EssC is a specificity determinant for *Staphylococcus aureus* type VII secretion

Franziska Jäger, Holger Kneuper and Tracy Palmer*

**Abstract**

The type VII protein secretion system (T7SS) is found in actinobacteria and firmicutes, and plays important roles in virulence and interbacterial competition. A membrane-bound ATPase protein, EssC in *Staphylococcus aureus*, lies at the heart of the secretion machinery. The EssC protein from *S. aureus* strains can be grouped into four variants (EssC1–EssC4) that display sequence variability in the C-terminal region. Here we show that the EssC2, EssC3 and EssC4 variants can be produced in a strain deleted for essC1, and that they are able to mediate secretion of EssA, an essential component of the secretion apparatus. They are, however, unable to support secretion of the substrate protein EssX, which is only encoded in essC1-specific strains. This finding indicates that EssC is a specificity determinant for T7 protein secretion. Our results support a model in which the C-terminal domain of EssC interacts with substrate proteins, whereas EssA interacts elsewhere.

The type VII secretion system (T7SS) is found primarily in bacteria of the actinobacteria and firmicutes phyla and secretes proteins that lack cleavable N-terminal signal peptides. The system is best characterized in mycobacteria, where it is designated ESX, and pathogenic members of the genus des. The system is best characterized in mycobacteria, where it is designated ESX, and pathogenic members of the genus...
strains. Genes coding for the core components EssA-EssB are highly conserved (Fig. 1b), as is the 5‘ end of essC, but the 3‘ portion of the gene falls into one of four sequence groupings [27]. The essC sequence type strictly co-varies with the sequence of adjacent 3‘ genes, some of which are known or strongly predicted to encode secreted substrates. This would be consistent with the C-terminal variable region of EssC playing a role in substrate recognition. In this study we have addressed this hypothesis directly by assessing whether EssC proteins from the EssC2, EssC3 and EssC4 classes can support the secretion of the EssC1 substrate, EsxC [28], and of the core component, EssA.

*S. aureus* EssC proteins are approximately 1480 amino acids in length and have a common domain organization, with two forkhead-associated (FHA) domains at their N-termini, followed by two transmembrane domains, and three repeats of a P-loop ATPase domain at their C-termini ([29, 30]; Fig. 1a). Sequence analysis indicates that *S. aureus* EssC proteins are almost sequence invariant until part way through the second ATPase domain, where the EssC1 variant, found in strains such as RN6390, Newman and USA300, starts to diverge (Fig. 1c, d). The EssC2, EssC3 and EssC4 variants are more similar to one another, and share almost identical sequences until ATPase domain 3, where they also start to vary (Fig. 1c, d). Of the four ATPases, variants 2 (from strain ST398) and 3 (from strain MRSA252) are the most similar (Fig. 1d).

We have previously constructed an in-frame deletion of essC in strain RN6390 and shown that this results in the inability to export both the core machinery component,
EsxA, and the substrates EssC and EsaD [14, 20]. This secretion deficiency could be rectified by the reintroduction of EssC1 encoded on plasmid pRMC2 [31]. Fig. 2(a) shows that production of EssC1 could be also restored when it was encoded on the expression vector pRAB11 [32], and that the reintroduction of plasmid-encoded EssC1 resulted in strong secretion of both EsxA and EssC in the RN6390 ΔessC strain.

Next, we amplified the genes for essC2 (from strain ST398), essC3 (from strain MRSA252) and essC4 (from strain EMRSA15), and also cloned these into pRAB11 (see Table S1 for the oligonucleotides used for these experiments, available in the online version of this article). We first confirmed that the three variant EssC proteins could be stably produced in the RN6390 ΔessC strain background. To this end, anhydrotetracycline (ATC) was added to induce plasmid-encoded production of EssC and whole-cell samples were analysed by blotting with an EssC antiserum. It should be noted that the antiserum used was raised against a truncated protein covering the last two ATPase domains of the EssC1 variant [20]. As shown in Fig. 2(a), each of the EssC2, EssC3 and EssC4 variants could be recognized by this antibody, but not so strongly as the cognate EssC1, probably due to a lack of conservation of epitopes in this region of the protein. We conclude that all EssC variants can be produced in strain RN6390.

Next, we asked whether the variant EssC proteins in RN6390 could support T7 protein secretion. Fig. 2(b) (top panel) shows that secretion of the EsxA core component was indeed supported by each of these EssC proteins, indicating that each EssC variant was functional in the heterologous strain background. However, none of the EssC variants were able to support secretion of the substrate protein, EsxC. Taken together, these results confirm that EssC is a specificity determinant for substrate secretion by the S. aureus T7SS. The findings strongly suggest that the sequence-invariant regions of EssC proteins are involved in mediating interactions with the conserved T7 core components, including the secreted protein EsxA (which has >99% sequence identity across all sequenced S. aureus strains), and that the sequence-variable region is involved in substrate recognition. This might imply that EsxA and EssC are secreted by different mechanisms.

Finally, it is interesting to note that the secretion of all known substrates mediated by the EssC1 variant is dependent on a chaperone protein, EsaE/EssE [14, 26]. Some substrates of the actinobacterial T7SS also interact with specific chaperones of the EspG family to ensure delivery to the cognate secretion machinery [13, 33], although other substrates appear to be exported independently of a specific chaperone [2]. No protein with any detectable sequence homology to either EsaE or EspG is encoded at the ess loci of the essC2, essC3 or essC4 strain variants. In future it will be interesting to identify the specific chaperone(s) responsible for the secretion of these substrates.
to determine whether the mechanism of substrate targeting differs across the Ess subtypes in S. aureus.

Funding information
This study was supported by the Wellcome Trust (through Investigator Award 10183/2/15/2 to T.P.), the Biotechnology and Biological Sciences Research Council (through EASTBIO Doctoral Training Partnership award number BB/J01446X/1), which provided a PhD studentship to F.J., and through grant BB/H007571/1) and by the Medical Research Council (through grant MR/M011224/1).

Acknowledgements
Dr Jon Cherry is thanked for his help with generating the structural model in Fig. 1(c).

Conflicts of interest
The authors declare that there are no conflicts of interest.

Fig. 2. Non-cognate EssC variants support secretion of EssA but not EssC. (a, b) Strain RN6390 or the isogenic essC deletion strain carrying pRAB11 (empty) or pRAB11 encoding the indicated essC variant was subcultured into TSB medium supplemented with 1 µM haemin [34] and either 25 ng ml⁻¹ (RN6390 ΔessC/pEssC_GAL3/110) or 100 ng ml⁻¹ (RN6390 ΔessC/pEssC_ESP3/12/5/pEssC_ST38/p/essC_MRSA12) anhydrotetracycline (ATC) to induce plasmid-encoded gene expression. The strains were grown aerobically until an OD₆₀₀ of 2 was reached, after which (a) 10 µl of OD₆₀₀ 1 adjusted cells was separated on an 8 % bis-Tris acrylamide gel and analysed by Western blotting using anti-EssC antiserum [20], or (b) the cultures were separated into supernatant and whole-cell fractions and the equivalent of 200 µl of culture supernatant (sn) and 10 µl of resuspended cell sample adjusted to an OD₆₀₀=1 were separated on a 15 % bis-Tris gel and immunoblotted using the antisera raised against EssA [20], EssC [20] or the cytosolic control, TrxA [35].

References


Edited by: P. Serror and J. Stülke