EssC is a specificity determinant for Staphylococcus aureus type VII secretion

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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 EsxA, an essential component of the secretion apparatus. They are, however, unable to support secretion of the substrate protein EsxC, 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 EsxA 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 Mycobacterium contain at least one protein of the WXG100 superfamily. Proteins of this family are small helical hairpins that have a conserved WXG amino acid motif in a short loop between the two helices [3, 4]. A non-cleaved sequence located close to the C-termini of some WXG100 proteins acts as a signal for T7 secretion [5–8]. Some studies have suggested that the WXG motif may act alongside the C-terminal region as a bipartite targeting signal [9, 10]. WXG100 proteins are secreted as folded dimers; in actinobacteria these are heterodimers of paired WXG100 proteins, whereas in firmicutes they may also be homodimers [9]. The T7SS also secretes much larger substrates that share a similar four-helical bundle arrangement of the WXG100 protein dimers [10–12]. Some T7 substrates interact with chaperones prior to secretion and there is evidence that secretion of LXG domain substrates in firmicutes is dependent on complex formation with a WXG100 protein partner [13–15].

There are commonalities and differences between the T7SS of actinobacteria and firmicutes [16]. A membrane-embedded ATPase of the FtsK/SpoIIIE family termed EccC/EssC is found in all T7SSs. In both systems the protein shares a similar overall topology, with two transmembrane domains that are usually followed by three P-loop ATPase domains at the C-terminus. Although all three P-loop ATPase domains are capable of binding ATP, mutagenesis studies have indicated that only ATP hydrolysis by domain 1 is essential for T7 secretion [8, 17]. In actinobacteria, a hexameric arrangement of the EccC ATPase lies at the centre of a 1.8MDa complex that also contains six copies of the EccB, EccD and EccE proteins [18]. In firmicutes, homologues of EccB, D and E are absent and a distinct set of membrane proteins, EsaA, EssA and EssB, work alongside the ATPase, EssC, to mediate T7 secretion [19–23]. In Staphylococcus aureus and Bacillus subtilis a secreted WXG protein, EsxA, and a small cytoplasmic protein, EsaB, are also required for T7SS activity [19, 20, 22–24] (Fig. 1a).

The EccC/EssC ATPase has previously been implicated in substrate recognition. It was shown that the C-terminal domain of EccC1 interacted with the EsaB substrate [5, 25], while the EccC ATPase domains have been co-crystallized with a peptide from the EsaD C-terminus [8]. Cross-linking and co-purification experiments have identified complexes of S. aureus EssC with substrates EsaD (also called EssD) and EsxC [14, 26]. Further evidence in support of a role for EssC in substrate recognition comes from genomic analysis of S. aureus [27]. It was noted that there was sequence variability at the ess locus across different S. aureus.
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 determine whether the mechanism of substrate targeting differs across the Ess subtypes in S. aureus.

**References**


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