A high throughput assay provides novel insight into type VII secretion in *Staphylococcus aureus*

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Abstract

Successful colonisation by the opportunistic pathogen *Staphylococcus aureus* depends on its ability to interact with other microorganisms. *S. aureus* strains harbour a T7b-subtype type VII secretion system (T7SSb), a protein secretion system found in a wide variety of Bacillota which functions in bacterial antagonism and virulence. Assessment of T7SSb activity in *S. aureus* has been hampered by low secretion activity under laboratory conditions, and the lack of a sensitive assay to measure secretion. Here we have utilised NanoLuc Binary Technology to develop a simple assay to monitor protein secretion via detection of bioluminescence. Fusion of the 11 amino acid NanoLuc fragment to the conserved substrate EsxA permits its extracellular detection upon supplementation with the large NanoLuc fragment and luciferase substrate. Following miniaturisation of the assay to 384 well format, we use high-throughput analysis to demonstrate that T7SSb-dependent protein secretion differs across strains and growth temperature. We further show that the same assay can be used to monitor secretion of the surface-associated toxin substrate TspA. Using this approach we identify three conserved accessory proteins required to mediate TspA secretion. Co-purification experiments confirm that all three proteins form a complex with TspA.

36 **Background**

37

38 The opportunistic pathogen *Staphylococcus aureus* is a major cause of both nosocomial and
39 community infections. It is a frequent coloniser of animals and humans and infections can
40 result in a range of life-threatening diseases such as pneumonia, endocarditis and sepsis. It is
41 notorious for developing resistance to frontline antimicrobials, with methicillin resistance
42 now widespread and vancomycin resistance also increasing [1, 2]. Like many Gram-positive
43 bacteria, *S. aureus* carries a specialised protein secretion machinery called the type VII
44 secretion system (T7SS), which it utilises to secrete antibacterial toxins targeting competitor
45 bacteria (reviewed in [3]). T7SS-mediated antagonism is likely to be important for colonisation
46 [4], and the *S. aureus* T7SS has been shown to be required for virulence in mouse infection
47 models [5-9]. There are several distinct T7SS subtypes, of which the T7SSa and T7SSb are the
48 best characterised [10]. The T7SSb is widely distributed among Bacillota, and has been studied
49 to date in species of *Streptococcus*, *Staphylococcus*, *Bacillus* and *Enterococcus* [8, 11-13].
50 However, in many cases functional and mechanistic studies have been hindered by low
51 secretion activity in laboratory conditions.

52

53 The *S. aureus* T7SSb is encoded on the core genome at the *ess* locus, and four variant locus
54 types have been identified, termed *essC-1 – essC-4*. The genes at the 5' end of the *ess* locus
55 are highly conserved and encode core components of the secretion machinery, while the 3'
56 end codes for different complements of variant-specific substrate and accessory genes [4,
57 14]. The primary component of the T7SSb transmembrane channel is the ATPase EssC,
58 inferred from its homology to the EccC component of the mycobacterial T7SSa channel whose
59 structure has been solved by cryo-electron microscopy [15, 16]. EssC has a conserved N-
60 terminus, two transmembrane domains which likely form the secretion channel, and four C-
61 terminal ATPase domains, the latter two of which are variant-specific and provide substrate
62 specificity [17]. Three additional membrane proteins, EsaA, EssA and EssB, and two small
63 globular proteins, EsxA and EsaB, are further essential components of the T7SSb secretion
64 system [6, 8, 18].

65

66 In addition to being a core component of the T7SSb secretion system, EsxA is itself a T7SS
67 secretion substrate. EsxA secretion is essential for the export of other T7SSb substrates, and

68 it appears to be co-secreted with at least some of them [8, 19]. EsxA is a member of the
69 WXG100 protein family, which are helical hairpin proteins that often, but not always, harbour
70 a central W-x-G sequence motif [20, 21]. In addition to EsxA, other WXG100-like proteins,
71 with more specialised functions, are also found in T7SSb$^+$ strains. These are usually encoded
72 at genetic loci with genes for larger T7SSb-secreted toxins. T7SSb toxins contain an LXG
73 domain, which can be located either at the protein N-terminus (for example TelB, EsaD and
74 TspA) or C-terminus (e.g. TslA) [6, 11, 18, 22-25]. Prior to secretion, the co-encoded WXG100-
75 like proteins, which have been termed Laps (for LXG-associated α-helical proteins) bind to the
76 LXG domain of the cognate toxin partner. This interaction generates a secretion-competent
77 rod-shaped complex, and conserved sequence motifs on both the LXG domain and one of the
78 Laps constitute a T7SSb-targeting signal [23, 24, 26, 27]. In some instances, a globular protein
79 is also encoded at the toxin genetic locus, which also facilitates toxin secretion [24, 27].
80
81 TspA is a membrane-depolarising toxin with antibacterial activity that is encoded by all *S.*
82 *aureus* strains analysed to date. Following secretion, TspA associates with the cell surface and
83 can only be released experimentally by digestion of the cell wall [22]. Unlike other
84 characterised LXG toxins, no WXG100-like partner proteins are encoded at the *tspA* locus, and
85 its secretion requirements are unclear. Assessing secretion by the *S. aureus* T7SS is hampered
86 by low secretion activity in laboratory growth media. Moreover, the lack of cleavable signal
87 peptides on T7SSb substrates means there is no size difference between the exported and
88 cytosolic forms making cellular lysis a confounding issue, particularly where cell wall digestion
89 is also required. To circumvent these difficulties, we have developed a novel secretion assay
90 that makes use of NanoLuc binary technology [28]. We show that this is a robust reporter
91 system that can be used to monitor both secreted and cell-surface localised T7SSb substrates
92 and which is amenable to high-throughput analysis. Using this assay we identify three genes
93 that are required for efficient export of TspA, and demonstrate that the encoded proteins
94 directly interact with the toxin.

95 **Methods**

96

97 ***Bacterial strains, plasmids and growth conditions***

98 *E. coli* was cultured in LB medium (Melford) and *S. aureus* was cultured in tryptic soy broth
99 (TSB; Oxoid) with chloramphenicol (10 µg/ml) where required. *E. coli* strains DH5α (*Δ(argF-*
100 *lac)169, φ80dlacZ58(M15), ΔphoA8, glnX44(AS), deoR481, rfbC1, gyrA96(NalR), recA1,*
101 *endA1, thiE1 and* hsdR17) and JM110 *rpsL thr leu thi lacY galK galT ara tonA tsx dam dcm*
102 *glnV44 Δ(lac-proAB)* e14- [F' *traD36 proAB*$^{+}$ *lacI*^{q} *lacZ*ΔM15] *hsdR17*(rK^{-}mK^{+}) were used for
103 cloning and preparation of plasmids for electroporation, respectively, and M15 harbouring
104 pREP4 (*F-, lac, ara, gal, mtl* [*KanR, lacI*]) was used for protein overproduction. *S. aureus* strains
105 and plasmids used are listed in Tables 1 and 2, respectively. Chromosomal deletion of *essC*
106 was accomplished by allelic exchange using plasmid pIMAY [29] carrying the *essC* flanking
107 regions. Insertion of the pep86 tag on the chromosome of COL and COLΔ*essC* used an updated
108 system with plasmid pIMAY-Z [30]. pIMAY plasmids were created using standard restriction
109 cloning techniques, and pIMAY-Z-esxApep86 was created using HiFi assembly (NEB). Plasmids
110 pRab11-esxApep86 and pRab11-tspA$_{1\text{-}328}$-pep86_2603_2602_2601 for NanoLuc secretion
111 assays, and plasmid pQE70-$_{Tstrep}$SACOL2603$_{myc}$-2602-2601$_{HA\text{-}his}$TspA for protein purification
112 were created using HiFi assembly (NEB, E0554S). *SACOL2603*, *SACOL2602* and *SACOL2601*
113 were individually deleted from pRab11-tspA$_{1\text{-}328}$-pep86_2603_2602_2601 using the Q5 site-
114 directed mutagenesis kit (NEB). Oligonucleotides are listed in Table S1.

115

116 ***NanoLuc secretion assays***

117 Furimazine was purchased from Promega (N1610) and used according to the manufacturer's
118 instructions. The NanoBit large subunit 11S was purified from *E. coli* BL21(DE3) [pBAD-$_{6H}$11S]
119 using the published protocol [28] and was used at a final concentration of 5 µM. For assay of
120 5 ml cultures, *S. aureus* strain COL were cultured as described above. Once cultures had
121 reached a density of 0.5 (OD_{600}), anhydrotetracycline (ATC; 250ng/ml) was added to induce
122 expression of EsxA-pep86. Cells were harvested at a density of 2 (OD_{600}) by centrifugation at
123 16,000 x g, 20°C. Cell pellets were resuspended in TBS + 50µg/ml lysostaphin (LSPN, Ambi)
124 and incubated for 10 minutes at 37°C. 100µl of each sample was supplemented with 5µM 11S
125 and 2µl furimazine (1:100 dilution of stock solution) in a 96-well plate (Greiner, REF655073)

126 with an approximate final well volume of 105µl. Luminescence at 460 nm was read 3 minutes
127 after incubation of the plate at room temperature, with a FLUOstar Omega using a gain value
128 of 3,000. Samples from the same cultures were prepared for immunoblot and analysed with
129 antibodies as previously described [24]. For assays in 384-well plates, overnight *S. aureus*
130 cultures were subcultured in fresh TSB at a density (OD_{600}) of 0.001 for assay of EsxA-pep86
131 secretion or 0.01 for assay of $TspA_{NT}$-pep86 secretion and cultured at 37 °C/34 °C/30 °C with
132 shaking at 200rpm in a 384-well plate (Greiner, REF781098), with a well volume of 50µl. In
133 general, for each assay one row of the 384 well plate was used (16 wells, i.e. 16 technical
134 replicates). This was repeated in triplicate with three separate starter cultures (i.e. three
135 biological replicates). The exceptions to this are in Fig. 2 D and E, where in Fig 2D, there is one
136 biological replicate but 64 technical replicates and Fig 2E where there are three biological
137 repeats each of 64 technical replicates. For COL strains grown at 37 °C, 250 ng/ml ATC was
138 added after 320 min and 11S and furimazine were added after 380 min. For all other strains
139 500 ng/ml ATC was added after 180 minutes (37°C cultures), 220 minutes (34°C cultures), or
140 240 minutes (30°C cultures). 11S and furimazine were added at 220 minutes (37°C cultures),
141 280 minutes (34°C cultures) or 300 minutes (30°C cultures). OD_{600} and luminescence readings
142 were taken every 10 minutes. To measure total luminescence (i.e. combined extracellular and
143 cytoplasmic levels), 50µg/ml lysostaphin in TBS was added to each well and incubated for 10
144 minutes at 37°C. luminescence readings were taken after supplementation of 11S and
145 furimazine.
146
147 ***Cell fractionation and western blot***
148 Single colonies of *S. aureus* harbouring the appropriate pRab11-based plasmid were cultured
149 in TSB medium with chloramphenicol. The following day, 600 µl of overnight culture was sub-
150 cultured into 10 ml of fresh TSB. When the culture reached OD_{600nm} = 0.5-0.6, 500 ng/ml of
151 ATC was added to induce plasmid-encoded gene expression. Cells were harvested at OD_{600} =
152 2. A 1.8 ml aliquot of the culture supernatant was retained, filtered through a 0.22µM sterile
153 filter and the filtrate supplemented with 50 µg/ml sodium deoxycholate (Merck, 89904) and
154 10% TCA (Cambridge Bioscience, 700016-10 ml-CAY). Proteins were precipitated on ice
155 overnight and subsequently pelleted by centrifugation. After washing with 80% ice-cold
156 ethanol, the pellet was resuspended in resuspension buffer (50mM Tris-HCl, pH 8.0, 4% SDS,
157 10% glycerol) then boiled with 1/3 volume of 4x Laemmli sample buffer (Bio-Rad, 1610747)

158 for 10 mins. This was taken as the supernatant fraction. The harvested cells were resuspended
159 in Tris-buffered saline containing 100 µg/ml lysostaphin and incubated at 37 °C for 30 mins.
160 This was then supplemented with 1/3 volume of 4x Laemmli sample buffer and boiled for 10
161 min. This was taken as the whole cell fraction.
162
163 For Western blot analysis, samples were separated by SDS PAGE, transferred to nitrocellulose
164 membrane using a Trans-Blot (Bio-Rad) with Whatman paper soaked in transfer buffer
165 composed of 25 mM Tris, 192 mM glycine pH 8.3, 10% methanol, and analysed by incubation
166 with one of the following primary antibodies: anti-EsxA [8], anti-TrxA [31], anti-HA tag (Merck,
167 H9658), anti-strep tag (Qiagen, 34850), anti-6*his tag (Thermo Fisher Scientific,11533923),
168 anti-myc tag (Abcam, Ab23), and an HRP-linked secondary antibody. A goat anti-mouse
169 antibody (Bio-Rad, 1706516) was used with primary antibodies against the strep, myc and HA
170 tags. A goat anti-rabbit antibody (Bio-Rad, 1721019) was used with all other primary
171 antibodies. All uncropped western blots are provided in the supplementary materials.
172
173 ***Protein purification***
174 LB medium containing ampicillin (0.125 mg/ml) was inoculated 1/100 with an overnight
175 preculture of strain M15 strain carrying pQE70-$_{Tstrep}$SACOL2603-$_{myc}$2602-2601$_{HA-his}$TspA and
176 grown at 37°C for 2 to 3 h with aeration, until OD_{600} reached ~0.5. Cultures were then
177 supplemented with 0.5 mM isopropyl β-d-1-thiogalactopyranoside (IPTG), and grown at 18°C
178 overnight, then harvested by centrifugation. Cell pellets were resuspended in binding buffer
179 W (100 mM Tris/HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA). Cells were lysed by sonication,
180 the resulting cell lysate was clarified by centrifugation at 20,000 g for 30 min, and the
181 supernatant was applied to a 1ml Strep-tactin XT column (IBA, 2-4021-001), washed with
182 bugger W (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA) and the bound proteins were
183 eluted in buffer BXT (IBA, 2-1042-025). Proteins eluted from the Strep-tactin XT column were
184 concentrated using a 50 MWCO Vivaspin centrifugal concentrator (Global Life Sciences
185 Solutions Operations, 28932318) before injection onto a Superdex S200 10/300 GL column
186 (Cytiva) equilibrated in 50 mM HEPES pH 7.5, 150 mM NaCl. Samples were analysed by SDS
187 PAGE using Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad).
188
189 ***Other methods***

190 Secretion data were analysed and presented using Prism (Graphad). Raw data is available for
191 download from FigShare (DOI 10.6084/m9.figshare.25368598). Gene neighbourhood
192 digrammes were generated using clinker (https://cagecat.bioinformatics.nl; [32]).

193 **Results**

194

195 ***A NanoLuc assay for measuring Type VII-dependent secretion in* Staphylococcus aureus**

196 Previous methods to monitor *S. aureus* T7SSb secretion activity have relied almost exclusively
197 on western blotting. Assessing secretion of endogenous WXG100-family proteins, in
198 particular EsxA, involves isolating and concentrating culture supernatants, usually by
199 precipitation with trichloroacetic acid [6, 8, 19, 33]. Furthermore, detection of natively
200 produced LXG toxins is generally too low to detect by western blotting even from
201 concentrated supernatant samples, and assessing their secretion has required
202 overproduction of epitope-tagged variants from an inducible plasmid [22-25]. These methods
203 depend on the availability of good quality antibodies, are often poorly reproducible, and are
204 difficult to quantify.

205

206 To circumvent these issues we utilised NanoLuc binary technology (abbreviated to NanoBit)
207 to develop a new assay to monitor T7 secretion. NanoLuc is an engineered variant of a deep
208 sea shrimp luciferase protein. It is relatively small (19 kDa) and has superior biophysical
209 characteristics compared to other luciferases [34]. NanoBit is a complementation reporter
210 system which has previously been used to investigate protein secretion via the Sec and Tat
211 translocons in *E. coli* [28, 35, 36]. Two separate NanoLuc subunits (11S, a 156 amino acid large
212 subunit and pep86, an 11 amino acid small subunit) combine with high affinity to generate
213 the functional enzyme, which luminesces with high intensity at 460 nm in the presence of the
214 substrate furimazine and molecular oxygen [34].

215

216 We first selected EsxA as our model substrate (Fig 1A). EsxA is produced at significantly higher
217 copy than other T7SS components [8] and can be detected by Western blot in culture
218 supernatants of the *essC-1* strain COL relatively reliably, allowing us to correlate our new
219 assay with data from traditional immunoblot assays. We constructed plasmid pRab11-
220 esxApep86 which permits inducible overproduction of EsxA with the NanoBit small subunit,
221 pep86, fused to its C-terminus. This plasmid was transferred into wild-type and Δ*essC* variants
222 of COL, and the wild-type strain was also transformed with empty pRab11 as a further control.
223 Cultures (5ml volume in 50ml falcon tubes) were supplemented with anhydrotetracycline
224 (ATC) and grown at 37°C to mid logarithmic phase ($OD_{600} \cong 2.0$). At this point samples of

225 whole cell culture, culture supernatant and lysostaphin-lysed cells were prepared. The
226 NanoBit large subunit, 11S, and the NanoLuc substrate furimazine were added to serial two-
227 fold dilutions of these samples, and luminescence readings were taken (Fig. 1B). Cell lysate
228 and culture supernatant samples from the same cultures were also analysed by immunoblot
229 (Fig. 1C).
230
231 Luminescence was detected in all samples except those from cells carrying empty vector,
232 signifying an EsxA-pep86–specific luminescence signal (Fig. 1B). Similar luminescence
233 readings were obtained for both the whole cell culture and the supernatant samples,
234 demonstrating that external pep86 can be measured without centrifugal separation of culture
235 supernatants (Fig. 1B). Deletion of *essC* resulted in a dramatic reduction in the level of
236 extracellular EsxA-pep86 compared to the wild-type strain, while the cytoplasmic level was
237 similar in both strains. This confirms that the extracellular EsxA-pep86-dependent
238 luminescence signal is due to secretion by the T7SS. We assign that residual low level of
239 extracellular EsxA-pep86 in the *essC* mutant strain to cell leakage/lysis.
240
241 When fractions of the same cultures were analysed by immunoblot a single band at ~11 kDa
242 was detected with antibodies against EsxA in all three strains (Fig. 1C), regardless of whether
243 the cells harboured pRab11 or pRab11-esxApep86. Since two of the three strains analysed
244 produce both endogenous chromosomally-encoded EsxA (11 kDa) and plasmid-encoded
245 EsxA-pep86 (12.3 kDa), we infer that this 11 kDa band corresponds to endogenous EsxA, and
246 that the pep86 fusion inhibits detection by the EsxA antibodies (see also Fig. 2C where
247 chromosomal tagging of *esxA* with pep86 inhibits antibody detection).
248
249 As an additional control, we constructed a similar fusion of a cytoplasmic protein, TrxA with
250 an N-terminal pep86 tag (pRab11-pep86-TrxA) and assayed this in the same way.
251 Luminescence readings showed that while pep86-TrxA was produced, barely any signal was
252 detected outside the cells (Fig. 1D). Immunoblots confirmed the cytoplasmic localisation of
253 TrxA and showed that EsxA was secreted normally in these strains, although similarly to EsxA-
254 pep86, the pep86-TrxA fusion was not detected by our TrxA antibodies (Fig. 1E). In summary,
255 Figure 1 demonstrates that luminescence readings correlate with T7-dependent secretion of

256 EsxA-pep86, and that extracellular EsxA-pep86 can be measured in unprocessed whole cell
257 cultures.
258
259
260 ***High throughput analysis of EsxA secretion***
261 Having established that we can use the NanoBit assay to measure T7SS secretion in *S. aureus*,
262 and that we can directly detect extracellular EsxA-pep86 from growing cultures, we next
263 sought to miniaturise it for high throughput format to improve quantitation and sensitivity.
264 To this end we engineered a C-terminal pep86 fusion onto chromosomally-encoded EsxA, to
265 avoid the need for an inducible plasmid. This ensures the assay is undertaken using a system
266 that is as close to native as possible. To assess whether the chromosomally-encoded EsxA-
267 pep86 fusion was competent to support T7 secretion, we transformed this strain with a
268 plasmid encoding a HA-tagged copy of the T7 LXG toxin substrate EsaD alongside its accessory
269 proteins. Immunoblot data for cell lysate and culture supernatant samples (Fig. 2A) showed
270 that although the EsxA-pep86 fusion was not detected by our antibodies, as we had seen
271 previously with the plasmid-encoded fusion, secretion of EsaD-HA was unaffected by the
272 presence of the pep86 tag on EsxA. We conclude that EsxA-pep86 is functional (Fig. 2A).
273
274 We next performed growth curves for 50 µl cultures of $COL_{esxApep86}$ and an otherwise isogenic
275 *essC* mutant in 384-well plate format. Strains grew slowly under these conditions, with
276 cultures reaching stationary phase approximately 20 hours (1200 minutes) after inoculation
277 at an OD_{600} of 0.001 (Fig. 2B). The *essC* mutant strain grew slightly more slowly in exponential
278 phase but reached the same final OD_{600} as the $essC^{+}$ strain. The half-life of the NanoLuc
279 luminescence signal is reported to be approximately 120 minutes (Promega) meaning that
280 continuous monitoring of secreted EsxA-pep86 throughout exponential growth would not be
281 possible. We therefore chose to monitor secretion during early exponential phase, and to this
282 end furimazine and 11S were added to cultures at the beginning of exponential phase, after
283 six hours (360 minutes) growth (Fig. 2B, dotted line) to initiate luminescence. Luminescence
284 readings were then taken at ten minute intervals. The luminescence signal for $COL_{esxApep86}$
285 cultures initially rose, peaking 70 minutes after initiation of luminescence, indicating that cells
286 were actively secreting protein during this time (Fig. 2C). The subsequent decline in
287 luminescence intensity is likely due to signal decay, although degradation of pep86 might also

288 contribute. As seen in the previous assay shown in Figure 1, some leakage of EsxA-pep86
289 occurred in the Δ*essC* strain (Fig. 2C).
290
291 To analyse the distribution of secretion activity within a clonal population, a single colony of
292 each of COL$_{esxApep86}$ and COLΔ*essC*$_{esxApep86}$ was used to inoculate overnight cultures, and these
293 were each used to inoculate 64 x 50 µl cultures and assayed as described above.
294 Luminescence readings taken at 430 minutes (OD_{600} = 0.23) were used to generate a scatter
295 plot of individual data points (Fig. 2D). This analysis reveals a moderate distribution of
296 secretion activity in replicate cultures of COL$_{esxApep86}$, however the distribution of extracellular
297 EsxA-pep86 levels in the control COLΔ*essC*$_{esxApep86}$ strain was quite broad, overlapping the
298 *essC*$^{+}$ strain and highlighting the requirement for a high throughput assay. The same analysis
299 was repeated for three independent cultures of each strain and this did not increase
300 variability (Fig. 2E).
301
302
303 ***Variable secretion characteristics of different* S. aureus *strains***
304 It has previously been reported that T7SS activity varies across different *S. aureus* isolates and
305 growth conditions [8, 19, 33]. However direct comparison across different *S. aureus* strains
306 has been hampered by low secretion activity (often below the detection limit) and low
307 reproducibility (our unpublished observations). We therefore used the NanoBit assay to
308 compare secretion across cell cultures of five additional *S. aureus* isolates, two of which (like
309 COL) carry the *essC-1* variant *ess* locus (RN6390 and JP5347), and one representative isolate
310 for each of *essC-2* (10.125.2X), *essC-3* (MRSA252) and *essC-4* (EMRSA15). Each strain, along
311 with its otherwise isogenic Δ*essC* mutant, was transformed with pRab11-esxApep86 and
312 assayed for EsxA-pep86 secretion in 384-well plates. Growth curves showed that each Δ*essC*
313 strain grew similarly to its parental strain (Fig. S1A). The five additional strains tested here all
314 entered exponential phase faster than COL, and so the timing of addition of ATC (to induce
315 EsxA-pep86 production) and of 11S and furimazine was adjusted accordingly (see methods).
316 T7SS-dependent secretion of EsxA-pep86 was observed, but to varying degree, in the three
317 *essC-1* isolates and the *essC-2* strain 10.1252.X. However no T7-secreted EsxA could be
318 detected in the *essC-3* (MRSA252) and *essC-4* (EMRSA-15) strains (Fig. 3A, S1B). To confirm
319 that EsxA-pep86 was being produced in all of the strains we measured total luminescence in

320 each culture following in-well cellular lysis (a combined measure of cytoplasmic and secreted
321 EsxA-pep86 levels (Fig. S1C). Substantive luminescence could be detected even in lysed
322 cultures of MRSA252 and EMRSA-15, indicating that these strains produce but do not secrete
323 EsxA-pep86.
324
325 Since no T7SS activity was detected for strains MRSA252 and EMRSA15 grown at 37°C we
326 wondered whether these isolates might exhibit a different temperature dependence for
327 secretion. We therefore repeated the NanoBit assay with these strains, alongside RN6390 as
328 an *essC-1* control, using cell cultures growing at 30°C and 34°C (Fig. 3B and S2). For EMRSA15,
329 EsxA-pep86 was secreted at both of the lower temperatures, while for MRSA252 the lower
330 temperatures made little difference to secretion activity, which was negligible at all
331 temperatures tested. EsxA was secreted similarly at all three temperatures in RN6390 (Fig. 3B
332 and S2). Temperature therefore contributes to the regulation of T7SS activity in *S. aureus* in a
333 strain-specific manner. Of note, EsxA-pep86 leakage was markedly lower at 30°C and 34°C
334 than at 37°C in each of the Δ*essC* strains (Fig. S2B).
335
336
337 ***Identification of TspA secretion partners***
338 TspA is a membrane-depolarising LXG toxin found across all *S. aureus essC* variant strains. It
339 is encoded at a locus remote from the T7SS structural genes and precedes a string of non-
340 identical genes coding for DUF443 membrane proteins which provide protection against TspA
341 activity [22] (Fig. 4A). Recent analysis of other T7SSb LXG toxins has revealed a common
342 mechanistic feature for secretion, involving formation of a hetero-oligomeric pre-secretion
343 complex between the LXG domain and helical hairpin partner proteins encoded at the same
344 genetic locus as the toxin [24, 26, 27, 37]. Logically, the TspA LXG domain should also be
345 expected to require similar helical binding partners to generate a secretion-competent
346 structure. However, there are no candidate genes coding for predicted helical proteins in the
347 immediate neighbourhood of *tspA* (*SACOL0643*) (Fig. 4A).
348
349 Genomic analysis previously identified a conserved module of three small genes, potentially
350 implicated in T7 secretion, at a locus distant from both the *tspA* and *ess* gene clusters [4] (Fig.
351 4A, shaded orange, yellow and blue). In a handful of *S. aureus* strains, for example SID53, a

352 second homologue of *tspA* is present at this locus, along with a DUF443-encoding immunity
353 gene (Fig. 4A). However, in most well-studied strains, including COL, USA300 and RN6390
354 there is no *tspA* homologue or immunity gene at this locus, although the small genes are
355 always present. This raised the possibility that the conserved cluster may be required for the
356 biogenesis of TspA. Analysis of the encoded proteins indicates that SACOL2603 and
357 SACOL2602 are predicted to be helical hairpin proteins belonging to the Lap1 (DUF3130) and
358 Lap2 (DUF3958) families of WXG100-like export factors, respectively. Members of these
359 protein families have been characterised in *Streptococcus intermedius* and shown to interact
360 with the LXG domain of the lipid II phosphatase toxin, TelC [11, 26]. Following the
361 nomenclature developed in *S. intermedius* and based on results described below we have
362 renamed SACOL2603 and SACOL2602 LapT1 and LapT2, respectively (for LXG-associated
363 alpha helical protein for TspA). The third protein encoded at this locus, SACOL2601, has a
364 DUF4176 domain. Genes encoding DUF4176 domains are frequently associated with T7SS loci
365 [3] and the involvement of a DUF4176 domain-protein in LXG toxin secretion was recently
366 determined in *S. intermedius* [27].
367
368 We therefore adapted the NanoBit assay to test the prediction that LapT1, LapT2 and
369 SACOL2601 function in TspA secretion. We constructed a plasmid carrying *tspA*$_{1\text{-}328}$-*pep86*,
370 *SACOL2603*/*lapT1, SACOL2602*/*lapT2* and *SACOL2601*, avoiding issues arising from TspA
371 toxicity by using a truncated version lacking the toxin domain but supplied with a C-terminal
372 pep86 tag. Due to the slow growth kinetics of strain COL in 384-well plates we carried out
373 assays in RN6390, whose sequence is identical to that of COL at the *tspA* and *SACOL2603* loci,
374 except for a single (synonymous) nucleotide change in *tspA*. Following our observation of
375 much lower EsxA-pep86 leakage into the supernatant at lower temperatures, we opted to
376 conduct the secretion assays at 34°C.
377
378 After introduction of the plasmid into RN6390 wild-type and Δ*essC* strains, we measured
379 luminescence in whole cell cultures, and in clarified culture supernatant following pelleting of
380 cells (Fig. 4B). The presence of pep86-tagged TspA$_{NT}$ in whole cell cultures was clearly
381 detected, dependent on an active T7SS. However little or no TspA$_{NT}$ was detected in the
382 fractionated culture supernatant, confirming the previous finding that TspA is cell surface-

383 associated rather than fully secreted [22]. These results also indicate that the TspA toxin
384 domain (residues 329-469) is dispensable for cell surface attachment.
385
386 To determine whether any of LapT1, LapT2 and SACOL2601 are required to support secretion
387 of TspA, we individually deleted each of the encoding genes from the plasmid and used the
388 high throughput assay in whole cell cultures to assess export of pep86-tagged $TspA_{NT}$. Fig. 4C
389 indicates that in the absence of each gene, significantly less $TspA_{NT}$-pep86 could be detected
390 in whole cell cultures, while the cytoplasmic levels (deduced from measuring total
391 luminescence per well following lysis) were not much affected (Fig S3B). We conclude that
392 each of LapT1, LapT2 and SACOL2601 support TspA secretion. It should be noted the
393 chromosomal copy of the *SACOL2601-2603* gene cluster is present in these strains and may
394 account for some of the residual secretion seen from the plasmid deletion constructs.
395
396 Previous work has reported that LXG proteins form a stable pre-secretion complex with their
397 cognate helical hairpin secretion partners [23, 24, 26, 27]. To investigate whether any of
398 LapT1, LapT2 or SACOL2601 interact with TspA, we co-produced all four proteins in *E. coli*.
399 The recombinant proteins carried an N-terminal twinstrep tag on LapT1 for purification, and
400 a C-terminal HA, N-terminal myc and N-terminal his tag on SACOL2601, LapT2 and TspA,
401 respectively, for detection. Complexes were purified by streptactin affinity chromatography
402 and analysed by size exclusion chromatography (SEC; Fig. 4D) and both SDS PAGE and western
403 blotting (Fig. 4E). All four proteins co-eluted in the SEC main peak confirming that they exist
404 as a tetrameric complex. Taken together our results indicate that the *SACOL2601-2603* gene
405 cluster is required for the biogenesis of TspA.

406 **Discussion**

407

408 Here we report a novel, sensitive assay for monitoring protein secretion by the T7SS in *S.*
409 *aureus*, using the NanoBit split luciferase system. The 11 amino acid pep86 fragment of
410 NanoLuc is small enough to be compatible with secretion when fused to the WXG100 protein
411 EsxA, or to a larger LXG protein substrate. As the presence of luminescence from secreted
412 proteins can be measured directly in unprocessed cell cultures, this enables high throughput
413 screening of large numbers of strains and growth conditions.

414

415 Most studies of the *S. aureus* T7SS to date have focussed on secretion by *essC-1* strains, using
416 a handful of isolates. Strains exhibit high genetic diversity at their T7 loci [14], but
417 experimental investigation of *essC-2* - *essC-4* variant strains has previously been constrained
418 by low activity in lab conditions, and the lack of a sensitive secretion assay [33]. Using the
419 NanoBit assay we found substantial variability in the secretion efficiencies of such strains, and
420 their temperature dependence. In particular, the EMRSA15 T7SS (an *essC-4* variant strain),
421 which was inactive at 37°C, exhibited a significant level of secretion at 30°C and 34°C
422 suggesting a role for temperature in T7SSb regulation. Interestingly, *ess* transcription and T7
423 secretion were previously shown to be activated by reducing membrane fluidity, which was
424 achieved by treating cells with *cis*-unsaturated fatty acids [38, 39]. Temperature-induced
425 changes in membrane fluidity may potentially account for the differences in secretion
426 observed here.

427

428 A number of pathways have been implicated in transcriptional regulation of the *S. aureus*
429 T7SS, including the SaeRS and ArlRS two-component systems, the Agr quorum-sensing system
430 and the alternative sigma factor SigB [19, 38, 40-43]. However, the complex regulatory picture
431 is far from complete and key questions around how, why and when the secretion system is
432 expressed remain unanswered. Moreover, additional factors may activate secretion at the
433 post transcriptional level, for example the presence of haemin has been linked to post-
434 transcriptional activation of the T7SS in some strains [33, 44]. The availability of a high
435 throughput assay will facilitate screening of a range of conditions, paving the way for a much
436 more detailed analysis of T7 regulation than has been possible to date.

437

438 We employed the NanoBit assay to explore the secretion of the surface-attached LXG toxin,
439 TspA. While other LXG toxins characterised to date are encoded alongside accessory proteins
440 essential for their biogenesis [24, 26, 27], secretion partners for TspA had not been identified.
441 A conserved cluster of three genes remote from the *tspA* locus had previously been
442 bioinformatically linked to the T7SS [4]. We showed that each of these genes supports
443 secretion of a TspA-pep86-fusion suggesting that their genomic conservation is because they
444 are required for TspA biogenesis. This conclusion was further supported by co-purification
445 experiments, where tagged variants of the three proteins formed a complex with TspA. Two
446 of these genes, *SACOL2603* and *SACOL2602* code for helical hairpin proteins of the DUF3130
447 and DUF3958 families, respectively. The LXG toxins, TelC and TelD, from *S. intermedius* both
448 require proteins from each of these families for secretion, with Lap1, being a DUF3130 protein
449 and Lap2 a DUF3958. In each case the Lap1 and Lap2 proteins were shown to interact with
450 the LXG domain of their cognate toxin partners. In keeping with this nomenclature, we have
451 renamed SACOL2603 as LapT1 and SACOL2602 as LapT2. Klein *et al*. [26] identified a
452 conserved FxxD motif close to the C-terminus of LapD1, showing that individual alanine
453 substitutions of either conserved residue was sufficient to prevent TelD secretion. LapD1 and
454 LapT1 share only limited sequence similarity (Fig. S4) and no FxxD motif is present in the
455 equivalent position on LapT1, although an FxxxD motif is present within 15 amino acids of the
456 C-terminus. This motif is conserved across LapT1 homologues in other staphylococci and in
457 enterococci. It remains to be determined whether this serves as a secretion motif for TspA
458 export.
459
460 The third protein gene, *SACOL2601*, codes for a DUF4176 protein. Interestingly, proteins of
461 this family are also encoded in the *S. intermedius telA*, *telB* and *telD* gene clusters, but not
462 *telC*. While the homologue encoded at the *telA* locus was shown to be essential for TelA
463 secretion, it was not required for TelC export [27]. It is not clear whether TelC requires a
464 DUF4176 protein encoded by a different cluster, or whether its secretion occurs
465 independently of such a protein. In addition to TspA, a variant-specific LXG protein coding
466 gene is found at the *ess* loci of *essC-1*, essC-*2*, *essC-3* and *essC-4 S. aureus* strains [4]. However,
467 no DUF4176-encoding genes are found at any *ess* loci, suggesting either they are not
468 universally required, or that a single DUF4176 protein might function in secretion of multiple
469 substrates. Further work would be required to investigate this.

470

471 In conclusion we describe a novel, sensitive assay to assess secretion of T7 substrate proteins
472 in *S. aureus*. We anticipate this approach should be broadly applicable for the study of type
473 VII secretion across a range of organisms.

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479 José Penadés and Ross Fitzgerald for *S. aureus* strain JP5347, and Sharon Peacock for strain
480 EMRSA15.

481

482 **Data, code and materials**

483 The datasets supporting this article have been uploaded to FigShare (DOI
484 10.6084/m9.figshare.25368598).

485 **Table 1. *S. aureus* strains used in this study**

Strain	Description	Reference
COL	MRSA, *agr, essC-1* variant strain	[45, 46]
COLΔ*essC*	COL with markerless deletion of *essC*	[22]
RN6390	NCTC8325 derivative. *rbsU, tcaR,* cured of ϕ11, ϕ12, ϕ13. *essC-1* variant strain	[47, 48]
RN6390Δ*essC*	RN6390 with markerless deletion of *essC*	[8]
JP5347	Human isolate, *essC-1* variant strain	This work
JP5347Δ*essC*	JP5347 with markerless deletion of *essC*	This work
MRSA252	Nosocomial HA-MRSA isolate, representative of Epidemic MRSA-16. *essC-2* variant strain	[49]
MRSA252Δ*essC*	MRSA252 with markerless deletion of *essC*	This work
10.1252.X	Livestock-associated ST398 isolate. *essC-3* variant strain	[33]
10.1252.XΔ*essC*	10.1252.X with markerless deletion of *essC*	This work
EMRSA15	EMRSA-15 clonal complex 22 human isolate. *essC-4* variant strain.	[50]
EMRSA15Δ*essC*	EMRSA15 with markerless deletion of *essC*	This work
COL$_{esxA\text{-}pep86}$	Pep86 coding sequence (VSGWRLFKKIS) fused to the 3' end of *esxA* in COL	This work
COLΔ*essC*$_{esxA\text{-}pep86}$	Pep86 coding sequence (VSGWRLFKKIS) fused to the 3' end of *esxA* in COLΔ*essC*	This work

486

487 **Table 2. Plasmids used in this study**

Plasmid	Details	Ref/source
pRab11	*E. coli*/*S. aureus* shuttle vector, carries $P_{xyl/tet}$ for inducible expression. Amp^r, cml^r.	[51]
pRab11-esxApep86	For ATC-inducible expression of EsxA with a C-terminal pep86 tag (VSGWRLFKKIS)	This study
pRab11-pep86trxA	For ATC-inducible expression of TrxA with a N-terminal pep86 tag (MVSGWRLFKKIS)	This study
pRab11-CBED-DG	Plasmid for overproduction of the *essC-1* substrate EsaD with a C-terminal HA tag, along with its immunity protein EsaG and secretion factors EsxB, EsxC, EsxD and EsaE.	[24]
pRab11-tspA$_{1-328}$-pep86_2603_2602_2601	For ATC-inducible production of $TspA_{NT}$ with a C-terminal pep86 tag (VSGWRLFKKIS), along with LapT1 (SACOL2603), LapT2 (SACOL2602) and SACOL2601.	This work
pRab11-tspA$_{1-328}$-pep86_2602-2601	For ATC-inducible production of $TspA_{NT}$ with a C-terminal pep86 tag (VSGWRLFKKIS), along with LapT2 (SACOL2602) and SACOL2601.	This work
pRab11-tspA$_{1-328}$-pep86_2603_2601	For ATC-inducible production of $TspA_{NT}$ with a C-terminal pep86 tag (VSGWRLFKKIS), along with LapT1 (SACOL2603) and SACOL2601.	This work
pRab11-tspA$_{1-328}$-pep86_2603-2602	For ATC-inducible production of $TspA_{NT}$ with a C-terminal pep86 tag (VSGWRLFKKIS), along with LapT1 (SACOL2603) and LapT2 (SACOL2602).	This work
pIMAY	*E. coli*/*S. aureus* shuttle vector, temperature sensitive, cml^r.	[29]
pIMAYessC	pIMAY carrying the flanking regions of *essC-1*	[8]
pIMAYessC-MRSA252	pIMAY carrying the flanking regions of *essC-2*	This work
pIMAYessC-ST398	pIMAY carrying the flanking regions of *essC-3*	This work
pIMAYessC-EMRSA15	pIMAY carrying the flanking regions of *essC-4*	This work
pIMAY-Z	*E. coli*/*S. aureus* shuttle vector, temperature sensitive, cml^r.	[30]
pIMAY-Z_esxApep86	pIMAY-Z carrying *esxA-pep86* and chromosomal flanking regions.	This work
pBAD-$_{6H}$11S	Expression vector for purification of 11S	[28]
pQE70	*E. coli* expression vector	Qiagen

pQE70-$_{Tstrep}$SACOL2603$_{myc}$-2602-2601$_{HA}$-$_{his}$TspA	For production of TspA with an N-terminal his tag, alongside LapT1 with an N-terminal twinstrep tag, LapT2 with an N-terminal myc tag and SACOL2601 with a C-terminal HA tag	This work

488

489 **Figure legends**

490

491 ***Figure 1. The NanoBit T7-secretion assay***

492 A. Schematic representation of the NanoBit secretion assay. The T7SSb substrate EsxA with a
493 C-terminal pep86 tag is produced in the *S. aureus* cytoplasm, and 11S and furimazine (the
494 NanoLuc large subunit and substrate) are provided outside the cell. Secretion of EsxA-pep86
495 via the T7SS results in functional complementation of 11S, which luminesces in the presence
496 of furimazine and O_2. Note that the cartoon is not an accurate representation of the
497 stoichiometry and architecture of the T7SSb, which are unknown.

498 B, D. COL wild-type or Δ*essC* strains carrying the indicated plasmids were grown in 5 ml
499 cultures. At OD_{600} = 0.5 ATC was added to induce gene expression from pRab11. When cells
500 reached OD_{600} = 2, samples corresponding to the whole cell culture, the clarified culture
501 medium ('supernatant') and lysed cells ('cytoplasm') were prepared. Serial two-fold dilutions
502 of whole cell cultures, clarified supernatant and cytoplasmic fractions were prepared, and 11S
503 and furimazine were added to each. The data presented correspond to the mean
504 luminescence readings of five biological replicates and error bars represent the SEM. Two-
505 way ANOVA was performed to determine statistical significance (ns $p>0.05$; **** $p<0.0001$).

506 C, E. Lysed whole cell (wc) and clarified culture supernatant (sn) fractions from (B) and (D)
507 were analysed by immunoblot with antibodies against EsxA (top) or TrxA (bottom).
508 Supernatant samples were concentrated by TCA precipitation prior to analysis, and the
509 amount analysed corresponds to 6 x more culture volume than for lysed cell samples.

510

511 ***Figure 2. High throughput secretion analysis of strains producing chromosomally-encoded***
512 ***EsxA-pep86.***

513 A. The indicated strains were grown in 5 ml cultures and ATC was added at OD_{600} = 0.5 to
514 induce gene expression from pRab11. 'pEsaD-HA' denotes plasmid pRab11-CBED-DEG,
515 carrying an HA-tagged copy of the T7 substrate EsaD, along with its accessory secretion
516 factors [24]. At OD_{600} = 2, lysed whole cell (wc) and clarified supernatant (sn) fractions were
517 prepared and analysed by immunoblot with antibodies against the HA tag (to detect EsaD-
518 HA), EsxA or TrxA. Supernatant samples were concentrated by TCA precipitation prior to
519 analysis, and the amount analysed corresponds to 6 x more culture volume than for lysed cell
520 samples.

521 B. Growth curve for cultures of COL wild-type and Δ*essC* strains, each producing
522 chromosomally encoded EsxA with a C-terminal pep86 tag from the native locus. 192 replicate
523 50 µl cultures (64 technical replicates x 3 biological replicates) were grown in 384-well plates
524 at 37°C. Furimazine and 11S were added at 360 min (dotted line). Mean OD_{600} values are
525 plotted and error bars correspond to the SEM (n=3).
526 C. Luminescence readings at ten minute intervals for the cultures in (B) following initiation of
527 luminescence with furimazine and 11S at 360 minutes. Error bars correspond to the SEM
528 (n=3).
529 D. A single overnight culture of each $COL_{esxA\text{-}pep86}$ and $COL\Delta essC_{esxA\text{-}pep86}$ was diluted to an
530 OD_{600} of 0.1 and used to inoculate 64 individual wells of a 384 well plate. After 360 minutes
531 growth, furimazine and 11S were added and a single luminescence reading from each well
532 was taken at 430 minutes.
533 E. As for (D) except that three overnight cultures of each $COL_{esxA\text{-}pep86}$ and COL $\Delta essC_{esxA\text{-}pep86}$
534 were used, giving 192 individual data points for each strain. Statistical significance in (D) and
535 (E) were assessed with the Mann-Whitney test, with **** indicating $p < 0.0001$ for each case.
536
537 ***Figure 3. EsxA secretion in different S. aureus strains and at different temperatures***
538 A. Wild-type and Δ*essC* variants of the indicated strains were transformed with pRab11-
539 esxApep86 and cultured in 384-well plates at 37°C. Expression of *esxA-pep86* was induced by
540 addition of ATC at 180 minutes, and luminescence was initiated by addition of furimazine and
541 11S at 220 minutes. Luminescence readings taken at ten minute intervals (shown in Fig. S1B)
542 were divided by OD_{600} readings to give the relative luminescence. The values for Δ*essC* relative
543 luminescence were subtracted from those of the wild-type relative luminescence to give a
544 relative secretion value for each time point. For each strain pair the time point with the peak
545 value for relative secretion is presented. Data represent the mean of three biological
546 replicates each with 16 technical replicates. Error bars correspond to the SEM (n=3).
547 B. Secretion assays were performed as in (A), but each strain pair was cultured at 30°C, 34°C
548 and 37°C (data for 37°C is reproduced from (A) for the sake of comparison). The timings of
549 ATC induction and luminescence initiation were adjusted for the slower growth rates at 30°C
550 and 34°C (see Fig S2A and methods) and raw luminescence readings are shown in Figure S2B.
551
552 ***Figure 4. Identification of TspA secretion factors***

553 A. Locus diagrams depicting the genetic neighbourhood of *tspA* (*SACOL0643*, red – top) and
554 the T7SS-associated genes *SACOL2603*, *SACOL2602* and *SACOL2601* (orange, yellow and blue
555 – middle) in strain COL. Bottom – the *SACOL2603* equivalent locus in strain SID53 has a *tspA2-*
556 *tsaI2* insertion between the homologues of *SACOL2602* and *SACOL2601*. *SACOL2603* encodes
557 a DUF3130/TIGR04917 family protein.
558 B. COL wild-type or Δ*essC* strains carrying plasmid pRab11-tspA1-328-
559 pep86_2603_2602_2601 were grown in 5 ml cultures at 37°C. At OD_{600} = 0.5 ATC was added
560 to induce gene expression from pRab11. When cells reached OD_{600} = 2, samples
561 corresponding to the whole cell culture ('WC') and the clarified culture medium ('SP') were
562 prepared and 11S and furimazine were added to each. The data presented correspond to the
563 mean luminescence readings of three biological replicates and error bars represent the SEM.
564 Statistical significance for whole cell culture of the wild-type vs clarified culture medium was
565 assessed with the Mann-Whitney test, with *** indicating $p< 0.001$. There was no significant
566 difference between values for the whole cell vs clarified culture medium for the Δ*essC* strain.
567 C. Wild-type and Δ*essC* variants of strain RN6390 were transformed with the indicated
568 plasmids and cultured in 384-well plates at 34°C. Expression from pRab11 was induced by
569 addition of ATC at 120 minutes, and luminescence was initiated by addition of furimazine and
570 11S at 260 minutes. Luminescence readings were taken at the 280 minute timepoint and were
571 divided by OD_{600} readings to give the relative luminescence. Data represent the mean of five
572 biological replicates each with 16 technical replicates. Error bars correspond to the SEM (n=5).
573 The relative luminescence of RN6390 harbouring each plasmid containing the indicated
574 individual gene deletion was compared with the relative luminescence signal of RN6390
575 harbouring pRab11-$tspA_{1\text{-}328}$-pep86_2603-2602-2601 using the Mann-Whitney test, * $p<$
576 0.05; ** $p<0.01$.
577 D. *E. coli* M15 cells carrying pQE70-$_{Tstrep}$SACOL2603$_{myc}$-2602-2601$_{HA\text{-}his}$TspA were grown in LB
578 medium. Following induction of gene expression with IPTG, complexes were purified by
579 streptactin- affinity purification, and the resulting sample was analysed by size-exclusion
580 chromatography (SEC).
581 E. Fractions from within the dotted lines in (D) were subject to SDS-PAGE and analysed by
582 coomassie staining (left panel) or by western blotting with antibodies against the tags on each
583 component (right panel).

584 **References**

585 1. Turner, NA, Sharma-Kuinkel, BK, Maskarinec, SA, Eichenberger, EM, Shah, PP, Carugati, M,
586 Holland, TL, Fowler, VG, Jr. 2019 Methicillin-resistant *Staphylococcus aureus*: an overview of
587 basic and clinical research. *Nat Rev Microbiol*. **17**, 203-218. (10.1038/s41579-018-0147-4)
588
589 2. Shariati, A, Dadashi, M, Moghadam, MT, van Belkum, A, Yaslianifard, S, Darban-Sarokhalil,
590 D. 2020 Global prevalence and distribution of vancomycin resistant, vancomycin intermediate
591 and heterogeneously vancomycin intermediate *Staphylococcus aureus* clinical isolates: a
592 systematic review and meta-analysis. *Sci Rep*. **10**, 12689. (10.1038/s41598-020-69058-z)
593
594 3. Boardman, ER, Palmer, T, Alcock, F. 2023 Interbacterial competition mediated by the type
595 VIIb secretion system. *Microbiology*. **169**, (10.1099/mic.0.001420)
596
597 4. Bowman, L, Palmer, T. 2021 The type VII secretion system of *Staphylococcus*. *Annu Rev*
598 *Microbiol*. **75**, 471-494. (10.1146/annurev-micro-012721-123600)
599
600 5. Burts, ML, DeDent, AC, Missiakas, DM. 2008 EsaC substrate for the ESAT-6 secretion
601 pathway and its role in persistent infections of S*taphylococcus aureus*. *Mol Microbiol*. **69**, 736-
602 746. (DOI 10.1111/j.1365-2958.2008.06324.x)
603
604 6. Burts, ML, Williams, WA, DeBord, K, Missiakas, DM. 2005 EsxA and EsxB are secreted by an
605 ESAT-6-like system that is required for the pathogenesis of *Staphylococcus aureus* infections.
606 *Proc Natl Acad Sci U S A*. **102**, 1169-1174.
607
608 7. Ishii, K, Adachi, T, Yasukawa, J, Suzuki, Y, Hamamoto, H, Sekimizu, K. 2014 Induction of
609 virulence gene expression in *Staphylococcus aureus* by pulmonary surfactant. *Infect Immun*.
610 **82**, 1500-1510. (10.1128/IAI.01635-13)
611
612 8. Kneuper, H, Cao, ZP, Twomey, KB, Zoltner, M, Jager, F, Cargill, JS, Chalmers, J, van der Kooi-
613 Pol, MM, van Dijl, JM, Ryan, RP*, et al.* 2014 Heterogeneity in *ess* transcriptional organization
614 and variable contribution of the Ess/Type VII protein secretion system to virulence across

615 closely related *Staphylocccus aureus* strains. *Mol Microbiol*. **93**, 928-943.
616 (10.1111/mmi.12707)
617
618 9. Wang, Y, Hu, M, Liu, Q, Qin, J, Dai, Y, He, L, Li, T, Zheng, B, Zhou, F, Yu, K*, et al.* 2016 Role of
619 the ESAT-6 secretion system in virulence of the emerging community-associated
620 *Staphylococcus aureus* lineage ST398. *Sci Rep*. **6**, 25163. (10.1038/srep25163)
621
622 10. Garrett, SR, Higginson, AB, Palmer, T. 2024 Multiple variants of the type VII secretion
623 system in Gram-positive bacteria. *bioRxiv*. **BIORXIV/2024/577966**
624 (https://doi.org/10.1101/2024.01.30.577966)
625
626 11. Whitney, J.C, Peterson, SB, Kim, J, Pazos, M, Verster, AJ, Radey, MC, Kulasekara, HD, Ching,
627 MQ, Bullen, NP, Bryant, D*, et al.* 2017 A broadly distributed toxin family mediates contact-
628 dependent antagonism between gram-positive bacteria. *Elife*. **6**, (10.7554/eLife.26938)
629
630 12. Kobayashi, K. 2021 Diverse LXG toxin and antitoxin systems specifically mediate
631 intraspecies competition in *Bacillus subtilis* biofilms. *PLoS Genet*. **17**, e1009682.
632 (10.1371/journal.pgen.1009682)
633
634 13. Chatterjee, A, Willett, JLE., Dunny, GM, Duerkop, BA 2021 Phage infection and sub-lethal
635 antibiotic exposure mediate *Enterococcus faecalis* type VII secretion system dependent
636 inhibition of bystander bacteria. *PLoS Genet*. **17**, e1009204. (10.1371/journal.pgen.1009204)
637
638 14. Warne, B, Harkins, CP, Harris, SR, Vatsiou, A, Stanley-Wall, N, Parkhill, J, Peacock, SJ,
639 Palmer, T, Holden, MT. 2016 The Ess/Type VII secretion system of *Staphylococcus aureus*
640 shows unexpected genetic diversity. *BMC Genom*. **17**, 222. (10.1186/s12864-016-2426-7)
641
642 15. Bunduc, CM, Fahrenkamp, D, Wald, J, Ummels, R, Bitter, W, Houben, ENG., Marlovits, TC.
643 2021 Structure and dynamics of a mycobacterial type VII secretion system. *Nature*. **593**, 445-
644 448. (10.1038/s41586-021-03517-z)
645

646 16. Beckham, KSH., Ritter, C, Chojnowski, G, Ziemianowicz, DS, Mullapudi, E, Rettel, M,
647 Savitski, MM, Mortensen, SA, Kosinski, J, Wilmanns, M. 2021 Structure of the mycobacterial
648 ESX-5 type VII secretion system pore complex. *Sci Adv*. **7**, (10.1126/sciadv.abg9923)
649
650 17. Jager, F, Kneuper, H, Palmer, T. 2018 EssC is a specificity determinant for *Staphylococcus*
651 *aureus* type VII secretion. *Microbiology*. **164**, 816-820. (10.1099/mic.0.000650)
652
653 18. Huppert, L. A., Ramsdell, T. L., Chase, M. R., Sarracino, D. A., Fortune, S. M., Burton, B. M.
654 2014 The ESX system in *Bacillus subtilis* mediates protein secretion. *PLoS One*. **9**, e96267.
655 (10.1371/journal.pone.0096267)
656
657 19. Anderson, M, Aly, KA, Chen, YH, Missiakas, D. 2013 Secretion of atypical protein substrates
658 by the ESAT-6 secretion system of *Staphylococcus aureus*. *Mol Microbiol*. **90**, 734-743.
659 (10.1111/mmi.12395)
660
661 20. Renshaw, PS, Lightbody, KL, Veverka, V, Muskett, FW, Kelly, G, Frenkiel, TA, Gordon, SV,
662 Hewinson, RG, Burke, B, Norman, J*, et al.* 2005 Structure and function of the complex formed
663 by the tuberculosis virulence factors CFP-10 and ESAT-6. *EMBO J*. **24**, 2491-2498.
664 (10.1038/sj.emboj.7600732)
665
666 21. Pallen, MJ. 2002 The ESAT-6/WXG100 superfamily -- and a new Gram-positive secretion
667 system? *Trends Microbiol*. **10**, 209-212.
668
669 22. Ulhuq, FR, Gomes, MC, Duggan, GM, Guo, M, Mendonca, C, Buchanan, G, Chalmers, JD,
670 Cao, Z, Kneuper, H, Murdoch, S*, et al.* 2020 A membrane-depolarizing toxin substrate of the
671 *Staphylococcus aureus* type VII secretion system mediates intraspecies competition. *Proc Natl*
672 *Acad Sci U S A*. **117**, 20836-20847. (10.1073/pnas.2006110117)
673
674 23. Garrett, SR, Mietrach, N, Deme, J, Bitzer, A, Yang, Y, Ulhuq, FR, Kretschmer, D, Heilbronner,
675 S, Smith, TK, Lea, SM*, et al.* 2023 A type VII-secreted lipase toxin with reverse domain
676 arrangement. *Nat Commun*. **14**, 8438. (10.1038/s41467-023-44221-y)
677

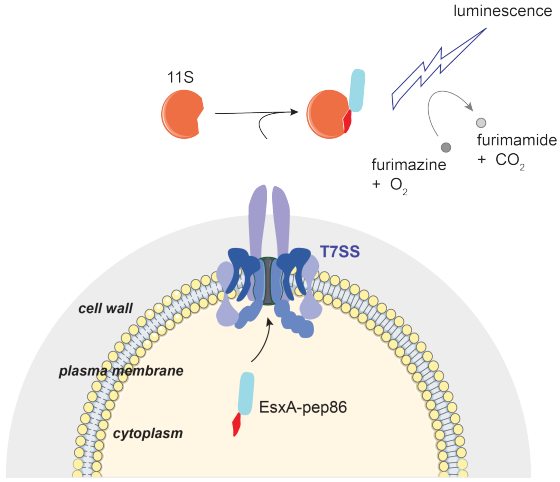
678 24. Yang, Y, Boardman, E, Deme, J, Alcock, F, Lea, S, Palmer, T. 2023 Three small partner
679 proteins facilitate the type VII-dependent secretion of an antibacterial nuclease. *mBio*. **14**,
680 e0210023. (10.1128/mbio.02100-23)
681
682 25. Cao, Z, Casabona, MG, Kneuper, H, Chalmers, JD, Palmer, T. 2016 The type VII secretion
683 system of *Staphylococcus aureus* secretes a nuclease toxin that targets competitor bacteria.
684 *Nat Microbiol*. **2**, 16183. (10.1038/nmicrobiol.2016.183)
685
686 26. Klein, TA, Grebenc, DW, Shah, PY, McArthur, OD, Dickson, BH, Surette, MG, Kim, Y,
687 Whitney, JC. 2022 Dual targeting factors are required for LXG toxin export by the bacterial
688 type VIIb secretion system. *mBio*. **13**, e0213722. (10.1128/mbio.02137-22)
689
690 27. Klein, TA, Shah, PY, Gkragkopoulou, P, Grebenc, DW, Kim, Y, Whitney, JC. 2024 Structure
691 of a tripartite protein complex that targets toxins to the type VII secretion system. *Proc Natl*
692 *Acad Sci U S A*. **121**, e2312455121. (10.1073/pnas.2312455121)
693
694 28. Pereira, GC, Allen, WJ, Watkins, DW, Buddrus, L, Noone, D, Liu, X, Richardson, AP,
695 Chacinska, A, Collinson, I. 2019 A high-resolution luminescent assay for rapid and continuous
696 monitoring of protein translocation across biological membranes. *J Mol Biol*. **431**, 1689-1699.
697 (10.1016/j.jmb.2019.03.007)
698
699 29. Monk, IR, Shah, IM, Xu, M, Tan, MW, Foster, TJ. 2012 Transforming the untransformable:
700 application of direct transformation to manipulate genetically *Staphylococcus aureus* and
701 *Staphylococcus epidermidis*. *mBio*. **3**, (10.1128/mBio.00277-11)
702
703 30. Monk, IR, Stinear, TP. 2021 From cloning to mutant in 5 days: rapid allelic exchange in
704 *Staphylococcus aureus*. *Access Microbiol*. **3**, 000193. (10.1099/acmi.0.000193)
705
706 31. Miller, M, Donat, S, Rakette, S, Stehle, T, Kouwen, TR, Diks, SH, Dreisbach, A, Reilman, E,
707 Gronau, K, Becher, D*, et al.* 2010 Staphylococcal PknB as the first prokaryotic representative
708 of the proline-directed kinases. *PLoS ONE*. **5**, e9057. (10.1371/journal.pone.0009057)
709

710 32. Gilchrist, CLM., Chooi, YH. 2021 Clinker & clustermap.js: Automatic generation of gene
711 cluster comparison figures. *Bioinformatics*. (10.1093/bioinformatics/btab007)
712
713 33. Casabona, MG, Kneuper, H, Alferes de Lima, D, Harkins, CP, Zoltner, M, Hjerde, E, Holden,
714 MTG., Palmer, T. 2017 Haem-iron plays a key role in the regulation of the Ess/type VII
715 secretion system of *Staphylococcus aureus* RN6390. *Microbiology*. **163**, 1839-1850.
716 (10.1099/mic.0.000579)
717
718 34. England, CG, Ehlerding, EB, Cai, W. 2016 NanoLuc: A small luciferase Is brightening up the
719 field of bioluminescence. *Bioconjug Chem*. **27**, 1175-1187.
720 (10.1021/acs.bioconjchem.6b00112)
721
722 35. Allen, WJ, Watkins, DW, Dillingham, MS, Collinson, I. 2020 Refined measurement of SecA-
723 driven protein secretion reveals that translocation is indirectly coupled to ATP turnover. *Proc*
724 *Natl Acad Sci U S A*. **117**, 31808-31816. (10.1073/pnas.2010906117)
725
726 36. Zhou, WJ, Hao, BH, Bricker, TM, Theg, SM. 2023 A real-time analysis of protein transport
727 via the twin arginine translocation pathway in response to different components of the
728 protonmotive force. *J Biol Chem*. **299**, (ARTN 105286 10.1016/j.jbc.2023.105286)
729
730 37. Spencer, BL, Job, AM, Robertson, CM, Hameed, ZA, Serchejian, C, Wiafe-Kwakye, CS,
731 Mendonça, JC, Apolonio, MA, Nagao, PE, Neely, MN*, et al.* 2023 Heterogeneity of the group
732 B streptococcal type VII secretion system and influence on colonization of the female genital
733 tract. *Mol Microbiol*. **120**, 258-275. (10.1111/mmi.15115)
734
735 38. Kenny, JG, Ward, D, Josefsson, E, Jonsson, IM, Hinds, J, Rees, HH, Lindsay, JA, Tarkowski,
736 A, Horsburgh, MJ. 2009 The response to unsaturated long chain free fatty acids: Survival
737 mechanisms and virulence implications. *Plos One*. **4**, (ARTN e4344
738 10.1371/journal.pone.0004344)
739

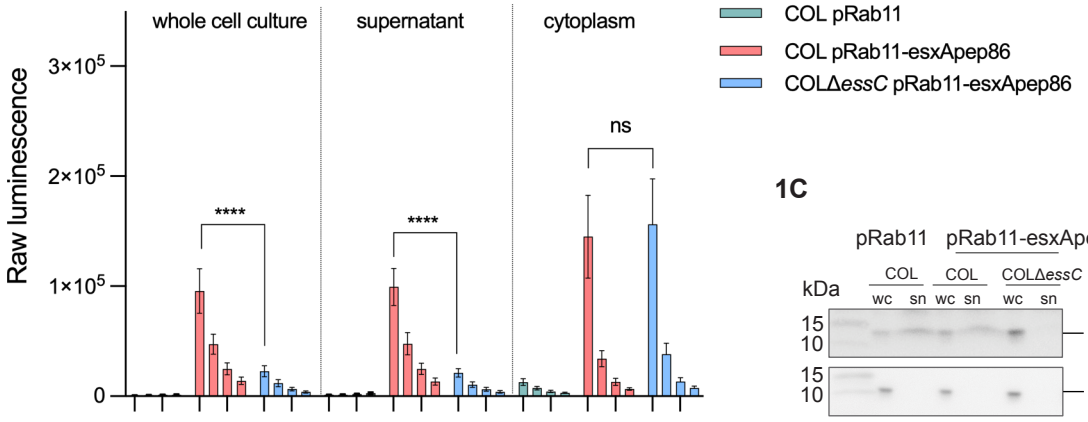
740 39. Lopez, MS, Tan, IS, Yan, D., Kang, J, McCreary, M, Modrusan, Z, Austin, CD, Xu, M, Brown,
741 EJ. 2017 Host-derived fatty acids activate type VII secretion in *Staphylococcus aureus. Proc*
742 *Natl Acad Sci U S A*. **114**, 11223-11228. (10.1073/pnas.1700627114)
743
744 40. Bischoff, M, Entenza, JM, Giachino, P. 2001 Influence of a functional *sigB* operon on the
745 global regulators *sar* and *agr* in *Staphylococcus aureus*. *J Bacteriol*. **183**, 5171-5179.
746 (10.1128/jb.183.17.5171-5179.2001)
747
748 41. Crosby, HA, Tiwari, N, Kwiecinski, JM, Xu, Z., Dykstra, A, Jenul, C, Fuentes, EJ, Horswill, AR.
749 2020 The *Staphylococcus aureus* ArlRS two-component system regulates virulence factor
750 expression through MgrA. *Mol Microbiol*. **113**, 103-122. (10.1111/mmi.14404)
751
752 42. Dunman, PM, Murphy, E, Haney, S, Palacios, D, Tucker-Kellogg, G, Wu, S, Brown, EL,
753 Zagursky, RJ, Shlaes, D, Projan, SJ. 2001 Transcription profiling-based identification of
754 *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. *J Bacteriol*. **183**, 7341-
755 7353. (10.1128/JB.183.24.7341-7353.2001)
756
757 43. Schulthess, B, Bloes, DA, Berger-Bachi, B. 2012 Opposing roles of sigmaB and sigmaB-
758 controlled SpoVG in the global regulation of *esxA* in *Staphylococcus aureus*. *BMC Microbiol*.
759 **12**, 17. (10.1186/1471-2180-12-17)
760
761 44. Casabona, MG, Buchanan, G, Zoltner, M, Harkins, CP, Holden, MTG., Palmer, T. 2017
762 Functional analysis of the EsaB component of the *Staphylococcus aureus* Type VII secretion
763 system. *Microbiology*. **163,** 1851-1863. (10.1099/mic.0.000580)
764
765 45. Dyke, KG, Jevons, MP, Parker, MT. 1966 Penicillinase production and intrinsic resistance
766 to penicillins in *Staphylococcus aures*. *Lancet*. **1**, 835-838.
767
768 46. Gill, SR, Fouts, DE, Archer, GL, Mongodin, EF, Deboy, RT, Ravel J, Paulsen, IT, Kolonay, JF,
769 Brinkac, L, Beanan, M*, et al.* 2005 Insights on evolution of virulence and resistance from the
770 complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and

771 a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *J Bacteriol*. **187**,
772 2426-2438. (10.1128/JB.187.7.2426-2438.2005)
773
774 47. Novick, RP, Ross, HF, Projan, SJ, Kornblum, J, Kreiswirth, B, Moghazeh, S. 1993 Synthesis
775 of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *EMBO J*. **12**,
776 3967-3975.
777
778 48. Garrett, SR, Mariano, G, Palmer, T. 2022 Genomic analysis of the progenitor strains of
779 *Staphylococcus aureus* RN6390. *Access Microbiology*. **4,** 11.
780
781 49. Holden, MT, Feil, EJ, Lindsay, JA, Peacock, SJ, Day, NP, Enright, MC, Foster, TJ, Moore, CE,
782 Hurst, L, Atkin, R*, et al.* 2004 Complete genomes of two clinical *Staphylococcus aureus* strains:
783 evidence for the rapid evolution of virulence and drug resistance. *Proc Natl Acad Sci U S A*.
784 **101**, 9786-9791. (10.1073/pnas.0402521101)
785
786 50. Donker, T, Reuter, S, Scriberras, J, Reynolds, R, Brown, NM, Torok, ME, James, R, Network,
787 E, Aanensen, DM, Bentley, SD*, et al.* 2017 Population genetic structuring of methicillin-
788 resistant *Staphylococcus aureus clone* EMRSA-15 within UK reflects patient referral patterns.
789 *Microb Genom*. **3**, e000113. (10.1099/mgen.0.000113)
790
791 51. Helle, L, Kull, M, Mayer, S, Marincola, G, Zelder, ME, Goerke, C, Wolz, C, Bertram, R. 2011
792 Vectors for improved Tet repressor-dependent gradual gene induction or silencing in
793 *Staphylococcus aureus*. *Microbiology*. **157**, 3314-3323. (10.1099/mic.0.052548-0)

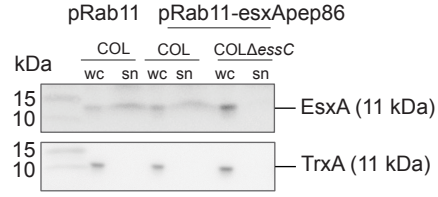
1A


luminescence
11S
furimamide
+ CO_2
furimazine
+ O_2
T7SS
cell wall
plasma membrane
cytoplasm
EsxA-pep86

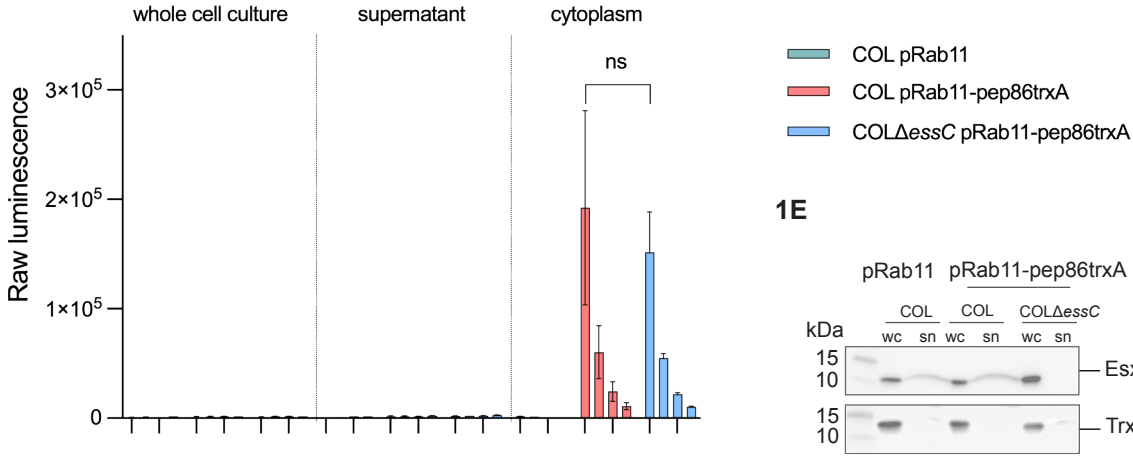
1B


whole cell culture
supernatant
cytoplasm
Raw luminescence
3×10^5
2×10^5
1×10^5
0
****
****
ns
COL pRab11
COL pRab11-esxApep86
COLΔ*essC* pRab11-esxApep86

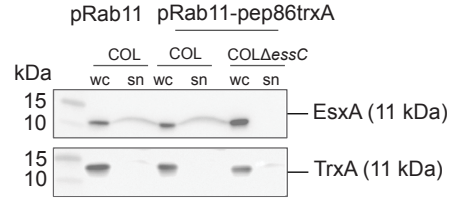
1C

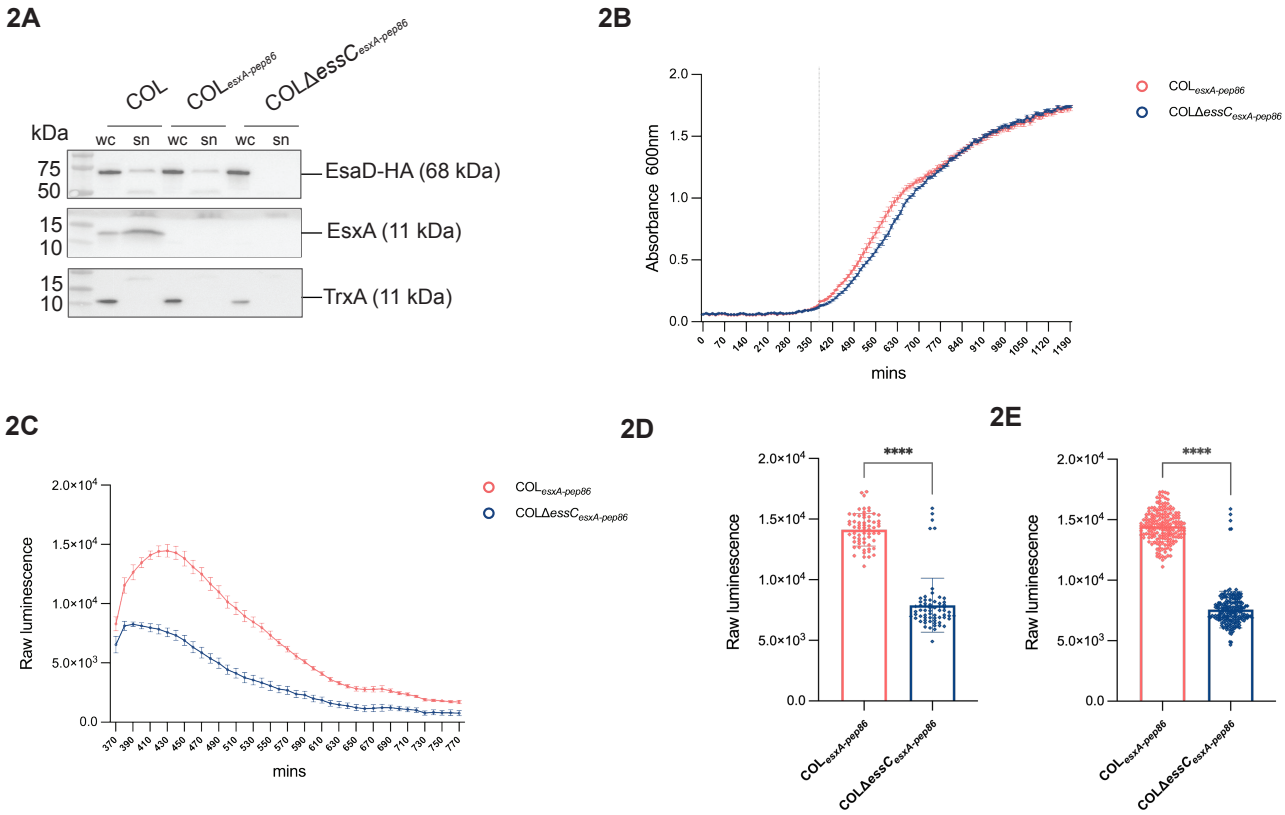

pRab11
pRab11-esxApep86
COL
COL
COLΔ*essC*
kDa
wc
sn
wc
sn
wc
sn
15
10
EsxA (11 kDa)
15
10
TrxA (11 kDa)

1D

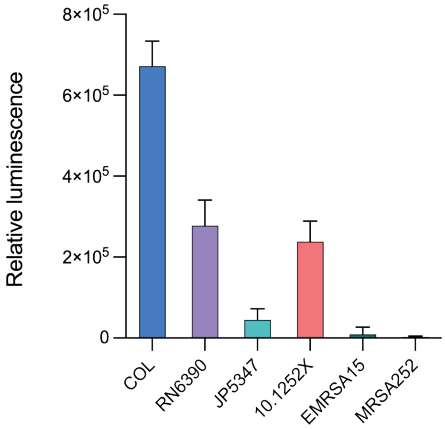

whole cell culture
supernatant
cytoplasm
Raw luminescence
3×10^5
2×10^5
1×10^5
0
ns
COL pRab11
COL pRab11-pep86trxA
COLΔ*essC* pRab11-pep86trxA

1E

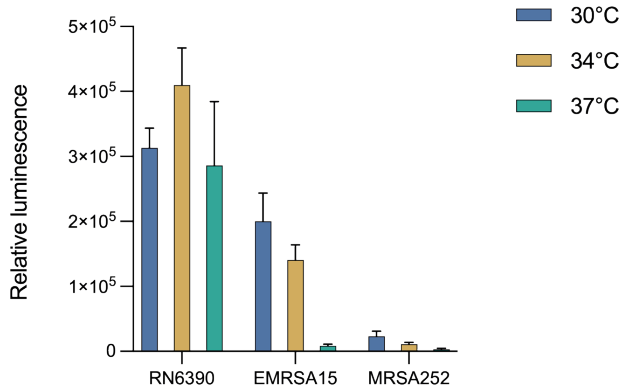

pRab11
pRab11-pep86trxA
COL
COL
COLΔ*essC*
kDa
wc
sn
wc
sn
wc
sn
15
10
EsxA (11 kDa)
15
10
TrxA (11 kDa)

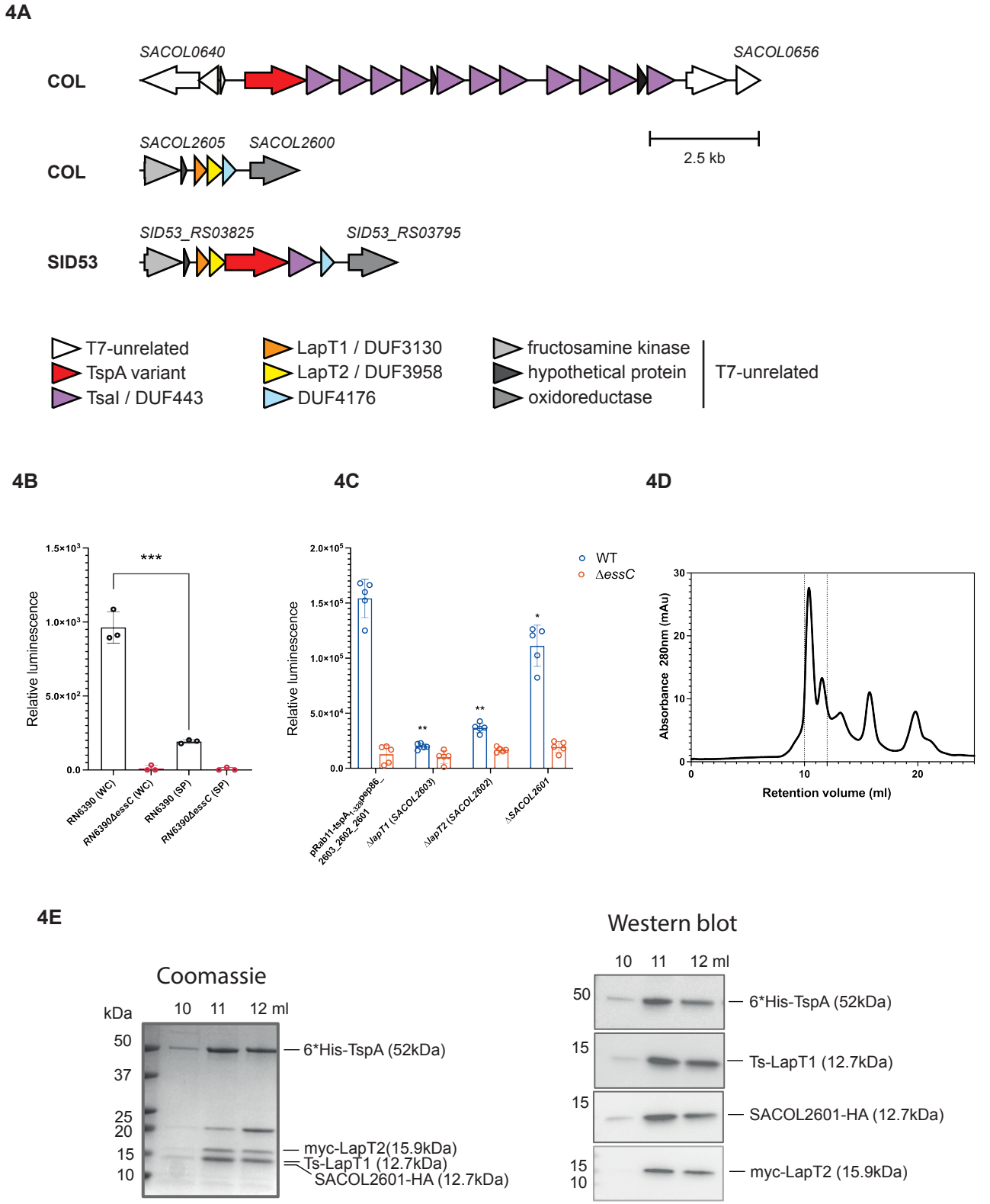

2A
COL
COLesxA-pep86
COLΔessCesxA-pep86
kDa
wc sn wc sn wc sn
75
50
EsaD-HA (68 kDa)
15
10
EsxA (11 kDa)
15
10
TrxA (11 kDa)
2B
Absorbance 600nm
mins
COLesxA-pep86
COLΔessCesxA-pep86
2C
Raw luminescence
mins
COLesxA-pep86
COLΔessCesxA-pep86
2D
Raw luminescence
****
COLesxA-pep86
COLΔessCesxA-pep86
2E
Raw luminescence
****
COLesxA-pep86
COLΔessCesxA-pep86

3A


Relative luminescence
8×10^5
6×10^5
4×10^5
2×10^5
0
COL
RN6390
JP5347
10.1252X
EMRSA15
MRSA252

3B


Relative luminescence
5×10^5
4×10^5
3×10^5
2×10^5
1×10^5
0
RN6390
EMRSA15
MRSA252
30°C
34°C
37°C


4A
COL
SACOL0640
SACOL0656
2.5 kb
COL
SACOL2605
SACOL2600
SID53
SID53_RS03825
SID53_RS03795
T7-unrelated
TspA variant
TsaI / DUF443
LapT1 / DUF3130
LapT2 / DUF3958
DUF4176
fructosamine kinase
hypothetical protein
oxidoreductase
T7-unrelated
4B
Relative luminescence
***
RN6390 (WC)
RN6390ΔessC (WC)
RN6390 (SP)
RN6390ΔessC (SP)
4C
Relative luminescence
WT
ΔessC
pRab11-tspA1-328pep86_2603_2602_2601
ΔlapT1 (SACOL2603)
ΔlapT2 (SACOL2602)
ΔSACOL2601
4D
Absorbance 280nm (mAu)
Retention volume (ml)
4E
Coomassie
kDa
10 11 12 ml
6*His-TspA (52kDa)
myc-LapT2(15.9kDa)
Ts-LapT1 (12.7kDa)
SACOL2601-HA (12.7kDa)
Western blot
10 11 12 ml
6*His-TspA (52kDa)
Ts-LapT1 (12.7kDa)
SACOL2601-HA (12.7kDa)
myc-LapT2 (15.9kDa)