A high throughput assay to measure 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 Firmicutes which functions in bacterial antagonism and virulence. Traditional assays for monitoring protein secretion by the T7SSb rely on concentrating cell culture supernatants for immunoblot analysis, a procedure which is time consuming, low throughput, non-quantitative and has low sensitivity. To overcome some of these disadvantages we have utilised NanoLuc Binary Technology to develop a simple assay to monitor protein secretion via detection of bioluminescence in 384-well plates. High-throughput analysis of secretion in replicate cultures reveals overlapping distributions for $T7^+$ and $T7^-$ populations highlighting a significant limitation of lower-throughput methods. We also demonstrate the utility of the NanoLuc assay in comparing T7-dependent protein secretion across different strains and find differences in the temperature dependence of T7 secretion across *S. aureus* isolates.

32 **Introduction**

33

34 The opportunistic pathogen *Staphylococcus aureus* is a major cause of both nosocomial and
35 community-acquired infections. It is a frequent coloniser of animals and humans, and
36 infections can result in a range of life-threatening diseases such as pneumonia, endocarditis
37 and sepsis. It is notorious for developing resistance to front line antimicrobials, with
38 methicillin resistance now widespread and vancomycin resistance also increasing (1, 2). Like
39 many Gram-positive bacteria, *S. aureus* carries a specialised protein secretion machinery
40 called the Type VII secretion system (T7SS), which it utilises to secrete antibacterial toxins
41 targeting competitor bacteria (3). T7SS-mediated antagonism is likely to be important for
42 colonisation (4), and the *S. aureus* T7SS has been shown to be required for virulence in mouse
43 infection models (5-9). There are two distinct T7SS subtypes, T7SSa and T7SSb, with the latter
44 being widely distributed among firmicutes. The T7SSb has been studied to date in species of
45 *Streptococcus*, *Staphylococcus*, *Bacillus* and *Enterococcus* (7, 10-12), however in many cases
46 functional and mechanistic studies are hindered by low secretion activity in laboratory
47 conditions.

48

49 The *S. aureus* T7SSb is encoded on the core genome at the *ess* locus, and four variant locus
50 types have been identified, *essC-1* – *essC-4* (13). The genes at the 5' end of the *ess* locus are
51 highly conserved and encode core components of the secretion machinery, while the 3' end
52 codes for different complements of variant-specific substrate and accessory genes. The
53 primary component of the T7SSb transmembrane channel is the ATPase EssC, inferred from
54 homology to the EccC component of the mycobacterial T7SSa machinery whose structure has
55 been solved by cryo-electron microscopy (14, 15). EssC has a conserved N-terminus, two
56 transmembrane domains which form the secretion channel, and four C-terminal ATPase
57 domains, the latter two of which are variant-specific and provide substrate specificity (16).
58 Two types of T7SSb-secreted substrates have been identified to date: Small WXG100 family,
59 or WXG100-like, proteins such as EsxA, and larger LXG domain-containing toxins such as TelB
60 and TspA (6, 10, 17, 18). EsxA is itself a highly conserved core functional component of the
61 T7SS machinery (6, 7).

62

63 Secretion of endogenous *S. aureus* WXG100-family proteins has previously been observed by
64 centrifuging exponential phase cultures, concentrating filtered supernatants (usually by
65 precipitation with trichloroacetic acid) and immunoblotting (6, 7, 19, 20). For LXG proteins a
66 similar protocol has been followed but generally with an epitope-tagged substrate
67 overproduced from an inducible plasmid (3, 18, 21). These assays are laborious, time
68 consuming and lack sensitivity – millilitres of culture supernatant are often required, and
69 detection depends on the availability of good quality antibodies. The immunoblot data are
70 poorly reproducible and are difficult to quantify, and the lack of cleavable signal peptides on
71 T7SSb substrates means there is no size difference between the exported and cytosolic forms
72 making cellular lysis a confounding issue. T7 activity across different *S. aureus* isolates and
73 growth conditions has previously been assessed by monitoring secretion of EsxA by
74 immunoblot (6, 7, 19, 20). While some strains clearly have a higher basal T7SS activity than
75 others in laboratory conditions, low activity is a problem in all strains and differences are
76 difficult to quantify given the shortcomings of current assay methods.
77
78 Here we have utilised NanoLuc binary technology (abbreviated to NanoBit) to develop a novel
79 assay for T7SS secretion in *S. aureus* which overcomes many of the limitations of immunoblot-
80 based assays. NanoLuc is an engineered variant of a deep-sea shrimp luciferase protein. It is
81 relatively small (19 kDa) and has superior biophysical characteristics compared to other
82 luciferases (22). NanoBit is a complementation reporter system which has previously been
83 used to investigate protein secretion via the Sec and Tat translocons in *E. coli* (23-25). Two
84 separate NanoLuc subunits (11S, a 156 amino acid 'large' subunit and pep86, an 11 amino
85 acid 'small' subunit) combine with to generate the functional enzyme, which luminesces with
86 high intensity at 460 nm in the presence of the substrate furimazine and molecular oxygen
87 (22). We therefore anticipated that secretion would be straightforward to monitor by
88 expressing a pep86-tagged substrate within *S. aureus* cells, and provision of purified 11S and
89 furimazine externally (Fig. 1A). We also reasoned that the short pep86 tag is unlikely to
90 interfere with substrate translocation as other short peptide tags are tolerated on T7SSb
91 substrates (e.g. (3, 18)). We show that the NanoLuc assay can be used to monitor and quantify
92 T7-dependent secretion of the model substrate EsxA. We use this to develop a high
93 throughput NanoLuc secretion assay to assess the distribution of EsxA secretion levels within
94 a clonal population and compare EsxA secretion levels across different *S. aureus* isolates.

95 **Results**

96

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

98

99 We selected the conserved, ubiquitous T7-secreted protein EsxA as our model substrate. EsxA
100 is produced at significantly higher copy number than other T7 components (7) and can be
101 detected by Western blot in culture supernatants of the *essC-1* strain COL relatively reliably,
102 allowing us to correlate the NanoLuc assay with data from traditional immunoblots. We first
103 constructed plasmid pRab11-*esxAp86* which permits inducible overproduction of EsxA with
104 the NanoBit small subunit, pep86, fused to its C-terminus. This plasmid, or an empty vector
105 control was transferred into wild-type and Δ*essC* variants of the *S. aureus essC-1* strain COL,
106 expression of plasmid-encoded *esxAp86* was induced and cells were grown at 37°C to mid
107 logarithmic phase. At this point, samples of whole cells, culture supernatant or lysostaphin-
108 lysed cells were prepared, serially diluted and incubated with the NanoBit large subunit, 11S
109 in 96 well plates. Following addition of the NanoLuc substrate furimazine, OD_{600nm} and
110 luminescence were measured (Fig. 1B), and the whole cell and supernatant samples were also
111 analysed by immunoblot (Fig. 1C).

112

113 Fig. 1B shows that luminescence can be detected in all samples except those derived from
114 cells carrying the empty vector, signifying an EsxA-pep86–specific luminescence signal. Similar
115 luminescence readings were obtained for whole cell culture or supernatant samples,
116 demonstrating that external pep86 can be measured without centrifugal separation of cells
117 from culture supernatants (Fig. 1B). Deletion of *essC* drastically reduces the level of
118 extracellular EsxA-pep86, and we presume that the low level of extracellular EsxA-pep86
119 detected from the mutant strain results from leakage or cell lysis. Two-fold dilution of the
120 cytoplasmic fractions indicate that EsxA-pep86 is present at similar levels in both strains.
121 When fractions of the same cultures were analysed by immunoblot, a single band at ~11 kDa
122 was detected with antibodies against EsxA in all samples, even those that did not harbour
123 plasmid-encoded *esxAp86* (Fig. 1C). Since all strains have chromosomally encoded *esxA*, we
124 infer that this band corresponds to endogenous EsxA (which has a mass of 11 kDa), and that
125 the pep86 fusion (which increases the mass to 12.3 kDa) inhibits detection by our EsxA

126 antibodies (see also Fig. 2C where chromosomal tagging of *esxA* with pep86 inhibits antibody
127 detection).
128
129 For comparison with a non-secreted control protein, we constructed a plasmid for production
130 of the cytoplasmic protein TrxA with an N-terminal pep86 fusion (pRab11-pep86-TrxA) and
131 assayed this in a similar way. Luminescence readings showed that while the cytoplasmic
132 control pep86-TrxA was produced, barely any protein was detected outside the cells (Fig. 1D).
133 Immunoblots confirmed the cytoplasmic localisation of TrxA and showed that EsxA was
134 secreted normally in these strains, although similar to EsxA-pep86, the pep86-TrxA fusion did
135 not appear to be detected by our TrxA antibodies (Fig. 1E). While cytoplasmic readings for
136 TrxA-pep86 in the Δ*essC* strain were comparable to those for EsxA-pep86 (compare Fig. 1B
137 and 1D 'cytoplasmic' blue bars), the luminescence signal corresponding to extracellular
138 protein in the Δ*essC* strain was much higher for EsxA-pep86 (compare Fig. 1B and 1D
139 'supernatant' blue bars). This might imply that overproduction of EsxA (but not TrxA) in cells
140 lacking EssC causes some cytoplasmic leakage – the definitive cause requires further
141 investigation, although it should be noted that EsxA has been implicated in membrane
142 destabilisation (26). In summary, Figure 1 demonstrates that luminescence readings correlate
143 with T7-dependent secretion of EsxA-pep86, and that extracellular EsxA-pep86 can be
144 measured in unprocessed whole cell cultures.
145
146
147 ***High throughput analysis of EsxA secretion***
148
149 Having established a simple, quantitative NanoLuc assay we next sought to convert it to high
150 throughput format to improve quantitation and sensitivity. Traditional blotting-based assays
151 are not amenable to this format due to both the large culture volumes and laborious sample
152 processing they require. To ensure our system was as close to native as possible, we
153 engineered the C-terminal pep86 fusion onto chromosomally-encoded EsxA at the *ess* locus,
154 avoiding the need for an inducible plasmid.
155
156 To assess whether the chromosomally encoded EsxA-pep86 fusion was functional, we
157 transformed this strain with a plasmid carrying an HA-tagged copy of the T7SS substrate EsaD

158 alongside its partner proteins EsxB, EsxC, EsxD, EsaE and EsaG (21). Immunoblot analysis of
159 cell and culture supernatant samples showed that when COL produced EsxA-pep86 as the
160 sole form of EsxA, secretion of EsaD-HA was similar to that seen for the wild type strain (Fig.
161 2A). This indicates that the pep86 tag does not detectably impede EsxA activity. However, the
162 EsxA-pep86 fusion was not detected by our EsxA antibodies, suggesting that the tag masks
163 the major antigenic epitope of EsxA.
164
165 We analysed growth kinetics of the COL$_{esxAp86}$ and COL$_{esxAp86}$ Δ*essC* strains in 50 µl culture
166 volumes in 384-well plate format. The strains showed slow growth kinetics in these
167 conditions, with cultures reaching stationary phase approximately 48 hours (2880 minutes)
168 following initial inoculation to an OD_{600} of 0.1 (Fig. 2B). Since the half-life of the NanoLuc
169 luminescence signal is reported to be approximately 120 minutes (Promega), continuous
170 monitoring of secreted EsxA-p86 throughout exponential growth was not possible. We
171 therefore initially chose to monitor secretion during early exponential phase, and to this end
172 furimazine and 11S were added to initiate luminescence measurement after 360 minutes of
173 growth (dotted line on Fig. 2B). Luminescence readings of the whole cell culture were
174 subsequently taken every ten minutes after supplementation. The luminescent signal for the
175 COL$_{esxAp86}$ culture initially rose, peaking 70 minutes after initiation of luminescence, indicating
176 that cells were actively secreting protein during this time (Fig. 2C). The subsequent decline of
177 the luminescence readings is likely due to signal decay, although potentially degradation of
178 pep86 could also contribute. As seen with the plasmid-based assay, there was some EssC-
179 independent leakage of EsxA-p86 in the Δ*essC* strain but the luminescent signal was much
180 lower than that of the *essC*$^{+}$ strain (Fig. 2C).
181
182 To analyse the distribution of secretion activity within a clonal population, a single colony
183 of each of COL$_{esxAp86}$ and COL$_{esxAp86}$ Δ*essC* was used to inoculate overnight cultures, and these
184 were subsequently used to inoculate 64 x 50 µl cultures in 384 well format. Furimazine and
185 11S were added to initiate luminescence measurement after 360 minutes of growth as
186 before, and luminescence was read at a single timepoint of 430 minutes (A_{600nm} = 0.23), which
187 is where we had observed the greatest difference in luminescence signal between the two
188 strains in Fig. 2C. These individual data points were used to generate a scatter plot (Fig. 2D).
189 This analysis reveals a relatively narrow distribution of secretion activity in replicate cultures

190 of $COL_{esxAp86}$, however the distribution of extracellular EsxA-p86 levels in the control $COL_{esxAp86}$
191 Δ*essC* strain was quite broad, overlapping the *essC*$^{+}$ strain and highlighting the requirement
192 for a high throughput assay. The same analysis was repeated using three independent
193 overnight cultures of each strain but this did not increase variability (Fig. 2E).
194
195
196 ***Variable secretion characteristics of different* S. aureus *strains***
197
198 Direct comparison of T7SS activity across different *S. aureus* strains has been hampered by
199 low secretion activity (often below the detection limit (7, 20)) and low reproducibility (our
200 unpublished observations). We used the NanoLuc assay to compare secretion across five
201 additional *S. aureus* isolates, two of which (like COL) carry the *essC-1* allele (RN6390 and
202 JP5347), and one representative isolate for each of *essC-2* (10.125.2X), *essC-3* (MRSA252) and
203 *essC-4* (EMRSA15). Each strain and its isogenic Δ*essC* variant was transformed with pRab11-
204 *esxAp86*, and these were cultured in 384-well plates in the presence of anhydrotetracycline
205 (ATC) to induce expression of plasmid-encoded EsxA-pep86.
206
207 Although growth curves revealed different growth kinetics for the six isolates, each Δ*essC*
208 variant grew similarly to the otherwise isogenic parental strain, allowing for meaningful
209 comparison of secreted EsxA (Fig S1A). We noted that each of these five additional strains
210 entered exponential phase earlier than COL, so we adjusted the timing of addition of 11S and
211 furimazine to these cultures to 230 minutes, measuring luminescence at ten minute intervals
212 afterwards (Fig. 3, Fig. S1B). Our analysis revealed that EsxA-p86 was secreted at variable
213 levels in the three *essC-1* isolates and the *essC-2* representative strain 10.1252.X. However,
214 T7SS-dependent secretion of EsxA was not detected in the *essC-3* and *essC-4* strains,
215 MRSA252 and EMRSA15 (Fig. 3A, B). We also noted that the level of EssC-independent leakage
216 of EsxA-p86 varied substantially between strains (Fig. 3A, Fig. S2). Since lysostaphin treatment
217 of cells for measurement of cytoplasmic EsxA-pep86 is not possible in the 384-well plate
218 format, we also conducted a NanoLuc assay for each strain in 5 ml cultures to ensure that
219 EsxA-pep86 was produced in all strains, observing that cytoplasmic EsxA-pep86 was present
220 in excess over the secreted protein in all cases (Fig. S2). The lack of secreted protein in
221 MRSA252 and EMRSA15 is therefore not due to a lack of EsxA in the cytosol.

222

223 Since no T7SS activity was detected in MRSA252 and EMRSA15 at 37°C we wondered whether
224 these strains might exhibit a different temperature dependence and so we repeated the
225 growth curves and NanoLuc secretion assay for these strains, alongside RN6390 as an *essC-1*
226 control, at 30°C and 34°C (Fig. 4, Fig S3). For EMRSA15, EsxA-p86 was secreted at both 30°C
227 and 34°C, while for MRSA252 the lower temperatures made negligible difference to secretion
228 activity. EsxA was secreted to a similar extent in RN6390 at all three temperatures.
229 Temperature therefore contributes to the regulation of T7 activity in *S. aureus* in a strain-
230 specific manner.

231

232

233 **Discussion**

234

235 The NanoLuc system is a powerful tool which can be adapted to a wide range of biological
236 applications and is gaining broad utility in the field of protein translocation (23-25, 27). Here
237 we develop a novel assay for monitoring protein secretion by the T7SS in *S. aureus*, using the
238 NanoBit split luciferase system. Luminescence from secreted proteins can be measured
239 directly in unprocessed cell cultures, enabling the high throughput screening of large numbers
240 of strains and growth conditions. EssC is the primary component of the T7 machinery, and our
241 initial experiments in the *essC*-1 strain COL clearly demonstrate that extracellular localisation
242 of EsxA is dependent on EssC. However, we do observe some extracellularly localised EsxA in
243 a Δ*essC* strain, and this proportion is higher than would be expected from a 'regular' cell lysis,
244 as predicted from similar analysis of the control cytoplasmic protein TrxA. Given the central
245 role of EssC in forming the secretion channel this is unlikely to result from EssC-independent
246 secretion of EsxA by the T7SS. Instead, we hypothesise that the elevated levels of EsxA-pep86
247 present in these experiments might destabilise cell membranes causing leakage of some
248 cytoplasmic components. In support of this hypothesis, EsxA has previously been reported to
249 have pore-forming activity in synthetic lipid bilayers (26), and this could be particularly
250 relevant when the EssC component, where EsxA might normally be expected to dock, is
251 absent.

252

253 Most studies of the *S. aureus* T7SS have focussed on secretion by *essC-1* type systems, using
254 a handful of isolates. Further isolates exhibit high genetic diversity at their T7 loci (13), but
255 experimental investigation of alternate systems has been constrained by their low activity in
256 laboratory growth conditions, and the lack of a sensitive secretion assay. We have used the
257 NanoLuc assay to analyse six different strains and find substantial variability in their secretion
258 efficiencies. A number of pathways have been implicated in transcriptional regulation of the
259 *S. aureus* T7SS, including SigB, the SaeRS and ArlRS two-component systems, and the Agr
260 quorum-sensing system (19, 28-30), however the complex regulatory picture is far from
261 complete and key questions around how, why and when secretion is activated remain
262 unanswered. Although no EsxA-pep86 secretion was detected in strain MRSA252, our analysis
263 was limited to a very early time window at the start of exponential phase; the NanoLuc assay
264 can be used for future analyses of secretion at different growth stages for this and other
265 strains. Interestingly, the EMRSA15 T7SS was inactive at 37°C, but was active at the lower
266 temperatures of 30°C and 34°C suggesting a role for temperature in T7b regulation. This new
267 assay will facilitate high throughput screening of a range of conditions, paving the way for a
268 much more detailed analysis of T7 secretion regulation.

269 **Methods**

270

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

272 *E. coli* was cultured in LB medium (Melford) and *S. aureus* was cultured in tryptic soy broth
273 (TSB; Oxoid) with chloramphenicol (10 µg/ml) where required. *E. coli* strains DH5α and JM110
274 were used for cloning and preparation of plasmids for electroporation respectively. *S. aureus*
275 strains and plasmids used are listed in Tables S1 and S2, respectively. Chromosomal deletion
276 of *essC* was accomplished by allelic exchange using plasmid pIMAY (31) carrying the flanking
277 regions of the relevant *essC* allele. Insertion of the pep86 tag on the chromosome of COL and
278 COLΔ*essC* used an updated system with plasmid pIMAY-Z (32). pIMAY plasmids were created
279 using standard restriction cloning techniques, and pIMAY-Z-esxAp86 and pRab11-esxAp86
280 were created using HiFi assembly (NEB). Oligonucleotides are listed in Table S3.

281

282

283 ***NanoLuc secretion assays***

284 Furimazine was purchased from Promega (Cat. # N1610) and used according to the
285 manufacturer's instructions. The NanoBit large subunit 11S was purified from BL21(DE3)
286 [pBAD-$_{6H}$11S] using the published protocol (24) and was used at a final concentration of 5 µM.
287 For assay of 5 ml cultures, *S. aureus* COL cells were cultured as described above. Once cultures
288 had reached a density of 0.5 (OD_{600}), anhydrotetracycline (ATC; 250ng/ml) was added to
289 induce expression of EsxA-pep86. Cells were harvested at a density of 2 (OD_{600}) by
290 centrifugation at 16,000 x g, 20°C. Cell pellets were resuspended in TBS + 50µg/ml lysostaphin
291 (Ambi) and incubated for 10 minutes at 37°C. 100µl of each sample was supplemented with
292 11S and furimazine in a 96-well plate (Greiner, REF655073) with an approximate final well
293 volume of 105µl. Luminescence was read 3 minutes after incubation of the plate at room
294 temperature, with a FLUOstar Omega using a gain value of 3,000. Samples from the same
295 cultures were prepared for immunoblot and analysed with antibodies as previously described
296 (21). For assays in 384-well plates, overnight *S. aureus* cultures were subcultured into fresh
297 TSB at a density of 0.01 (OD_{600}) and cultured at 37 ^{o}C/34 ^{o}C/30 ^{o}C with shaking at 200rpm in a
298 384-well plate (Greiner, REF781098), with a well volume of 50µl. ATC was added after 180
299 minutes (37°C cultures), 270 minutes (34°C cultures), or 310 minutes (30°C cultures), at 250

300 ng/ml for COL, 500 ng/ml for all other strains. 11S and furimazine were added at 220 minutes
301 (37°C cultures), 280 minutes (34°C cultures) or 310 minutes (30°C cultures). OD_{600nm} and
302 luminescence readings were taken every 10 minutes.
303
304
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309 NanoLuc assay, Ian Collinson for plasmid pBAD-$_{6H}$11S for purification of 11S, José Penadés for
310 *S. aureus* strain JP5347, Simon Heilbronner for USA300 LAC and Sharon Peacock for strain
311 EMRSA-15.

312 **Figure legends**

313

314 ***Figure 1. The NanoLuc T7-secretion assay***

315 A. Schematic representation of the NanoLuc assay. The T7 substrate EsxA with a C-terminal
316 pep86 tag is produced in the *S. aureus* cytoplasm, and 11S and furimazine (the NanoLuc large
317 subunit and substrate) are provided outside the cell. Secretion of EsxA-pep86 via the T7SS
318 results in functional complementation of 11S, which luminesces in the presence of furimazine
319 and O_2. Note that the cartoon is not an accurate representation of the stoichiometry and
320 architecture of the T7SSb, which are unknown. B., D. *S. aureus* strain COL or an isogenic Δ*essC*
321 mutant carrying the indicated plasmids were grown aerobically in 5 ml culture volumes. At
322 OD_{600nm} = 0.5, anhydrotetracycline (ATC) was added to induce expression from pRab11. When
323 cells reached OD_{600nm} = 2, samples corresponding to the culture medium ('supernatant') and
324 lysed cells ('cytoplasm') were prepared. Serial two-fold dilutions of unprocessed whole cell
325 cultures, supernatant and cytoplasmic fractions were subsequently supplemented with 11S
326 and furimazine. The data presented correspond to the mean luminescence readings of five
327 biological replicates and error bars represent the SEM. Two-way ANOVA was used to
328 determine statistical significance, **** $p < 0.0001$, ns - not significant. C., E. Lysed cell (wc)
329 and supernatant (sn) fractions from (B) and (D) were analysed by immunoblot with antibodies
330 against EsxA (top panles) or TrxA (bottom panels). Supernatant samples were concentrated
331 by TCA precipitation prior to analysis, and the amount analysed corresponds to 6 x more
332 culture volume than for lysed cell samples.

333

334 ***Figure 2. High throughput secretion analysis of chromosomally-encoded EsxA-pep86.***

335 A. *S. aureus* COL, COL encoding chromosomal pep86-tagged EsxA ($COL_{esxA\text{-}pep86}$) or COL Δ*essC*
336 encoding chromosomally pep86-tagged EsxA ($COL_{esxA\text{-}pep86}$ Δ*essC*) harbouring plasmid
337 pRab11-CBED-DEG (annotated as pEsaD-HA) were grown aerobically in 5 ml culture volumes
338 and ATC was added at OD_{600nm} = 0.5 to induce expression from pRab11. At OD_{600nm} = 2, lysed
339 whole cell (wc) and supernatant (sn) fractions were prepared and analysed by immunoblot
340 with antibodies against the HA tag (to detect EsaD-HA), EsxA or TrxA as indicated. Supernatant
341 samples were concentrated by TCA precipitation prior to analysis, and the amount analysed
342 corresponds to 6 x more culture volume than for lysed cell samples. B. Growth curves for
343 $COL_{esxA\text{-}pep86}$ and $COL_{esxA\text{-}pep86}$ Δ*essC*. 192 replicate 50 µl cultures (64 x 3 biological replicates)

344 for each strain were grown in 384-well plates at 37°C. Furimazine and 11S were added at 6h
345 (dotted line). Mean OD_{600nm} values were plotted and error bars correspond to the SEM (n=3).
346 C. Luminescence readings were recorded at ten minute intervals for each of the cultures in
347 (B) following addition of furimazine and 11S. D. A single overnight culture of each of COL_{esxA-}
348 $_{pep86}$ and $COL_{esxA-pep86}$ Δ*essC* was diluted to an OD_{600} of 0.1 and used to inoculate 64 individual
349 wells of a 384 well plate (Fig. 2B). The plate was cultures for 360 minutes after which
350 furimazine and 11S were added and a single luminescence reading from each well taken at
351 430 minutes. E. As part D except that three overnight cultures of each of $COL_{esxA-pep86}$ and
352 $COL_{esxA-pep86}$ Δ*essC* were used, giving 192 individual data points for each strain. Statistical
353 significance in D and E were assessed with the Mann-Whitney test, **** $p < 0.0001$.
354
355
356 ***Figure 3. Comparison of EsxA secretion in different* S. aureus *strains.***
357 A. The six pairs of wild-type / Δ*essC* strains, as indicated, were transformed with pRab11-
358 EsxAp86 and cultured in 384-well plates at 37°C. Expression of *esxA-p86* was induced by
359 addition of ATC at 180 minutes, and luminescence was initiated by addition of furimazine and
360 11S at 220 minutes. Graphs show mean luminescence readings for each pair of strains taken
361 every ten minutes following initiation of luminescence. Data represent the mean of three
362 biological replicates with 64 cultures per replicate. Error bars correspond to the SEM (n=3). B.
363 Raw luminescence values from (A) were normalised for OD_{600nm}. Normalised values for each
364 Δ*essC* were subtracted from normalised values for the respective wild type to give a relative
365 secretion value for each time point. For each strain pair the time point showing the largest
366 luminescence difference between the wild type and Δ*essC* is presented.
367
368 ***Figure 4. Temperature dependence of EsxA secretion***
369 A. Three pairs of wild-type / Δ*essC* strains, as indicated were transformed with pRab11-
370 EsxAp86 and cultured in 384-well plates at 30°C and 34°C. Expression of *esxA-pep86* was
371 induced by addition of ATC at the beginning of exponential phase (see Fig. S3), and
372 luminescence was initiated by addition of furimazine and 11S. Graphs show mean
373 luminescence readings for each pair of strains taken every ten minutes following initiation of
374 luminescence. B. Raw luminescence values from (A) were normalised for OD_{600nm}. Normalised
375 Δ*essC* values were subtracted from normalised WT values to give a relative secretion value

376 for each time point. For each strain pair the time point showing the largest luminescence
377 difference between the wild type and Δ*essC* is presented. Values for secretion at 37°C are
378 reproduced from Fig. 3B for comparison. In all cases, data represent the mean of three
379 biological replicates with 64 cultures per replicate. Error bars correspond to the SEM.

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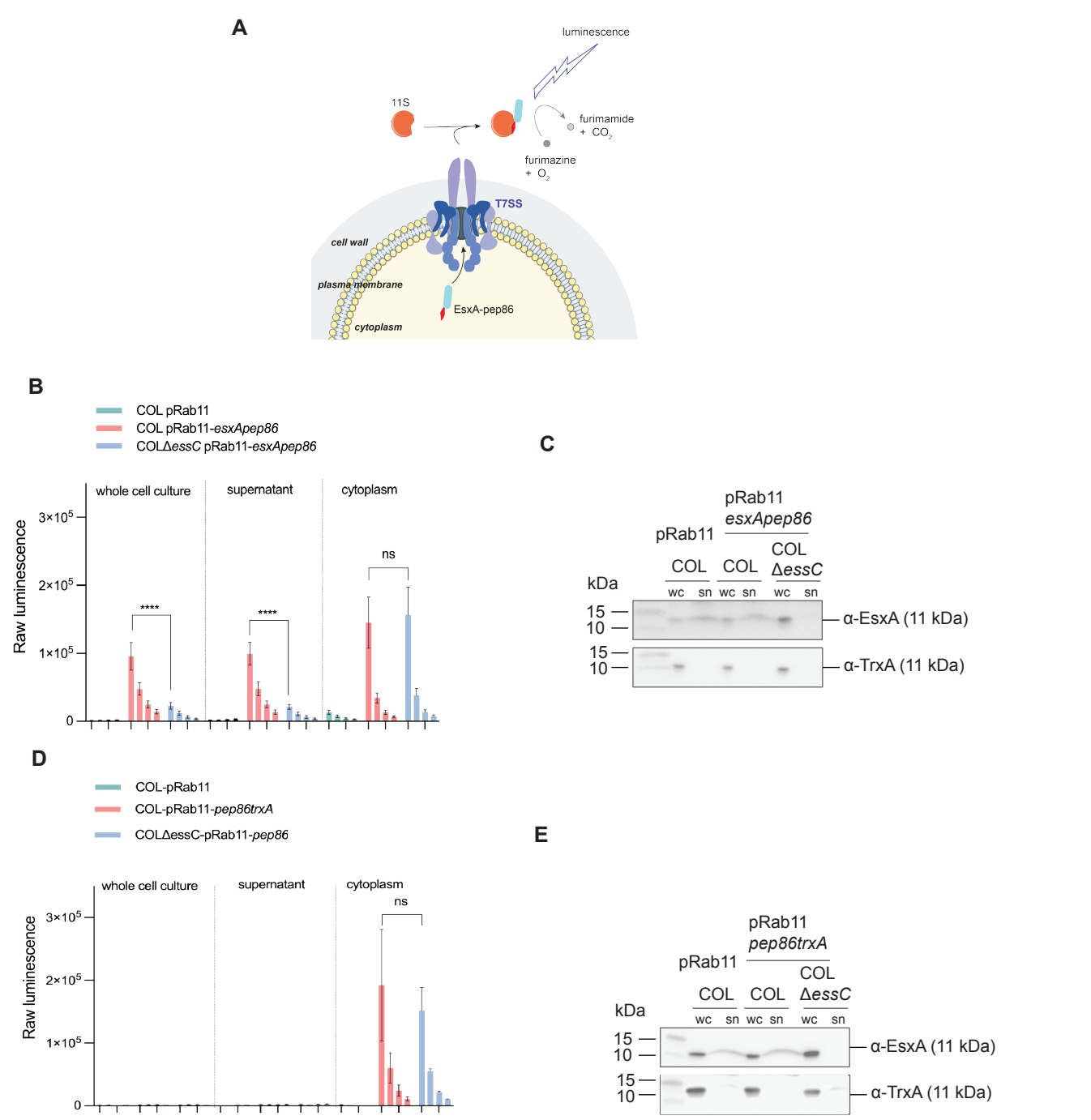
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luminescence
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+ CO_2
furimazine
+ O_2
T7SS
cell wall
plasma membrane
cytoplasm
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whole cell culture
supernatant
cytoplasm
Raw luminescence
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2×10^5
1×10^5
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COL
COL
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10
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15
10
α-TrxA (11 kDa)
D
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whole cell culture
supernatant
cytoplasm
Raw luminescence
3×10^5
2×10^5
1×10^5
0
ns
E
pRab11
pRab11
pep86trxA
COL
COL
COL
Δ*essC*
kDa
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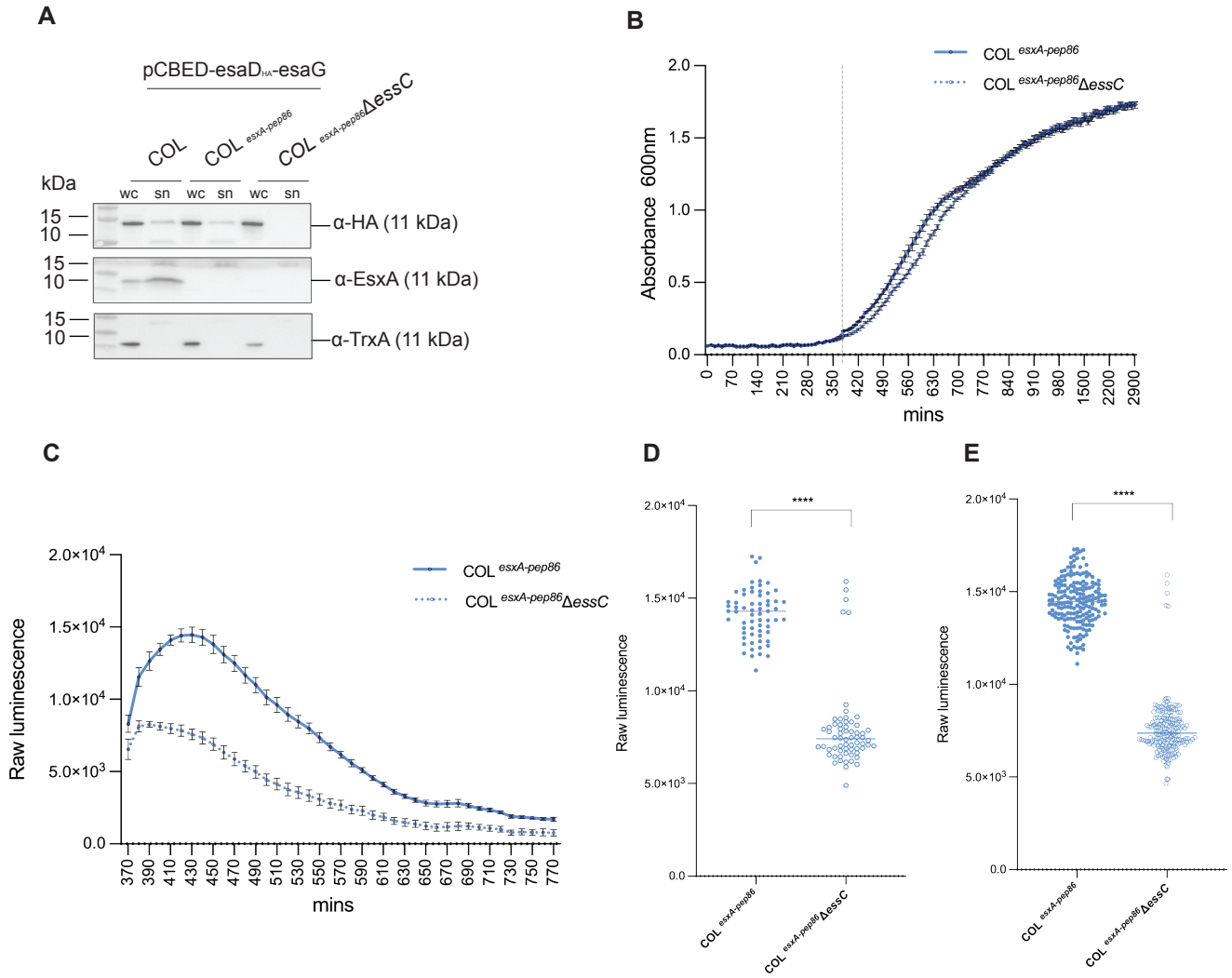
15
10
α-EsxA (11 kDa)
15
10
α-TrxA (11 kDa)

Figure 1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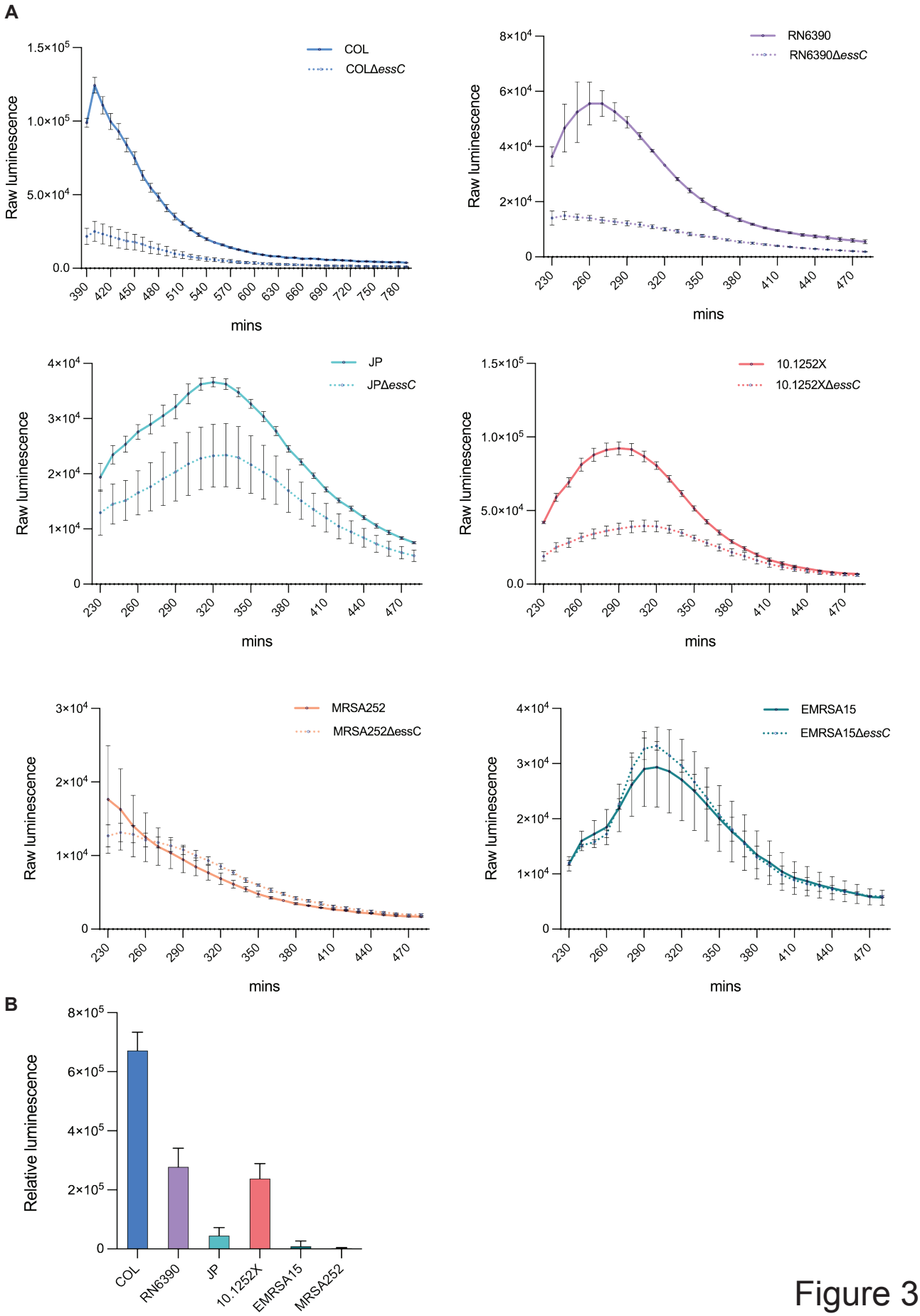
COL esxA-pep86ΔessC
kDa
wc sn wc sn wc sn
15
10
α-HA (11 kDa)
α-EsxA (11 kDa)
α-TrxA (11 kDa)
B
COL esxA-pep86
COL esxA-pep86ΔessC
Absorbance 600nm
mins
C
Raw luminescence
mins
D
E
Raw luminescence
****
COL esxA-pep86
COL esxA-pep86ΔessC

Figure 2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COLΔessC
RN6390
RN6390ΔessC
JP
JPΔessC
10.1252X
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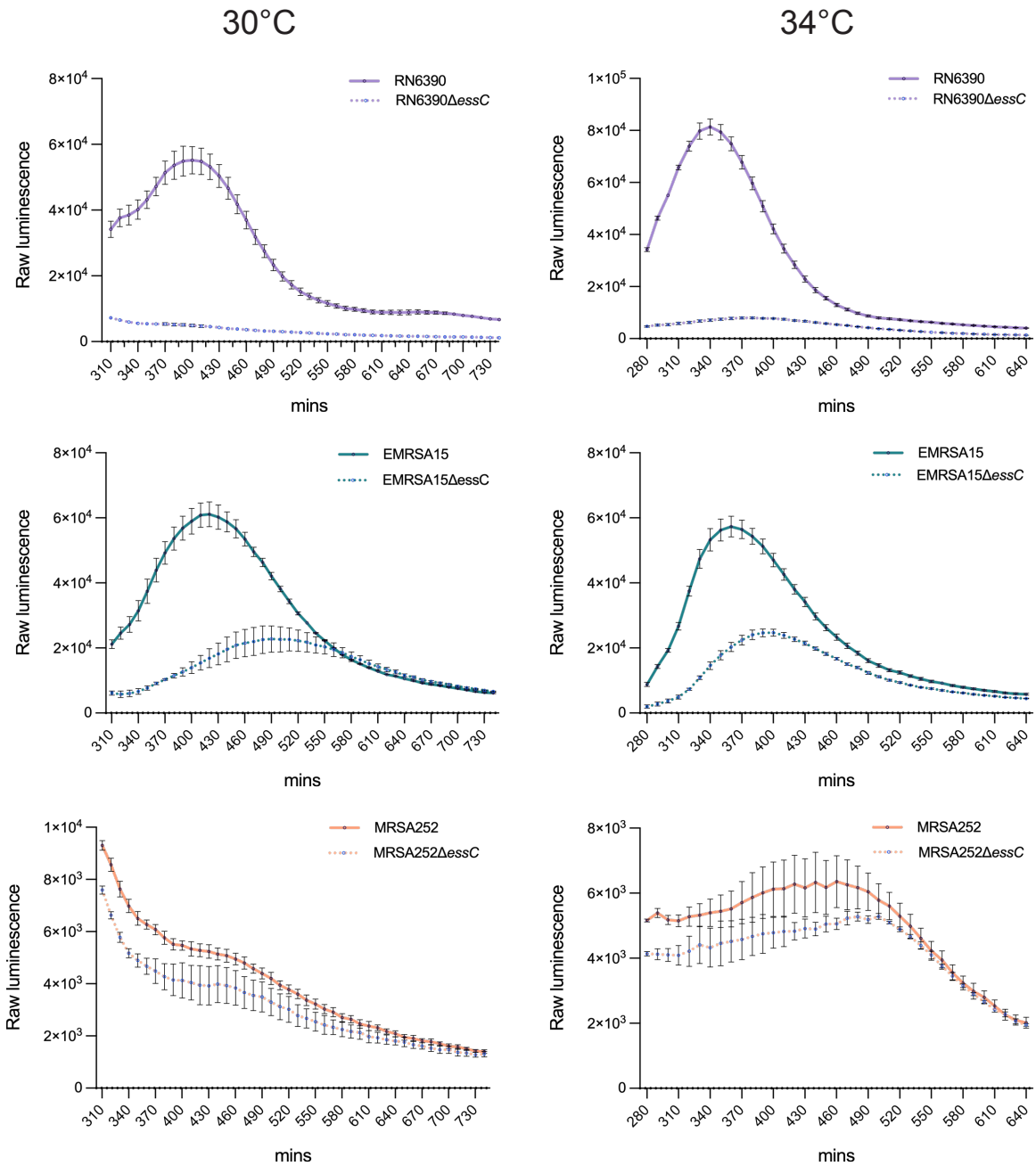
MRSA252
MRSA252ΔessC
EMRSA15
EMRSA15ΔessC
Raw luminescence
mins
B
Relative luminescence
COL
RN6390
JP
10.1252X
EMRSA15
MRSA252

Figure 3

A


30°C
34°C
RN6390
RN6390ΔessC
EMRSA15
EMRSA15ΔessC
MRSA252
MRSA252ΔessC
Raw luminescence
mins

B

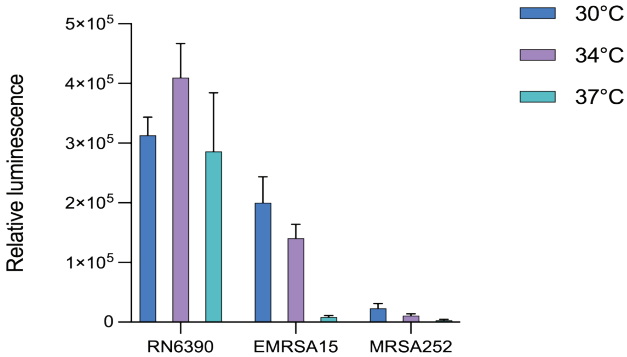

30°C
34°C
37°C
Relative luminescence
RN6390
EMRSA15
MRSA252

Figure 4