UNIVERSITY OF DUNDEE

University of Dundee

The type VII secretion system of Staphylococcus aureus secretes a nuclease toxin that targets competitor bacteria

Cao, Zhenping; Kneuper, Holger; Casabona, M. Guillermina; Chalmers, James D.; Palmer, Tracy

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

Zhenping Cao[1], M. Guillermina Casabona[1], Holger Kneuper[1], James D. Chalmers[2] and Tracy Palmer[1]

Division of [1]Molecular Microbiology, School of Life Sciences, and [2]Division of Cardiovascular & Diabetes Medicine, School of Medicine, University of Dundee, Dundee, UK.

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   envelope[1]. 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 EsxB[2-4]. In addition to secreting EsxA/B proteins,

39   Mycobacterial T7SSs can also secrete much larger proteins of the PE/PPE family[5], which are

40   highly abundant in the genomes of some species[6].

41   A distantly related T7SS, termed T7b[7], 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 protein[11]. 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 EsxA[10] and the predicted cytoplasmic protein EsaB[16]. 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 proteins[18]. 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 cells[22]. 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 factor[23], however its

67   absence from some *S. aureus* strains indicates it is unlikely to be a critical component of the

68   secretion machinery[18]. 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 protein[23]. 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 pT7.5[24]. 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   RNA[29]. 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 (EsaD$_{421-614}$, harbouring the

117   H528A codon substitution) and N-terminal region (EsaD$_{1-420}$). 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 EsaD$_{421-614}$ (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

5

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 Mg^{2+} or Zn^{2+} ions. Fig 2f shows that wild type

126   EsaD specifically degraded plasmid DNA in the presence of Mg^{2+},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 Mg^{2+}-

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 complexes[15], 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 secretion[30-32]. 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 $EsaD_{1-420}$ or $EsaD_{421-614}$(H528A) (Fig 3c). The

151   results show that EsaE specifically interacts with the non-nuclease region of EsaD. We tried

6

152 to confirm these genetic observations with co-purification experiments but were not able to

153 stably produce the truncated $EsaD_{1-420}$.

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 $EsaD_{1-420}$ 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

7

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 cells[34-38]. 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 clusters[18] (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 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 number[18]. 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   from[18] 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 media[16] 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 state[32] and deliver them to the cognate secretion machinery[31]. 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 activity[43], 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　systems[1]. 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/ml[46]) or for induction of target gene

287　expression from the pRAB11 plasmid[47]; 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 described[48]; quantitative assessment of protein interactions was undertaken by

297   plating onto MacConkey medium[49] containing 0.4% maltose as carbon source, and quantified

298   by β-galactosidase assays (according to the method of[50]) 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 OD_{600} of 2, as

304   described previously[16] 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 pIMAY[46]. 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 8325-4[51]. 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 described[52]. 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 OD_{600} 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 OD_{600} 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 OD_{600} of 0.5, EsaG-His production was initiated

338 by the addition of 250 ng/ml of ATC and cultured until an OD_{600} 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 $^{\circ}$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-EsaD$_{421-614}$-His or pQE70-EsaG-EsaG-EsaD$_{421-614}$(H528A)-His was cultured to

358   OD$_{600}$ 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 with some modifications, and was undertaken

364   once for each variant (Fig 2e). Briefly, the thawed pellets were resupended in lysis buffer (8 M

365   Urea, 100 mM NaH$_2$PO$_4$, 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 NaH$_2$PO$_4$, 150mM NaCl, 20mM imidazole, 1 mM

370   PMSF, pH 8.0) followed by five washes with native wash buffer (50 mM NaH$_2$PO$_4$, 500mM

371 NaCl, 20mM imidazole, 1 mM PMSF, pH 8.0). Finally, the bound protein was eluted into 200

372 μl 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 μg of purified $EsaD_{421-}$

374 $_{614}$-His, purified $EsaD_{421-614}$(H528A)-His or an equivalent volume of elution buffer at 37°C for 1

375 hour in a final volume of 20μl 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 OD_{600nm} of 2, cells were

381 harvested and the supernatant samples precipitated with trichloroacetic acid in the presence

382 of deoxycholate, as described previously[16]. 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 μg/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 by[16]. Carbonate-washing of membranes was undertaken according

390 to[53]. 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 previously[17] 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-EsxA[16] 1:2500, α-EsxC[16] 1:2000, α-EssC[16]

396 1:10000, α-TrxA[54] 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 µl 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 µg/µl 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 µl 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 µl 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 µg/µl in 200 µl 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

428   All correspondence and requests for materials should be addressed to Tracy Palmer

429

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

References

1   Costa, T. R. *et al.* Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nat Rev Microbiol* **13**, 343-359, doi:10.1038/nrmicro3456 (2015).

2   Pym, A. S. *et al.* Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. *Nature medicine* **9**, 533-539 (2003).

3   Hsu, T. *et al.* The primary mechanism of attenuation of bacillus Calmette-Guerin is a loss of secreted lytic function required for invasion of lung interstitial tissue. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 12420-12425 (2003).

4   Stanley, S. A., Raghavan, S., Hwang, W. W. & Cox, J. S. Acute infection and macrophage subversion by Mycobacterium tuberculosis require a specialized secretion system. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 13001-13006 (2003).

5   Abdallah, A. M. *et al.* A specific secretion system mediates PPE41 transport in pathogenic mycobacteria. *Molecular microbiology* **62**, 667-679 (2006).

6   Cole, S. T. *et al.* Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537-544, doi:10.1038/31159 (1998).

7   Abdallah, A. M. *et al.* Type VII secretion--mycobacteria show the way. *Nat Rev Microbiol* **5**, 883-891 (2007).

8   Baptista, C., Barreto, H. C. & Sao-Jose, C. High levels of DegU-P activate an Esat-6-like secretion system in *Bacillus subtilis*. *PLoS ONE* **8**, e67840, doi:10.1371/journal.pone.0067840 (2013).

9   Huppert, L. A. *et al.* The ESX system in *Bacillus subtilis* mediates protein secretion. *PLoS ONE* **9**, e96267, doi:10.1371/journal.pone.0096267 (2014).

10   Burts, M. L., Williams, W. A., DeBord, K. & Missiakas, D. M. EsxA and EsxB are secreted by an ESAT-6-like system that is required for the pathogenesis of *Staphylococcus aureus* infections. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 1169-1174 (2005).

11   Pallen, M. J. The ESAT-6/WXG100 superfamily -- and a new Gram-positive secretion system? *Trends in microbiology* **10**, 209-212 (2002).

12   Rosenberg, O. S. *et al.* Substrates Control Multimerization and Activation of the Multi-Domain ATPase Motor of Type VII Secretion. *Cell* **161**, 501-512, doi:10.1016/j.cell.2015.03.040 (2015).

13   Renshaw, P. S. *et al.* Structure and function of the complex formed by the tuberculosis virulence factors CFP-10 and ESAT-6. *The EMBO journal* **24**, 2491-2498, doi:10.1038/sj.emboj.7600732 (2005).

14   Sundaramoorthy, R., Fyfe, P. K. & Hunter, W. N. Structure of *Staphylococcus aureus* EsxA suggests a contribution to virulence by action as a transport chaperone and/or adaptor protein. *Journal of molecular biology* **383**, 603-614 (2008).

15   Sysoeva, T. A., Zepeda-Rivera, M. A., Huppert, L. A. & Burton, B. M. Dimer recognition and secretion by the ESX secretion system in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 7653-7658, doi:10.1073/pnas.1322200111 (2014).

16   Kneuper, H. *et al.* Heterogeneity in ess transcriptional organization and variable contribution of the Ess/Type VII protein secretion system to virulence across closely related *Staphyloccus aureus* strains. *Molecular microbiology* **93**, 928-943, doi:10.1111/mmi.12707 (2014).

17   Jäger, F., Zoltner, M., Kneuper, H., Hunter, W. N. & Palmer, T. Membrane interactions and self-association of components of the Ess/Type VII secretion system of *Staphylococcus aureus*. *FEBS letters* **590**, 349-357, doi:10.1002/1873-3468.12065 (2016).

18   Warne, B. *et al.* The Ess/Type VII secretion system of *Staphylococcus aureus* shows unexpected genetic diversity. *BMC genomics* **17**, 222, doi:10.1186/s12864-016-2426-7 (2016).

504   19   Burts, M. L., DeDent, A. C. & Missiakas, D. M. EsaC substrate for the ESAT-6 secretion
505   pathway and its role in persistent infections of *Staphylococcus aureus*. *Molecular*
506   *microbiology* **69**, 736-746, doi:DOI 10.1111/j.1365-2958.2008.06324.x (2008).
507   20   Anderson, M., Aly, K. A., Chen, Y. H. & Missiakas, D. Secretion of atypical protein
508   substrates by the ESAT-6 secretion system of *Staphylococcus aureus*. *Molecular*
509   *microbiology* **90**, 734-743, doi:10.1111/mmi.12395 (2013).
510   21   Wang, Y. *et al.* Role of the ESAT-6 secretion system in virulence of the emerging
511   community-associated *Staphylococcus aureus* lineage ST398. *Scientific reports* **6**,
512   25163, doi:10.1038/srep25163 (2016).
513   22   Korea, C. G. *et al.* Staphylococcal Esx proteins modulate apoptosis and release of
514   intracellular *Staphylococcus aureus* during infection in epithelial cells. *Infection and*
515   *immunity* **82**, 4144-4153, doi:10.1128/IAI.01576-14 (2014).
516   23   Anderson, M., Chen, Y. H., Butler, E. K. & Missiakas, D. M. EsaD, a Secretion Factor
517   for the Ess Pathway in *Staphylococcus aureus*. *Journal of bacteriology* **193**, 1583-
518   1589, doi:Doi 10.1128/Jb.01096-10 (2011).
519   24   Tabor, S. & Richardson, C. C. A bacteriophage T7 RNA polymerase/promoter system
520   for controlled exclusive expression of specific genes. *Proceedings of the National*
521   *Academy of Sciences of the United States of America* **82**, 1074-1078 (1985).
522   25   Butala, M., Zgur-Bertok, D. & Busby, S. J. The bacterial LexA transcriptional repressor.
523   *Cell Mol Life Sci* **66**, 82-93, doi:10.1007/s00018-008-8378-6 (2009).
524   26   Hill, T. M., Sharma, B., Valjavec-Gratian, M. & Smith, J. *sfi*-independent filamentation
525   in *Escherichia coli* Is *lexA* dependent and requires DNA damage for induction. *Journal*
526   *of bacteriology* **179**, 1931-1939 (1997).
527   27   Gavrieli, Y., Sherman, Y. & Ben-Sasson, S. A. Identification of programmed cell death
528   in situ via specific labeling of nuclear DNA fragmentation. *The Journal of cell biology*
529   **119**, 493-501 (1992).
530   28   Bos, J., Yakhnina, A. A. & Gitai, Z. BapE DNA endonuclease induces an apoptotic-like
531   response to DNA damage in *Caulobacter*. *Proceedings of the National Academy of*
532   *Sciences of the United States of America* **109**, 18096-18101,*
533   doi:10.1073/pnas.1213332109 (2012).
534   29   Blower, T. R., Salmond, G. P. & Luisi, B. F. Balancing at survival's edge: the structure
535   and adaptive benefits of prokaryotic toxin-antitoxin partners. *Curr Opin Struct Biol* **21**,
536   109-118, doi:10.1016/j.sbi.2010.10.009 (2011).
537   30   Daleke, M. H. *et al.* Specific chaperones for the type VII protein secretion pathway.
538   *The Journal of biological chemistry* **287**, 31939-31947, doi:10.1074/jbc.M112.397596
539   (2012).
540   31   Ekiert, D. C. & Cox, J. S. Structure of a PE-PPE-EspG complex from *Mycobacterium*
541   *tuberculosis* reveals molecular specificity of ESX protein secretion. *Proceedings of the*
542   *National Academy of Sciences of the United States of America* **111**, 14758-14763,
543   doi:10.1073/pnas.1409345111 (2014).
544   32   Korotkova, N. *et al.* Structure of the *Mycobacterium tuberculosis* type VII secretion
545   system chaperone EspG5 in complex with PE25-PPE41 dimer. *Molecular microbiology*
546   **94**, 367-382, doi:10.1111/mmi.12770 (2014).
547   33   Zoltner, M. *et al.* EssC: domain structures inform on the elusive translocation channel
548   in the Type VII secretion system. *The Biochemical journal*, doi:10.1042/BCJ20160257
549   (2016).
550   34   Russell, A. B. *et al.* Type VI secretion delivers bacteriolytic effectors to target cells.
551   *Nature* **475**, 343-347, doi:10.1038/nature10244 (2011).
552   35   Murdoch, S. L. *et al.* The opportunistic pathogen *Serratia marcescens* utilizes type VI
553   secretion to target bacterial competitors. *Journal of bacteriology* **193**, 6057-6069,
554   doi:10.1128/JB.05671-11 (2011).
555   36   Souza, D. P. *et al.* Bacterial killing via a type IV secretion system. *Nature*
556   *communications* **6**, 6453, doi:10.1038/ncomms7453 (2015).
557   37   Hood, R. D. *et al.* A type VI secretion system of *Pseudomonas aeruginosa* targets a
558   toxin to bacteria. *Cell Host Microbe* **7**, 25-37, doi:10.1016/j.chom.2009.12.007 (2010).

559	38	Aoki, S. K. *et al.* Contact-dependent inhibition of growth in *Escherichia coli. Science (New York, N.Y* **309**, 1245-1248, doi:10.1126/science.1115109 (2005).
561	39	Zhang, D., de Souza, R. F., Anantharaman, V., Iyer, L. M. & Aravind, L. Polymorphic toxin systems: Comprehensive characterization of trafficking modes, processing, mechanisms of action, immunity and ecology using comparative genomics. *Biology direct* **7**, 18, doi:10.1186/1745-6150-7-18 (2012).
565	40	Jamet, A. & Nassif, X. New players in the toxin field: polymorphic toxin systems in bacteria. *MBio* **6**, e00285-00215, doi:10.1128/mBio.00285-15 (2015).
567	41	Ma, A. T., McAuley, S., Pukatzki, S. & Mekalanos, J. J. Translocation of a *Vibrio cholerae* type VI secretion effector requires bacterial endocytosis by host cells. *Cell Host Microbe* **5**, 234-243, doi:10.1016/j.chom.2009.02.005 (2009).
570	42	Akpe San Roman, S. *et al.* A heterodimer of EsxA and EsxB is involved in sporulation and is secreted by a type VII secretion system in *Streptomyces coelicolor. Microbiology (Reading, England)* **156**, 1719-1729, doi:10.1099/mic.0.037069-0 (2010).
573	43	Holberger, L. E., Garza-Sanchez, F., Lamoureux, J., Low, D. A. & Hayes, C. S. A novel family of toxin/antitoxin proteins in *Bacillus* species. *FEBS letters* **586**, 132-136, doi:10.1016/j.febslet.2011.12.020 (2012).
576	44	Housden, N.G. & Kleanthous, C. Colicin translocation across the *Escherichia coli* outer membrane. *Biochemical Society Transactions* **40**, 1475-1479, doi: 10.1042/BST20120255 (2012).
579	45	Willett, J.L., Gucinski, G.C., Fatherree, J.P., Low, D.A. & Hayes, C.S. Contact-dependent growth inhibition toxins exploit multiple independent cell-entry pathways. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 11341-11346 (2015).
583	46	Monk, I. R., Shah, I. M., Xu, M., Tan, M. W. & Foster, T. J. Transforming the untransformable: application of direct transformation to manipulate genetically *Staphylococcus aureus* and *Staphylococcus epidermidis. MBio* **3**, doi:10.1128/mBio.00277-11 (2012).
587	47	Helle, L. *et al.* Vectors for improved Tet repressor-dependent gradual gene induction or silencing in Staphylococcus aureus. *Microbiology (Reading, England)* **157**, 3314-3323, doi:10.1099/mic.0.052548-0 (2011).
590	48	Karimova, G., Pidoux, J., Ullmann, A. & Ladant, D. A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 5752-5756 (1998).
593	49	Sambrook, J. & Russell, D. W. *Molecular cloning : a laboratory manual.* 3rd edn, (Cold Spring Harbor Laboratory Press, 2001).
595	50	Miller, J. H. *A short course in bacterial genetics: A laboratory manual and handbook for Escherichia coli and related bacteria.* (Cold Spring Harbor Laboratory Press, 1992).
597	51	Lei, M. G. *et al.* A single copy integration vector that integrates at an engineered site on the *Staphylococcus aureus* chromosome. *BMC Res Notes* **5**, 5, doi:10.1186/1756-0500-5-5 (2012).
600	52	Novick, R. P. Genetic systems in staphylococci. *Methods Enzymol* **204**, 587-636 (1991).
602	53	Keller, R., de Keyzer, J., Driessen, A.J.M & Palmer, T. Co-operation between different targeting pathways during integration of a membrane protein. *J of Cell Biol* **199,** 303-315 (2012).
605	54	Miller, M. *et al.* Staphylococcal PknB as the first prokaryotic representative of the proline-directed kinases. *PLoS ONE* **5**, e9057, doi:10.1371/journal.pone.0009057 (2010).

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 OD_{600} of 2 or (c) the indicated strains harbouring pRAB11 (empty) or

615 pRAB11-EsxC were cultured in TSB medium to OD_{600} of 0.5, then supplemented with ATC

616 (50ng/ml; to induce plasmid-encoded gene expression) until OD_{600} 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 OD_{600} 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 OD_{600} 0.5, supplemented with 1 mM IPTG (time zero) and OD_{600} measured at 1 hr

635 intervals (*n*=3 biological replicates, error bars are $\pm$ SD). b. – d. EsaG interacts with the

nuclease domain of EsaD. b. Interactions between pT25-EsaG and EsaD variants fused to pT18 assessed by β-galactosidase activity assay in *E. coli* BTH101. BTH101 harbouring pT25 and pT18 was the negative control. Error bars are $\pm$ SD (*n*=3 biological replicates). Student's *t*-test gives *p* values < 0.00001 for EsaD/EsaG and $EsaD_{421-614}$/EsaG relative to the negative control. Inset shows the same strain/plasmid combinations on MacConkey maltose plates. c. and d. Top two panels: *S. aureus* RN6390 carrying c. pRAB11 (empty vector), pRAB11-EsaG-HA or pRAB11-EsaD(H528A)-His-EsaG-HA, or e. pRAB11 (empty), pRAB11-EsaG-HA, $pRAB11-EsaD_{421-614}(H528A)$-His or $pRAB11-EsaD_{421-614}(H528A)$-His-EsaG-HA was cultured to OD_{600} of 0.5, then supplemented with ATC (500ng/ml). Cells were harvested at OD_{600} 3, lysed and histidine-tagged EsaD purified. Cell lysate (load) and eluted fractions (20μl of each) were analysed by western blot with anti-His and anti-HA antisera. Bottom two panels show repeat experiments of: c. the EsaD(H528A)-His-EsaG-HA co-purification or d. the $EsaD_{421-614}(H528A)$-His-EsaG-HA co-purification. Samples of load (10μl), flow through (20μl), final wash (30μl) and elution fraction (30μl) were analysed using the same antisera. Coomassie-stained samples of the load and elute fractions are shown in Fig S4. e. *E. coli* M15[prep4] harbouring pQE70 alone (empty) $pQE70-EsaG-EsaG-EsaD_{421-614}$-His or pQE70-EsaG-EsaG-$EsaD_{421-614}(H528A)$-His were cultured to OD_{600} of 0.5 and supplemented with 1mM IPTG. An aliquot was harvested after 4 hours' induction and resuspended in lysis buffer. His-tagged EsaD nuclease domain (wild type or H528A variant) was purified in the presence of 8M urea, refolded and eluted as described in Methods. 10μl of each sample were separated (12% bis-Tris gel) and stained using coomassie instant blue. f. EsaD is a Mg^{2+}-dependent DNase. Plasmid DNA was incubated with purified $EsaD_{421-614}$-His, $EsaD_{421-614}(H528A)$-His or buffer alone with either 50mM $MgCl_2$ or $ZnCl_2$, after which the DNA was analysed by agarose gel electrophoresis.

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 EsaD$_{1-420}$ or EsaD$_{421-614}$(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 EsaD$_{1-420}$/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 OD$_{600}$ of 0.5

672 supplemented with 500ng/ml ATC and harvested at OD$_{600}$ of 3. Cells were lysed and histidine-

673 tagged EsaD(H528A) was purified. Cell lysate (load) and eluted fractions (20μl 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 (10μl), flow through (20μl), final wash (30μl) and

677 elution fraction (30μl) 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 OD$_{600}$ of 0.5 supplemented with 250ng/ml

680 ATC and harvested at OD$_{600}$ of 3. Samples of supernatant and cells (equivalent to 250μl

681 supernatant and 10μl cells adjusted to OD$_{600}$ 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 OD$_{600}$ of 0.5, supplemented with 2 mM IPTG for 4 hours, harvested and

685 lysed. HA-tagged EsaE was purified and 10μl, 25μl and 35μl 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 (10μl), flow through (20μl), final wash (30μl) and elution fraction (30μl) 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.

691

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 OD_{600} of

695   0.5, supplemented with ATC (250ng/ml) and harvested at OD_{600} 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 Na_2CO_3 (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 OD_{600} of 0.5 supplemented with ATC (250ng/ml) and at OD_{600} 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.
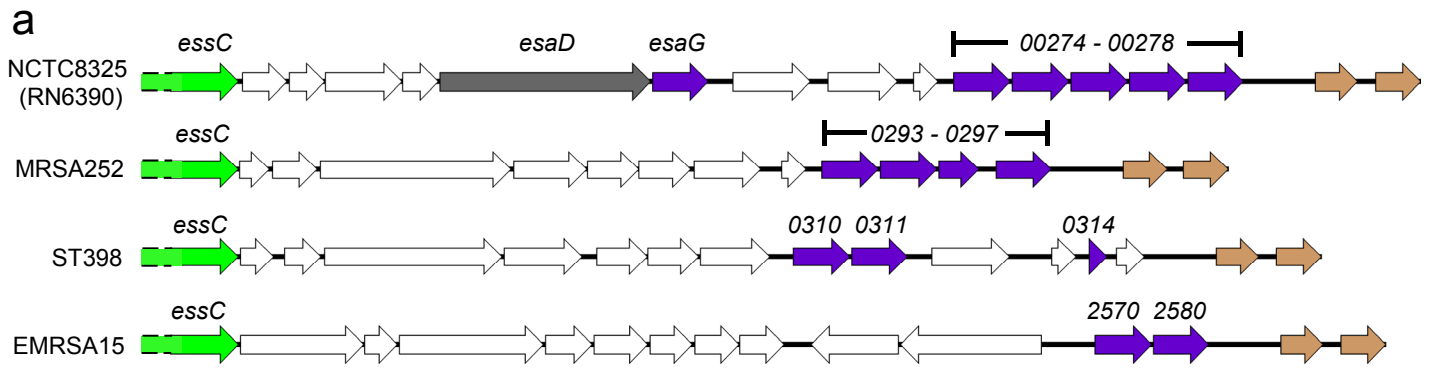
714

715

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* locus[18]. 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 COLΔ*ess*) overproduced EsaD-HA or EsaD(H528A)-

730   HA along with EsaG-His as described in Methods, and was incubated with either RN6390,

731   RN6390Δ*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   RN6390Δ*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).

a

b

c

EsaD (H528A)-His +

d

e

f

a

β-galactosidase activity (Miller Units)

Negative EsxA EsxB
EsaB EsxC EsaD
EsaE EsxD EsaG

EsaD(H528A)+ negative EsxA EsxB EsaB EsxC EsaD(H528A) EsaE EsxD EsaG

b

kD
EsaD(H258A)-His⁺ empty EsaD(H258A)-His⁺ EsaE-HA empty EsaD(H258A)-His⁺ EsaE-HA

EsaE-HA
load elute

75
50
25

← EsaD-His
← EsaE-HA

load elute

kD load flow through wash elute
75
50
25

← EsaD(H528A)-His
← EsaE-HA

c

β-galactosidase activity (Miller Units)

Negative D:E
D₁₋₄₂₀:E D₄₂₁₋₆₁₄:E

EsaE+ negative EsaD(H528A) EsaD₁₋₄₂₀ EsaD₄₂₁₋₆₁₄(H528A)

d

kD empty EsaE-His
sn c sn c

25 α-His
10 α-TrxA
10 α-EsxA

e

kD empty EsaD(H528A)-HA EsaE-His
sn c sn c

75
50 α-HA
37
25 α-His
10 α-TrxA
10 α-EsxA

f

kD empty D(H528A)-Myc:E-HA: G-His empty D(H528A)-Myc:E-HA: G-His empty D(H528A)-Myc:E-HA: G-His

100
75
50 ← EsaD(H528A)-Myc
37
25 ← EsaE-HA
20 ← EsaG-His

α-HA α-His α-Myc

g

kD load flow through wash elute
25 ← EsaE-HA
20 ← EsaG-His
75 ← EsaD(H528A)-Myc

a

NCTC8325 (RN6390) *essC* *esaD* *esaG* *00274 - 00278*

MRSA252 *essC* *0293 - 0297*

ST398 *essC* *0310* *0311* *0314*

EMRSA15 *essC* *2570* *2580*

b

| Negative | 0310 | 0311 | 0314 | 0293 | 0294 |
| 0295 | 0297 | 2570 | 2580 | EsaG | |

β-galactosidase activity (Miller Units)

EsaD(H528A)+   negative   0310   0311   0314   0293   0294   0295   0297   2570   2580   EsaG

ST398   MRSA252   EMRSA15

c

| Negative | 00274 | 00275 | 00276 |
| 00277 | 00278 | EsaG | |

β-galactosidase activity (Miller Units)

EsaD(H528A)+   negative   00274   00275   00276   00277   00278   EsaG

d

Attacker strain

- COL pEsaD-HA-EsaG-His
- COLΔ*ess* pEsaD-HA-EsaG-His
- COL pEsaD(H528A)-HA-EsaG-His

Log(c.f.u.)

Prey   RN6390   RN6390 pEsaG-His   Δ*00268-00278*

*** ** * ***

Δ*00268-00278*   RN6390   RN6390 pEsaG-His

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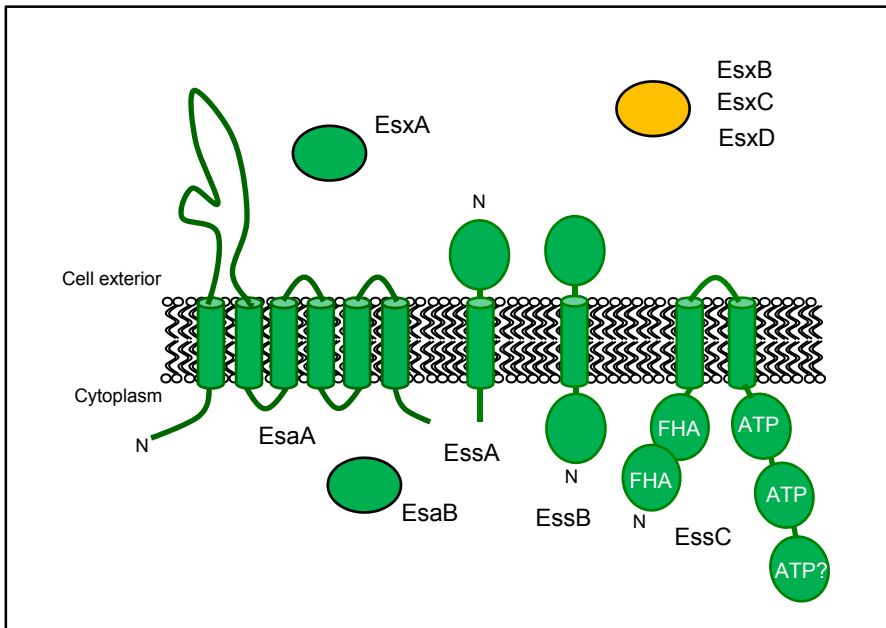
Supplementary Figure 1. Localization and predicted topologies of Ess components in *S. aureus* strain RN6390. Essential components of the secretion machinery are shown in green and known secreted substrates in yellow. FHA – forkhead associated domain. ATP – P-loop ATP-binding domain.

Supplementary Figure 2. EsaD has a predicted nuclease domain at its C-terminus. Alignment of *S. aureus* EsaD with Orthologs identified using BlastP [10]. Multiple sequence alignment was generated using Clustal Omega [11] with default settings. The *Streptococcus pyogenes* Spd1 protein, for which nuclease activity has been described [12], 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^{0}C to OD_{600} 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.
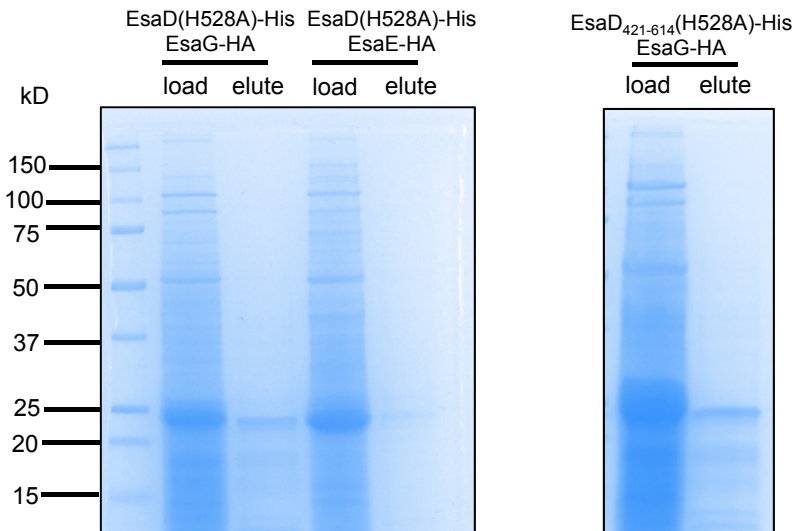
Supplementary Figure 4. Analysis of EsaD(H528A)-His-EsaG-HA, EsaD(H528A)-His-EsaE-HA and EsaD$_{421-614}$(H528A)-His-EsaG-HA co-purification by coomassie staining. The *S. aureus* wild type strain, RN6390, carrying EsaD(H528A)-His-EsaG-HA, pRAB11-EsaD(H528A)-His-EsaE-HA or pRAB11-EsaD(H528A)(421-614)-His-EsaG-HA was cultured in TSB medium to OD$_{600}$ of 0.5 supplemented with ATC (500ng/ml) and harvested at OD$_{600}$ 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.
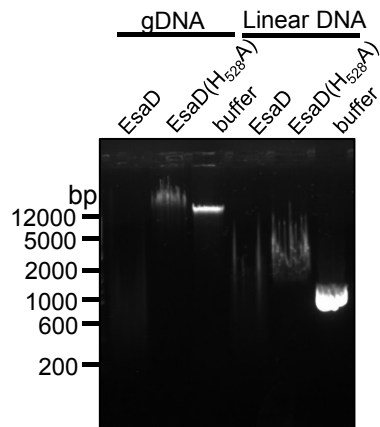
Supplementary Figure 5. EsaD is a Mg^{2+}-dependent DNase. 200ng of *S. aureus* gDNA or 400ng of a linear DNA PCR product were incubated with 0.4 μg purified EsaD$_{421-614}$-His, EsaD$_{421-614}$(H528A)-His or an equivalent volume of elution buffer, each supplemented with 50 mM MgCl$_2$ in a final volume of 20 μl at 37 °C for 20 mins after which the DNA was analysed by 1% agarose gel electrophoresis.
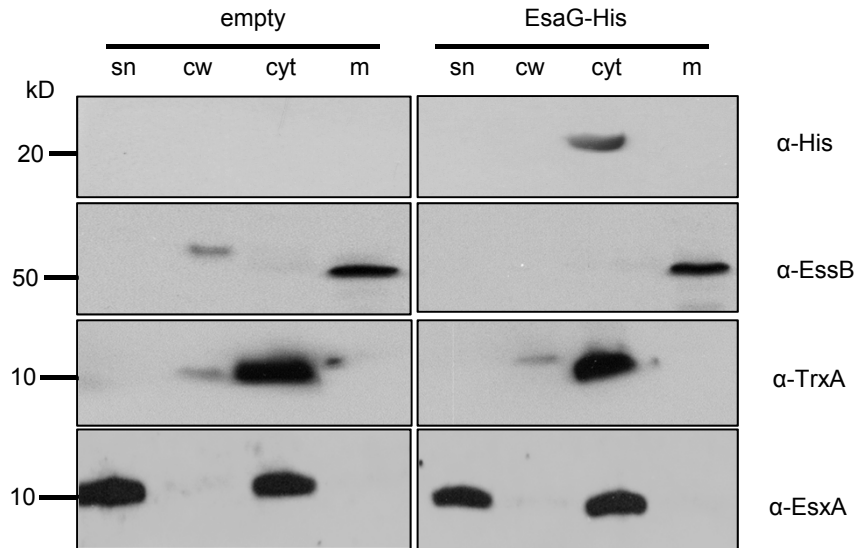
Supplementary Figure 6. EsaG is a cytoplasmic protein. The *S. aureus* wild type strain, RN6390, harbouring pRAB11 (empty) or pRAB11-EsaG-His was cultured in TSB medium to OD_{600} of 0.5, supplemented with ATC (250ng/ml) and harvested at OD_{600} of 2. A sample of the supernatant (sn) was retained as 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.
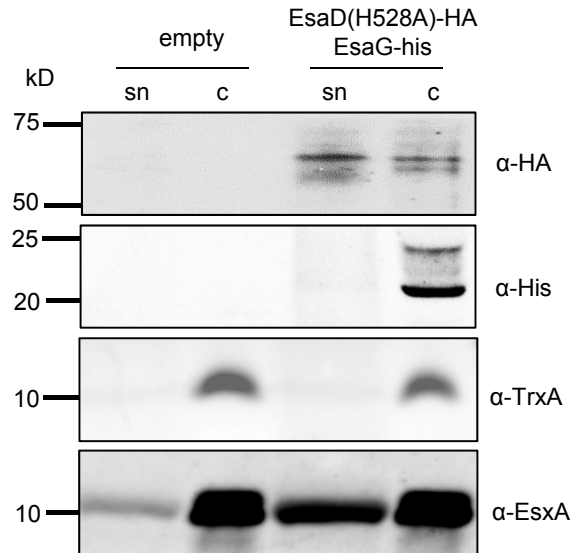
Supplementary Figure 7. EsaG is not co-secreted with EsaD. The *S. aureus* wild type strain, RN6390, harbouring pRAB11 (empty) or pRAB11-EsaD(H528A)-HA-EsaG-His were cultured in TSB medium to OD_{600} of 0.5, supplemented with ATC (250ng/ml) and harvested at OD_{600} of 2. Samples of the supernatant (sn) and cellular (c) fractions (an equivalent of 250μl of supernatant and 10μl of cells adjusted to OD_{600} of 1) were separated on 12% bis-Tris gels and immunoblotted using the indicated antisera.

ΔesaDG   ΔesaE

EsaD(H528A)-HA   empty   EsaD(H528A)-HA   empty

kD   sn   c   sn   c   sn   c   sn   c

75 —   α-HA

10 —   α-TrxA

10 —   α-EsxA
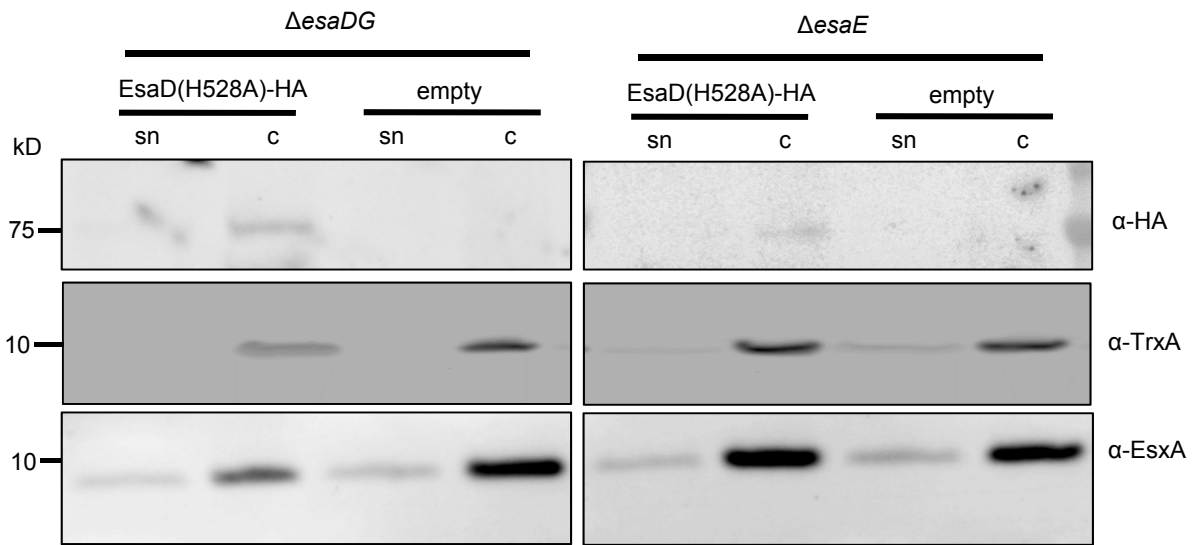
Supplementary Figure 8. EsaD-HA is not detected in the supernatant when *esaE* or *esaG* are deleted. The indicated *S. aureus* strains harbouring pRAB11 (empty) or pRAB11-EsaD(H528A)-HA were cultured in TSB medium until to OD_{600} of 0.5, supplemented with ATC (250ng/ml) and harvested at OD_{600} of 2. Samples of the supernatant (sn) and cellular (c) fractions (an equivalent of 250μl of supernatant and 10μl of cells adjusted to OD_{600} of 1) were separated on 12% bis-Tris gels and immunoblotted using the indicated antisera.
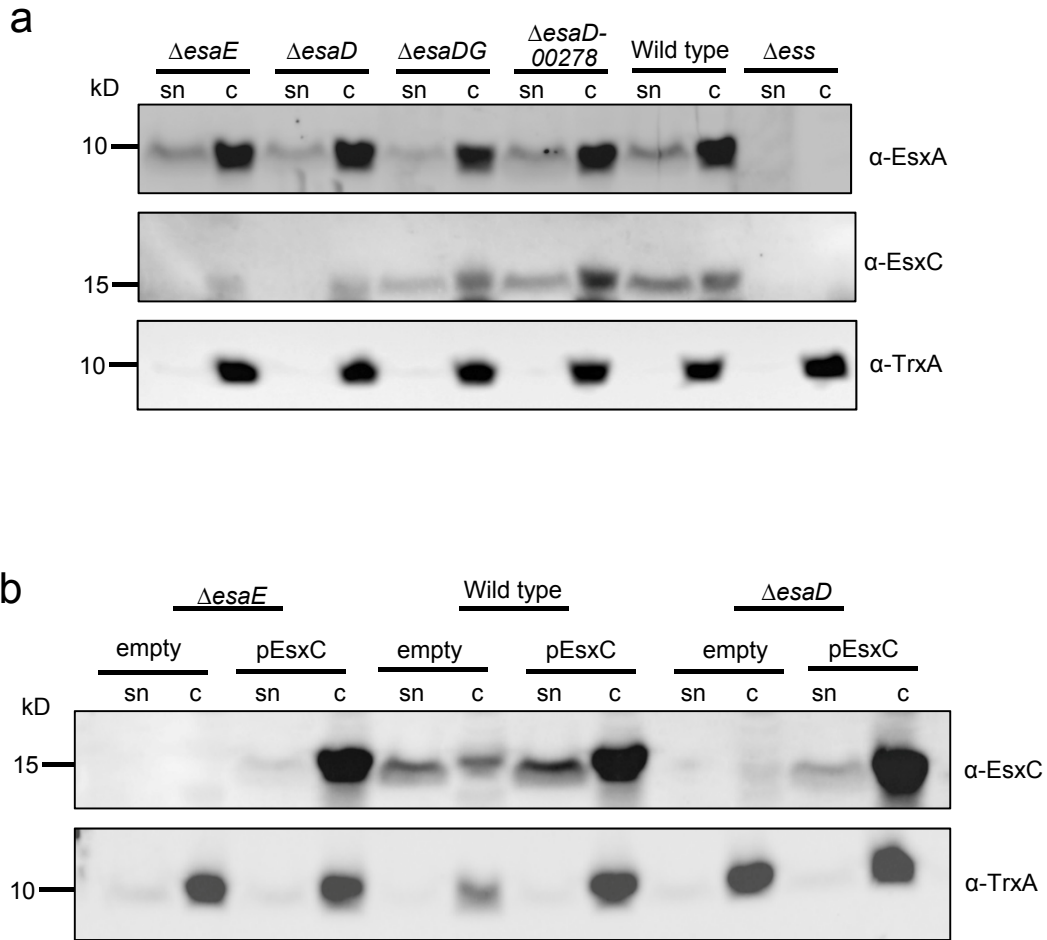
Supplementary Figure 9. EsaD and EsaG proteins are not required for the secretion of EsxA and EsxC. a. The RN6390 wild-type or isogenic deletion strains, as indicated, were cultured in TSB medium or b. The indicated strains harbouring pRAB11 (empty) or pRAB11-EsxC were cultured in TSB medium until an OD_{600} of 0.5 and supplemented with ATC (50ng/ml). a. and b. When cultures reached OD_{600} 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 OD_{600} of 1) were separated on 12 % bis-Tris gels and immunoblotted with the indicated antisera.
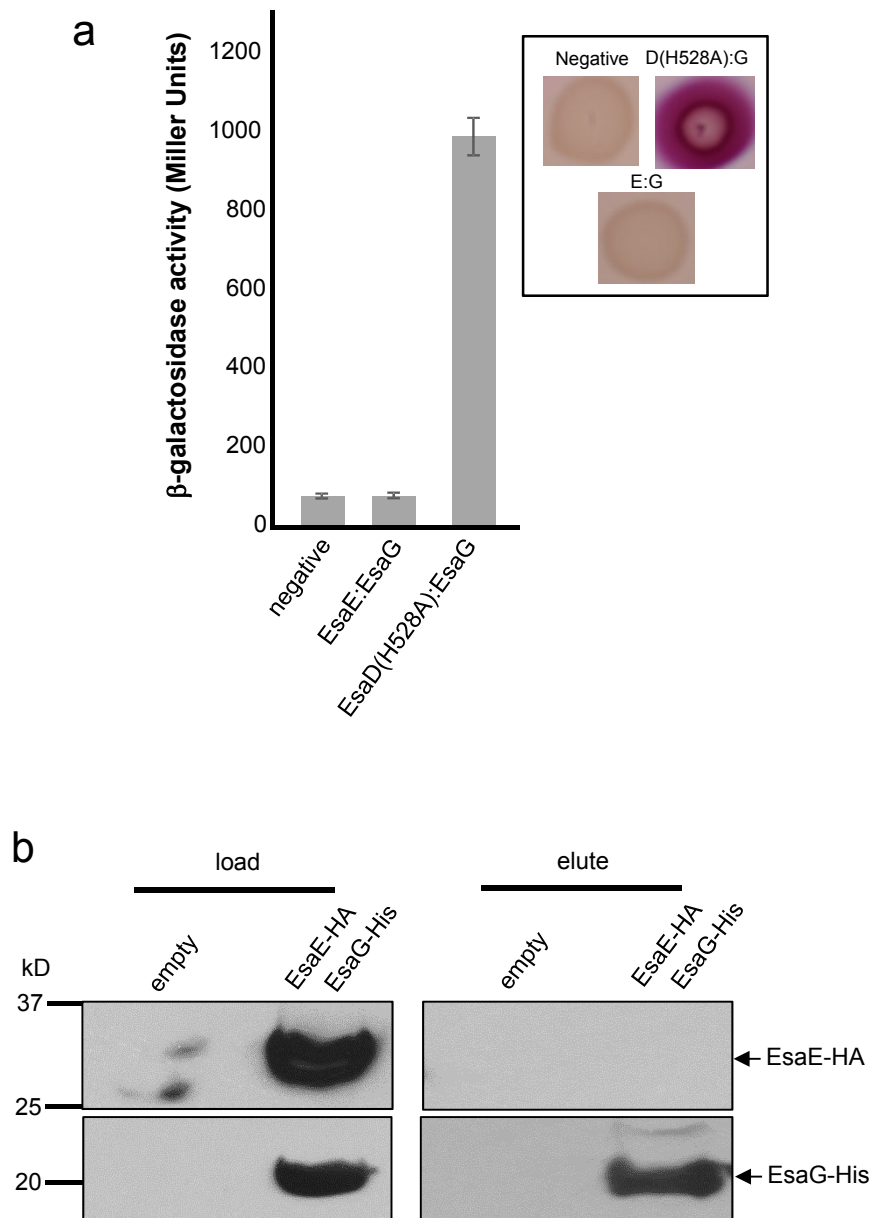
Supplementary Figure 10. EsaE and EsaG do not interact with each other. a. Bacterial two-hybrid analysis of strain BTH101 harbouring pT25 and pT18 (negative), pT25-EsaE and pT18-EsaG, or pT25-EsaG and pT18-EsaD(H528A) assessed by β-galactosidase activity assay. Error bars represent the standard deviation (*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 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 in each case the elution fractions (10μl aliquots of each) were separated by SDS PAGE (12%) and analysed by western blot with anti-HA or anti-His antibodies.
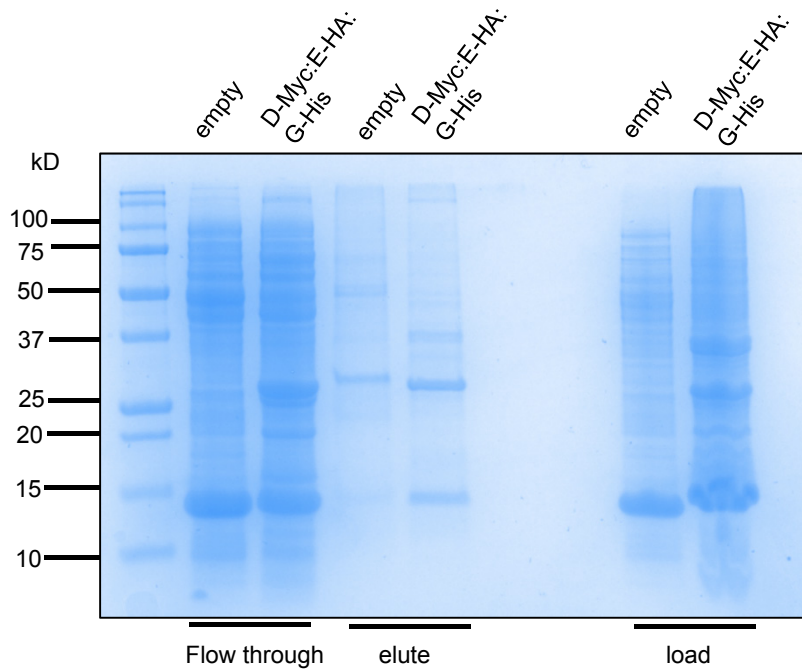
Supplementary Figure 11. Analysis of EsaE-HA-EsaD(H528A)-Myc-EsaG-His co-purification by coomassie staining. *E. coli* M15[pRep4] carrying pQE70 (empty) or pQE70- EsaE-HA-EsaD(H528A)-Myc-EsaG-His was cultured, lysed and EsaE-HA was purified using anti-HA antibody-coupled agarose beads as described in Methods and in the legend to Fig 3. The same samples (10μl 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.
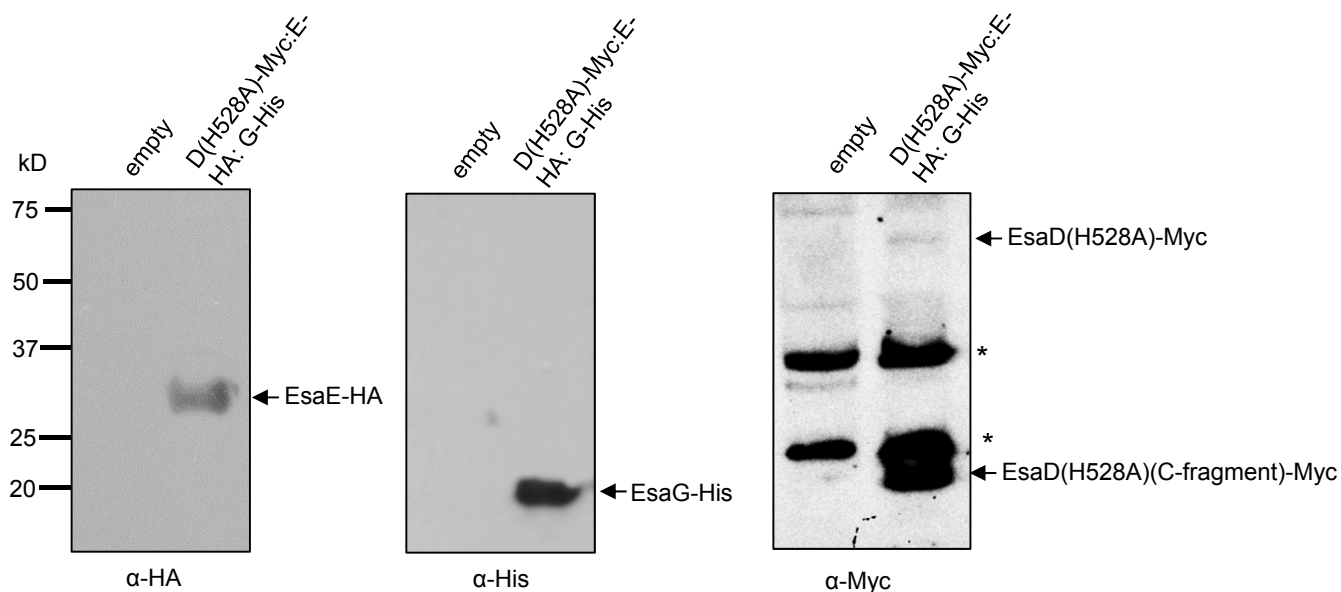
Supplementary Figure 12. EsaD, EsaE and EsaG form a ternary complex. *E. coli* strain M15[pRep4] carrying pQE70 (empty) or pQE70-EsaE-HA-EsaD(H528A)-Myc-EsaG-His was cultured in LB medium to OD_{600} of 0.5, and then supplemented with 2 mM IPTG for 4 hours, after which cells were harvested and lysed. His-tagged EsaG was purified using Ni-affinity beads, and 20μl 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.

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.
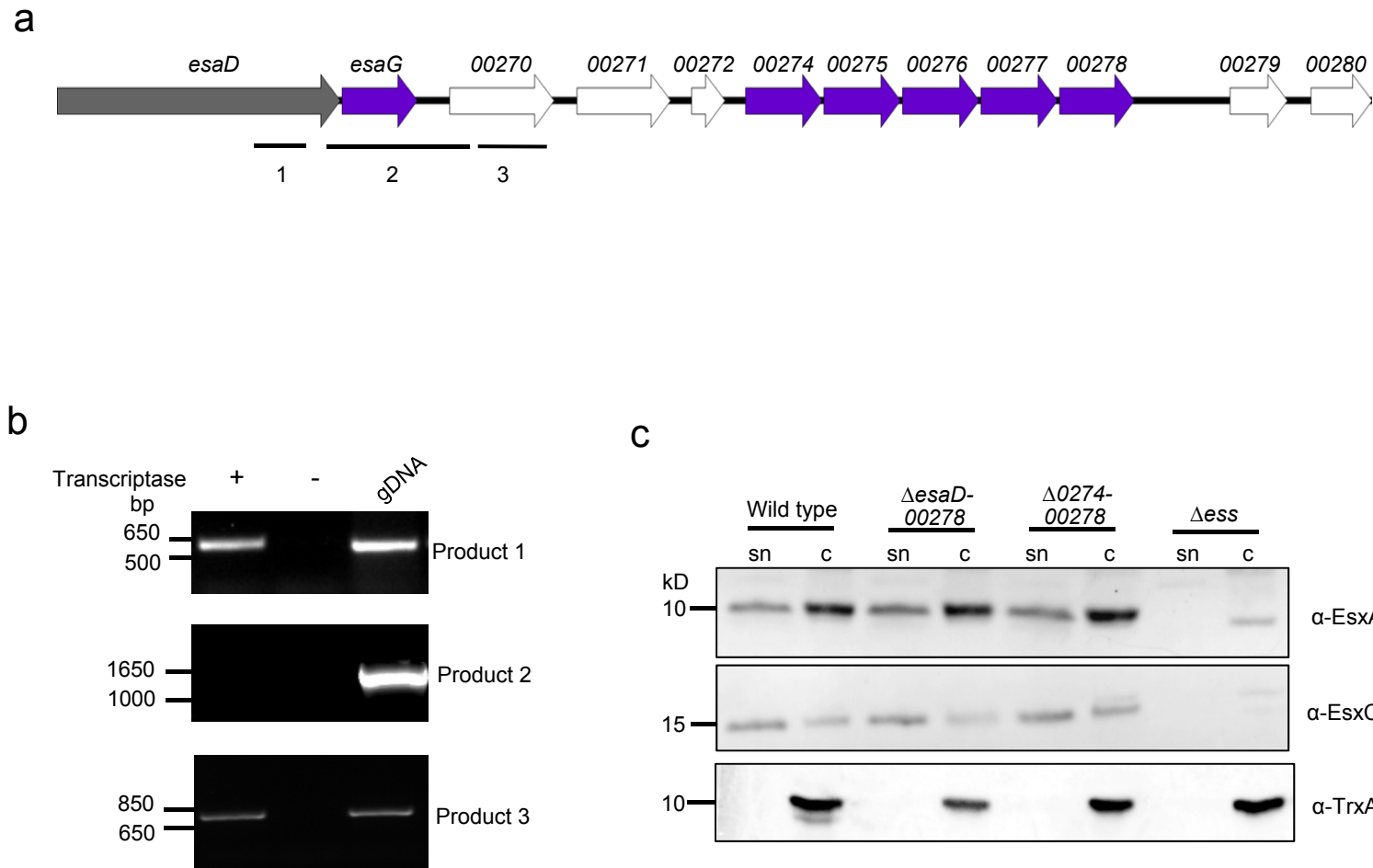
Supplementary Figure 14. Genes encoding additional DUF600 domain proteins are not co-transcribed with *esaG* and are not required for EsxA and EsxC secretion. a. Genetic organisation at the 3' end of the *ess* locus in S. aureus strain NCTC8325, with genes encoding DUF600 proteins are shaded in purple. 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 250μl of supernatant and 10 μl of cells adjusted to OD_{600} 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
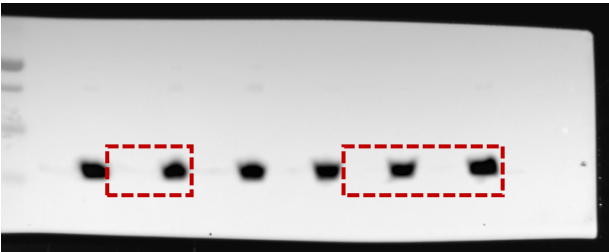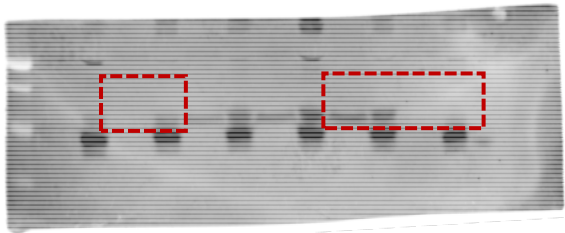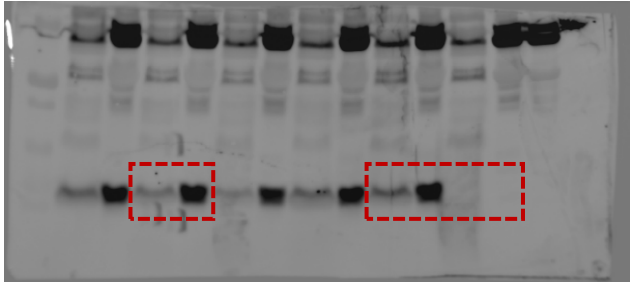
Uncropped Figure 1c
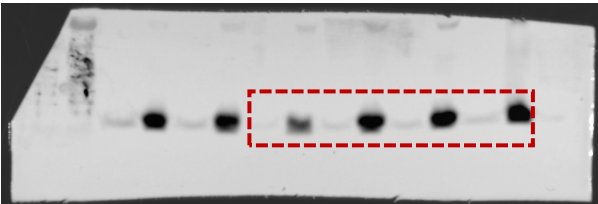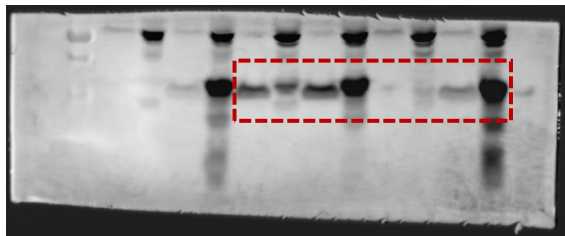
Uncropped Figure 1d
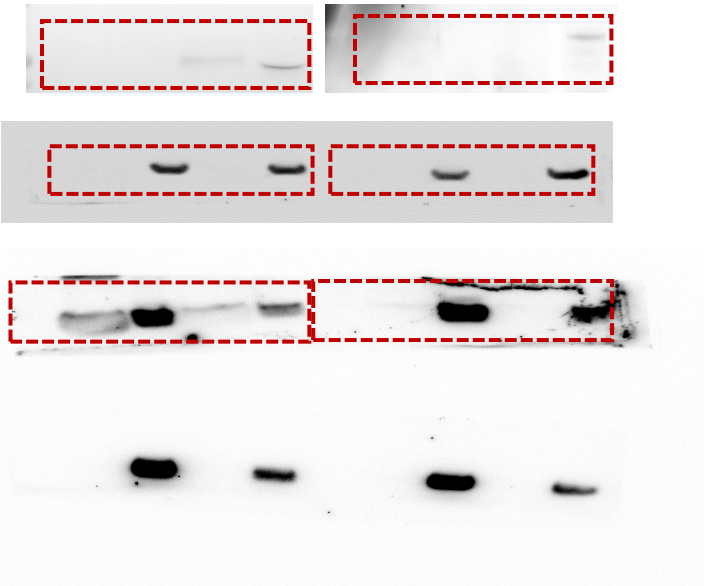
Uncropped Figure 1e
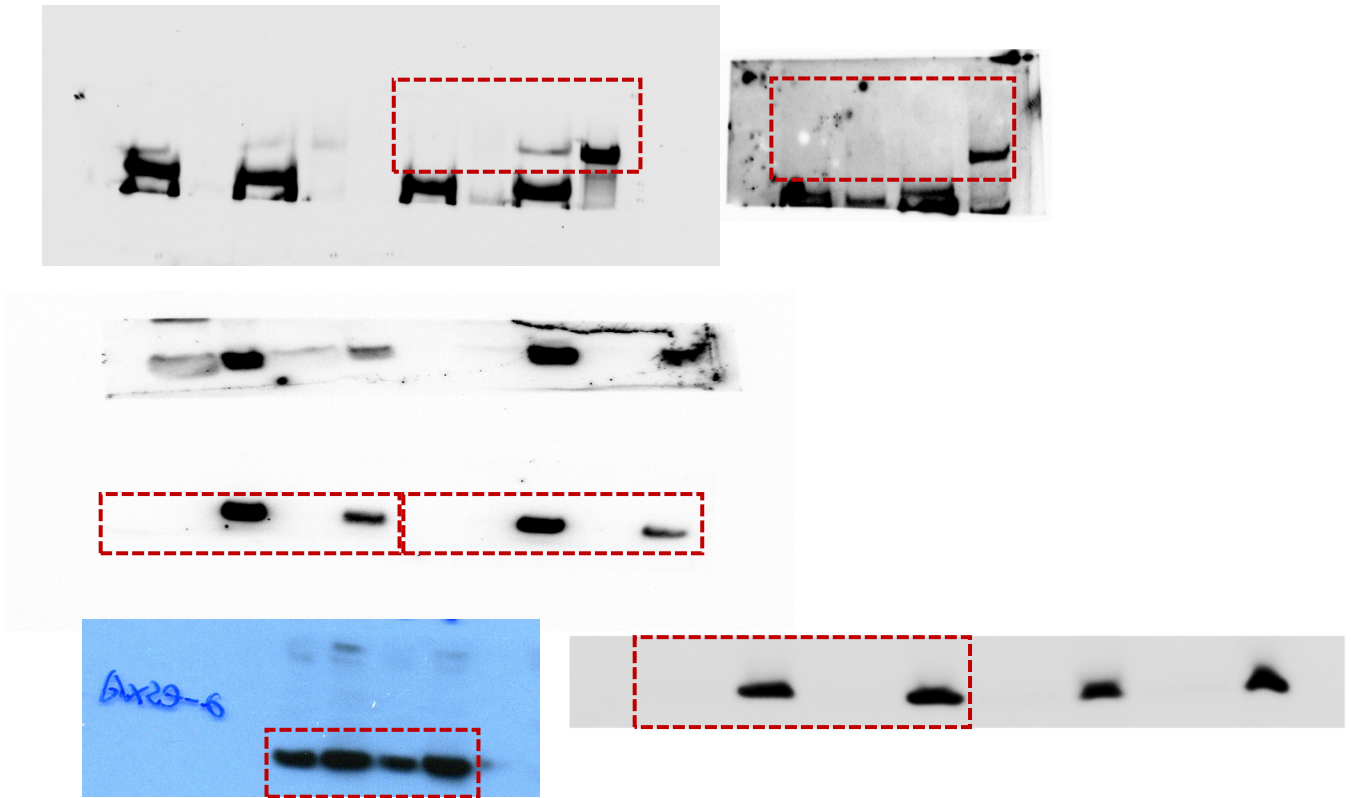
Uncropped Figure 1f
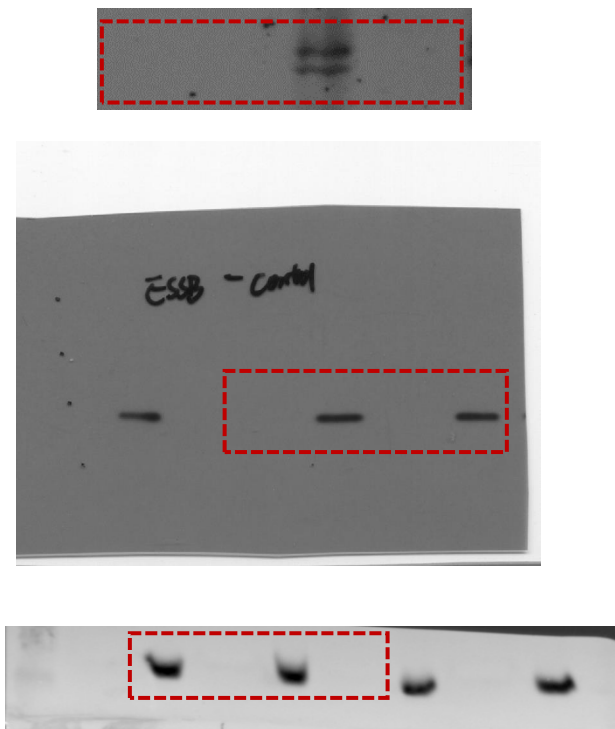
Uncropped Figure 2c
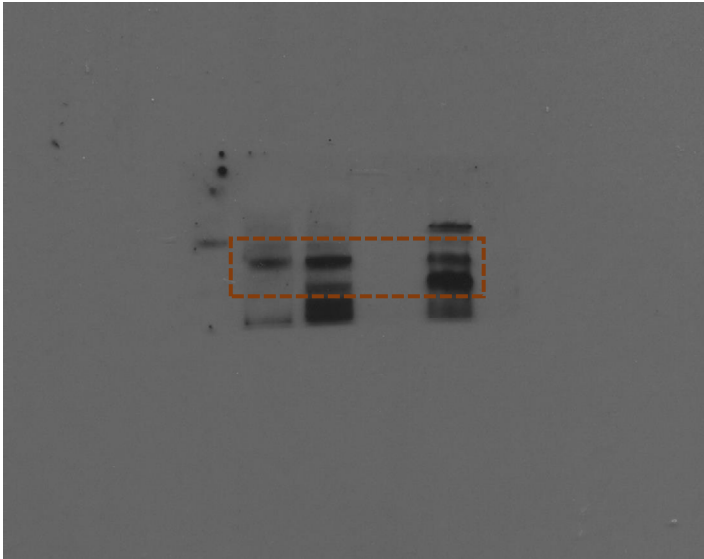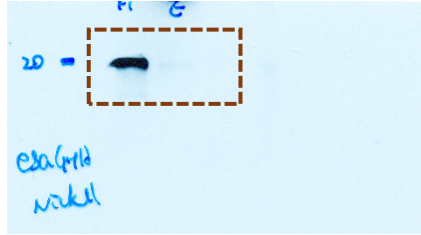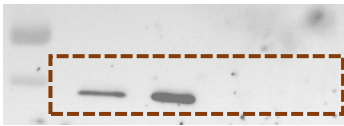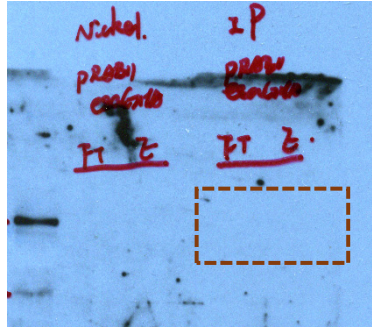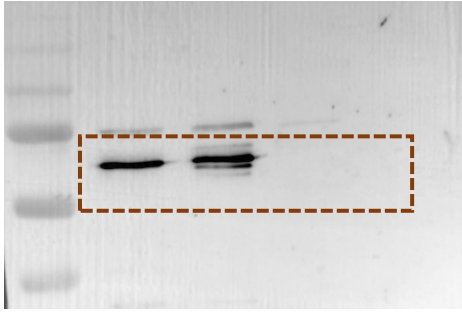
Uncropped Figure 2d
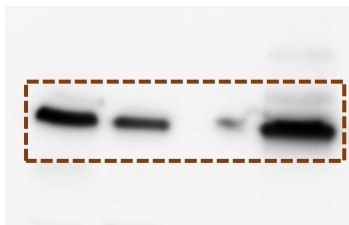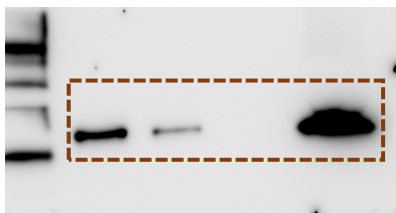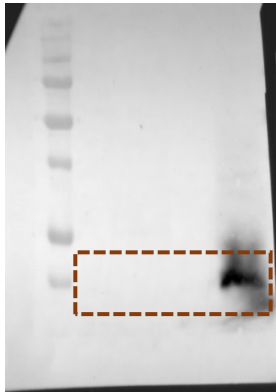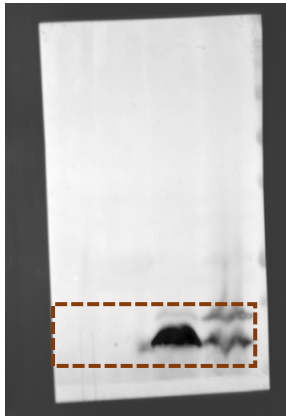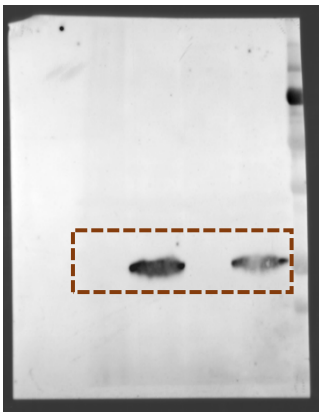
Uncropped Figure 2e
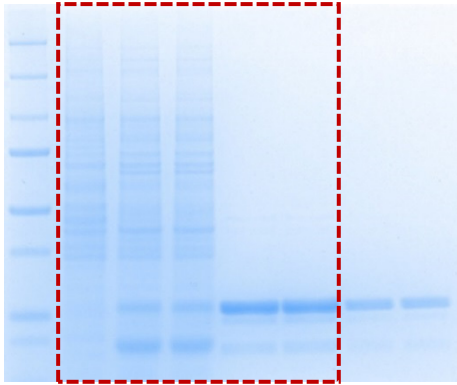
Uncropped Figure 2g
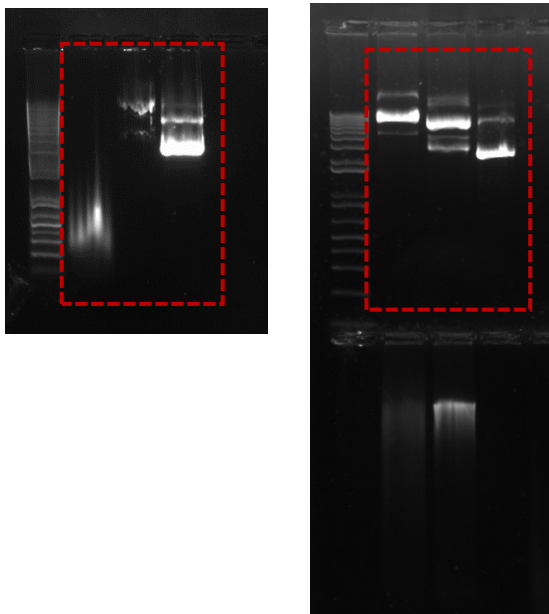
Uncropped Figure 3b
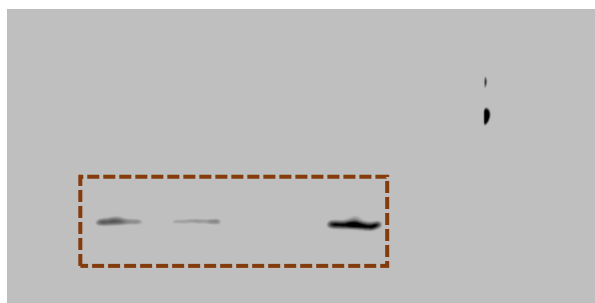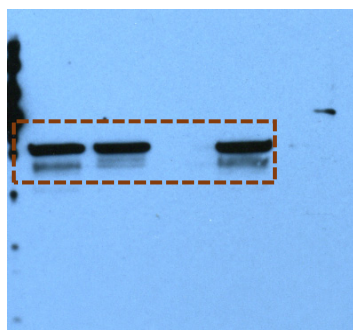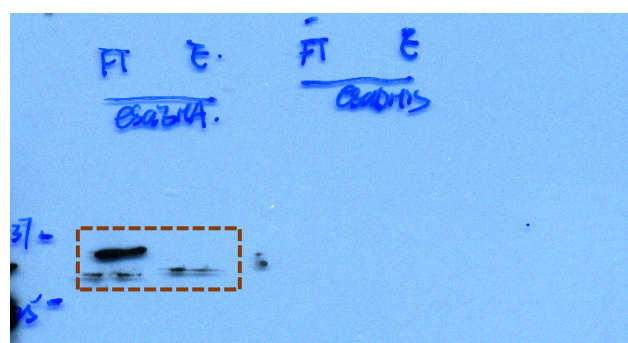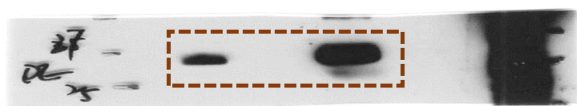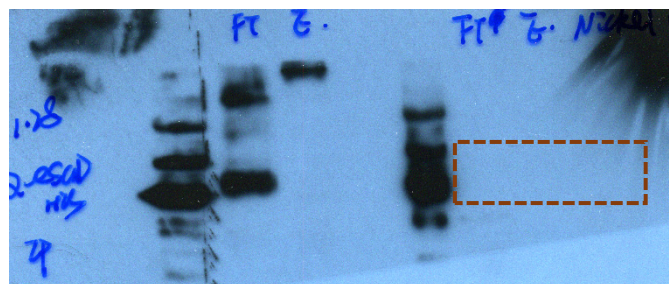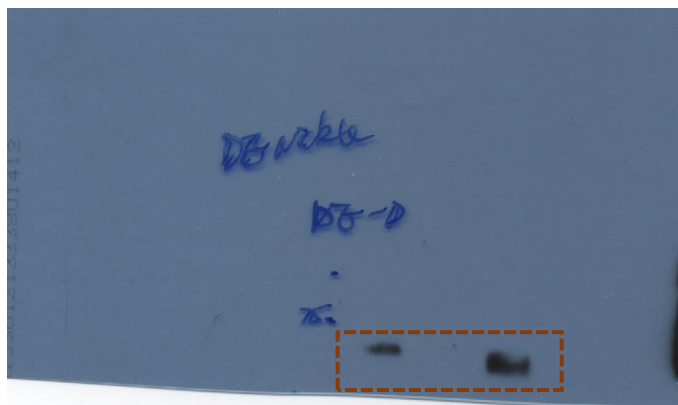
Uncropped Figure 3d
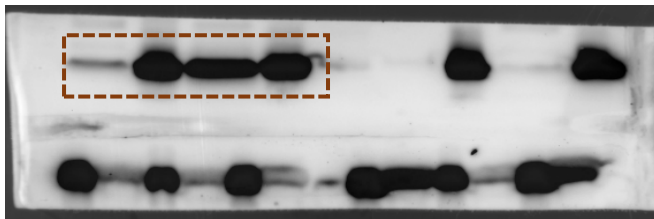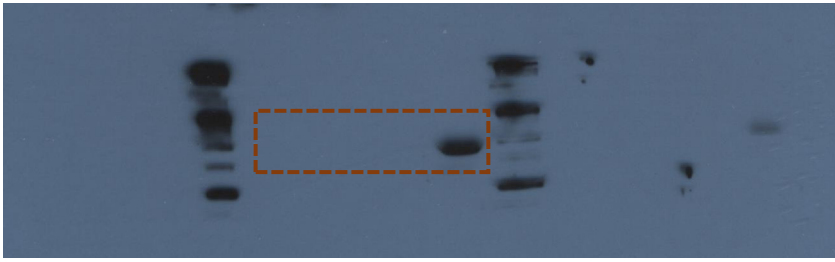
Uncropped Figure 3e
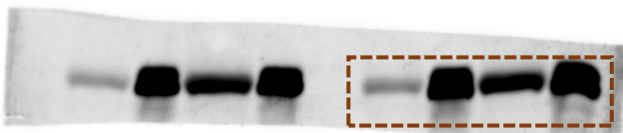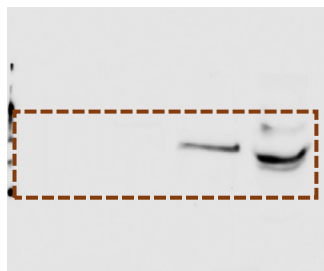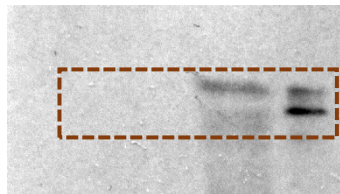
Uncropped Figure 3f
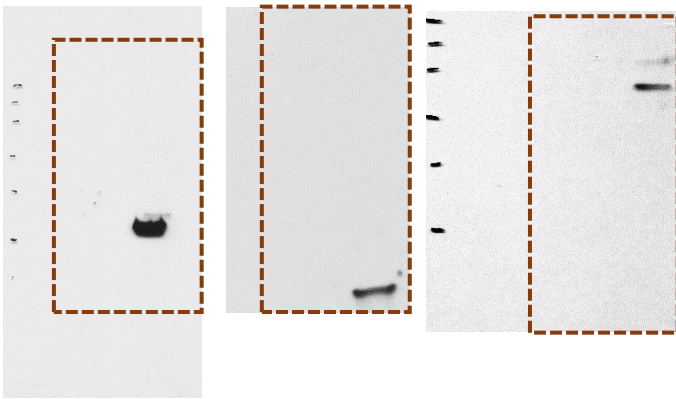
Uncropped Figure 3g
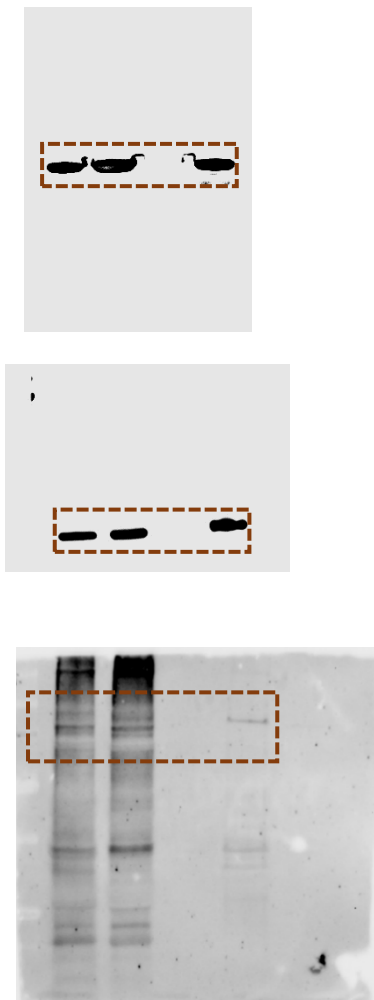
Uncropped Figure 4a
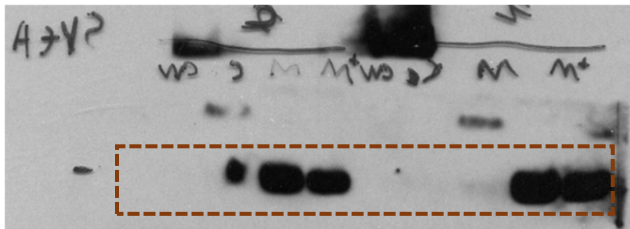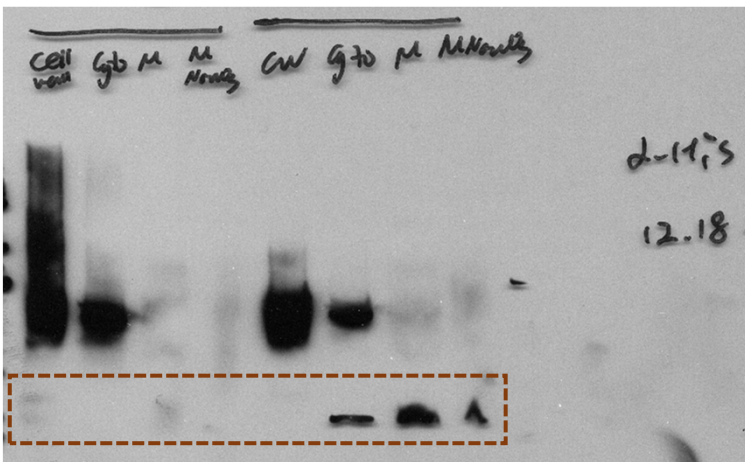
Uncropped Figure 4b
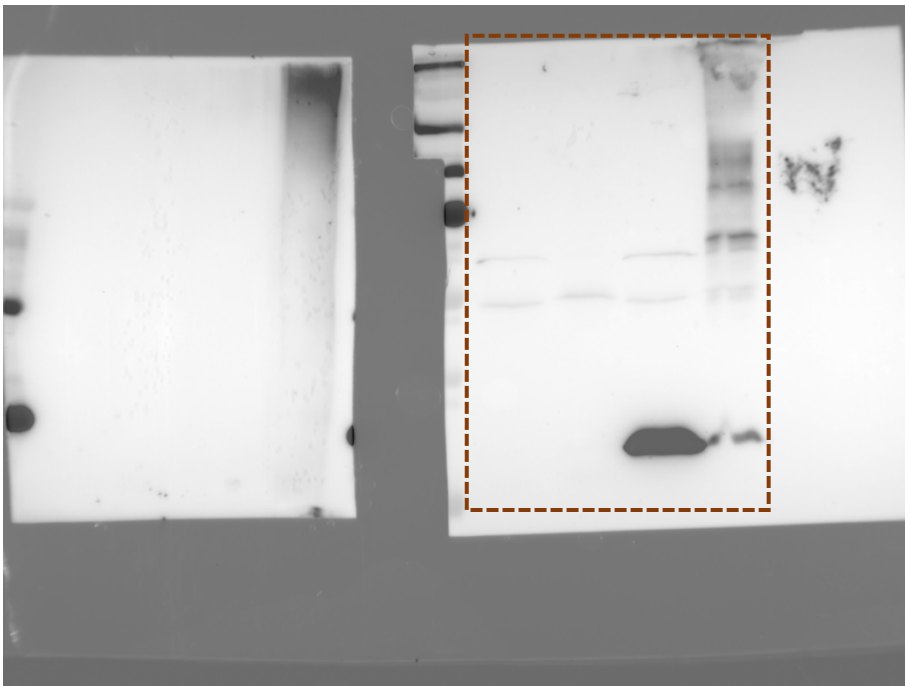
Uncropped Figure 4c
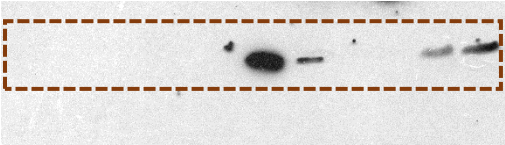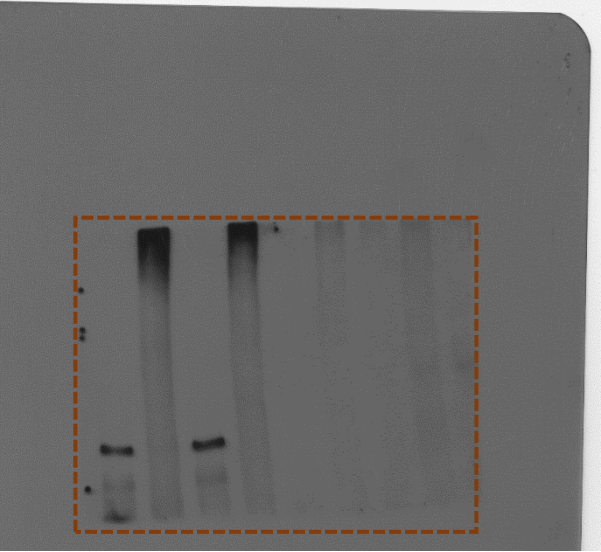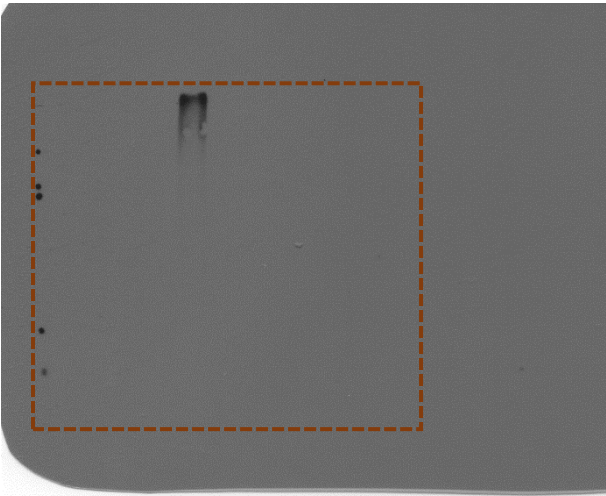
Uncropped Supplementary Figure 5
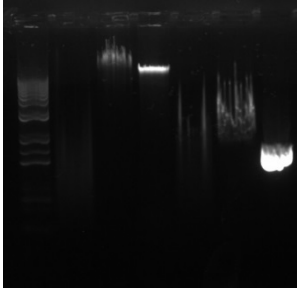
Uncropped Supplementary Figure 6
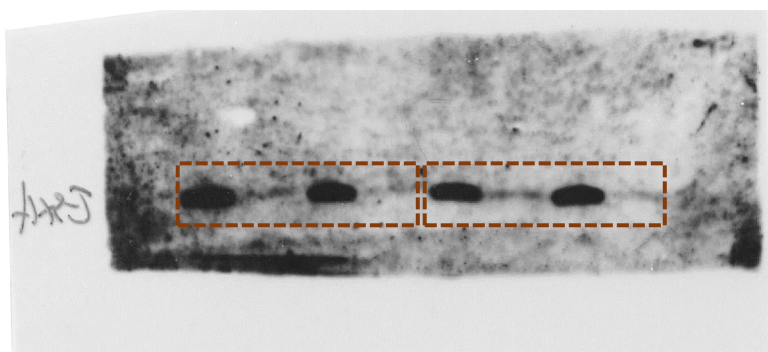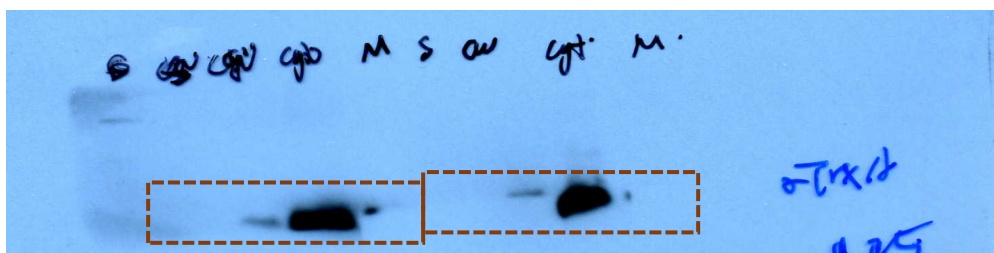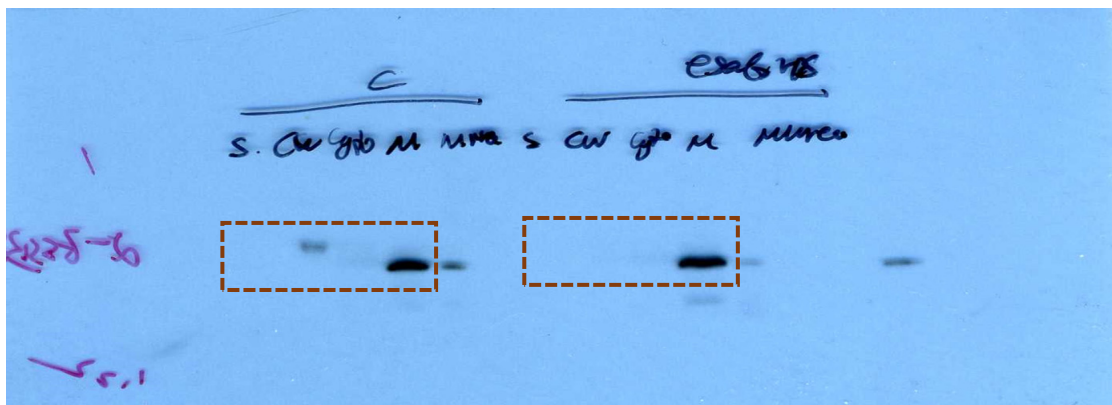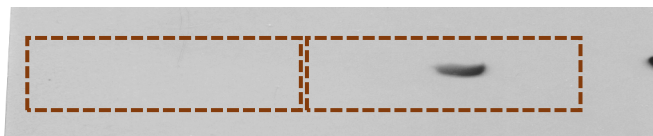
As a shorter exposure
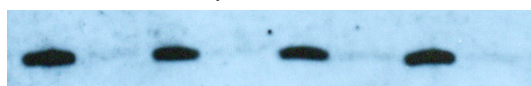
Uncropped Supplementary Figure 7
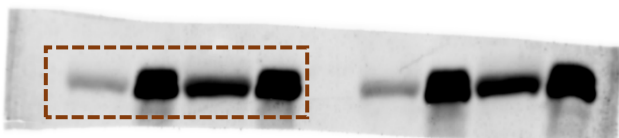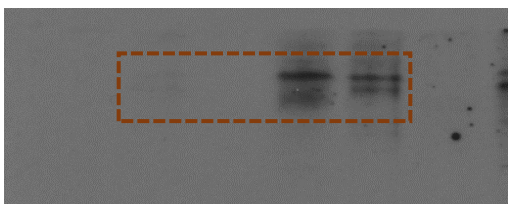
Uncropped Supplementary Figure 8
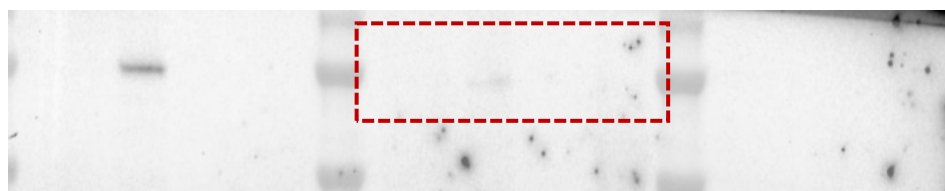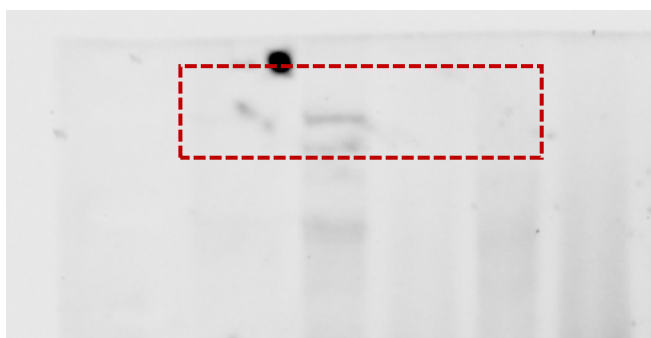
Uncropped Supplementary Figure 9a
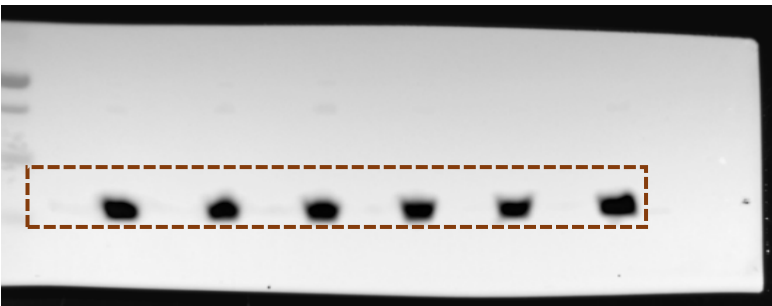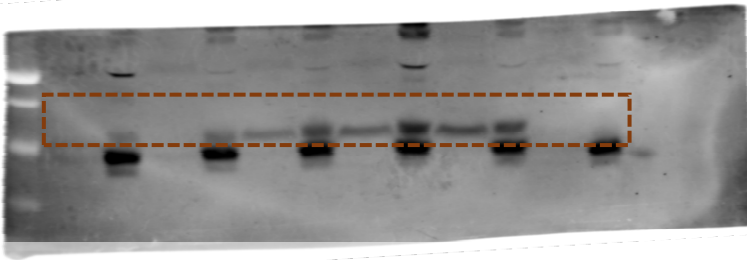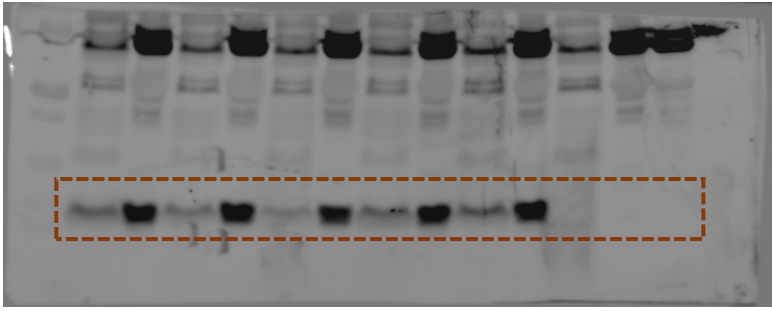
Uncropped Supplementary Figure 9b
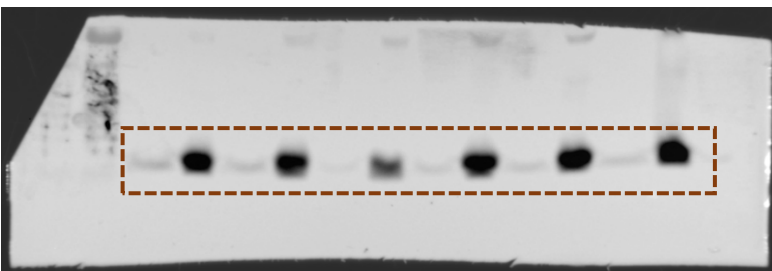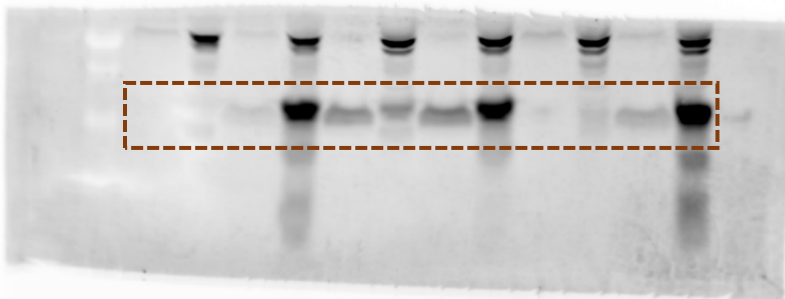
Uncropped Supplementary Figure 10
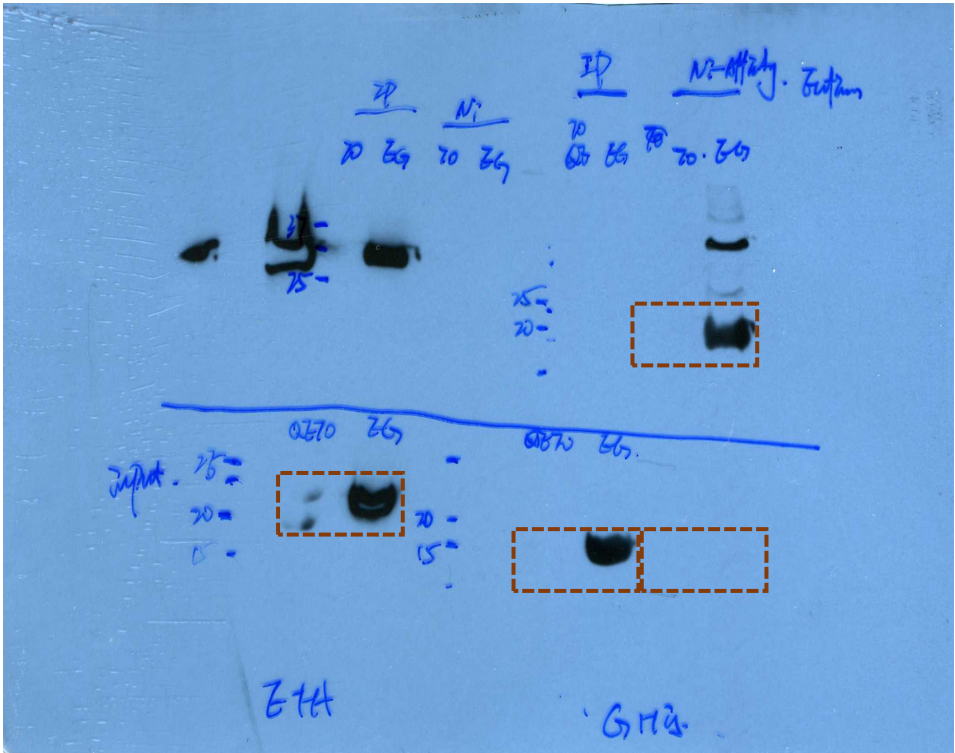
Uncropped Supplementary Figure 12
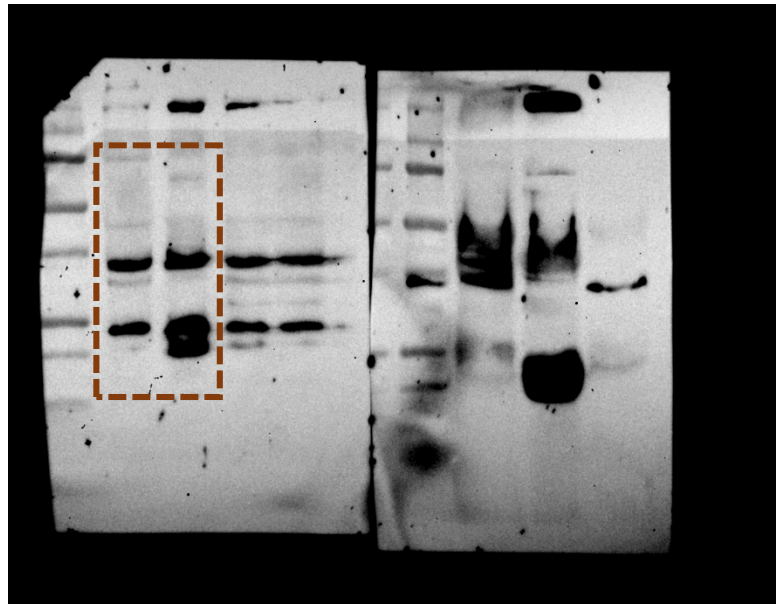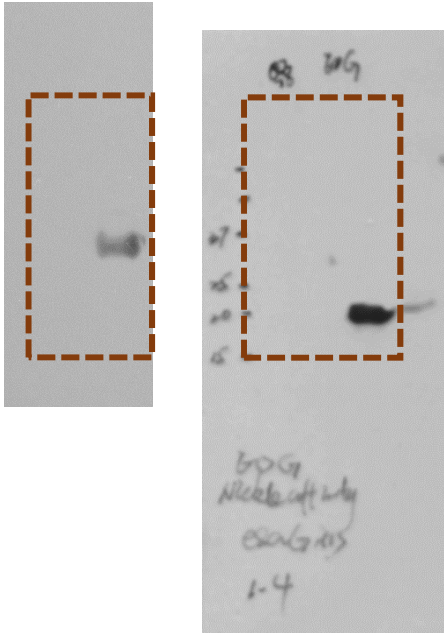
Uncropped Supplementary Figure 14b
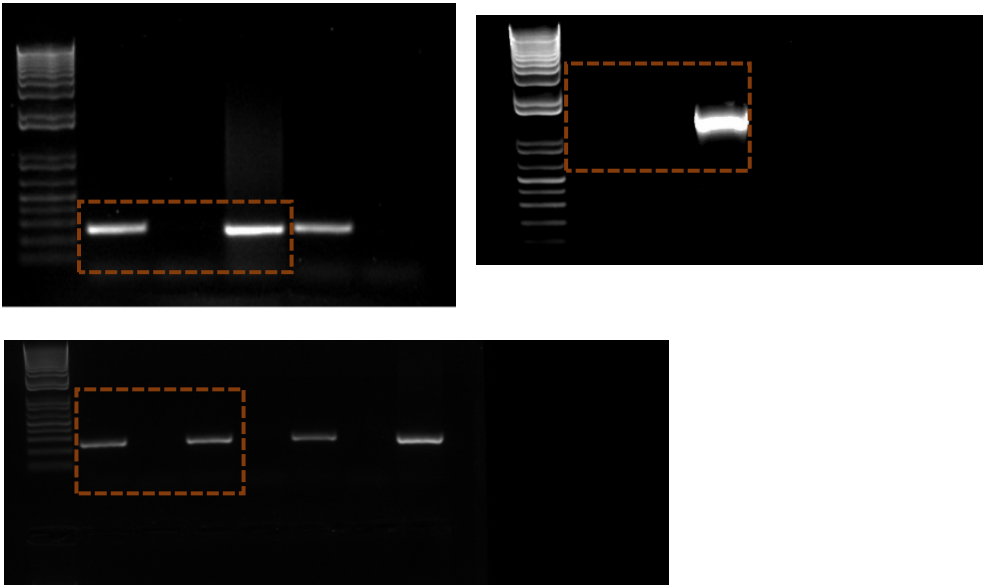
Uncropped Supplementary Figure 14c
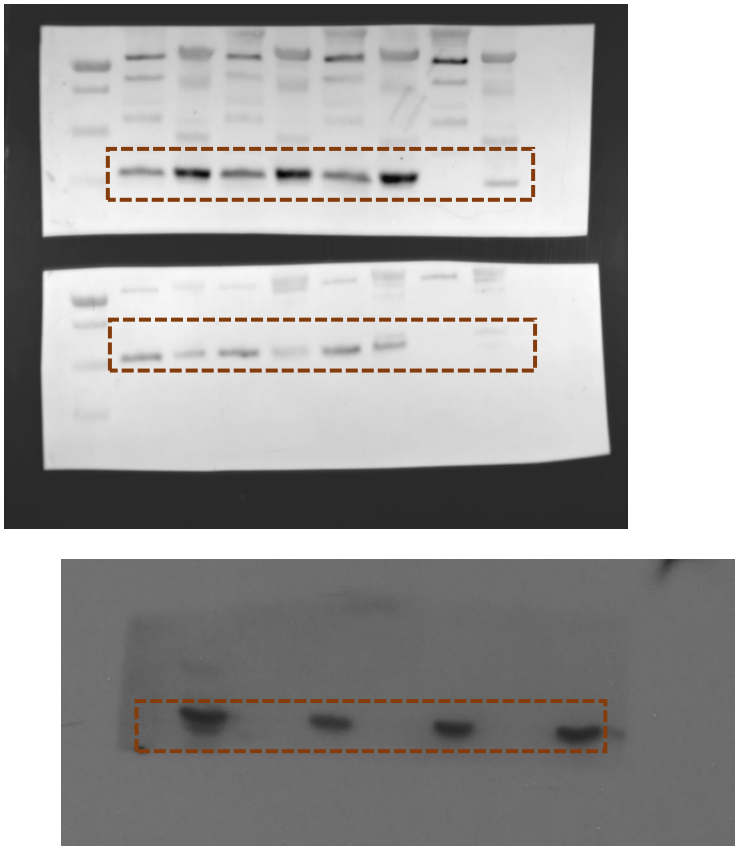
Supplementary Figure 16. Synthetic gene sequences used in this study.

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

<u>GGATCC</u>*ATGAAAGACGTTAAACGTATCGACTACTTCTCTTACGAAGAACTGACCATCCT*
GGGTGGTTCTAAACTGCCGCTGGTTAACTTCGAACTGTTCGACCCGTCTAACTTCGAAG
AAGCGAAAGCGGCGCTGATCGAAAAGAACTGGTTACCGAAAACGACAAACTGACCGA
CGCGGGTTTCAAAGTTGCGACCCTGGTTCGTGAATACATCTCTGCGATCGTTAACATCC
GTATCAACGACATGTACTTCGCGCCGTTCTCTTACGAAAAAGACGAATACATCCTGCTG
TCTCGTTTCAAAAACAACGGTTTCCAGATCCGTATCATCAACAAAGACATCGCGTGGTG
GTCTATCGTTCAGTCTTACCCGCTGCTGATGCGTCAGGAAAAATCTAACGACTGGGACT
TCAAACAGATCGACGACGAAACCCTGGAAAACCTGAACAACGAATCTATCGACACCATC
GGTCGTGTTCTGGAAATCGAAATCTACAACCACCAGGGTGACCCGCAGCAGTCTCTGT
ACAACATCTACGAACAGAACGACCTGCTGTTCATCCGTTACCCGCTGAAAGACAAAGTT
CTGAACGTTCACATCGGTGTTATCAACACCTTCATCCGTGAACTGTTCGGTTTCGACAC
CGACGAAACCACATCAACAAAGCGGAAGAA<u>TATCCATATGATGTTCCAGATTATGCA</u>T
AAT<u>GGATCC</u>ATTAAAGAGGAGAAATTAACC*ATGACCAAAGACATCGAATACCTGACCGC*
GGACTACGACAACGAAAAATCTTCTATCCAGTCTGTTATCGACGCGATCGAAGGTCAGG
ACTTCCTGGACGTTGACACCACCATGGACGACGCGGTTTCTGACGTTTCTTCTCTGGAC
GAAGACGGTGCGATCTCTCTGACCTCTTCTGTTGTTGGTCCGCAGGGTTCTAAACTGAT
GGGTTACTACCAGAACGAACTGTACGACTACGCGTCTCAGCTGGACTCTAAAATGAAAG
AAATCATCGACACCCCGTTCATCGAAGACATCGACAAAGCGTTCAAAGGTATCACCAAC
GTTAAACTGGAAAACATCCTGATCAAAAACGGTGGTGGTCACGGTCGTGACACCTACG
GTGCGTCTGGTAAAATCGCGAAAGGTGACGCGAAAAAATCTGACTCTGACGTTTACTCT
ATCGACGAAATCCTGAAATCTGACCAGGAATTCGTTAAAGTTATCGACCAGCACTACAA
AGAAATGAAAAAGAAGACAAAAAACTGTCTAAATCTGACTTCGAAAAAATGATGACCCA
GGGTGCGTCTTGCGACTACATGACCGTTGCGGAAGCGGAAGAACTGGAAGAACAGAAA
AAAAAAGAAGAAGCGATCGAAATCGCGGCGCTGGCGGGTATGGTTGTTCTGTCTTGCA
TCAACCCGGTTGCGGGTGCGGTTGCGATCGGTGCGTACTCTGCGTACTCTGCGGCGA
ACGCGGCGACCGGTAAAAACATCGTTACCGGTCGTAAACTGTCTAAAGAAGAACGTAT
CATGGAAGGTCTGTCTCTGATCCCGCTGCCGGGTATGGGTTTCCTGAAAGGTGCGGGT
AAATCTCTGATGAAACTGGGTTTCAAAGGTGGTGAAAAATTCGCGGTTAAAACCGGTCT
GCAGAAAACCATGCAGCAGGCGGTTTCTCGTATCTCTCCGAAAATGGGTATGATGAAAA
ACTCTGTTCTGAACCAGTCTCGTAACTTCGCGCAGAACACCCACGTTGGTCAGATGCTG
TCTAACATGCGTGGTCAGGCGACCCACACCGTTCAGCAGTCTCGTAACTGGATCGGTC
AGCAGGCGCAGAACGTTAAACGTATCGTTAACAACGGTCTGGACAAAGAAATCGCGCA
CCCGTTCAAACAGCAGCTGGCGCCGGCGGGTATGGGTGGTATCAAATTCGCGGAAAC
CACCACCCTGCGTAACATGGGTCAGAACATCAAACGTGCGGTTACCCCGCAGAACCAC
GTTACCCACGGTCCGAAAGACTCTATGGTTCGTTCTGAAGGTAAACACTCTATCTCTTC
TCACGAAATGAACTCTTCTAAATACGTTGAATCTCCGAACTACACCAAAGTTGAATTCGG
TGAACACTACGCGCGTCTGCGTCCGAAAAAACTGAAAGCGAACATCGAATACACCACC
CCGACCGGTCACATCTACCGTACCGACCACAAAGGTCGTATCAAAGAAGTTTACGTTGA
CAACCTGTCTCTGAAAGACGGTGACCGTAACTCTCACGCGCAGCGTACCGTTGGTGGT
GAAGACCGTCTGCCGGACGACGACGGTGGTCACCTGATCGCGCGTATGTTCGGTGGT
TCTAAAGACATCGACAACCTGGTTGCGCAGTCTAAATTCATCAACCGTCCGTTCAAAGA
AAAAGGTCACTGGTACAACCTGGAAAAAGAATGGCAGGAATTCCTGAACTCTGGTAAAG
AAGTTAAAAACATCAAAATGGAAGTTAAATACTCTGGTAACTCTCAGCGTCCGACCATCT
TCAAAGTTGAATACGAAATCAACGGTGAACGTAACATCCGTCGTATCCTGAACAAA<u>GAA</u>
<u>CAAAAACTTATTTCTGAAGAAGACCTG</u>TAATATTAAAGAGGAGAAATTAACC*ATGACCTT*
CGAAGAAAAACTGTCTAAATCTACAACGAAATCGCGAACGAAATCTCTTCTATGATCCC
GGTTGAATGGGAAAAAGTTTACACCATGGCGTACATCGACGACGGTGGTGGTGAAGTT
TTCTTCAACTACACCAAACCGGGTTCTGACGACCTGAACTACTACACCAACATCCCGAA
AGAATACAACATCTCTGTTCAGGTTTTCGACGACCTGTGGATGGACCTGTACGACCTGT
TCGAAGAACTGCGTGACCTGTTCAAAGAAGAAGACCTGGAACCGTGGACCTCTTGCGA
ATTCGACTTCACCCGTGAAGGTGAACTGAAAGTTTCTTTCGACTACATCGACTGGATCA

ACTCTGAATTCGGTCAGATCGGTCGTCAGAACTACTACAAATACCGTAAATTCGGTATC
CTGCCGGAAACCGAATACGAAATCAACAAAGTTAAAGAAATCGAACAGTACATCAAAGA
ACTGGAA<u>AGATCT</u>

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
<u>GGATCC</u>CACTTTCGAAGAAAAATTAAGTGAAATGTATAGCGAGATTGCGAATAAGATTA
GCAGCATGATACCGGTAGAGTGGGAGCAAGTATATGCAATGGCATATGTAACTGATCAA
GCTGGAGAAGTCATCTTTAATTATACTAAACCAGATAGTGATGAATTAAATTATTATTCAG
ACATACCTAAAGATTGCAATGTCTCAAAAGATATTTTTAAGAATTCATGGTTTAAAGTTTA
TCGAATGTTTGATGAGTTAAGAGAAACTTTTAAAGAAGAAGGGCTTGAACCATGGACAT
CATGCGAATTTGACTTTACAAGAGATGGCAAATTGAATGTATCTTTTGATTATATAGATT
GGATAAATACAGAGTTTGATCAATTGGGCCGTCAAAATTATTATATGTACAAAAAATTTG
GGGTTATACCAGAAATGGAATATGAAATGGAAGAAGTTAAAGAAATCGAACAATATATTA
AAGAGCAAGAAGAAGCTGAACAA<u>GGTACC</u>

pT25-00275
<u>GGATCC</u>CACTTTCGAAGAGAAAATAAGCAAATTATATAATGAGATTGCGAATGAGATTAG
CAGTATGATACCGGTAGAGTGGGAAAAGTATATACAATGGCTTATATAGATGATGGAG
GAGGTGAAGTATTCTTTAATTATACTAAACCAGGTAGTGATGACTTGAATTATTACACCG
ATATACCTAAGGAGTATAACATCTCTGTGCAAGTATTTGATGATTTATGGATGGATTTAT
ATGATTTGTTTGAGGAATTAAGAGATTTATTTAAAGAAGAAGGGCTTGAACCATGGACAT
CATGTGAATTTGACTTTACAAGCGAAGGTAAATTAAAAGTTTCATTTGATTATATAGATTG
GATAAATACAGAGTTTGATCAATTAGGCCGTGAAAATTATTATATGTATAAAAAATTTGG
GGTTTTACCAGAAATGGAATATGAAATGGAAGAAATTAAAGAAATCGATCAATATATTAA
AGAGCAAGATGAAGCTGAAATA<u>GGTACC</u>

pT25-00276
<u>GGATCC</u>CACTTTCGAAGAGAAAATAAGCAAATTATATAATGAGATTGCGAATGAGATTAG
CAGTATGATACCGGTAGAGTGGGAAAAGTATATACAATGGCTTATATAGATGATGGAG
GAGGTGAAGTATTCTTTAATTATACTAAACCAGGAAGTGAAGATTTGAATTATTATACCG
ATATACCTAAGGAGTATAATGTTTCTGTGCAAGTATTTGATGATTTATGGATGGATTTATA
TGATTTGTTTAAGAATTTAAGAAATTTATTTAAAGAAGAAGGACTTGAACCATGGACATC
ATGTGAATTTGACTTTACAAGAGACGGCAAATTGAATGTTTCATTTGATTATATTGATTG
GGCGAATTCAGAGTTTGGACAAATGGGAAGAGAACATTATTACATGTATAAAAAATTTG
GAATTTGGCCTGAAAAGAATATGCCATAAATTGGGTAAAAAAAATAAAAGATTATGTTA
AAGAGCAAGATGAAGCTGAACTA<u>GGTACC</u>

pT25-00277
<u>GGATCC</u>CACTTTCGAAGAAAACTAAGTCAAATGTACAATGAAATTGCAAATGAAATCAG
TGGAATGATACCAGTTGAATGGGAAAATATATATACAATTGCCTATGTAACTGATCAAGG
TGGAGAGGTCATTTTTAATTATACTAAACCAGGTAGCGATGAATTGAATTATTACACATA
TATCCCTAGAGAGTATAATGTCTCTGAAAAGTATTTTATGATTT
GTGGACGGATTTATATAGATTGTTTAAGAAGTTAAGAGAAACTTTTAAAGAAGAAGGGCT
TGAACCATGGACATCAAGTGAATTTGACTTTACAAGCGAAGGTAAATTAAAAGTTTCATT
TGATTATATTGATTGGATAAATACAGAGTTTGATCAATTAGGCCGTGAAAACTATTATAT
GTATAAAAAGTTTGGTGTTTTACCAGAAATGGAATACGAAATGGAAGAAGTTAAAGAAAT
CGAGCAATATATTAAAGAGCAAGATGAAGCTGAACTA<u>GGTACC</u>

pT25-00278
<u>GGATCC</u>CACTTTCGAAGAAAAGCTAAGTCAAATGTACAATGAAATTGCAAATGAAATCA
GTGGAATGATACCAGTAGAATGGGAAAAGTATATACAATTGCCTACGTAGATGATGAA
GGTGGAGAGGTTGTTTTTAATTATACTAAACCAGGAAGTGAAGATTTGAATTATTATTCA
GATATTCCTAAAGATTGCAATGTCTCAAAGATATTTTTAAGAATTCATGGTTTAAAGTTT
ATCGAATGTTTGATGAGTTAAGAGAAACTTTTAAAAAAGAAGATTTAGAACCGTGGACAT
CATGTGAATTTGACTTTACAAGAAAGGGAAATTTAAAAGTATCATTTGATTATATAGATTG
GATTAAATTAGGTTTTGGCCCATCAGGAAAGGAAACTACTATATGTACAAAAAATTTGG
TATTTTACCAGATATGGAATATGAAATGGAAGAAATTCGAGCAGTAGAGAAGTATGTTAA
AGAGCAAGAG<u>GGTACC</u>

pT25-SAPIG0310
<u>GGATCC</u>CACTTTCGAAGAAAACTAAGTCAAATGTACAACGAGATTGCGAATGAGATCA
ATGGAATGATACCAGTAGACTGGGAAAAGGTATATGCAATGGCATATATAGATGATGGA
GGAGGAGAAGTGTTCTACTATTACACAGAACCTGGAAGGAATGAATTATACTACTATAC
TAGTGTATTAAATAAATATGATATCAGAATCAGAATTTATGGACTCAGAGTATGAGTT
GTATAAACAATTTCAAAAGTTAAGAAATATATTTAAAGAAGAAGGACATGAACCATGGAC
ATCATGCGAATTTGATTTTACAAGAGAAGGTAAATTAAAAGTTTCATTTGATTATATAGAT
TGGATAAATTCAGAATTTGGTCAAATAGGTCGACAAAATTACTATAAGTATAGAAAATTT
GGAATTTTACCAGAAACGGAATATGAAATTAATAAAGTTAAAGAAATCGAGCAATATGTT
AAAGAACAAGAAGAAGCTGAAATA<u>GGTACC</u>

pT25-SAPIG0311
<u>GGATCC</u>CAATTTCGAAGAAAACTAAGTCAAAAGTACAACGAGATTGCGAATAAAATTA
GTAGCATGATACCAGTAGAGTGGGAAAAGGTATATGCAATGGCTTATATAAATGAAAGA
AATGGAGAAGTTTTCTACAATTATACTGAGCCAAGCAGTGATGAATTGTTTTACTATACG
AGCGTGTTAAATAAATATAATATACCAAGATCAGAATTTATGGACTCAGTATATGAATTAT
ATAAGCAATTTGATAATTTAAGAGAATTGTTTATAGAAGAAGGACTCGAGCCATGGACAT
CATGCGAATTTGACTTTACAAGAGAGGGTAAATTAAACGTATCTTTTGATTATATTGATT
GGACTAAATTAGAATTTGGTCAAATAGCAAAAGAAAATTATTATATGTATAAAAAATTTGG
AGTTATGCCAGAAATGAAATATGAAATTAATAAAGTTAAAGAAGTAGAGAAGTGTATTAA
AGAGCAAGAAGAA<u>GGTACC</u>

pT25-SAPIG0314
<u>GGATCC</u>CACTTTCGAAGAGAAATTAAGTGAAATGTATAACGAGATTGCGAATGAGATCA
GTGGGATGATACCAGTAGAATGGGAGCAAGTATTTACAATAGCCTATGTAACTGATCAA
GCTGGAGAAGTCATTTTTAATTATACTAAACTGGTAGTGATGAAT<u>GGTACC</u>

pT25-SAR0293
<u>GGATCC</u>CACTTTCGAAGAGAAGTTAAGTCAAATGTACAACGAGATTGCGAATGAGATCA
GTGGGATGATACCGATAGAGTGGGAAAAGTATATACAATGGCTTATATAGATGATGAA
GGTGGAGAAGTGTTCTACTATTACACAGAACCTGGAAGCAATGAATTATACTACTATACT
AGTGTATTAAATAAATATGATATCGGAATCAGAATTTATGGACTCAGCGTATGAGTTG
TATAAACAATTTCAAAATTTAAGAAATATATTTAAAGAAGAAGGATATGAACCATGGACAT
CATGCGAATTTGATTTTACAAAGAAGGTGAATTAAAAGTTTCATTTGATTATATAGATTG
GATCAATACAGAGTTTGATCAATTGGGCCGTCAAAATTATTATATGTACAAAAAATTTGG
GGTTATACCAGAAATGGAATATGAAATGGAAGAAGTTAAAGAAATCGAGCAATATATTAA
AGAGCAAGATGAAGCTGAACAA<u>GGTACC</u>

pT25-SAR0294
<u>GGATCC</u>CACTTTCGAAGAAAAATTAAGTGAAATGTACAATGAAATTGCGAATAAAATTAG
TAGCATGATACCAGTAGAATGGGAAAAGGTATATACAATGGCTTATATAGATGATGGAG
GAGGTGAAGTATTCTTTAATTATACTAAAATAAACAGCGATGAATTGAATTATTACACCG
ATATACCTAAGGAGTATAACATTTCTGTGCAAGTATTTGATGATTTATGGATGGATTTATA
TGATTTGTTTGAGGAATTAAGAAATTTATTTAAAGAAGAAGGACATGAACCATGGACATC

ATGCGAATTTGATTTTACAAGAGACGGCAAATTGAATGTTTCATTTGATTATATTGATTG
GGCGAATTCAGAGTTTGGGCCAATGGGAAGAGAACATTATTATATGTATAAAAAATTTG
GAATTTGGCCTGAAAAGAATATGCCATAAATTGGGTTGAAAAAATAAAAGATTATGTTA
AAGAGCAAGAAGAAGCTGAACTA<u>GGTACC</u>

pT25-SAR0295
<u>GGATCC</u>CACTTTCGAAGAAAAACTAAGTGAAATGTACAACAAGATTGCAAATGAGATTG
TTGGCATGATACCTGTAGAATGGGAAAAGGTATATACAATAGCCTATGTAAATGATAGA
GGTGGAGAGGTCGTTTTTAATTATACTAAACCAGGTAGCGATGAATTGAATTATTACATG
AATATATCTAGAGATTATAATGTTTCGGAAGAAATATTTGATGATTTATGGATGGAACTTT
ATAGATCATTTAAAAAATTAAGAAATATATTTAAAGAAGAAGGACATGAACCATGGACAT
CATGCGAATTTGATTTTACAAACGAAGGTAAATTAAAAGTTTCATTTGATTATATTGATTG
AAAGAATACAGAATTTGATCAATTGAGTCTTGAAAATTATTATATGTACAAAAAATTTGGG
GTTATACCAGAAATGGAAGAAATTAAAGAAATCGAGCAATATATTAAAGAGCAAGAAGAA
GCTGAACTA<u>GGTACC</u>

pT25-SAR0297
<u>GGATCC</u>CACTTTCGAAGAAAAACAAAGTGAAATGTACAATAAAATTGCAAATGAGATTAG
TGGGATGATACCAGTAGAGTGGGAAAAGGTATATACAATTGCCTACCTAGATGATGAAG
GTGGAGAAGTCGTTTTTAATTATACTAAACCAGGTAGCGATGAATTGAATTATTACACGG
ATATATCTAGAGATTATAATATTTCAGAAAAAATATTTGATGATTTATGGATGAATCTTTAT
TACTTGTTTATGAATTTAAGGGATTTATTTAAATAAGAAGATTTAGAACCATGGACATCAT
GTGAATTTGACTTTACAAGCGAAGGTGATTTAAACGTATCTTTTGATTATATAGATTGGA
TTAAATTAGGTTTTGGCCCATCAGGAAAGGAAACTACTATATGTATAAAAAGTTTGGTG
TTTTACCAGAAATGGAATATGAAATGGAAGAAATTCGAGCAGTAGAGAAGTATGTTAAA
GAGCAAGAG<u>GGTACC</u>

pT25-SAEMRSA15_02570
<u>GGATCC</u>CACTTTCGAAGAAAAATTAAGTGAAATGTACAATGAAATTGCGAATGAGATTAG
CAGTATGATACCAGTAGAGTGGGAAAAGGTATATGCAATTGCCTATGTAGATGATCAAG
GTGGAGAGGTCGTTTTTAATTATACAAAACCAGGTAGTGATGAATTGAATTATTACACGA
ATATATCTAGAGGTTATAATGTTTCGGAAGAAATATTTGATGATTTATGGATGAATCTGTA
TTACTTGTTTAAGAATTTAAGGAATTTATTTAAAGAAGAAGGACTCGAACCATGGACATC
ATGCGAATTTGACTTTACAAGAGACGGCAAATTGAAAGTATCATTTGATTATATAGATTG
GATTAATACAGAGTTTGATCAATTGGGCCGTGAAAATTACTATATGTACAAAAAGTTTGG
TGTTTTACCAGAAATGGAATACGAGATGGAAGAAATTAAAGAAATCGAGCAATATATTAA
AGAGCAAGATGAAGCTGAACTA<u>GGTACC</u>

pT25-SAEMRSA15_02580
<u>GGATCC</u>CACTTTCGAAGAAAAACTAAGTCAAATGTACAACGAGATTGCGAATGAGATTA
GCAGCATGATACCAGTAGAGTGGGAAAAGGTATATGTAATTGCCTATGTAGATGATGGA
GGTGGAGAGGTCATTTTTAATTATACTAAACCAGGTAGCGATGAATTGAATTATTACACA
TATATCCCTAGAGAGTATAATGTCTCTGAAAAGTATTTTATGATTTGTGGACGGATTTAT
ATAGATTGTTTAAGAAGTTAAGAAATGCATTTAAAGAAGAAGGACTTGAACCATGGACAT
CATGTGAATTTGACTTTGCAAGAGATGGCAAATTAAATGTATCTTTTGATTATATTGATTG
GGTAAATACAGAGTTTGATCAATTGGGCCGTGAAAACTATTATATGTATAAAAAGTTTGG
TGTTTTACCAGAAACGGAATATGAAATGGAAGAAATTCGAGCAGTAGAGAAGTATGTTA
AAGAGCAAGA<u>GGTACC</u>

Restriction sites are underlined

***ermC* integration synthetic construct**

GTCGACGCAGGTAGAGATACAAGAGGATTAATTCGTTTACATCAATTCGATAAAGTGGA
AATGGTACGTTTTGAACAACCTGAAGATTCATGGAATGCTTTAGAAGAAATGACAACAAA
CGCAGAAGCAATTCTAGAAGAGTTAGGTTTACCATACCGTCGTGTTATTTTATGTACAG
GTGATATTGGATTTAGTGCAAGCAAACATATGATTTAGAAGTTTGGTTACCAAGCTACA
ATGATTATAAAGAAATTAGTTCATGCTCAAACTGTACGGATTTCCAAGCGCGTCGTGCTA
ACATCCGCTTCAAGCGTGACAAAGCAGCTAAACCAGAATTAGCACATACATTAAATGGT
AGTGGTTTAGCAGTTGGACGTACATTTGCTGCTATTGTTGAAAATTACCAAATGAAGAT
GGAACAGTAACAATTCCAGAAGCATTAGTACCATTTATGGGTGGTAAAACACAAATTTCA
AAACCAGTTAAATAAAGGCTTTAGCTACAAGCTTTAAAAAGTATATATCTACGTATACTTA
AAGCAAGGGCAAGATACTTTAAATAATATTTTAAAAAGTGGTGACGAAGCTGTCGCCAC
TTTTTTTGTGCTGTAAAAATATAATAGTGA**G**GATGCAGTTGTAAAGGGACAAGAGCTTTG
GTATAATATAAAATTGTGAGTAATAGAATTATTGCTCCTTGCCCATTATGGGCCGCTTAG
TCCAAAAGGAGGTGCAACAGATGAACGAGAAAAATATAAAACACAGTCAAAACTTTATT
ACTTCAAAACATAATATAGATAAAATAATGACAAATATAAGATTAAATGAACATGATAATA
TCTTTGAAATCGGCTCAGGAAAAGGGCATTTTACCCTTGAATTAGTACAGAGGTGTAATT
TCGTAACTGCCATTGAAATAGACCATAAATTATGCAAAACTACAGAAAATAAACTTGTTG
ATCACGATAATTTCCAAGTTTTAAACAAGGATATATTGCAGTTTAAATTTCCTAAAAACCA
ATCCTATAAAATATTTGGTAATATACCTTATAACATAAGTACGGATATAATACGCAAAATT
GTTTTTGATAGTATAGCTGATGAGATTTATTTAATCGTGGAATACGGGTTTGCTAAAAGA
TTATTAAATACAAAACGCTCATTGGCATTATTTTTAATGGCAGAAGTTGATATTTCTATAT
TAAGTATGGTTCCAAGAGAATATTTTCATCCTAAACCTAAAGTGAATAGCTCACTTATCA
GATTAAATAGAAAAAAATCAAGAATATCACACAAAGATAAACAGAAGTATAATTATTTCGT
TATGAAATGGGTTAACAAAGAATACAAGAAAATATTTACAAAAAATCAATTTAACAATTCC
TTAAAACATGCAGGAATTGACGATTTAAACAATATTAGCTTTGAACAATTCTTATCTCTTT
*TCAATAGCTATAAATTATTTAATAAGTAA*GTTACAACCAATGACGACTGGGGCATTTCTTT
AATGAATTGCTCCAGTTTTTGT**C**CAATGCACATAACAACAATAAATTAAGTTTGTGGTTTA
ATGGGGTGAACGCATTTCATTATAGCAACAATACGGGATAATTATGATGAACTAAAACAA
TCTAAAACGTAACAAGTTTGAGCATCACTAATATAGGAAAGGAAGCGATAAAATACTGAT
TTCGTTGATATGTAGTATGAGTTATATCGATGGAGTAGGGTAGGGGGAGGGGATGATTA
TAAGGGAGTGGTACATGAATCAATATCCCAGACTCATCATCAGATATAAAAATTTATAAA
ATTGATACTTAAAAACAACTACAAATCCATAGAAAATATGGAGGTAGTCTTAAATAAAAAA
TTGAAAATTCTCAAAAATAAAAAGTTAATATGAAGCTGACTAAAGACTCCGGAATGTCTA
ACCTCAGACAAACTGATGTCTAATGTTATTGCTTAGGGTATAGAACTGTATTAGACTAGG
TATATTATTTTTTTCGTAATTATATAAATATAAAGTGGCAAAGGAGGTAATTGAGATGACAA
CACATTTAAGTTTTAGACAAGGCGTGCAAGAGTGTATCCCAACATTATTGGGTTATGCC
GGTGTTGGTATTTCATTTGGTATTGTGGCTTCGTCTC GAATTC

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*.

gtcgacATGgcgatgattaaaatgagcccggaagaaattcgcgcgaaaagccagagctatggccagggcagcgatcagat
tcgccagattctgagcgatctgacccgcgcgcagggcgaaattgcggcgaactgggaaggccaggcgtttagccgctttgaag
aacagtttcagcagctgagcccgaaagtggaaaaatttgcgcagctgctggaagaaattaaacagcagctgaacagcaccgc
ggatgcggtgcaggaacaggatcagcagctgagcaacaactttggcctgcagTAAgcattctgaaattggcaaagtcacattt
tctaatgtggctttgcttatcattttttttaagaaaacaactgaaaggaaataagcATGaaaaaaaaaaaactggatttatgcgctgat
tgtgaccctgattattattattgcgattgtgagcatgatttttttttgtgcagaccaaatatggcgatcagagcgaaaaaggcagccag
agcgtgagcaacaaaaacaacaaaattcatattgcgattgtgaacgaagatcagccgaccacctataacggcaaaaaagtg
gaactgggccaggcgtttattaaacgcctggcgaacgaaaaaaactataaatttgaaaccgtgacccgcaacgtggcggaaa
gcggcctgaaaaacggcggctatcaggtgatgattgtgattccggaaaactttagcaaactggcgatgcagctggatgcgaaa
acgccgagcaaaattagcctgcagtataaaaccgcggtgggccagaaagaagaagtggcgaaaaacaccgaaaaagtgg

tgagcaacgtgctgaacgattttaacaaaaacctggtggaaatttatctgaccagcattattgataacctgcataacgcgcagaa
aaacgtgggcgcgattatgacccgcgaacatggcgtgaacagcaaatttagcaactatctgctgaacccgattaacgattttccg
gaactgtttaccgatacctggtgaacagcattagcgcgaacaaagatattaccaaatggtttcagacctataacaaaagcctgc
tgagcgcgaacagcgatacctttcgcgtgaacaccgattataacgtgagccacctgattgaaaaacagaacagcctgtttgatg
aacataacaccgcgatggataaaatgctgcaggattataaaagccagaaagatagcgtggaactggataactatattaacgcg
ctgaaacagatggatagccagattgatcagcagagcagtatgcaggataccggcaaagaagaatataaacagaccgtgaaa
gaaaacctggataaactgcgcgaaattattcagagccaggaaagcccgtttagcaaaggcatgattgaagattatcgcaaaca
gctgaccgaaagcctgcaggatgaactggcgaacaacaaagatctgcaggatgcgctgaacagcattaaaatgaacaacgc
gcagtttgcggaaaacctggaaaaacagctgcatgatgatattgtgaaagaaccggataccgataccacctttatttataacatg
agcaaacaggatttttattgcggcgggcctgaacgaagatgaagcgaacaaatatgaagcgattgtgaaagaagcgaaacgct
ataaaaacgaatataacctgaaaaaaccgctggcggaacatattaacctgaccgattatgataaccaggtggcgcaggatacc
agcagcctgattaacgatggcgtgaaagtgcagcgcaccgaaaccattaaaagcaacgatattaaccagctgaccgtggcga
ccgatccgcattttaactttgaaggcgatattaaaattaacggcaaaaaatatgatattaaagatcagagcgtgcagctggatacc
agcaacaaagaatataaagtggaagtgaacggcgtggcgaaactgaaaaaagatgcggaaaaagattttctgaaagataaa
accatgcatctgcagctgctgtttggccaggcgaaccgccaggatgaaccgaacgataaaaaagcgaccagcgtggtggatg
tgaccctgaaccataacctggatggccgcctgagcaaagatgcgctgagccagcagctgagcgcgctgagccgctttgatgcg
cattataaaatgtataccgataccaaaggccgcgaagataaaaccgtttgataacaaacgcctgattgatatgatggtggatcagg
tgattaacgatatggagagctttaaagatgataaagtggcggtgctgcatcagattgatagcatggaagaaaacagcgataaac
tgattgatgatattctgaacaacaaaaaaaacaccaccaaaaacaaagaagatattagcaaactgattgatcagctggaaaac
gtgaaaaaaacctttgcggaagaaccgcaggaaccgaaaattgataaaggcaaaaacgatgaatttaacaccatgagcagc
aacctggataaagaaattagccgcattagcgaaaaaagcacccagctgctgagcgatacccaggaaagcaaaagcattgcg
gatagcgtgagcggccagctgaaccaggtggataacaacgtgaacaaactgcacgcgacgggccgcgcgctgggcgtgcg
cgcgaacgatctgaaccgccagatggcgaaaaacgataaagataacgaactgtttgcgaaagaatttaaaaaagtgctgcag
aacagcaaagatggcgatcgccagaaccaggcgctgaaagcgtttatgagcaacccggtgcagaaaaaaaaacctggaaaa
cgtgctggcgaacaacggcaacaccgatgtgattagcccgaccctgtttgtgctgctgatgtatctgctgagcatgattaccgcgta
tatttttatagctatgaacgcgcgaaaggccagatgaactttattaaagatgattatagcagcaaaaaccatctgtggaacaacgt
gattaccagcggcgtgattggcaccaccggcctggtggaaggcctgattgtgggcctgattgcgatgaacaaatttcatgtgctgg
cgggctatcgcgcgaaatttattctgatggtgattctgaccatgatggtgtttgtgctgattaacacctatctgctgcgccaggtgaaa
agcattggcatgtttctgatgattgcggcgctgggcctgtattttgtggcgatgaacaacctgaaagcggcgggccagggcgtgac
caacaaaattagcccgctgagctatattgataacatgttttttaactatctgaacgcggaacatccgattggcctggtgctggtgattc
tgaccgtgctggtgattattggctttgtgctgaacatgtttattaaacattttaagaaagagagatTAAtctaATGctgatgaacag
cgtgattgcgctgacctttctgaccgcgcagcagcaacaacggcggcctgaacattgatgtgcagcaggaagaagaaaaacgc
attaacaacgatctgaaccagtatgataccacccctgtttaacaaagatagcaaagcggtgaacgatgcgattgcgaaacagaa
aaaagaacgccagcagcagattaaaaacgatatgtttcagaaccaggcgagccatagcacccgcctgaacgaaaccaaaa
aagtgctgtttagcaaaagcaacctggaaaaaaccagcgaaagcgataaaagcccgtatattcagaacaaacaggaaaaa
aaaattttccgtatattctgatgagcgtggggggcttttttgactttaggatTTGtcattttttcaattcataaagggagacgaacgaaa
aatgaatcagcacgtaaaagtaacattTGAttttaccaactataactatggcacctatgatctggcggtgccggcgtatctgccga
ttaaaaacctgattgcgctggtgctggatagcctggatattagcattttttgatgtgaacacccagattaaagtgatgaccaaaggcc
agctgctggtggaaaacgatcgcctgattgattatcagattgcggatggcgatattctgaaactgctgTAGgaggaaaaatagA
TGgtgaaaaaccataacccgaaaaacgaaatgcaggatatgctgacccgctggatgcggaagaagcggcgaaaaccaa
actgcgcctggatatgcgcgaaattccgaaaagcagcattaaaccggaacattttcatctgatgtatctgctggaacagcatagc
ccgtattttattgatgcggaactgaccgaactgcgcgatagctttcagattcattatgatattaacgataaccataccccgtttgataa
cattaagagctttaccaaaaacgaaaaactgcgctatctgctgaacattaaaaacctggaagaagtgaaccgcacccgctata
cctttgtgctggcgccggatgaactgtttttttacccgcgatggcctgccgattgcgaaaacccgcggcctgcagaacgtggttgatc
cgctgccggtgagcgaagcggaatttctgacccgctataaagcgctggtgatttgcgcgtttaacgaaaaacagagctttgatgc
gctggtggaaggcaacctggaactgcataaaggcaccccgtttgaaaccaaagtgattgaagcggcgaccctggatctgctga
ccgcgtttctggatgaacagtatcagaaacaggaacaggattatagccagaactatgcgtatgtgcgcaaagtgggccataccg
tgtttaaatgggtggcgattggcatgaccacccctgagcgtgctgctgattgcgtttctggcgtttctgtattttagcgtgatgaaacata
acgaacgcattgaaaaaggctatcaggcgtttgtgaaagatgattatacccaggtgctgaacacctatgatgatctggatggcaa
aaaactggataaagaagcgctgtatatttatgcgaaaagctatattcagaccaacaaacagggcctggaaaaagataaaaaa
gaaaacctgctgaacaacgtgaccccgaacagcaacaaagattatctgctgtattggatggaactgggccagggccatctgga
tgaagcgattaacattgcgacctatctggatgataacgatattaccaaactggcgctgattaacaaactgaacgaaattaaaaac
aacggcgatctgagcaacgataaacgcagcgaagaaaccaaaaaatataacgataaactgcaggatattctggataaagaa
aaacaggtgaaagatgaaaaagcgaaaagcgaagaagaaaaagcgaagcgaaagatgaaaaactgaaacagcagg
aagaaaacgaaaaaaaacagaaagaacaggcgcagaaagataaagaaaaacgccaggaagcggaacgcaaaaaaT

AGtataggactgaggcaaagacaATGcataaactgattattaaatataacaaacagctgaaaatgctgaacctgcgcgatg
gcaaaacctataccattagcgaagatgaacgcgcggatattaccctgaaaagcctgggcgaagtgattcatctggaacagaac
aaccagggcacctggcaggcgaaccataccagcattaacaaagtgctggtgcgcaaaggcgatctggatgatattaccctgc
agctgtataccgaagcggattatgcgagctttgcgtatccgagcattcaggataccatgaccattggcccgaacgcgtatgatgat
atggtgattcagagcctgatgaacgcgattattattaaagattttcagagcattcaggaaagccagtatgtgcgcattgtgcatgata
aaaacaccgatgtgtatattaactatgaactgcaggaacagctgaccaacaaagcgtatattggcgatcatatttatgtggaagg
catttggctggaagtgcaggcggatggcctgaacgtgctgagccagaacaccgtggcgagcagcctgattcgcctgacccagg
aaaatgccgcacgcgcaggcggatgattataacacctatcatcgcagcccgcgcattattcatcgcgaaccgaccgatgatatta
aaattgaacgcccgccgcagccgattcagaaaaacaacaccgtgatttggcgcagcattattccgccgctggtgatgattgcgct
gaccgtggtgattttctggtgcgcccgattggcatttatattctgatgatgattggcatgagcaccgtgaccattgtgtttggcattacc
acctattttagcgaaaaaaaaaatataacaaagatgtggaaaaacgcgaaaaagattataaagcgtatctggataacaaaa
gcaaagaaattaacaaagcgattaaagcgcagcgctttagcctgaactatcattatccgaccgtggcggaaattaaagatattgt
ggaaaccaaagcgccgcgcatttatgaaaaaaccagccatcaccatgatttTctgcattataaactgggcattgcgaacgtgga
aaagagctttaaactggattatcaggaagaagaatttaaccagcgccgcgatgaactgtttgatgatgcgaaagaactgtatga
attttataccgatgtggaacaggcgccgctgattaacgatctgaaccacggcccgattgcgtatattggcgcgcgccatctgattct
ggaagaactggaaaaaatgctgattcagctgagcacctttcatagctatcacgatctggaatttctgtttgtgacccgcgaagatg
aagtggaaaccctgaaatgggcgcgctggctgccgcacatgaccctgcgcggccagaacattcgcggctttgtgtataaccag
cgcacccgcgatcagattctgaccagcatttatagcatgattaaagaacgcattcaggcggtgcgcgaacgcagccgcagcaa
cgaacagattattttacccccgcagctggtgtttgtgattaccgatatgagcctgattattgatcatgtgattctggaatatgtgaacca
ggatctgagcgaatatggcattagcctgatttttgtggaagatgtgattgaaagcctgccggaacatgtggataccattattgatatt
aaaagccgcaccgaaggcgaactgattaccaaagaaaaagaactggtgcagctgaaatttacccccgaaaacattgataac
gtggataaagaatatattgcgcgccgcctggcgaacctgattcatgtggaacatctgaaaaacgcgattccggatagcattacctt
tctggaaatgtataacgtgaaagaagtggatcagctggatgtggtgaaccgctggcgccagaacgaaacctataaaacgatgg
cggtgccgctgggcgtgcgcggcaaagatgatattctgagcctgaacctgcatgaaaaagcgcatggcccgcatggcctggtg
gcgggcaccaccggcagcggcaaaagcgaaattattcagagctatattctgagcctggcgattaactttcatccgcatgaagtg
gcgtttctgctgattgattataaaggcggcggcatggcgaacctgtttaaagatctggtgcatctggtgggcaccattaccaacctg
gatggcgatgaagcgatgcgcgcgctgaccagcattaaagcggaactgcgcaaacgccagcgcctgtttggcgaacatgatg
tgaaccatattaaccagtatcataaactgtttaaagaaggcattgcgaccgaaccgatgccgcatctgtttattattagcgatgaatt
tgcggaactgaaaagcgaacagccggatttttatgaaagaactggtgagcaccgcgcgcattggccgcagcctgggcattcatc
tgattctggcgacccagaaaccgagcggcgtggtggatgatcagatttggagcaacagcaaatttaaactggcgctgaaagtg
caggatcgccaggatagcaacgaaattctgaaaaaccccggatgcggcggatattaccctgccgggccgcgcgtatctgcaggt
gggcaacaacgaaatttatgaactgtttcagagcgcgtggagcggcgcgacctatgatattgaaggcgatataactggaagtgg
aagataaaaccatttatatgattaacgattatggccagctgcaggcgattaacaaagatctgagcggcctggaagatgaagaaa
ccaaagaaaaccagaccgaactggaagcggtgattgatcatattgaaagcattaccacccgcctggaaattgaagaagtgaa
acgcccgtggctgccgccgctgccggaaaacgtgtatcaggaagatctggtggaaaccgattttcgcaaactgtggagcgatg
atgcgaaagaagtggaactgaccctgggcctgaaagatgtgccggaagaacagtatcagggcccgatggtgctgcagctga
aaaaagcgggccatattgcgctgattggcagtccgggctatggccgcaccacctttctgcataacattattttttgatgtggcgcgcc
atcatcgcccggatcaggcgcacatgtatctgtttgattttggcaccaacggcctgatgccggtgaccgatattccgcatgtggcgg
attattttaccgtggatcaggaagataaaattgcgaaagcgattcgcatttttaacgatgaaattgatcgccgcaaaaaaattctga
gccagtatcgcgtgaccagcattagcgaatatcgcaaactgaccggcgaaaccattccgcatgtgtttattctgattgataactttg
atgcggtgaaagatagcccgtttcaggaagtgtttaaaacatgatgattaaaatgacccgcgaaggcctggcgctggatatgc
aggtgaccctgaccgcgagccgcgcgaacgcgatgaaaaccccgatgtatattaacatgaaaacccgcattgcgatgtttctgt
atgataaaagcgaagtgagcaacgtggtgggccagcagaaatttgcggtgaaagatgtggtgggccgcgcgctgctgagcag
cgatgataacgtgagctttcatattggccagccgtttaaacatgatgaaaccaaaagctataacgatcagattaacgatgaagtg
agcgcgatgaccgaattttataaaggcgaaacccccgaacgatattccgatgatgccggatgaaattaaatatgaagattatcgc
gaaagcctgaacctgccggatattgtggcgaacggcgcgctgccgattggcctggattatgaaggcgtgaccctgcagaaaatt
aaactgaccgaaccggcgatgattagcagcgaaaacccgcgcgaaattgcgcatattgcggaaattatgatgaaagaaattg
atattctgaacgaaaaatatgcgatttgcattgcggatagcagcggcgaatttaaagcgtatcgccatcaggtggcgaactttgcg
gaagaacgcgaagatattaaagcgattcatcagctgatgattgaagatctgaaacagcgcgaaatggatggcccgtttgaaaa
agatagcctgtatattattaacgattttaaaaccttTattgattgcacctatattccggaagatgatgtgaaaaaactgattaccaaag
gcccggaactgggcctgaacattctgtttgtgggcattcataaagaactgattgatgcgtatgataaacagattgatgtggcgcgc
aaaatgattaaccagtttagcattggcattcgcattagcgatcagcagttttttaaatttcgctttattcagcgcgaaccggtgattaaa
gaaaacgaagcgtatatggtggcgaaccaggcgtatcagaaaattcgctggtttaaaTAAcaatgaattaaataggagggag
gtatgttATGaactttaacgatattgaaacgatggtgaaaagcaaatttaaagatattaaaaaacatgcggaagaaattgcgca
tgaaattgaagtgcgcagcggctatctgcgcaaagcggaacagtataaacgcctggaatttaacctgagctttgcgctggatgat

attgaaagcaccgcgaaagatgtgcagaccgcgaaaagcagcgcgaacaaagatagcgtgaccgtgaaaggcaaagcgc
cgaacaccctgtatattgaaaaacgcaacctgatgaaacagaaactggaaatgctgggcgaagatattgataaaaacaaaga
aagcctgcagaaagcgaaagaaattgcgggcgaaaaagcgagcgaatattttaacaaagcgatgaacTAAtattgaggtg
aagatATGggcggctataaaggcattaaagcggatggcggcaaagtggatcaggcgaaacagctggcggcgaaaaccgc
gaaagatattgaagcgtgccagaaacagacccagcagctggcggaatatattgaaggcagcgattgggaaggccagtttgcg
aacaaagtgaaagatgtgctgctgattatggcgaaatttcaggaagaactggtgcagccgatggcggatcatcagaaagcgatt
gataacctgagccagaacctggcgaaatatgataccctgagcattaaacagggcctggatcgcgtgaacccgTAAaagctt

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
S. aureus strains		
RN6390	NCTC8325 derivative, *rbsU*, *tcaR*, cured of φ11, φ12, φ13	Refence[1]
ΔessC	As RN6390, ΔessC	Reference[2]
Δess	Complete deletion from *esxA* – *esaG*	Reference[2]
ΔesaE	As RN6390, ΔesaD	This work
ΔesaD	As RN6390, ΔesaE	This work
ΔesaDG	As RN6390, ΔesaDG	This work
Δsaouhsc00268-00278	As RN6390, ΔesaD-saouhsc00278	This work
Δsaouhsc00274-00278	As RN6390, Δsaouhsc00274-saouhsc00278	This work
RN6390::*ermC*	As RN6390, with *ermC* resistance gene chromosomal insertion	This work
ΔesaDG::*ermC*	As ΔesaDG with *ermC* resistance gene from RN6390::*ermC* (phage φ11 transduction)	This work
Δsaouhsc00268-00278::*ermC*	As ΔesaD-saouhsc00278 with *ermC* resistance gene from RN6390::*ermC* (phage φ11 transduction)	This work
Δsaouhsc00274-00278::*ermC*	As Δsaouhsc00274-saouhsc00278 with *ermC* resistance gene from RN6390::*ermC* (phage φ11 transduction)	This work
COL	MRSA, *agr*	Reference[3]
COLΔess	Complete deletion from *Sacol0271* (*esxA*) - *Sacol0282*	Reference[2]
E. coli strains		
JM110	*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⁺)	Stratagene
BL21(DE3)	*E. coli* B: F⁻, *dcm*, *ompT*, *hsdS*(rB-, mB-), *gal*, λDE3	Reference[4]

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

Supplementary Table 2. Plasmids used in this study

Plasmid	Relevant genotype or description	Source or reference
pIMAY	*E. coli/S. aureus* shuttle vector, temperature sensitive, cml[r]	[6]
pIMAY-esaE	pIMAY carrying *esaE* deletion allele	This work
pIMAY-esaD	pIMAY carrying *esaD* deletion allele	This work
pIMAY-esaDG	pIMAY carrying *esaDG* deletion allele	This work
pIMAY-saouhsc00268-00278	pIMAY carrying *esaD-saouhsc00278* deletion allele	This work
pIMAY-saouhsc00274-00278	pIMAY carrying *saouhsc00274-saouhsc00278* deletion allele	This work
pRAB11	*E. coli/S. aureus* shuttle vector, inducible protein expression, amp[r], cml[r]	Reference[7]
pRAB11-EsaD(H528A)-HA	pRAB11 producing C-terminally HA-tagged EsaD	This work
pRAB11-EsaE-his	pRAB11 producing C-terminally His-tagged EsaE	This work
pRAB11-EsaG-his	pRAB11 producing C-terminally His-tagged EsaG	This work
pRAB11-EsaD-His-EsaG-HA	pRAB11 producing C-terminally His-tagged EsaD and C-terminally HA-tagged EsaG	This work
pRAB11-EsaD-HA-EsaG-His	pRAB11 producing C-terminally HA-tagged EsaD and C-terminally His-tagged EsaG	This work
pRAB11-EsaD(H528A)-His	pRAB11 producing C-terminally His-tagged H528A substituted EsaD	This work
pRAB11-EsaG-HA	pRAB11 producing C-terminally HA-tagged EsaG	This work
pRAB11-EsaE-HA	pRAB11 producing C-terminally HA-tagged EsaE	This work
pRAB11-EsaD(H528A)-His-EsaE-HA	pRAB11 producing C-terminally His-tagged H528A substituted EsaD and C-terminally HA-tagged EsaE	This work
pRAB11-EsaD(H528A)-HA-EsaE-His	pRAB11 producing C-terminally HA-tagged H528A-substituted EsaD and C-terminally His-tagged EsaE	This work
pRAB11-EsaD$_{421-614}$(H528A)-His-EsaG-HA	pRAB11 producing C-terminally His-tagged H528A substituted nuclease domain of EsaD (aa 421-614and C-terminally HA-tagged EsaG	This work
pRAB11-EsaD$_{421-614}$(H528A)-His	pRAB11 producing C-terminally His-tagged H528A substituted nuclease domain of EsaD (aa 421-614)	This work
pRAB11-EsxC	pRAB11 producing native (untagged) EsxC	This work
pQE70	Vector for regulatable protein overproduction in *E. coli* (T5 promoter). Amp[r]	Qiagen
pQE70-EsaE-HA-EsaD-Myc-EsaG-His[1]	pQE70 producing C-terminally HA-tagged EsaE, C-terminally Myc-tagged EsaD and C-terminally His-tagged EsaG	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 *E. coli* (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 *B. pertussis* *cyaA*; Amp^r	Reference[9]
pT18-EsaE	pT18 carrying *esaE*	This work
pT18-EsaD(H528A)	pT18 carrying *esaD* (H528A codon substitution)	This work
pT18-EsaD$_{1-420}$	pT18 carrying *esaD* (codons 1-420)	This work
pT18-EsaG	pT18 carrying *esaG*	This work
pT25	Vector encoding T18 fragment of *B. pertussis* *cyaA*; cml^r	Reference[9]
pT25-EsxA	pT25 carrying *esxA*	This work
pT25-EsxB	pT25 carrying *esxB*	This work
pT25-EsaB	pT25 carrying *esaB*	This work
pT25-EsxC	pT25 carrying *esxC*	This work
pT25-EsaE	pT25 carrying *esaE*	This work
pT25-EsaD(H528A)	pT25 carrying *esaD* (H528A codon substitution)	This work
pT25-EsaD$_{421-614}$	pT25 carrying *esaD* (codons 421-614, with H528A codon substitution)	This work
pT25-EsxD	pT25 carrying *esxD*	This work
pT25-EsaG	pT25 carrying *esaG*	This work
pT25-saouhsc00274*	pT25 carrying *saouhsc00274*	This work
pT25-saouhsc00275*	pT25 carrying *saouhsc00275*	This work
pT25-saouhsc00276*	pT25 carrying *saouhsc00276*	This work

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

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

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

Supplementary Table 3. Oligonucleotides and cloning strategies used in this study

Name	Nucleotide Sequence (5'-3')	Template	Restriction Enzyme	Usage
EsaE-for-bglIII-esxA-RBS	GC<u>AGATCT</u>AGGAGGTTTCTACTTATGAAAGATGTT AAGCGAAT	gDNA	*Bgl*II	Construction of pRAB11-EsaE-his[1] and pRAB11-EsaE-HA[1]
EasE-rev-sacI-HA	GC<u>GAGCTC</u>TTATGCATAATCTGGAACATCATATGGATACTCCT CTGCTTTATTAATATGAT	gDNA	*Sac*I	Construction of pRAB11-EsaE-HA[1]
EasE-rev-sacI-His	GC<u>GAGCTC</u>TTAGTGGTGGTGGTGGTGGTGCTCCTCTGCTTTATTA ATATGAT	gDNA	*Sac*I	Construction of pRAB11-EsaE-his[1]
EsaG-for-bgl II	GCGC<u>AGATCT</u>AGGAGGTTTCTACTTCAACATGACATTTGAAGAGA	gDNA	*Bgl*II	Construction of pRAB11-EsaG-his[1] and pRAB11-EsaG-HA[1]
esaG-rev-SacI-HA	GC<u>GAGCTC</u>TTATGCATAATCTGGAACATCATATGGATATTCTTCTA GCTCTTTAATATATT	gDNA	*Sac*I	Construction of pRAB11-EsaG-HA[1]
esaG-rev-his-EcoRI	GC<u>GAATTC</u>TTAGTGGTGGTGGTGGTGGTGTTCTTCTAGCTCTTTAA TATATT	gDNA	*Eco*RI	Construction of pRAB11-EsaG-his[1]
esaD-kpnI-for-esxA-RBS	GAAA<u>GGTACC</u>AGGAGTTTCTACTTATGACAAAAGA TATTGAATATCTAAC	gDNA	*Kpn*I	Construction of pRAB11-EsaD(H528A)-his[1] and pRAB11-EsaD(H528A)-HA[1]
esaD-his-to-ala-for	CGATGATGGAGGTGCATTAATCGCTAGAATG	gDNA		Change of His to Ala codon at codon 528 of *esaD*[2]
esaD-his-to-ala-rev	CATTCTAGCGATTAATGCACCTCCATCATCG	gDNA		Change of His to Ala codon at codon 528 of *esaD*[2]
EsaD-his-rev-bgl II	GCGC<u>AGATCT</u>CTAGTGGTGGTGGTGGTGGTGCTTATTTAATATTCT TCTAATATTTCT	gDNA/ *esaD(H528A)*	*Bgl*II	Construction of pRAB11-EsaD(H528A)-his[1] and pRAB11-EsaD$_{421-614}$(H528A)-His[1]
EsaD-HA-rev-bgl II	GCGC<u>AGATCT</u>CTATGCATAATCTGGAACATCATATGGATACTTATTT AATATTCTTCTAATATTTCT	gDNA /*esaD(H528A)*	*Bgl*II	Construction of pRAB11-EsaD(H528A)-HA[1]

esaD(421-614aa)-kpnI-for-esxA-RBS	GAAAGGTACCAGGAGGTTTCTACTTATGACACATGGTCCAAAAGATAGTATGGTGAG	*esaD(H528A)*	*Kpn*I	Construction of pRAB11-EsaD$_{421-614}$(H528A)-His[1]
00268-00278A1	CATGGAGCTCGATTGTACAATC	gDNA	*Sac*I	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	GCGCGAGCTCCATCTATTTCAGTGTTAATTTAC	gDNA	*Sac*I	Construction of pIMAY-saouhsc_00268-00278[3]
00268-00278-out1	TATGTATTTTGCACCATTTAGC			Sequencing primer
00268-00278-out2	CGTTTAAATGTTTGACGCAAGA			Sequencing primer
00274-00278A1	CATGGAGCTCGTATTACATATAGGTGTGGGT	gDNA	*Sac*I	Construction of pIMAY-saouhsc_00274-00278[3]
00274-00278A2	TGTCTGCTACATGTTATCGCCCCTATGTGTTGC	gDNA		Construction of pIMAY-saouhsc_0027400278[3]
00274-00278B1	GATAACATGTAGCAGACATGTTATAAAAGACTGTG	gDNA		Construction of pIMAY-saouhsc_00274-00278[3]
00274-00278B2	GCGCGGTACCCATCTATTTCAGTGTTAATTTAC	gDNA	*Kpn*I	Construction of pIMAY-saouhsc_00274-00278[3]
00274-00278-out1	GGAGCGTTTGCTTTTGTTGTAA	gDNA		Sequencing primer
0266A1	AGGATCCAGTGCTAATAAGGACAGTG	gDNA	*Bam*HI	Construction of pIMAY-esaE[3]
0266A2	CTCTGCTTTATCTTTCATCATGGGTTCAC	gDNA		Construction of pIMAY-esaE[3]
0266B1	ATGAAAGATAAAGCAGAGGAGTAATGACG	gDNA		Construction of pIMAY-esaE[3]
0266B2	TTCGGATCCAATTGAGATGCATAATC	gDNA	*Bam*HI	Construction of pIMAY-esaE[3]

0268A1	AGAA<u>GGATCC</u>AATGATTGGGACTTTAAAC	gDNA	*Bam*HI	Construction of pIMAY-esaD[3] and pIMAY-esaDG[3]
0268A2	CTTATTTAATTTTGTCATGTCATGCACC	gDNA		Construction of pIMAY-esaD[3] and pIMAY-esaDG[3]
0268B1	ATGACAAAATTAAATAAGTAGAGGTGCC	gDNA		Construction of pIMAY-esaD[3]
0268B2	TGA<u>GGATCC</u>ACTTTACGGTATCTATTG	gDNA	*Bam*HI	Construction of pIMAY-esaD[3]
0269A1	GGTT<u>GAATTC</u>GGAGAACACTATGC	gDNA	*Eco*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	AAA<u>GGATCC</u>ATGTTGCAAATACTGCG	gDNA	*Bam*HI	Construction of pIMAY-esaG[3] and pIMAY-esaD[3]
EsaG-out1	TGGGTCAAAACATAAAGCGTGC			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	GAAGAATATTAAATAAGTAGAGGTGC	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	GCGCGGATCCCGCGATGATTAAAATGAGCCCGGAAG	Synthetic *esxA* gene sequence[†]	*Bam*HI	Construction of pT25-EsxA[4]
pT25-EsxA-R	GCGCGGTACCTTACTGCAGGCCAAAGTTGTTGCTCAGC	Synthetic *esxA* gene sequence[†]	*Kpn*I	Construction of pT25-EsxA[4]
pT25-EsxB-F	GCGCGGATCCCGGGCGGCTATAAAGGCATTAAAGC	Synthetic *esxB* gene sequence[†]	*Bam*HI	Construction of pT25-EsxB[4]
pT25-EsxB-R	GCGCGGTACCTTACGGGTTCACGCGATCCAGGCCCTG	Synthetic *esxB* gene sequence[†]	*Kpn*I	Construction of pT25-EsxB[4]
pT25-EsaB-F	GCGCGGATCCCAATCAGCACGTAAAAGTAACATTTG	Synthetic *esaB* gene sequence[†]	*Bam*HI	Construction of pT25-EsaB[4]
pT25-EsaB-R	GCGCGGTACCTTACAGCAGCAGTTTCAGAATATCGCCATCC	Synthetic *esaB* gene sequence[†]	*Kpn*I	Construction of pT25-EsaB[4]
pT25-EsaC-F	GCGCGGATCCCAACTTTAACGATATTGAAACGATG	Synthetic *esxC* gene sequence[†]	*Bam*HI	Construction of pT25-EsxC[4]
pT25-EsaC-R	GCGCGGTACCTTAGTTCATCGCTTTGTTAAAATATTCG	Synthetic *esxC* gene sequence[†]	*Kpn*I	Construction of pT25-EsxC[4]
T25esaD-F	GCGCGGATCCCACAAAAGATATTGAATATCT	*esaD(H528A)*	*Bam*HI	Construction of pT25-EsaD(H528A)[4]
T25esaD-R	GCGCGGTACCATTACTTATTTAATATTCTTCTAAT	*esaD(H528A)*	*Kpn*I	Construction of pT25-EsaD(H528A)[4]
T25esaE-F	GCGCGGATCCCAAAGATGTTAAGCGAATAGAT	gDNA	*Bam*HI	Construction of pT25-EsaE[4]
T25esaE-R	GCGCGGTACCATTACTCCTCTGCTTTATTAATAT	gDNA	*Kpn*I	Construction of pT25-EsaE[4]
T25esaF-F	GCGCGGATCCCACGTTGAGTGGAAAAATTAGTG	gDNA	*Bam*HI	Construction of pT25-EsxD[4]
T25esaF-R	GCGCGGTACCATTATCCCTCAATATTATAGT	gDNA	*Kpn*I	Construction of pT25-EsxD[4]
T25esaG-F	GCGCGGATCCCACATTTGAAGAGAAGCTTAG	gDNA	*Bam*HI	Construction of pT25-EsaG[4]

T25esaG-R	GCGCGGTACCATTATTCTTCTAGCTCTTTAATA	gDNA	KpnI	Construction of pT25-EsaG[4]
T25esaD-421-614-F	GCGCGGATCCCACACATGGTCCAAAAGATAG	esaD(H528A)	KpnI	Construction of pT25-EsaD$_{421-614}$(H528A)[4]
T18esaD-F	GCGCGGGCCCCACAAAAGATATTGAATATCT	esaD(H528A)	ApaI	Construction of pT18-EsaD(H528A)[5]
T18esaD-R	GCGCCTCGAGGGCTTATTTAATATTCTTCTAAT	esaD(H528A)	XhoI	Construction of pT18 EsaD(H528A)[5]
T18esaE-F	GCGCGGGCCCCAAAGATGTTAAGCGAATAGAT	gDNA	ApaI	Construction of pT18-EsaE[5]
T18esaE-R	GCGCCTCGAGGGCTCCTCTGCTTTATTAATAT	gDNA	XhoI	Construction of pT18-EsaE[5]
T18esaG-F	GCGCGGGCCCCACATTTGAAGAGAAGCTTAG	gDNA	ApaI	Construction of pT18-EsaG[5]
T18esaG-R	GCGCCTCGAGGGTTCTTCTAGCTCTTTAATA	gDNA	XhoI	Construction of pT18-EsaG[5]
T18esaD-1-420-R	GCGCCTCGAGGGCACGTGATTTTGTGGTGTAACAG	gDNA	XhoI	Construction of pT18-esaD$_{1-420}$[5]
pQE70-esaE-for	GCGAGCATGCATGAAAGACGTTAAACGTATC	Synthetic esaE gene sequence*	SphI	Construction of pQE70-EsaE-HA-EsaG-his
pQE70-esaEG-overlapping-for	GAAATTAACCATGACCAAAAACAAATAATATTAAAGAGGAGAAATTA ACCATGACCTTCGAAGAAAAACTGTC	Synthetic esaEG gene sequence*		Construction of pQE70-EsaE-HA-EsaG-his
pQE70-esaEG-overlapping-rev	CATATGATGTTCCAGATTATGCATAATGGATCCATTAAAGAGGAGA AATTAACCATGACCAAAAACAAATAAT	Synthetic esaEG gene sequence*		Construction of pQE70-EsaE-HA-EsaG-his
EsaG-for-SphI	GCGAGCATGCCCTTCGAAGAAAAACTGTCTAAAATC	Synthetic esaG gene sequence*	SphI	Construction of pQE70-EsaG-EsaG-EsaD421-614-his and pQE70-EsaG-EsaG-EsaD421-614(H528A)-his[6]
EsaG-overlapping-for	GTACATCAAAGAA CTGGAATAATAA ATTAAAGAGG AGAAATTAACC ATGACCTTCGAAGAAAAACTG	Synthetic esaG gene sequence*		Construction of pQE70-EsaG-EsaG-EsaD421-614-his and pQE70-EsaG-EsaG-EsaD421-614(H528A)-his[6]
EsaG-overlapping-rev	CAGTTTTTCTTCGAAGGTCATGGTTAATTTCTCCTCTTTAATTTATTA TTCCAGTTCTTTGATGTAC	Synthetic esaG gene sequence*		Construction of pQE70-EsaG-EsaG-EsaD421-614-his and

				pQE70-EsaG-EsaG-EsaD421-614(H528A)-his[6]
EsaG-rev-apaI-bamHI	GCGA<u>GGATCCGGGCCC</u>TTATTATTCCAGTTCTTTGATGTACTGTTCG	Synthetic *esaG* gene sequence*	*Apa*I/*BamH*I	Construction of pQE70-EsaG-EsaG-EsaD421-614-his and pQE70-EsaG-EsaG-EsaD421-614(H528A)-his[6]
EsaDnu-for-ApaI	GCGA <u>GGGCCC</u> ATTAAAGAGGAGAAATTAACCATGCACGGTCCGAAAGACTCTATGGTTC	Synthetic *esaD* gene sequence*	*Apa*I	Construction of pQE70-EsaG-EsaG-EsaD421-614-his and pQE70-EsaG-EsaG-EsaD421-614(H528A)-his[6]
EsaDnu-rev-BgIII	GCGA<u>AGATCT</u> TTTGTTCAGGATACGACGGATGTTACG	Synthetic *esaD* gene sequence*	*Bgl*II	Construction of pQE70-EsaG-EsaG-EsaD421-614-his and pQE70-EsaG-EsaG-EsaD421-614(H528A)-his[6]
pQE70-esaD-qc-F	ACGACGACGGTGGTGCACTGATCGCGCGTATGTTCGGT	Synthetic *esaD* gene sequence*		Construction of pQE70-EsaE-HA-EsaD(H528A)-Myc-EsaG-his[7]
pQE70-esaD-qc-R	ACCGAACATACGCGCGATCAGTGCACCACCGTCGTCGT	Synthetic *esaD* gene sequence*		Construction of pQE70-EsaE-HA-EsaD(H528A)-Myc-EsaG-his[7]
pQE70-esaG-rev	GCGC<u>AGATCT</u>TTCCAGTTCTTTGATGTACTG	Synthetic *esaG* gene sequence*	*Bgl*II	Construction of pQE70-EsaE-HA-EsaG-his
pT7.5-esaD--for	AG<u>TCTAGA</u>AAGCTTTACTATAATATTGAGG	gDNA	*Xba*I	Construction of pT7.5esaD and pT7.5esaDG
pT7.5-esaD-xhoI-rev	CTA<u>CTCGAG</u>CTTATTTAATATTCTTCTAAT	gDNA	*Xho*I	Construction of pT7.5esaD(V584Y)
pT7.5-esaD/G- xhoI-rev	GC<u>CTCGAG</u>TTCTTCTAGCTCTTTAATATATT	gDNA	*Xho*I	Construction of pT7.5esaDG
Intctrl1	CCAACTGCTGAAGTACCATTAACG			Checking chromosomal *ermC* integration
Intctrl2	GTACCTGCTATAAACAACGCGCAC			Checking chromosomal *ermC* 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.

[3]The 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 using for sequencing to check the deletions, as shown schematically below.

[4]*esxA*, *esxB*, *esxC*, *esaB*, *esaD(H528A)*, *esaD_{421-614}(H528A)*, *esaE*, *esxD* and *esaG* were each cloned as *Bam*HI-*Kpn*I fragments into similarly digested pT25.

[5]*esaD(H528A)*, *esaD_{1-420}*, *esaE*, and *esaG,* were each cloned as *Apa*I-*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.

[6]Two copies of esaG were amplified, one with primer pair EsaG-for-SphI and EsaG-overlapping-for and the second with primer pair EsaG-overlapping-rev and EsaG-rev-apal-bamHI 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 *Apa*I-*Bgl*II fragment.

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

Supplementary References

1   Novick, R. P. *et al.* Synthesis of *staphylococcal* virulence factors is controlled by a regulatory RNA molecule. *The EMBO journal* **12**, 3967-3975 (1993).

2   Kneuper, H. *et al.* Heterogeneity in ess transcriptional organization and variable contribution of the Ess/Type VII protein secretion system to virulence across closely related *Staphylocccus aureus* strains. *Molecular microbiology* **93**, 928-943, doi:10.1111/mmi.12707 (2014).

3   Gill, S. R. *et al.* Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *Journal of bacteriology* **187**, 2426-2438, doi:10.1128/JB.187.7.2426-2438.2005 (2005).

4   Studier, F. W. & Moffatt, B. A. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *Journal of molecular biology* **189**, 113-130 (1986).

5   Karimova, G., Ullmann, A. & Ladant, D. A bacterial two-hybrid system that exploits a cAMP signaling cascade in Escherichia coli. *Methods Enzymol* **328**, 59-73 (2000).

6   Monk, I. R., Shah, I. M., Xu, M., Tan, M. W. & Foster, T. J. Transforming the untransformable: application of direct transformation to manipulate genetically *Staphylococcus aureus* and *Staphylococcus epidermidis*. *MBio* **3**, doi:10.1128/mBio.00277-11 (2012).

7   Helle, L. *et al.* Vectors for improved Tet repressor-dependent gradual gene induction or silencing in *Staphylococcus aureus*. *Microbiology (Reading, England)* **157**, 3314-3323, doi:10.1099/mic.0.052548-0 (2011).

8   Tabor, S. & Richardson, C. C. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 1074-1078 (1985).

9   Karimova, G., Pidoux, J., Ullmann, A. & Ladant, D. A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 5752-5756 (1998).

10   Altschul, S. F. *et al.* Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic acids research* **25**, 3389-3402 (1997).

11   Sievers, F. *et al.* Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular systems biology* **7**, 539, doi:10.1038/msb.2011.75 (2011).

12   Korczynska, J. E., Turkenburg, J. P. & Taylor, E. J. The structural characterization of a prophage-encoded extracellular DNase from *Streptococcus pyogenes*. *Nucleic acids research* **40**, 928-938, doi:10.1093/nar/gkr789 (2012).