

1     **EsaB is a core component of the *Staphylococcus aureus***  
2                     **Type VII secretion system**

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18

19 **Abstract**

20 Type VII secretion systems (T7SS) are found in many bacteria and secrete proteins involved  
21 in virulence and bacterial competition. In *Staphylococcus aureus* the small ubiquitin-like EsaB  
22 protein has been previously implicated as having a regulatory role in the production of the  
23 EsxC substrate. Here we show that in the *S. aureus* RN6390 strain, EsaB does not genetically  
24 regulate production of any T7 substrates or components, but is indispensable for secretion  
25 activity. Consistent with EsaB being a core component of the T7SS, loss of either EsaB or  
26 EssC are associated with upregulation of a common set of iron acquisition genes. However,  
27 a further subset of genes were dysregulated only in the absence of EsaB. In addition,  
28 fractionation revealed that although an EsaB fusion to yellow fluorescent protein partially  
29 localised to the membrane, it was still membrane-localised when the T7SS was absent. Taken  
30 together our findings suggest that EsaB has T7SS-dependent and T7SS-independent roles in  
31 *S. aureus*.

32

## 33 INTRODUCTION

34 Protein secretion systems are nanomachines employed by bacteria to transport protein  
35 substrates across their cell envelopes. Gram-negative bacteria produce a number of different  
36 secretion machineries that export proteins involved in a wide variety of processes including  
37 signalling, nutrient scavenging, host interaction and virulence (1). The type VII secretion  
38 system (T7SS) is found in some Gram-negative and many Gram-positive bacteria, and is  
39 particularly common among organisms of the actinobacteria and firmicutes phyla (2). The  
40 T7SS was initially described in the pathogenic mycobacteria *Mycobacterium tuberculosis* and  
41 *Mycobacterium bovis*, where the ESX-1 T7SS was shown to be essential for virulence, due to  
42 the secretion of two major T-cell antigens EsxA (formerly known as ESAT-6) and EsxB  
43 (formerly known as CFP-10) (3-5). EsxA and EsxB are founding members of the WXG100  
44 protein family that appear to be exclusively linked to T7SSs, and all characterised T7 systems  
45 are associated with at least one family member. The presence of a membrane-bound ATPase  
46 of the SpoIIIE/FtsK family (termed EccC in actinobacteria and EssC in firmicutes) is another  
47 hallmark of all T7SSs (6). In Mycobacteria, three further membrane proteins EccB, EccD and  
48 EccE assemble with EccC to form a large 1.5 MDa core complex (7, 8). This complex further  
49 associates with a membrane-bound mycosin serine protease, MycP, that is essential for T7  
50 protein secretion and for stability of the membrane complex (9).

51 *Staphylococcus aureus*, an opportunistic pathogen of humans and animals, also elaborates a  
52 T7SS that is distantly related to the T7SSs found in mycobacteria (10). Mutational analysis  
53 has indicated that it plays an important role in persistence in mouse models of infection, intra-  
54 species competition and potentially iron homeostasis (10-15). In commonly-studied strains of  
55 *S. aureus* such as Newman, USA300 and RN6390, the secretion system is encoded by the  
56 12 gene *ess* locus (10, 12, 16). The first six genes at this locus encode core components of  
57 the secretion machinery, including the WXG100 protein EsxA and the SpoIIIE/FtsK ATPase  
58 EssC (Fig 1A,B). However, *S. aureus* and other firmicutes lack homologues of EccB, EccD,  
59 EccE and MycP and instead have an apparently unrelated set of membrane-bound secretion

60 components (*EsaA*, *EssA* and *EssB* in *S. aureus*) (12, 17-19). The sixth component of the *S.*  
61 *aureus* T7SS is *EsaB*, which is predicted to be a small cytoplasmic protein of 80 amino acids  
62 that is structurally related to ubiquitin (20). In *S. aureus* strains Newman and USA300, a  
63 transposon insertion in *esaB* does not abolish secretion of T7 substrates but is linked with an  
64 increase in RNA transcripts covering the gene encoding the substrate *EsxC* (11). By contrast,  
65 in-frame deletion of *esaB* abolished *EsxA* and *EsxC* secretion in strain RN6390 but did not  
66 detectably affect production of these substrate proteins (12). Similarly, inactivation of *yukD*,  
67 which encodes the *Bacillus subtilis* *esaB* homologue, also abolished T7 secretion (17, 18).

68 In this study, we have addressed the role of *EsaB* in *S. aureus* T7 secretion using strain  
69 RN6390. We show that *EsaB* does not regulate *esxC* transcripts or those of other *ess*-encoded  
70 genes. Instead our findings show that *EsaB* behaves as a core component of the T7SS.  
71 Interestingly, however, RNA-Seq analysis identified a subset of genes from the AirSR regulon  
72 that showing altered regulation in the absence of *EsaB*, suggesting that it may play additional,  
73 T7SS-independent roles in *S. aureus* physiology.

74

## 75 METHODS

### 76 Bacterial strains and growth conditions.

77 *S. aureus* strain RN6390 (NCTC8325 derivative, *rbsU*, *tcaR*, cured of  $\phi$ 11,  $\phi$ 12,  $\phi$ 13; (21))  
78 and the isogenic  $\Delta$ *esaB* and  $\Delta$ *esx* ( $\Delta$ *esxA* – *esaG*) strains (12) were employed in this study.  
79 *S. aureus* strains were cultured in Tryptic Soy Broth (TSB) at 37°C with shaking unless  
80 otherwise stated. For calculation of cell numbers we estimated by dilution analysis that one  
81 unit at OD 600nm corresponds to  $6 \times 10^8$  CFU for strain. When required, chloramphenicol (Cml,  
82 final concentration 10  $\mu$ g/ml) was added for plasmid selection. *E. coli* strain JM110  
83 (Stratagene) was used for cloning purposes and BL21(DE3) (22) for *EsaB* overproduction and  
84 purification. *E. coli* was grown in Luria-Bertani (LB) medium at 37°C with agitation. When  
85 appropriate, ampicillin was used for plasmid selection (final concentration 125  $\mu$ g/ml).

86 **Genetic constructs.** All plasmids used in this study are listed in Table 1. The *esaB* gene with  
87 its own RBS was PCR amplified from *S. aureus* RN6390 genomic DNA using primers *EsaB*-  
88 fw and *EsaB*-rev (Table S1). The 0.3 kb *HpaI/EcoRI* restriction fragment was cloned into  
89 pRAB11 under control of the tetracycline inducible promoter, giving pRAB11-*esaB*. Clones  
90 were selected in *E. coli* and verified by DNA sequencing. Plasmid pRAB11-*esaB*-YFP was  
91 generated by cloning the 0.3 kb *HpaI/EcoRI* restriction fragment into pRAB11-YFP (15).  
92 Clones were selected in *E. coli* and verified by DNA sequencing. Nucleotide variants of *esaB*  
93 were generated by the Quickchange site-directed mutagenesis protocol (Stratagene) using  
94 pRAB11-*esaB* or pRAB11-*esaB*-YFP as a template and primers listed in Table S1. Modified  
95 plasmids were digested using *DpnI* for at least 1h at 37°C and transformed into *E. coli*. Single  
96 point mutations were verified by DNA sequencing.

97 **RNA isolation and RT-PCR.** For RNA-Seq analysis, three biological repeats of the *S. aureus*  
98 *esaB* strain was grown aerobically in TSB up to an OD<sub>600</sub> of 1 at which point mRNA was  
99 prepared (in three technical replicates). This experiment was carried out alongside the  
100 RN6390 and *essC* strains (15) and followed identical methodology.

101 For RT-PCR, the indicated *S. aureus* strains were grown aerobically in TSB and harvested at  
102 an OD<sub>600</sub> of 1. At this point, the mRNA was extracted using the SV total RNA Isolation Kit  
103 (Promega) with some minor modifications. Cell samples were stabilized in 5% phenol/95%  
104 ethanol on ice for at least 30 min and then centrifuged at 2770 g from 10 min. Cells were then  
105 resuspended in 100 µl of TE buffer containing 500 µg ml<sup>-1</sup> lysostaphin and 50 µg ml<sup>-1</sup> lysozyme  
106 and incubated at 37°C from 30 min. Subsequently, the manufacturer's instructions were  
107 followed. Isolated RNA was subjected to a second DNase treatment using the DNA-free kit  
108 (Ambion). RNA was stored at -80°C until use. RT-PCR to probe transcription of genes in the  
109 indicated strains was carried out using 500 ng of mRNA as template with the indicated primers  
110 (Table S1). PCR products were visualized on 1% agarose gels.

111 **Purification of 6His-EsaB and generation of polyclonal antisera.** The EsaB coding  
112 sequence (UniProt code ESAB\_STAAM) was PCR amplified from a synthetic gene (codon  
113 optimized for *Escherichia coli* K12 (Genscript)) using the primers EsaB-pET1 and EsaB-pET2  
114 (Table S1) and cloned into the *NdeI/XhoI* site of a modified pET15b vector (Novagen). The  
115 plasmid produces an N-terminal His<sub>6</sub>-tagged protein with a TEV (tobacco etch virus) protease  
116 cleavage site. The protein was expressed and purified as described previously (23), except  
117 the tag-free EsaB was not collected in the flow-through of the negative purification but required  
118 a 30mM imidazole elution. The final size exclusion chromatography step used a 24ml HR  
119 30/100 GL Superdex75 column (GE healthcare), equilibrated with 20 mM Tris pH 7.8, 100 mM  
120 NaCl and was calibrated with molecular mass standards (thyroglobulin, 670 kDa; γ-globulin,  
121 158 kDa; serum albumin, 67 kDa; ovalbumin; 44 kDa, myoglobin, 17 kDa; and vitamin B12, 1  
122 kDa). 2 mg purified EsaB (retaining a Gly–Ala–Ser–Thr sequence at the N-terminus after the  
123 cleavage step) was utilized as antigen to immunize rabbits for polyclonal antibody production  
124 in a standard three injections protocol (Seqlab, Goettingen, Germany).

125 **Secretion assays, subcellular fractionation and western blotting.** The indicated strains  
126 were grown overnight in TSB, diluted 1/100 in fresh medium and grown up to mid-log phase,  
127 at which point whole cells and supernatant fractions were harvested as described previously

128 (12). Briefly, cells and supernatant were separated by a 10 min centrifugation step at 2770 *g*.  
129 Cells were washed twice with PBS, adjusted to and OD<sub>600</sub> of 1 and digested using 50 µg/ml of  
130 lysostaphin by incubation at 37°C for 30 min. Supernatants were filtered using a 0.22 µm filter  
131 and TCA-precipitated in the presence of 50 µg/ml deoxycholate, as described. For *S. aureus*  
132 subcellular fractionation, cells were grown to mid-log phase with shaking and treated as  
133 previously described (12). Briefly, cells were harvested by centrifugation and resuspended in  
134 TSM buffer (50 mM Tris-HCl pH 7.6, 0.5 M sucrose, 10 mM MgCl<sub>2</sub>). Lysostaphin was added to  
135 a final concentration of 50 µg ml<sup>-1</sup> and cells were incubated at 37°C for 30 min to digest the  
136 cell wall. At this point, protoplasts were sedimented to recover the cell wall (supernatant  
137 fraction). Protoplasts were disrupted by sonication and the membrane was obtained after an  
138 ultracentrifugation step at 227 000 *g* for 30 min and at 4°C. The supernatant was retained as  
139 the cytoplasmic fraction. Samples were boiled for 10 min prior to separation in bis-Tris gels  
140 and subsequent western blotting.

141 Polyclonal antisera were used at the following dilutions: α-EsxA 1:2500 (12), α-EsxB 1:1000  
142 (15), α-EsxC 1:2000 (12), α-EsaB 1:500, α-TrxA 1:20000 (24) and α-SrtA (Abcam) 1:3000.  
143 Anti-GFP antibody was obtained from Roche and used according to manufacturer's  
144 instructions.

## 145 RESULTS

### 146 **EsaB does not regulate the level of *esxC* transcripts in strain RN6390**

147 A previous study has shown that a transposon insertion in the *esaB* gene results in an increase  
148 in *esxC* transcripts in the Newman and USA300 strain backgrounds, and a concomitant  
149 increase in the EsxC polypeptide, implicating it as a regulator (11). To investigate whether loss  
150 of *esaB* by in-frame deletion affects the level of *esxC* mRNA in strain RN6390, we isolated  
151 mRNA from the parental strain and the isogenic *esaB* mutant, prepared cDNA and undertook  
152 reverse transcriptase PCR with primers covering either *esxA* (the first gene at the *ess* locus,  
153 included as a negative control) or *esxC* (Fig 1A). It can be seen (Fig 1C) that the level of  
154 transcripts for each of these genes was qualitatively similar in the wild type and *esaB*  
155 backgrounds.

156 To examine this quantitatively, we undertook RNA-Seq analysis on RNA prepared from three  
157 biological repeats of the RN6390 and *esaB* strains grown aerobically in TSB to an OD<sub>600</sub> of 1.  
158 Note that these experiments were performed at the same time as the RN6390 vs *essC* RNA-  
159 Seq analysis described in (15) and used the same RN6390 dataset. Fig 1D shows that the  
160 level of *esxC* transcripts were indistinguishable between the wild type and *esaB* strains.  
161 Analysis of the transcript levels of the other genes at the *ess* locus indicates that in general  
162 they were also not significantly altered by the loss of *esaB* although there was a small increase  
163 in the level of *essB*. We conclude that there is no evidence that *esaB* regulates the level of  
164 *esxC* transcripts in RN6390.

165 We next examined the entire transcript profile of the *esaB* mutant to investigate the  
166 transcriptional/post-transcriptional response to the loss of this small protein. We found 101  
167 genes de-regulated in the *esaB* mutant compared to the parental strain (using a cut off of  
168 logFC > 2 or < -2 and qvalue < 0.05, as applied previously (15)), Fig 2A. Of these, 43 were  
169 upregulated by the loss of *esaB* whereas 58 were downregulated when *esaB* was absent –  
170 these genes are listed in Table 2. Interestingly, almost all of the genes that were differentially



171 regulated in the *essC* mutant (15) were also similarly regulated in the *esaB* strain (Fig 4B),  
172 although there was a substantive subset of genes that were differentially expressed in the  
173 *esaB* mutant but not the *essC* strain (Table 2). It can be seen that almost all of the iron  
174 acquisition genes, including those for heme acquisition, staphyloferrin synthesis and uptake  
175 and ferrichrome import were commonly upregulated by loss of either *esaB* or *essC* (Table 2).  
176 Furthermore six of the eight downregulated genes from the *essC* strain were also down  
177 regulated in the *esaB* strain (note that one of the two genes unaffected in the *esaB* dataset is  
178 *essC* itself, which appears downregulated in the *essC* dataset because it has been deleted).  
179 The finding that almost the entire subset of genes differentially regulated in the absence of  
180 *essC* is also similarly altered by loss of *esaB* strongly suggests that *EsaB* is, like *EssC*, a core  
181 component that is essential for activity of the secretion machinery in strain RN6390.

182 As mentioned above, a subset of transcripts were differentially expressed in the *esaB* but not  
183 the *essC* strain. These include downregulated genes required for anaerobic nitrate respiration  
184 (*narGHJ/narK*), some secreted proteases (*sspA/B/C*, *aur*), capsular polysaccharide synthesis  
185 (*capG/F/hysA*), lactose metabolism (*lacB/C/D*) and antimicrobial peptide synthesis  
186 (*epiA/C/D/P*). Many of these genes are under control of the essential two component  
187 regulatory system AirSR (formerly YhcSR) (25-28). These findings suggest that *EsaB* may  
188 have additional roles in the cell in addition to its requirement for T7 protein secretion.

189

## 190 ***EsaB* is present at low amounts in cells of *S. aureus* RN6390**

191 To explore the biological role of *EsaB* in T7 secretion, we firstly overproduced recombinant  
192 *EsaB* with a cleavable His-tag in *E. coli*, and following cleavage of the tag the protein was  
193 further purified by gel filtration chromatography (Fig 3A, B). The purified protein, which eluted  
194 with an estimated molecular mass of approximately 12.8 kDa, is close to the expected size of  
195 a monomer (9.1 kDa + 0.3 kDa retained following cleavage of the tag = 9.4 kDa). This is in  
196 agreement with structural analysis of the *B. subtilis* *EsaB* homologue, YukD, which also

197 appears to be monomeric (20).

198 Polyclonal antisera were raised against purified EsaB and the antibody was affinity purified  
199 against the EsaB antigen, before being used to detect the protein in whole cells of *S. aureus*.  
200 Fig 3C shows that although the purified antiserum could clearly recognize purified EsaB, it did  
201 not detect a band of the expected size of EsaB in whole cells. Probing a dilution series of  
202 purified EsaB indicated that the antibody was able to cross-react with as little as 25ng of  
203 protein, which is equivalent to  $1.6 \times 10^{11}$  EsaB molecules. Since the antibody was unable to  
204 detect EsaB in whole cells from  $9.6 \times 10^8$  colony forming units that were loaded onto the SDS  
205 gel, we conclude that there are less than 170 molecules of EsaB per cell.

206 Since we were unable to detect native EsaB in *S. aureus* cell extracts, we constructed a series  
207 of tagged variants for which commercial antisera were available. To this end we introduced  
208 His<sub>6</sub>, Myc, hemagglutinin (HA) and Strep epitopes onto the N-terminus of EsaB, and His<sub>6</sub>, Myc,  
209 HA, mCherry or FLAG epitopes onto the C-terminus, but in each case were unable to detect  
210 the tagged protein (not shown). We also introduced His<sub>6</sub> and His<sub>9</sub> epitopes into two predicted  
211 loop regions internal to the EsaB sequence but again were unable to detect tagged EsaB (not  
212 shown). The only tag we introduced that allowed detection of EsaB was a C-terminal yellow  
213 fluorescent protein (YFP) tag. Fig 3D shows that basal production of either native (untagged)  
214 EsaB or EsaB-YFP from plasmid vector pRAB11 was sufficient to restore secretion of the  
215 T7SS extracellular protein EsxA and of substrates EsxB and EsxC to the culture supernatant.  
216 Blotting the same cell samples for the presence of the YFP fusion protein (Fig 3E) showed  
217 that it migrated at close to the predicted mass (37 kDa) of the EsaB fusion. There was no  
218 evidence for degradation of the fusion protein even after prolonged exposure of the  
219 immunoblot (Fig 3E). We conclude that the YFP-tagged variant of EsaB probably retains  
220 functionality.

221

222 **EsaB-YFP partially localizes to the cell membrane**

223 EsaB is predicted to be a soluble cytoplasmic protein (10), and is known to share structural  
224 homology with ubiquitin (20). Interestingly, a domain sharing the same fold is also associated  
225 with the actinobacterial T7SS, being found at the cytoplasmic N-terminus of EccD (29),  
226 indicating that ubiquitin-like proteins are essential features of all T7SSs. To determine the  
227 subcellular location of EsaB-YFP, we blotted secreted and whole cell samples of the *esaB*  
228 mutant strain producing plasmid-encoded EsaB-YFP with the YFP antiserum. Fig 4A shows  
229 that EsaB-YFP was associated exclusively with the cellular fraction.

230 We next fractionated these cells to obtain cytoplasm, cell wall and membrane fractions.  
231 Immunoblotting with antisera to control proteins known to localize to the cell membrane (SrtA)  
232 and cytoplasm (TrxA) indicated that the fractionation had been largely successful, although  
233 some SrtA was found in the cell wall fraction (Fig 4B). Blotting these same fractions for the  
234 presence of EsaB-YFP showed that the protein localised to both the cytoplasm and membrane  
235 fractions. Some degradation of the fusion protein was also noted in these experiments which  
236 may result from the activation of proteases during fractionation. When unfused YFP was  
237 produced in the wild type strain it did not localise to the membrane (Fig 4C), indicating that  
238 membrane binding was unlikely to be mediated through the YFP portion of the fusion.

239 Next we tested whether EsaB-YFP localised to the membrane through interactions with  
240 membrane components of the T7SS. To this end we repeated the fractionation in a strain  
241 carrying a chromosomal deletion in all twelve genes at the *ess* locus (Fig 1A). However this  
242 did not alter the localization of EsaB-YFP, which was still detected in both cytoplasm and  
243 membrane fractions (Fig 4B). It may be that EsaB-YFP localises to the membrane through  
244 interaction with additional membrane proteins, consistent with additional, T7SS-independent  
245 roles for EsaB suggested through the RNA-Seq analysis. Alternatively, we cannot rule out that  
246 the membrane localization arises as an artifact of the C-terminal YFP tag, since this tag is  
247 known to influence protein behaviour (e.g. (30)).

248

## 249 **Mutagenesis of conserved hydrophilic and hydrophobic patches on EsaB**

250 An alignment of EsaB homologues encoded across firmicutes (Fig 5A) identifies a number of  
251 highly conserved amino acids. Many of these are hydrophilic and fall on one face of the  
252 predicted structure of EsaB including T8 (*S. aureus* numbering) which is highly conserved as  
253 either threonine or serine, and the invariant K56. The presence of an invariant lysine is  
254 particularly intriguing since there are a number of highly conserved lysine residues on the  
255 structurally-related protein ubiquitin, that are used to assemble polyubiquitin chains (31). To  
256 probe potential roles of these conserved residues we mutated each of T8, D10, L21, K30,  
257 K52, K56, L66, G74 and D75 to alanine on plasmid-encoded EsaB and assessed whether the  
258 variant EsaB proteins were able to restore T7 secretion activity to the *esaB* deletion strain.

259 Fig 5C shows that alanine substitutions of each of these conserved residues was tolerated by  
260 EsaB with the exception of T8A, which completely abolished EsaB activity. To test whether  
261 other side chain substitutions were permissive at T8, we subsequently constructed EsaB T8S,  
262 T8E, T8H, T8K and T8R substitutions. As seen in Fig 5D, in addition to T8A the T8R  
263 substitution also abolished EsaB activity, but the other substitutions resulted in active protein.

264 Ubiquitin has a conserved hydrophobic patch (Fig 6A, *left*) that forms a common site of  
265 interaction with many different binding partners (32). Analysis of the predicted structure of  
266 EsaB (Fig 6A, *right*) shows that there are some hydrophobic residues on the surface potentially  
267 at positions approximating the hydrophobic patch region of ubiquitin. To assess whether these  
268 hydrophobic residues may be involved in EsaB function, we firstly mutated V7, I44, I71 and  
269 L77 to alanine residues. Fig 6B shows that these substitutions did not detectably affect T7  
270 secretion indicating that the function of EsaB had not been compromised. We next substituted  
271 each of these residues for a positively-charged lysine. This more drastic change of amino acid  
272 side-chain was still tolerated at positions 44 and 77, but inactivated EsaB when substituted for  
273 V7 or I71.

274 Finally we attempted to assess whether any of the inactivating substitutions E7K, T8A, T8R  
275 or I71K altered the subcellular location of EsaB-YFP. However, when we introduced each of  
276 these substitutions into EsaB-YFP we found that they destabilised the protein as it was almost  
277 undetectable in whole cells (Fig 6D), precluding further analysis. We are therefore unable to  
278 determine whether these substitutions directly alter EsaB function or have an indirect effect  
279 by disrupting folding.

## 280 DISCUSSION

281 In this work we have investigated the role of EsaB in Type VII secretion. EsaB proteins are  
282 conserved in firmicutes that produce the T7SS and are encoded at the same loci. Previous  
283 work had implicated EsaB in the regulation of *esxC* transcripts (11), although this cannot be a  
284 conserved role for EsaB proteins as they are found in all *S. aureus* strains, including the subset  
285 that do not encode *esxC* (16). Here we show that EsaB does not regulate *esxC* in strain  
286 RN6390, nor any of the other genes encoded at the *ess* locus. Instead, deletion of *esaB* is  
287 associated with upregulation of genes involved in iron acquisition, mirroring the upregulation  
288 of iron-acquisition genes seen when the core T7 component, EssC, is absent (15). This  
289 supports the notion that EsaB is a core component of the secretion machinery in RN6390, and  
290 in agreement with this, deletion of *esaB* prevented export of the T7-dependent extracellular  
291 proteins EsxA, EsxB and EsxC. This conclusion is also in agreement with related studies in  
292 *B. subtilis*, where the EsaB homologue YukD was shown to be essential for secretion of the  
293 WXG100 protein Yuke (17, 18).

294 The precise role of EsaB in T7 secretion is unclear. Structural analysis of *B. subtilis* YukD  
295 shows that it shares a very similar fold to ubiquitin but that it lacks the ability to be conjugated  
296 with other proteins (20). Interestingly, a ubiquitin-like domain is also associated with the  
297 actinobacterial T7SS, being found at the cytoplasmic N-terminus of the polytopic EccD  
298 membrane component (29), suggesting that EsaB-like components are essential features of  
299 all T7SSs. Ubiquitin interacts directly with many different protein binding partners (32), and it  
300 is therefore likely that EsaB interacts with one or more components of the T7SS, potentially  
301 regulating activity. Post-translational regulation of the *S. aureus* T7SS has been suggested  
302 because in some growth conditions the secretion machinery is present but there is no or very  
303 little substrate secretion (12, 19). Other protein secretion systems are also post-translationally  
304 regulated, for example the flagellar type III secretion system is regulated through interaction  
305 of the FliI component with the second messenger cyclic di-GMP (33), and Type VI secretion  
306 systems are regulated by phosphorylation (34). In this context, EsaB proteins contain a highly

307 conserved threonine (or serine) residue close to their N-termini which we considered as a  
308 potential site for phosphorylation. Intriguingly, substitution of EsaB T8 for alanine abolished  
309 the function of EsaB, although introduction of either the phospho-mimetic glutamate at this  
310 position or a positively charged lysine did not affect EsaB activity.

311 The low cellular levels of EsaB precluded further analysis of the native protein, but a C-terminal  
312 fusion of EsaB with YFP partially localised to the cell membrane. We reasoned that binding of  
313 EsaB-YFP to membranes was mediated through interaction with one or more of the T7SS  
314 membrane proteins. However, some EsaB-YFP was still membrane associated when it was  
315 analysed in a strain lacking all of the core T7 components, suggesting that it may interact with  
316 additional membrane proteins potentially unrelated to the T7SS. Support for this suggestion  
317 comes from RNA-Seq analysis of the *esaB* mutant strain. In addition to a common set of genes  
318 showing similar regulation in the *esaB* and *essC* strains, a further subset of genes were  
319 uniquely deregulated in the *esaB* mutant. Many of the genes in this EsaB-specific subset are  
320 part of the AirSR regulon (25-28). The AirSR two component system responds to oxidation  
321 signals via a redox-active [2Fe-2S] cluster in the sensor kinase AirS to regulate diverse sets  
322 of genes involved in anaerobic respiration, lactose metabolism and capsule biosynthesis. In  
323 future it will be interesting to further decipher the roles of EsaB in T7 protein secretion and *S.*  
324 *aureus* physiology.

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333 interest.



334 **FIGURE LEGENDS**

335 **Figure 1. EsaB is not a transcriptional regulator.** (A) The *ess* locus in *S. aureus* RN6390.  
336 Genes encoding core components are shaded in grey, secreted proteins in blue and a  
337 cytoplasmic antitoxin in yellow. The regions analysed by RT-PCR are indicated. (B) Predicted  
338 subcellular locations of Ess-encoded components. cw – cell wall, cm – cytoplasmic  
339 membrane. (C) RT-PCR analysis of *esxA* (region 1) and *esxC/B* (region 2) from the RN6390  
340 and isogenic *esaB* and *esxA* mutant strains. Equivalent amounts of mRNA from each strain  
341 were used to generate cDNA. RT: reverse transcriptase. (D) Total mRNA counts of *ess* genes  
342 from RNA-Seq analysis of RN6390 and the *esaB* mutant strain. RPKM - reads of transcript  
343 per kilobase per million of mapped reads.

344 **Figure 2. RNA-Seq analysis of differentially regulated genes in the *esaB* mutant strain.**  
345 (A) Volcano plot representation of the differentially expressed genes in RN6390 strain  
346 compared to the isogenic *esaB* mutant. The orange and grey spots represent, respectively,  
347 genes that are up- or down-regulated in the *esaB* mutant relative to the parental strain. B.  
348 Overlap between up- and down-regulated genes in the *esaB* and *essC* datasets.

349 **Figure 3. EsaB is present in cells at low amounts.** (A) Purification of EsaB by gel filtration  
350 chromatography. Inset shows column calibration. (B) SDS PAGE analysis of EsaB during  
351 purification steps. Lanes labelled 1, 2 and 3 correspond to similarly-labelled fractions from the  
352 gel filtration column. (C) Titration of  $\alpha$ -EsaB antibodies. The indicated amounts of purified  
353 EsaB, alongside 30  $\mu$ l of OD<sub>600</sub> 5 adjusted cells were loaded on a SDS-PAGE as indicated  
354 and blotted using  $\alpha$ -EsaB antibodies. Two exposures of the blot are shown. (D) RN6390  
355 harbouring empty pRAB11, and the isogenic *esaB* deletion strain harbouring pRAB11, or  
356 pRAB11 encoding native EsaB or EsaB-YFP was cultured aerobically in TSB medium until an  
357 OD<sub>600</sub> of 2 was reached. Samples were fractionated to give cells and supernatant (sn), and  
358 supernatant proteins were precipitated using TCA. For each gel, 10  $\mu$ l of OD<sub>600</sub> 1 adjusted  
359 cells and 15  $\mu$ l of culture supernatant were loaded. Blots were probed with anti-EsxA, anti-

360 EsxB or anti-EsxC antisera, alongside anti-TrxA (cytoplasmic control). Cell and supernatant  
361 samples have been blotted on the same gel but intervening lanes have been spliced out. (E)  
362 EsaB-YFP can be detected in whole cells. RN6390 harbouring empty pRAB11, and the  
363 isogenic *esaB* deletion strain harbouring pRAB11, or pRAB11 encoding EsaB-YFP was  
364 cultured aerobically in TSB medium until an OD<sub>600</sub> of 2 was reached. Whole cell samples (20  
365 µl of OD<sub>600</sub> 2 adjusted cells) were loaded and blots were probed with anti GFP antibodies. Two  
366 exposures of the blot are shown.

367 **Figure 4. EsaB-YFP localizes to the cytoplasm and membrane.** (A) EsaB-YFP is not  
368 secreted in *S. aureus* strain RN6390. RN6390 harbouring empty pRAB11 and the isogenic  
369  $\Delta$ *esaB* strain harbouring empty pRAB11 or pRAB11 encoding EsaB-YFP were cultured in TSB  
370 medium until mid-log phase and separated into cellular and supernatant fractions (sn). For  
371 each gel, 10 µl of OD<sub>600</sub> 1 adjusted cells and 15 µl of TCA-precipitated culture supernatant  
372 were loaded. Blots were probed with anti-EsxA, anti-TrxA (cytoplasmic control) and anti-GFP  
373 antisera. Cell and supernatant samples have been blotted on the same gel but intervening  
374 lanes have been spliced out. Subcellular localisation of (B) EsaB-YFP in RN6390 and an  
375 isogenic  $\Delta$ *esx* ( $\Delta$ (*esxA-esaG*) strain or (C) YFP in RN6390. Cells were grown aerobically in  
376 TSB to mid-log phase and fractionated as indicated in the Methods. Equivalent amount of  
377 each fraction was probed with anti-TrxA (cytoplasmic control), anti-SrtA (membrane control),  
378 anti-EsxA and anti-GFP antisera.

379 **Figure 5. Site-directed mutagenesis of conserved residues of EsaB.** (A) Sequence  
380 alignment of EsaB homologues from: Sau - *Staphylococcus aureus*; Slu - *Staphylococcus*  
381 *lugdunensis*; Lmo - *Listeria monocytogenes*; Lgr - *Listeria grayi*; Bce - *Bacillus cereus*; Bam -  
382 *Bacillus amyloliquefaciens*; Bsu - *Bacillus subtilis*; Bli - *Bacillus licheniformis*; Bhc -  
383 *Bhargavaea cecembensis*; Ssi - *Solibacillus silvestris*; Sor - *Streptococcus oralis*; Sga -  
384 *Streptococcus gallolyticus*. \* indicate conserved residues and † indicates residues forming a  
385 potential hydrophobic patch that were mutated in this work. (B) Model of *S. aureus* EsaB with  
386 positions of conserved residues targeted for mutagenesis highlighted. The N- and C-termini

387 are also indicated. (C) and (D) RN6390 harbouring empty pRAB11, and the isogenic *esaB*  
388 deletion strain harbouring pRAB11, or pRAB11 encoding native the indicated variants of EsaB  
389 were cultured aerobically in TSB medium until an OD<sub>600</sub> of 2 was reached. Samples were  
390 fractionated to give cells and supernatant (sn), and supernatant proteins were precipitated  
391 using TCA. For each gel, 10 µl of OD<sub>600</sub> 1 adjusted cells and 15 µl of culture supernatant were  
392 loaded. Blots were probed with anti-EsxA, and anti-TrxA (cytoplasmic control) antisera.

393 **Figure 6. Mutagenesis of a hydrophobic patch on EsaB.** (A) Ribbon model of ubiquitin (left;  
394 PDB: 1ubi) with residues forming a conserved hydrophobic patch highlighted in purple and *S.*  
395 *aureus* EsaB (right) with positions of hydrophobic residues targeted for mutagenesis shown in  
396 pink. (B) and (C) RN6390 harbouring empty pRAB11, and the isogenic *esaB* deletion strain  
397 harbouring pRAB11, or pRAB11 encoding native EsaB or the indicated amino acid-substituted  
398 variants were cultured aerobically in TSB medium until an OD<sub>600</sub> of 2 was reached. Samples  
399 were fractionated to give cells and supernatant (sn), and supernatant proteins were  
400 precipitated using TCA. For each sample, 10 µl of OD<sub>600</sub> 1 adjusted cells and 15 µl of culture  
401 supernatant were loaded. Blots were probed with anti-EsxA, and anti-TrxA (cytoplasmic  
402 control) antisera. Cell and supernatant samples have been blotted on the same gel but  
403 intervening lanes have been spliced out. (D) The  $\Delta$ *esaB* strain harbouring pRAB11 encoding  
404 EsaB-YFP (WT-YFP) or the indicated amino acid-substituted variants were cultured and  
405 fractionated as in (B) and (C). For each sample, 10 µl of OD<sub>600</sub> 1 adjusted cells and 15 µl of  
406 culture supernatant were loaded and blots were probed with anti-EsxA, anti-TrxA or anti-GFP  
407 antisera.

<b>Plasmid</b>	<b>Relevant genotype or description</b>	<b>Source or reference</b>
pRAB11	<i>E. coli</i> / <i>S. aureus</i> shuttle vector, inducible protein expression, Amp <sup>r</sup> , Cml <sup>r</sup>	(35)
pRAB11-esaB	pRAB11 producing EsaB	This study
pRAB11-esaB-V7A	pRAB11 producing V7A-substituted EsaB	This study
pRAB11-esaB-V7K	pRAB11 producing V7K -substituted EsaB	This study
pRAB11-esaB-T8A	pRAB11 producing T8A-substituted EsaB	This study
pRAB11-esaB-T8E	pRAB11 producing T8E-substituted EsaB	This study
pRAB11-esaB-T8R	pRAB11 producing T8R-substituted EsaB	This study
pRAB11-esaB-T8H	pRAB11 producing T8H-substituted EsaB	This study
pRAB11-esaB-T8K	pRAB11 producing T8K-substituted EsaB	This study
pRAB11-esaB-T8S	pRAB11 producing T8S -substituted EsaB	This study
pRAB11-esaB-D10A	pRAB11 producing D10A-substituted EsaB	This study
pRAB11-esaB-D20A	pRAB11 producing D20A-substituted EsaB	This study
pRAB11-esaB-L21A	pRAB11 producing L21A-substituted EsaB	This study
pRAB11-esaB-K30A	pRAB11 producing K30A-substituted EsaB	This study
pRAB11-esaB-I44A	pRAB11 producing I44A-substituted EsaB	This study
pRAB11-esaB-I44K	pRAB11 producing I44K-substituted EsaB	This study
pRAB11-esaB-K52A	pRAB11 producing K52A-substituted EsaB	This study
pRAB11-esaB-K56A	pRAB11 producing K56A-substituted EsaB	This study
pRAB11-esaB-L66A	pRAB11 producing L66A-substituted EsaB	This study
pRAB11-esaB-I71A	pRAB11 producing I71A-substituted EsaB	This study
pRAB11-esaB-I71K	pRAB11 producing I71K -substituted EsaB	This study
pRAB11-esaB-G74A	pRAB11 producing G74A-substituted EsaB	This study
pRAB11-esaB-D75A	pRAB11 producing D75A-substituted EsaB	This study
pRAB11-esaB-L77A	pRAB11 producing L77A-substituted EsaB	This study
pRAB11-esaB-L77K	pRAB11 producing L77K -substituted EsaB	This study
pRAB11-esaB-YFP	pRAB11 producing EsaB-YFP	This study
pRAB11-esaB-V7K-YFP	pRAB11 producing V7K-substituted EsaB-YFP	This study
pRAB11-esaB-T8A-YFP	pRAB11 producing T8A-substituted EsaB-YFP	This study
pRAB11-esaB-T8R-YFP	pRAB11 producing T8R-substituted EsaB-YFP	This study

pRAB11-esaB-I71K-YFP	pRAB11 producing I71K-substituted EsaB-YFP	This study
pET15b-HISEsaB	pET15b expressing 6XHis-tagged EsaB	This study

408 **Table 1.** Plasmids used in this study.

Locus ID	Gene name	FC in <i>esaB</i> mutant	Proposed Function	FC in <i>essC</i> mutant
<b>Downregulated genes</b>				
SAOUHSC_00986	<i>sspC</i>	-23.7	Cysteine protease	n.s.
SAOUHSC_00988	<i>sspA</i>	-22.3	Glutamyl endopeptidase	n.s.
SAOUHSC_00987	<i>sspB</i>	-20.8	Cysteine protease	n.s.
SAOUHSC_01573	—	-19.0	Unknown, hypothetical protein	n.s.
SAOUHSC_01941	<i>spIB</i>	-18.8	Serine protease SpIB	-4.3
SAOUHSC_02971	<i>aur</i>	-17.1	Zinc metalloproteinase aureolysin	n.s.
SAOUHSC_01942	<i>spIA</i>	-16.4	Highly specific serine protease specific to <i>S. aureus</i>	-5.4
SAOUHSC_02680	<i>narH</i>	-15.7	Nitrate reductase subunit beta	n.s.
SAOUHSC_01944	—	-14.3	Unknown, hypothetical protein	-4.5
SAOUHSC_02681	<i>narG</i>	-14.3	Nitrate reductase subunit alpha	n.s.
SAOUHSC_01121	<i>hla</i>	-13.5	$\alpha$ -hemolysin	-4.1
SAOUHSC_02241	<i>lukF</i>	-13.0	Unknown, hypothetical protein	-3.3
SAOUHSC_02163	<i>hIb</i>	-12.3	$\beta$ -hemolysin	n.s.
SAOUHSC_01938	<i>spID</i>	-12.2	Serine protease SpID	-4.3
SAOUHSC_02679	<i>narJ</i>	-12.2	Nitrate reductase subunit delta	n.s.
SAOUHSC_02671	<i>narK</i>	-11.6	Putative nitrate transporter	n.s.
SAOUHSC_02455	<i>lacA</i>	-11.0	Galactose-6-phosphate isomerase subunit LacA	n.d.
SAOUHSC_01530	—	-10.9	Hypothetical phage protein	n.s.
SAOUHSC_01542	—	-10.9	Unknown, SNF2 family protein	n.s.
SAOUHSC_01535	—	-10.9	Phage capsid protein	n.s.
SAOUHSC_02240	<i>hIb</i>	-10.5	Truncated $\beta$ -hemolysin	n.s.
SAOUHSC_02243	<i>lukG</i>	-10.4	Leukocidin like toxin	-4.5
SAOUHSC_02685	<i>nirR</i>	-10.3	Unknown, hypothetical protein	n.s.
SAOUHSC_01939	<i>spIC</i>	-10.3	Serine protease SpIC	-3.2
SAOUHSC_01937	—	-10.3	Unknown, hypothetical protein	-2.8
SAOUHSC_02970	<i>argR</i>	-8.8	Arginine repressor family protein	n.s.
SAOUHSC_00113	<i>adhE</i>	-8.6	Bifunctional acetaldehyde-CoA/alcohol dehydrogenase	n.s.
SAOUHSC_00051	<i>plc</i>	-8.1	1-phosphatidylinositol phosphodiesterase	-2.5
SAOUHSC_00898	<i>argH</i>	-6.7	Argininosuccinate lyase	n.s.
SAOUHSC_02684	<i>nasD</i>	-6.6	Assimilatory nitrite reductase [NAD(P)H] large subunit	n.s.
SAOUHSC_02709	<i>hIlgC</i>	-6.5	$\gamma$ -hemolysin component C precursor	-1.8
SAOUHSC_02682	<i>nasF</i>	-6.4	Uroporphyrin-III C-methyltransferase	n.s.
SAOUHSC_02462	—	-6.4	Unknown, hypothetical protein	n.s.
SAOUHSC_00401	—	-6.3	Putative exported protein	-1.6
SAOUHSC_01950	<i>epiD</i>	-6.3	Flavoprotein	n.s.
SAOUHSC_01936	<i>spIE</i>	-6.3	Serine protease SpIE	-3.3
SAOUHSC_02454	<i>lacB</i>	-6.3	Galactose-6-phosphate isomerase subunit LacB	-3.4
SAOUHSC_00899	<i>argG</i>	-6.2	Argininosuccinate synthase	n.s.
SAOUHSC_02108	<i>ftn</i>	-6.1	Ferritin	n.s.
SAOUHSC_00368	—	-6.1	Unknown, hypothetical protein	n.s.
SAOUHSC_00411	—	-5.9	Unknown, hypothetical protein	-2.2
SAOUHSC_01951	<i>epiC</i>	-5.8	Epidermin biosynthesis protein EpiC	n.s.
SAOUHSC_02683	<i>nasE</i>	-5.6	Assimilatory nitrite reductase [NAD(P)H] small subunit	n.s.
SAOUHSC_01935	<i>spIF</i>	-5.3	Serine protease SpIF	-2.7
SAOUHSC_02452	<i>lacD</i>	-5.2	Tagatose 1,6-diphosphate aldolase	-2.6

SAOUHSC_01953	<i>epiA</i>	-5.2	Gallidermin superfamily EpiA protein	n.s.
SAOUHSC_02941	<i>nrdG</i>	-4.9	Anaerobic ribonucleotide reductase activating protein	n.s.
SAOUHSC_00717	<i>saeP</i>	-4.7	Putative lipoprotein	-1.4
SAOUHSC_01990	<i>glnQ</i>	-4.6	Glutamine transport ATP-binding protein	n.s.
SAOUHSC_02557	<i>yut</i>	-4.5	Putative urea transporter	n.s.
SAOUHSC_01949	<i>epiP</i>	-4.4	Intracellular serine protease	n.s.
SAOUHSC_00120	<i>capG</i>	-4.4	UDP-N-acetylglucosamine 2-epimerase	n.s.
SAOUHSC_01952	<i>bsaB</i>	-4.4	Lantibiotic epidermin biosynthesis protein EpiB	n.s.
SAOUHSC_03015	<i>hisZ</i>	-4.4	ATP phosphoribosyltransferase regulatory subunit	n.s.
SAOUHSC_00119	<i>capF</i>	-4.4	Capsular polysaccharide biosynthesis protein CapF	n.s.
SAOUHSC_02463	<i>hysA</i>	-4.3	Hyaluronate lyase	n.s.
SAOUHSC_02453	<i>lacC</i>	-4.1	Tagatose-6-phosphate kinase	-2.2
SAOUHSC_00608	<i>adh1</i>	-4.1	Alcohol dehydrogenase	n.s.
<b>Upregulated genes</b>				
SAOUHSC_02767	<i>opp-1A</i>	4.0	Peptide ABC transporter substrate-binding protein	2.6
SAOUHSC_02655	—	4.2	Unknown, hypothetical protein	6.3
SAOUHSC_01292	—	4.4	Putative DNA-binding protein	n.s.
SAOUHSC_00130	<i>isdI</i>	4.4	Heme-degrading monooxygenase IsdI	5.7
SAOUHSC_00176	—	4.5	Extracellular solute-binding protein	n.s.
SAOUHSC_02435	<i>sfaA</i>	4.5	Putative transporter	6.7
SAOUHSC_02799	<i>sarT</i>	4.6	Staphylococcal accessory regulator	n.s.
SAOUHSC_02432	—	4.8	Unknown, hypothetical protein	6.2
SAOUHSC_02245	—	4.9	Unknown, hypothetical protein	6.5
SAOUHSC_00652	<i>fhuA</i>	5.1	Ferrichrome ABC transporter ATP-binding protein FhuA	7.0
SAOUHSC_00071	<i>sirC</i>	5.3	Involved in staphyloferrin B transport into the cytoplasm	4.6
SAOUHSC_00131	—	5.3	Putative membrane protein	6.1
SAOUHSC_02821	—	5.8	Putative membrane protein	n.s.
SAOUHSC_02719	—	6.2	ABC transporter ATP-binding protein	5.5
SAOUHSC_01920	—	6.3	Putative lipoprotein	n.s.
SAOUHSC_02428	<i>htsB</i>	6.3	Heme transport system permease HtsB	5.4
SAOUHSC_00974	—	6.4	Unknown, hypothetical protein	n.s.
SAOUHSC_01081	<i>isdA</i>	6.5	Iron-regulated heme-iron binding protein	5.4
SAOUHSC_00072	<i>sirB</i>	6.5	Involved in staphyloferrin B transport into the cytoplasm	7.4
SAOUHSC_02554	<i>fhuD2</i>	6.5	Ferric hydroxamate receptor 1 FhuD2	6.8
SAOUHSC_01090	—	6.7	Unknown, hypothetical protein	3.9
SAOUHSC_00973	—	7.9	Unknown, hypothetical protein	n.s.
SAOUHSC_01086	<i>isdF</i>	8.5	ABC permease IsdF	6.1
SAOUHSC_01085	<i>isdE</i>	8.6	Heme-receptor lipoprotein IsdE	5.6
SAOUHSC_01089	<i>isdG</i>	8.7	Heme-degrading monooxygenase IsdG	4.7
SAOUHSC_01087	—	8.9	Iron compound ABC transporter permease	6.3
SAOUHSC_01082	<i>isdC</i>	8.9	Heme transporter IsdC	5.5
SAOUHSC_00748	<i>sstC</i>	9.6	Ferrichrome ABC transporter ATP-binding protein SstC	9.1
SAOUHSC_00545	<i>sdrD</i>	10.0	Fibrinogen-binding protein SdrD	n.s.
SAOUHSC_02246	<i>fhuD1</i>	10.0	Iron compound ABC transporter FhuD1	8.0
SAOUHSC_00972	—	10.1	Unknown, hypothetical protein	n.s.
SAOUHSC_01088	<i>srtB</i>	10.2	Sortase StrB	6.2
SAOUHSC_00747	<i>sstB</i>	10.4	Ferrichrome ABC transporter permease SstB	9.0

SAOUHSC_00070	<i>sarH1</i>	11.2	Unknown, hypothetical regulatory-like protein	n.s.
SAOUHSC_02430	<i>htsA</i>	11.2	Heme transport system lipoprotein HtsA	10.5
SAOUHSC_00746	<i>sstA</i>	11.9	Ferrichrome ABC transporter permease SstA	10.9
SAOUHSC_01084	<i>isdD</i>	13.3	ATP-hydrolysing and heme-binding protein IsdD	6.2
SAOUHSC_00074	<i>sirA</i>	13.6	Receptor component of staphyloferrin B	16.3
SAOUHSC_01514	—	15.6	Unknown, hypothetical protein	n.s.
SAOUHSC_02232	—	16.7	Hypothetical phage protein	n.s.
SAOUHSC_02084	—	17.7	Phage repressor protein	n.s.
SAOUHSC_02218	—	25.9	Unknown, hypothetical protein	n.s.
SAOUHSC_00069	<i>spa</i>	51.5	Protein A	n.s.

409 **Table 2.** Genes differentially regulated ( $>\log 2$  fold) in the RN6390 *esaB* deletion mutant,  
410 sorted by ascending fold change (FC). Genes highlighted in grey are also differentially  
411 regulated in the *essC* deletion strain. The column on the right shows the fold change (FC) of  
412 the same gene in the *essC* dataset where n.s. indicates no statistically significant change in  
413 expression level relative to the same gene in the wildtype dataset.



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Primer	Nucleotide Sequence (5'-3')	Usage
EsaB-pET1	GCGCGCCATATGAATCAGCACGTAAAAGT AAC	Amplification of <i>esaB</i> synthetic gene for cloning into pET15b
EsaB-pET2	GCGCGCCTCGAGTCACAGCAGTTTCAGAA TATCGCCATC	Amplification of <i>esaB</i> synthetic gene for cloning into pET15b
EsaB-fw	TTTTGTTAACTCATAAAGGGAGACGAACG	Amplification of <i>esaB</i> gene and its own RBS from RN6390 genome for cloning in pRAB11
EsaB-rev	TTAGAATTCCCTCCTATAGTAACTTCAAATA TC	Amplification of <i>esaB</i> gene and its own RBS from RN6390 genome for cloning in pRAB11
MGC137	CAGCACGTAAAAGCAACATTTGATTTT	Quick change mutagenesis EsaB V7A
MGC138	AAAATCAAATGTTGCTTTTACGTGCTG	Quick change mutagenesis EsaB V7A
MGC165	CAGCACGTAAAAAACATTTGATTTT	Quick change mutagenesis EsaB V7K
MGC166	AAAATCAAATGTTTTTTTACGTGCTG	Quick change mutagenesis EsaB V7K
MGC3	CACGTAAAAGTAGCATTTGATTTTACT	Quick change mutagenesis EsaB T8A
MGC4	AGTAAAATCAAATGCTACTTTTACGTG	Quick change mutagenesis EsaB T8A
MGC151	CACGTAAAAGTAGATTTTACT	Quick change mutagenesis EsaB T8D
MGC152	AGTAAAATCAAATCTACTTTTACGTG	Quick change mutagenesis EsaB T8D
MGC153	CACGTAAAAGTAAAATTTGATTTTACT	Quick change mutagenesis EsaB T8K
MGC154	AGTAAAATCAAATTTTACTTTTACGTG	Quick change mutagenesis EsaB T8K
MGC161	CACGTAAAAGTAAGATTTGATTTTACT	Quick change mutagenesis EsaB T8R
MGC162	AGTAAAATCAAATCTACTTTTACGTG	Quick change mutagenesis EsaB T8R
MGC163	CACGTAAAAGTACATTTTACT	Quick change mutagenesis EsaB T8H
MGC164	AGTAAAATCAAATGTACTTTTACGTG	Quick change mutagenesis EsaB T8H
MGC53	CACGTAAAAGTAGAATTTGATTTTACT	Quick change mutagenesis EsaB T8E
MGC54	AGTAAAATCAAATCTACTTTTACGTG	Quick change mutagenesis EsaB T8E
MGC174	CAGCACGTAAAAGTATCATTGATTTTACT	Quick change mutagenesis EsaB T8S
MGC175	AGTAAAATCAAATGATACTTTTACGTGCTG	Quick change mutagenesis EsaB T8S
MGC5	AAAGTAACATTTGCATTTACTAATTAT	Quick change mutagenesis EsaB D10A
MGC6	ATAATTAGTAAATGCAAATGTTACTTT	Quick change mutagenesis EsaB D10A
MGC7	TACGGCACATATGCATTAGCAGTACCA	Quick change mutagenesis EsaB D20A
MGC8	TGGTACTGCTAATGCATATGTGCCGTA	Quick change mutagenesis EsaB D20A
MGC133	GGCACATATGACGCAGCAGTACCAGCA	Quick change mutagenesis EsaB L21A

MGC134	TGCTGGTACTGCTGCGTCATATGTGCC	Quick change mutagenesis EsaB L21A
MGC9	TATTTACCGATAGCAAACCTTAATAGCT	Quick change mutagenesis EsaB K30A
MGC10	AGCTATTAAGTTTGCTATCGGTAAATA	Quick change mutagenesis EsaB K30A
MGC139	TTGGACATTTTCAGCATTGATGTCAAT	Quick change mutagenesis EsaB I44A
MGC140	ATTGACATCAAATGCTGAAATGTCCAA	Quick change mutagenesis EsaB I44A
MGC167	TTGGACATTTCAAAATTTGATGTCAAT	Quick change mutagenesis EsaB I44K
MGC168	ATTGACATCAAATTTTGAAATGTCCAA	Quick change mutagenesis EsaB I44K
MGC11	AATACACAAATTGCAGTGATGACGAAA	Quick change mutagenesis EsaB K52A
MGC12	TTTCGTCATCACTGCAATTTGTGTATT	Quick change mutagenesis EsaB K52A
MGC13	AAAGTGATGACGGCAGGTCAATTAATT	Quick change mutagenesis EsaB K56A
MGC14	AAGTAATTGACCTGCCGTCATCACTTT	Quick change mutagenesis EsaB K56A
MGC15	GAAAATGATCGAGCAATTGATTATCAA	Quick change mutagenesis EsaB L66A
MGC16	TTGATAATCAATTGCTCGATCATTTTC	Quick change mutagenesis EsaB L66A
MGC143	ATTGATTATCAAGCAGCTGATGGAGAT	Quick change mutagenesis EsaB I71A
MGC144	ATCTCCATCAGCTGCTTGATAATCAAT	Quick change mutagenesis EsaB I71A
MGC169	ATTGATTATCAAAAAGCTGATGGAGAT	Quick change mutagenesis EsaB I71K
MGC170	ATCTCCATCAGCTTTTTGATAATCAAT	Quick change mutagenesis EsaB I71K
MGC135	CAAATCGCTGATGCAGATATTTTGAAG	Quick change mutagenesis EsaB G74A
MGC136	CTTCAAAATATCTGCATCAGCGATTTG	Quick change mutagenesis EsaB G74A
MGC17	ATCGCTGATGGAGCAATTTTGAAGTTA	Quick change mutagenesis EsaB D75A
MGC18	TAACCTCAAAATTGCTCCATCAGCGAT	Quick change mutagenesis EsaB D75A
MGC147	GCTGATGGAGATGCATTGAAGTTACTA	Quick change mutagenesis EsaB L77A
MGC148	TAGTAACTTCAATGCATCTCCATCAGC	Quick change mutagenesis EsaB L77A
MGC171	GCTGATGGAGATAAATTGAAGTTACTA	Quick change mutagenesis EsaB L77K
MGC172	TAGTAACTTCAATTTATCTCCATCAGC	Quick change mutagenesis EsaB L77K
region-1-f	CAGGAGGTTTCTAGTTATGGC	RT-PCR, region 1
region-1-r	GTTCTTGAACGGCATCAGC	RT-PCR, region 1
region-2-f	GCATATGTACGCAAAGTAGGAC	RT-PCR, region 2
region-2-r	TCGTTAGTTGCTCTTGAGTTC	RT-PCR, region 2

508 **Table S1.** Oligonucleotides used in this study. Restriction enzyme sites have been underlined.

509

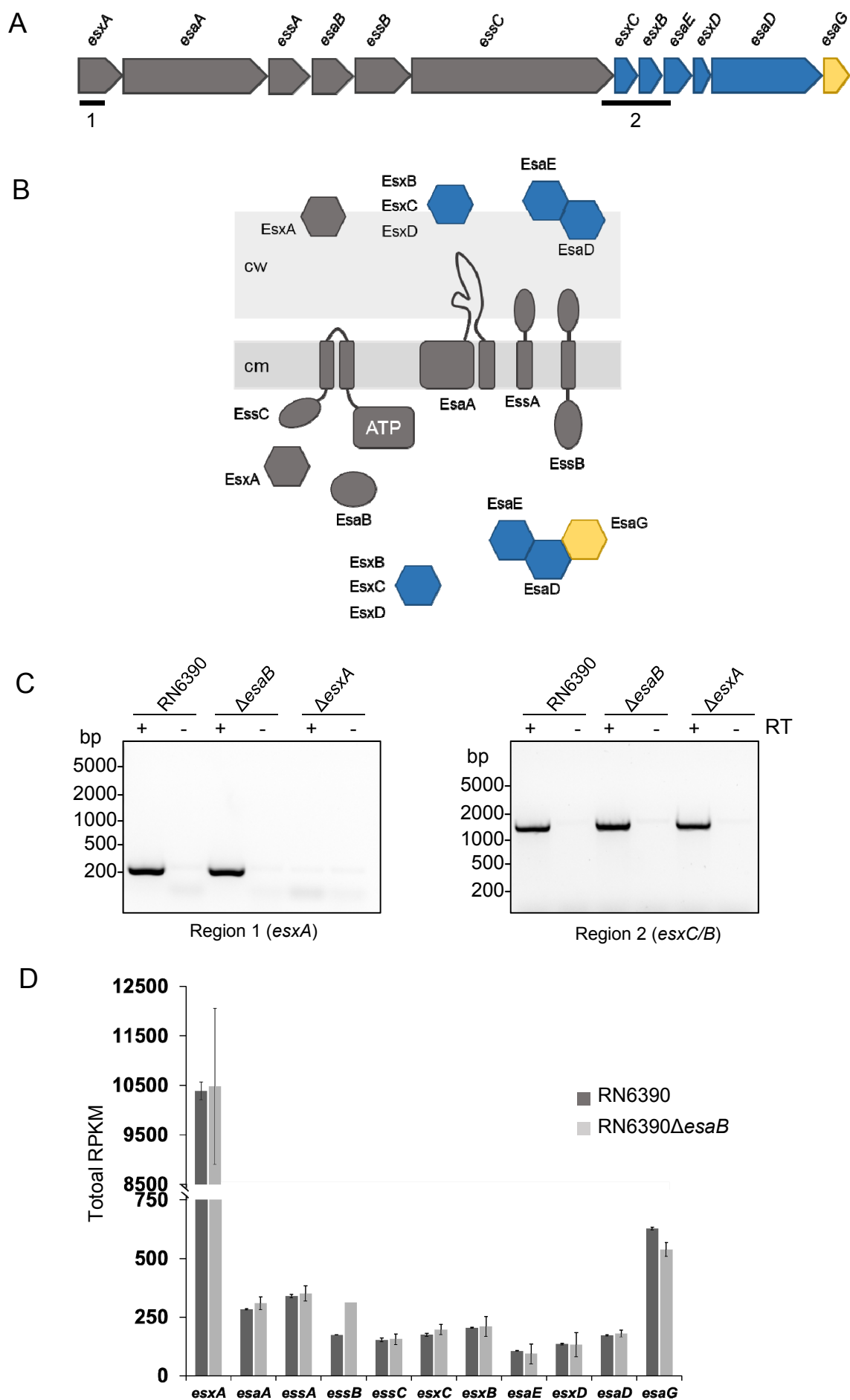
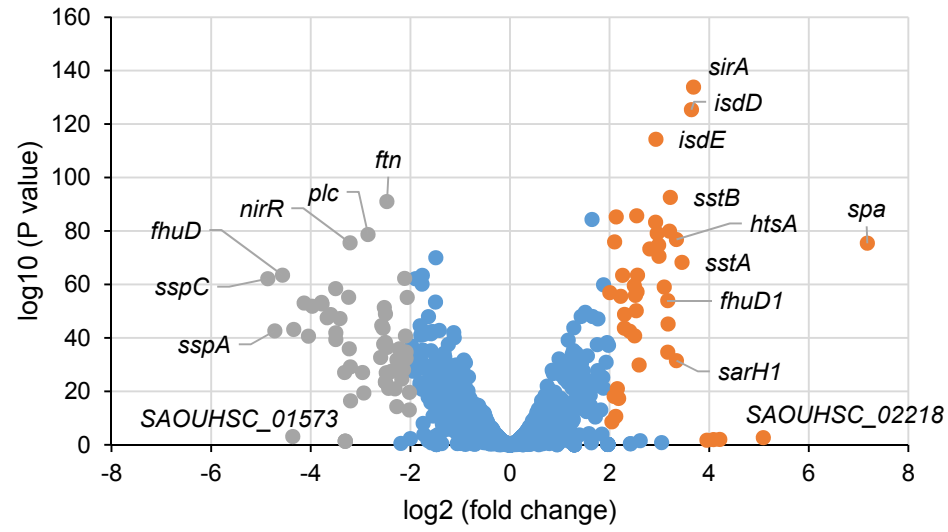


Fig 1

A

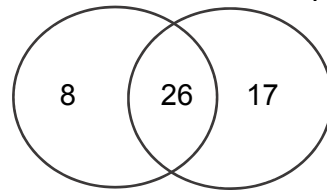


B

**Up-regulated**

RN6390 vs  $\Delta$ essC

RN6390 vs  $\Delta$ esaB



**Down-regulated**

RN6390 vs  $\Delta$ essC

RN6390 vs  $\Delta$ esaB

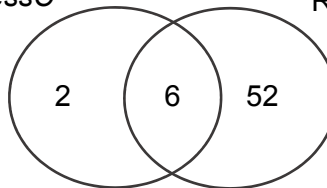


Fig 2



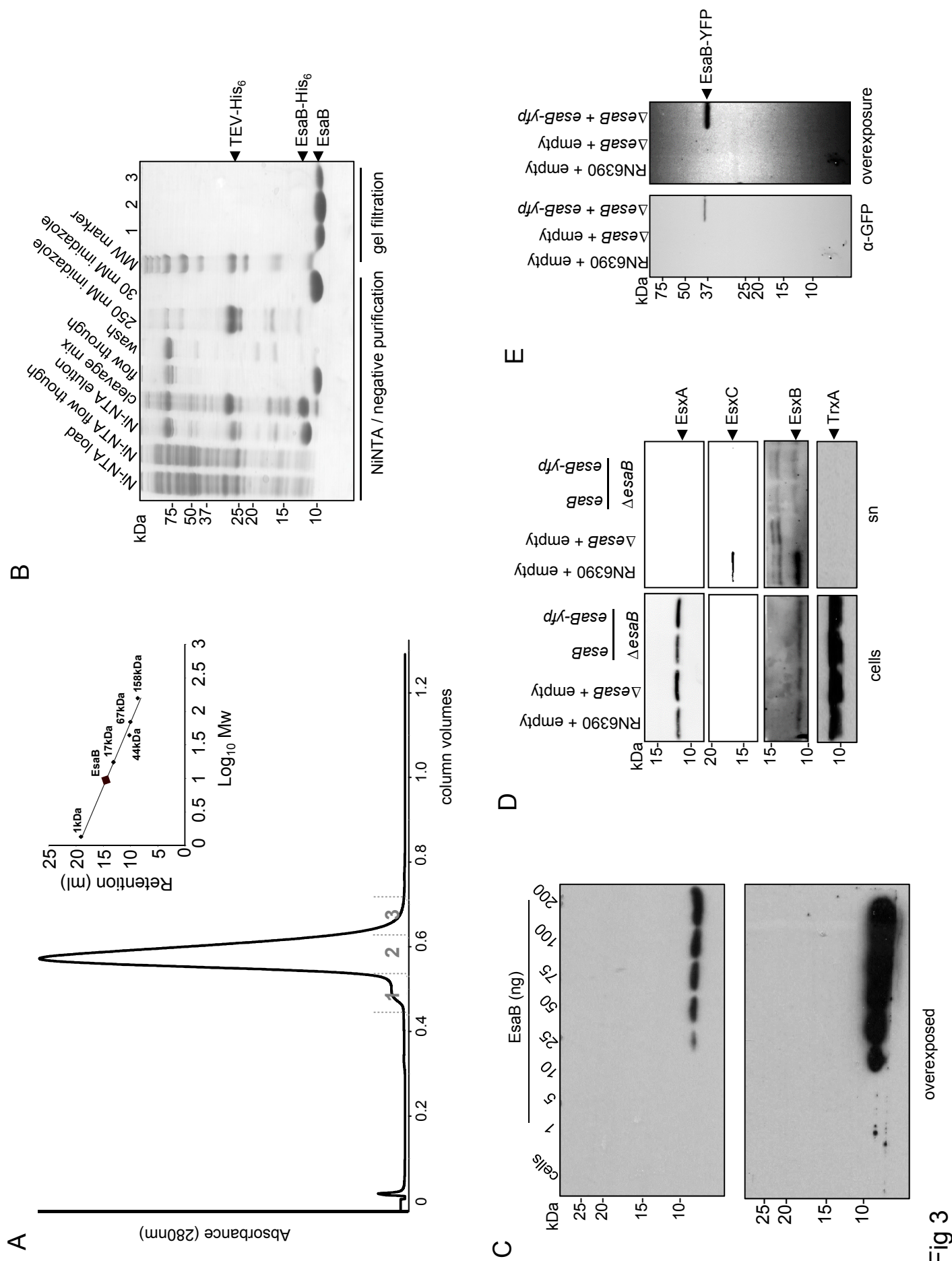


Fig 3

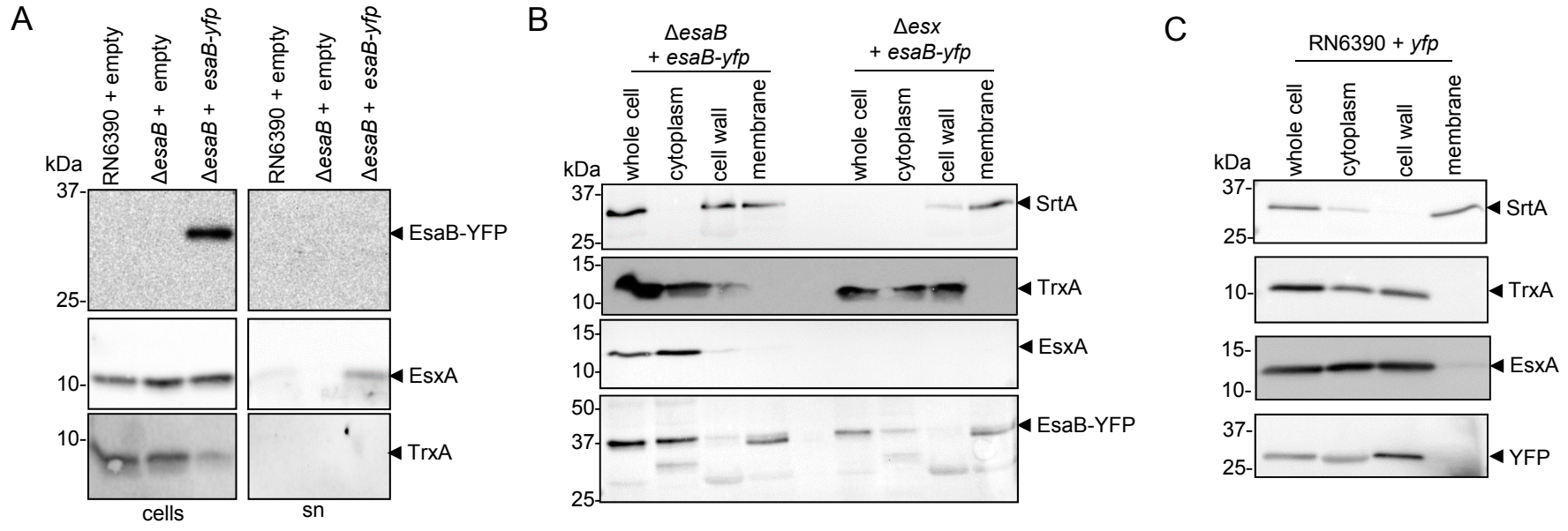


Fig 4

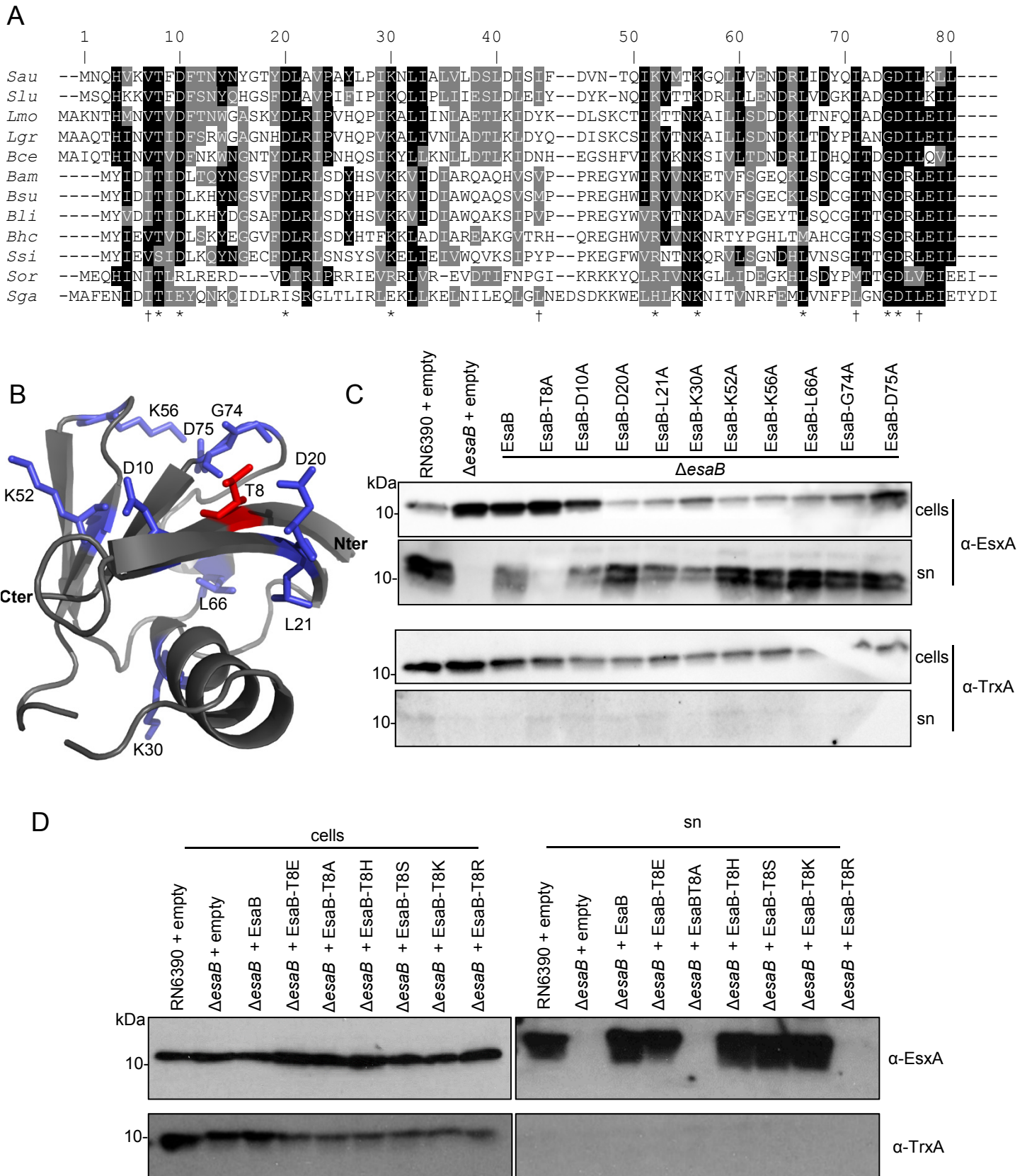


Fig 5

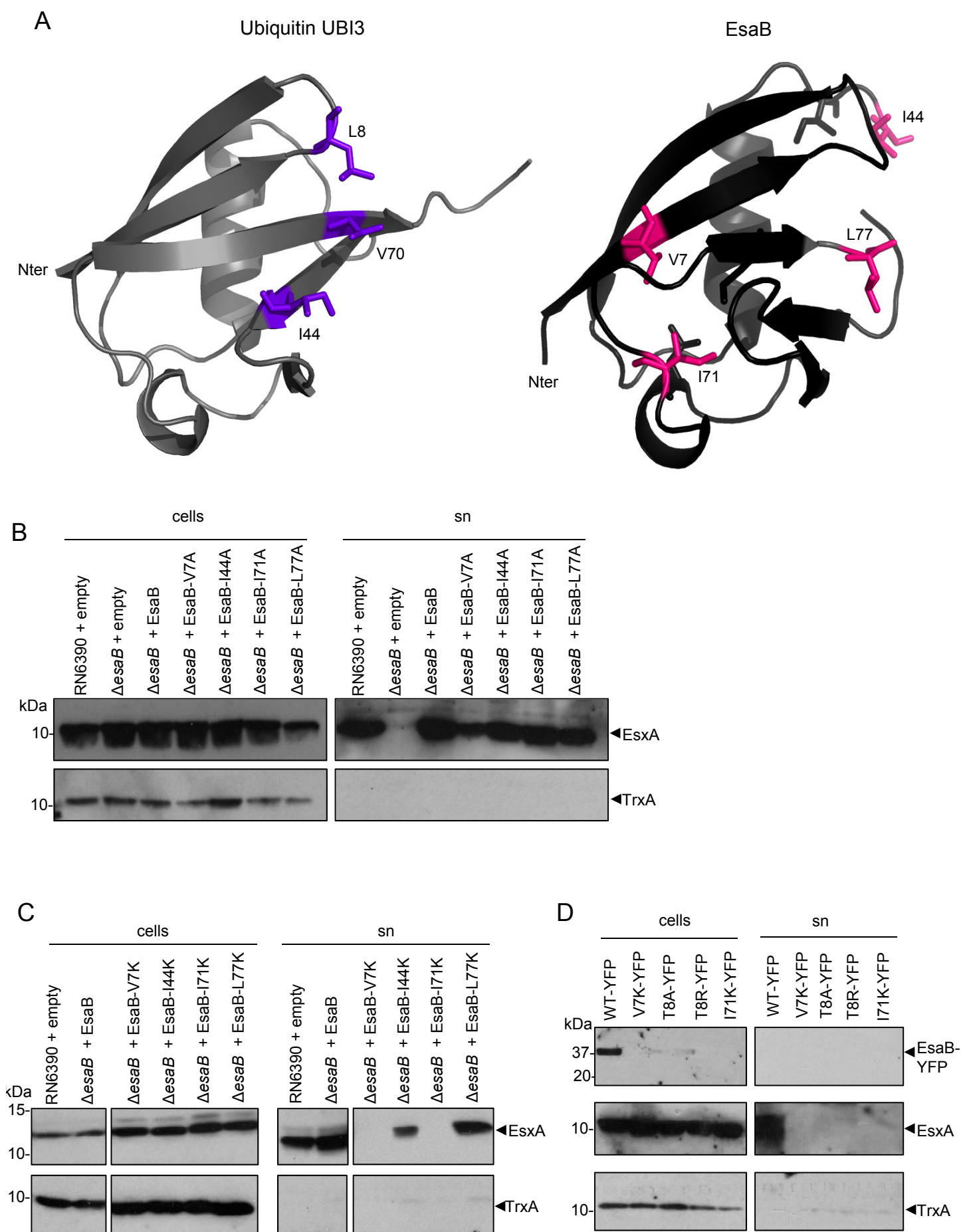


Fig 6