTCTAGAAAGCTTTACTATAATATTGAGG	gDNA	<i>XbaI</i>	Construction of pT7.5esaD and pT7.5esaDG
pT7.5-esaD-xhoI-rev	CTACTCGAGCTTATTTAATATTCTTCTAAT	gDNA	<i>XhoI</i>	Construction of pT7.5esaD(V584Y)
pT7.5-esaD/G- xhoI-rev	GCCTCGAGTTCTTCTAGCTCTTTAATATATT	gDNA	<i>XhoI</i>	Construction of pT7.5esaDG
Intctrl1	CCAAGTCTGAAGTACCATTAACG			Checking chromosomal <i>ermC</i> integration
Intctrl2	GTACCTGCTATAAACAACGCGCAC			Checking chromosomal <i>ermC</i> integration

Restriction enzyme sequences are shown in underline.

¹*esaE-His*, *esaE-HA* and *esaG-HA* were cloned as *Bgl*II-*Sac*I fragments, *esaG-His* was cloned as a *Bgl*II-*Eco*RI fragment and *esaD(H528A)-His*, *esaD(H528A)-HA* and *esaDNuc(aa 421-615; H528A)-His* were cloned as *Kpn*I-*Bgl*II fragments into similarly digested pRAB11. In each case the forward primer incorporated the *esxA* ribosome binding site (shown in red) to initiate translation.

²*esaD(H528A)* was generated by overlapping PCR generating DNA covering the N-terminus of *esaD* to just beyond codon 528 (amplified with primers *esaD-kpnI-for-esxA-RBS* and *esaD-his-to-ala-rev* introducing the H528A substitution) and from just prior to codon 258 to the end of *esaD* (amplified with primers *esaD-his-to-ala-for* and *EsaD-his-rev-bgl II* inducing H528A substitution). Subsequently the two fragments were spliced together by overlap PCR using primers *esaD-kpnI-for-esxA-RBS* and *EsaD-his-rev-bgl II*.

³The two flanking regions, upstream and downstream of the gene to be deleted including the first three and last three codons, were separately amplified with the A1/A2 and B1/B2 primer pairs. Next, overlapping PCR was performed with the A1 and B2 primers using the amplified flanking regions as template and the product was subsequently cloned into PIMAY (as a *Sac*I fragment for the *saouhsc_00268-saouhsc_00278* deletion, a *Sac*I-*Kpn*I fragment for the *saouhsc_00274-saouhsc_00278* deletion, a *Bam*HI fragment for the *esaD*, *esaDG* and *esaE* deletions and an *Eco*RI-*Bam*HI fragment for the *esaG* deletion). The primers out1 and out2 were used for sequencing to check the deletions, as shown schematically below.



⁴*esxA*, *esxB*, *esxC*, *esaB*, *esaD(H528A)*, *esaD₄₂₁₋₆₁₄(H528A)*, *esaE*, *esxD* and *esaG* were each cloned as *Bam*HI-*Kpn*I fragments into similarly digested pT25.

⁵*esaD(H528A)*, *esaD₁₋₄₂₀*, *esaE*, and *esaG*, were each cloned as *Apal*-*Xho*I fragments into similarly digested pT18.

[†]A 10,651bp synthetic DNA sequence coding for *EsxA*, *EsaA*, *EssA*, *EssB*, *EssC*, *EsxC*, *EsxB* from RN6390, codon optimised for expression in *E. coli* was used as template. The sequence of this synthetic DNA is given in Fig S12.

⁶Two copies of *esaG* were amplified, one with primer pair *EsaG-for-Sph*I and *EsaG-overlapping-for* and the second with primer pair *EsaG-overlapping-rev* and *EsaG-rev-apal-bam*HI and cloned into pQE70 by three way ligation as an *Sph*I-*Bam*HI fragment. DNA encoding *EsaD₄₂₁₋₆₁₄* or *EsaD₄₂₁₋₆₁₄(H528A)* was subsequently cloned into this as an *Apal*-*Bgl*II fragment.

^{*}A 3,125bp synthetic sequence coding for *EsaE*, *EsaD(H528A)* and *EsaG*. Sequence given in Fig S13. ⁷The (H528A) substitution of *EsaD* was introduced into this construct by Quickchange using the indicated primers.

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