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The Ess/Type VII secretion system of *Staphylococcus aureus* secretes a nuclease toxin that targets competitor bacteria

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Summary

The type VII protein secretion system (T7SS) plays a critical role in the virulence of human pathogens including *Mycobacterium tuberculosis* and *Staphylococcus aureus*. Here we report that the *S. aureus* T7SS secretes a large nuclease toxin, EsaD. The toxic activity of EsaD is neutralised during its biosynthesis through complex formation with an antitoxin, EsaG, which binds to its C-terminal nuclease domain. The secretion of EsaD is dependent upon a further accessory protein, EsaE, that does not interact with the nuclease domain, but instead binds to the EsaD N-terminal region. EsaE has a dual cytoplasmic/membrane localization and membrane-bound EsaE interacts with the T7SS secretion ATPase, EssC, implicating EsaE in targeting the EsaDG complex to the secretion apparatus. EsaD and EsaE are co-secreted whereas EsaG is found only in the cytoplasm and may be stripped off during the secretion process. Strain variants of *S. aureus* that lack *esaD* encode at least two copies of EsaG-like proteins most likely to protect themselves from the toxic activity of EsaD secreted by *esaD*$^+$ strains. In support of this, a strain overproducing EsaD elicits significant growth inhibition against a sensitive strain. We conclude that T7SSs may play unexpected and key roles in bacterial competitiveness.

Protein secretion systems are used by bacteria to interact with and manipulate their environments, and play critical roles in the secretion of virulence factors. Gram-negative bacteria produce numerous secretion systems that transport substrates across the cell envelope1. Many Gram-positive bacteria also produce a specialised protein secretion machinery termed the Type VII secretion system (T7SS). T7SSs are found in representatives of the Actinobacteria and Firmicutes phyla. The system was first described in the Actinobacterial pathogens *Mycobacterium tuberculosis* and *M. bovis* where T7SS ESX-1 was shown to be essential for virulence and to secrete two small proteins ESAT-6 and CFP-10, subsequently renamed EsxA and EsxB2–4. In addition to secreting EsxA/B proteins, Mycobacterial T7SSs can also secrete much larger proteins of the PE/PPE family5, which are highly abundant in the genomes of some species6.

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A distantly related T7SS, termed T7b7, is also found in Firmicutes such as *Bacillus subtilis*8,9 and *Staphylococcus aureus*10. T7SSs share two common types of components: a membrane-bound hexameric ATPase of the FtsK/SpoIIIE protein family11,12 and at least one EsxA/EsxB-related protein11. EsxA and EsxB are members of the WXG100 superfamily that form dimeric helical hairpins13,14, and in Firmicutes EsxA is exported as a folded homo-dimer14,15. The *S. aureus* Ess system comprises six core components10,16 (Figs 1A; S1). In addition to EssC, three further membrane proteins EsaA, EssA, EssB are essential for T7 secretion activity16,17 along with the secreted protein EsxA10 and the predicted cytoplasmic protein EsaB16. All except one strain of *S. aureus* examined to date encode the six core T7 components but there is strain variability in the repertoire of T7 substrate proteins18. Studies using strains with the NCTC8325 T7S gene cluster organisation, including Newman, USA300 and RN6390 have identified EsxB, EsxC and EsxD as secreted substrates10,16,19,20. These three proteins are small (~100-130 aa), and their precise functions remain to be elucidated.

The T7SS has previously been shown to contribute to virulence in mouse infection models10,16,21 and to facilitate release of intracellular *S. aureus* from epithelial cells22. Here we identify a further function for the *S. aureus* T7SS in bacterial competition. We show that EsaD is a T7 nuclease substrate that interacts with two Ess accessory proteins, EsaG, an antitoxin, and EsaE, a putative chaperone, during its biosynthesis. Strains of *S. aureus* that do not encode the EsaD substrate harbour *esaG* homologues, most likely to protect themselves from killing by EsaD-producing strains. In support of this we demonstrate EsaD-dependent growth inhibition of *S. aureus*. Our findings confirm that the Gram-positive T7SS has anti-bacterial activity in addition to anti-eukaryotic function.

Results

EsaD is not required for T7SS activity

EsaD is encoded within the T7SS gene cluster (Fig 1a) and has been reported to be a membrane-bound T7SS accessory factor23, however its absence from some *S. aureus* strains indicates it is unlikely to be a critical component of the secretion machinery18. We constructed an in-frame *esaD* deletion and asked whether it was required for secretion of the core component EsxA and the substrate protein EsxC. Fig 1b shows that EsxA was still secreted by the *esaD* strain, but very little EsxC was detected. To circumvent this we overproduced EsxC from a plasmid (Fig 1c), and in this case could clearly detect EsxC in culture supernatants of the *esaD* strain. We conclude that EsaD is not essential for T7-dependent secretion.

EsaD is a predicted nuclease that is secreted by the T7SS

EsaD encoded by strain NCTC8325 is predicted to be a 614 residue protein, and sequence analysis suggests that the C-terminal ~170 aa comprise a nuclease domain (Fig S2). In accord with EsaD having toxic activity, we were unable to clone *esaD* unless we introduced a H528A codon substitution at the predicted nuclease active site. When HA-tagged EsaD(H528A) was produced in either *S. aureus* RN6390 or COL strains, tagged protein was

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detected in the supernatant (Fig 1d, e). The lack of EsaD in supernatants of the cognate *essC* strains strongly suggests that EsaD is a T7SS secreted substrate.

EsaD has previously been reported as a membrane protein23. However, topology prediction programmes (e.g. TMHMM) do not predict transmembrane regions. To explore the location of cellular EsaD we fractionated cells producing EsaD(H528A)-HA and Fig 1f shows the tagged protein (migrating as a double band) was clearly detected only in the cytoplasmic fraction. We conclude EsaD is not a membrane protein.

EsaD has toxic activity that is neutralised by EsaG

To confirm that EsaD is a toxin, it was essential to clone the wild-type gene. We could readily clone *esaD* if the downstream gene, *esaG*, was included, but not if this sequence was omitted. We were eventually able to obtain a clone of *esaD* in pT7.524. Expression of genes from this vector is under control of the T7 promoter which is not recognised by *E. coli* RNA polymerase. Even so, the clone we obtained, which gave very small colonies, harboured a V584Y substitution. We reasoned that this might serve to lower the stability/toxicity of EsaD. When this construct was introduced into *E. coli* BL21(DE3) (that encodes an inducible copy of the phage T7 RNA polymerase), growth ceased when the inducer, IPTG was added (Fig 2a) but cells continued to grow if *esaG* was co-expressed with wild type *esaD*. We conclude that EsaD has toxic activity that is modulated by EsaG.

Examination of *E. coli* cells by microscopy showed that production of EsaD(V584Y) resulted in cell elongation (Fig S3a,b), a hallmark of the SOS response induced by DNA damage25,26, consistent with EsaD exhibiting DNase activity. We confirmed EsaD-induced DNA damage using TUNEL, which labels the ends of fragmented DNA27,28. Fluorescence microscopy showed TUNEL staining in a subset of cells producing EsaD(V584Y) (Fig S3c) that could also be detected by flow cytometry (red dots in FACS plot in Fig S3d). Production of EsaD was also associated with an increase in side scatter, consistent with changes in cellular morphology seen by microscopy. We conclude that EsaD results in DNA damage when produced in the cytoplasm of *E. coli*.

EsaG interacts with the nuclease domain of EsaD

The presence of *esaG* counteracts the toxic activity of EsaD suggesting that *esaG* is an antitoxic gene. Antitoxins may be proteins or RNA29. Inspection of *esaG* indicates that it is a probable protein-coding gene, producing a protein of the uncharacterised DUF600 family. To investigate whether EsaG interacts with EsaD we co-produced EsaD(H528A)-His with EsaG-HA in *S. aureus* and purified tagged EsaD from cell lysates. Fig 2c shows that EsaG co-purifies with EsaD indicating the proteins form a complex.

EsaD-EsaG interaction was also confirmed by bacterial two hybrid assay (Fig 2b). EsaG (163 aa) is significantly smaller than EsaD, suggesting it may interact with only part of EsaD. We genetically separated EsaD into predicted nuclease domain ($EsaD_{421-614}$, harbouring the H528A codon substitution) and N-terminal region ($EsaD_{1-420}$). Fig 2b shows that EsaG interacts specifically with the predicted nuclease domain. This was confirmed biochemically by co-purification of EsaG-HA with His-tagged $EsaD_{421-614}$ (Fig 2d). We

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conclude that EsaG is a proteinaceous antitoxin that blocks EsaD activity by direct interaction.

To investigate directly whether EsaD has DNase activity, we overproduced His-tagged wild type or H528A variants of the nuclease domain in *E. coli* in the presence of EsaG and purified His-tagged EsaD in the presence of 8M urea to unfold the protein and detach bound EsaG (Fig 2e). After refolding and eluting from the Ni-resin, the EsaD nuclease domains were incubated with plasmid DNA in the presence of Mg^{2+} or Zn^{2+} ions. Fig 2f shows that wild type EsaD specifically degraded plasmid DNA in the presence of Mg^{2+},and could also degrade genomic and linear DNA (Fig S5). The H528A variant showed some DNAse activity, but appeared to be much less potent than EsaD, as expected. We conclude that EsaD is a Mg^{2+}-dependent DNase.

Fractionation of cells overproducing tagged EsaG shows that it is found exclusively in the cytoplasm (Fig S6). However, as the T7SS can export protein complexes15, we tested whether it could be co-secreted if overproduced with EsaD. Fig S7 shows that in the presence of secreted EsaD(H528A), EsaG localised only to the cell fraction and does not appear to be co-secreted with its partner protein. We then asked whether EsaG was required for secretion of EsaD. We were not able to delete *esaG* from *S. aureus* unless we also deleted *esaD*, consistent with its antitoxic role. Production of HA-tagged EsaD(H528A) in the *esaDG* mutant strain resulted in no detectable EsaD in the supernatant and very little in the cells (Fig S8). We conclude that EsaG is required for the stability/secretion of EsaD. EsaG was not, however, required for the secretion of either EsxA or EsxC (Fig S9) and is therefore not a general T7S accessory factor.

EsaE also interacts with EsaD and is required for its secretion or stability

Studies of Mycobacterial T7SS have revealed that PE/PPE substrate proteins interact with specific chaperones that facilitate their secretion30–32. To investigate whether any additional soluble proteins encoded at the *S. aureus* T7SS locus interact with EsaD, we used bacterial two hybrid analysis. Fig 3a shows no evidence for EsaD interaction with any known secretion substrates (EsxB, EsxC or EsxD) or soluble machinery components (EsaB or EsxA). Interaction was detected between EsaD and EsaE, which was also confirmed biochemically as EsaE-HA co-purified with EsaD(H528A)-His when the two proteins were co-produced in *S. aureus* (Fig 3b). To determine whether EsaE interacted with the nuclease domain of EsaD or elsewhere on the protein, we screened EsaE interaction with $EsaD_{1\text{-}420}$ or $EsaD_{421\text{-}614}$(H528A) (Fig 3c). The results show that EsaE specifically interacts with the non-nuclease region of EsaD. We tried to confirm these genetic observations with co-purification experiments but were not able to stably produce the truncated $EsaD_{1\text{-}420}$.

We constructed an in-frame deletion of *esaE* to determine whether it was required for EsaD secretion. We were readily able to obtain the *esaE* mutant, consistent with EsaE not being an antitoxin but playing some other role in EsaD biosynthesis. Production of HA-tagged EsaD(H528A) in this strain resulted in no detectable EsaD in the supernatant and very little in the cells (Fig S8), suggesting EsaE is required for its stability or secretion. Although EsaE was not required for EsxA secretion, there was no apparent secretion of EsxC in the *esaE*

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mutant, even if EsxC was overproduced from a plasmid (Fig S9). Thus EsaE is required for efficient secretion of EsaD and at least one further T7SS substrate.

His-tagged EsaE was found almost exclusively in the cellular fraction, suggesting it is not, by itself, a T7SS substrate (Fig 3d). We did, however, routinely observe that overproduction of tagged EsaE led to a dramatic increase in the level of extracellular EsxA (Fig 3d), for reasons that are unclear. However, when EsaE-His was co-produced with EsaD(H528A)-HA, notable secretion of His-tagged EsaE could now be detected suggesting that these two proteins are co-exported as a complex.

EsaD/E/G form a ternary complex

We next assessed whether EsaD could form a ternary complex with EsaE and EsaG. Control experiments (Fig S10) showed no direct interaction between EsaE and EsaG by either two hybrid or co-purification experiments. When EsaD(H528A)-Myc, EsaE-HA and EsaG-His were co-produced in *E. coli* and EsaE-HA immunoprecipitated, EsaG-His and Myc-tagged EsaD were also co-precipitated, consistent with the three proteins forming a ternary complex (Fig 3f,g). Reciprocal experiments where His-tagged EsaG was isolated by Ni-affinity purification resulted in co-purification of EsaE-HA and EsaD-Myc (Fig S12). In this latter experiment, although some full length EsaD-Myc was detected, most of the protein was fragmented to the approximate size of the nuclease domain, consistent with the prior instability of $EsaD_{1\text{-}420}$ noted previously.

EsaE interacts with the multimeric form of EssC

Although EsaE is predicted to be soluble, subcellular fractionation showed a proportion of His-tagged EsaE localised to the membrane and was stable to carbonate washing (Fig 4a). As EsaE has a dual cytoplasmic-membrane location, we wondered whether it may play a role in targeting EsaD/EsaG to the membrane-bound secretion machinery. We undertook formaldehyde crosslinking experiments in whole cells of *S. aureus* producing EsaE-His, isolated membranes and blotted for EsaE. Fig 4b shows several EsaE-His crosslinks, including a particularly strong crosslink migrating above 250 kD. It has previously been shown that the *S. aureus* T7 ATPase, EssC, forms a high molecular weight multimer17,33. To ascertain whether this high molecular weight crosslink also contained EssC, we repeated the crosslinking experiments in the wild type and *essC* mutant strains. Fig 4c shows that the EsaE-His-containing crosslink migrated with an apparently identical mass as the EssC-containing crosslink and moreover, no such crosslink was detected when *essC* was deleted. We conclude that the membrane-bound form of EsaE interacts with the multimeric form of EssC.

These results suggest a model for the biosynthesis and secretion of EsaD (Fig 4d), whereby the interaction of EsaG and EsaE with their respective binding domains on EsaD is essential for maintaining EsaD in a catalytically-inactive, secretion-competent conformation. Our findings support the idea that targeting of the protein complex to the T7 secretion machinery is by virtue of the interaction between EsaE and the assembled EssC multimer and that EsaG is stripped from the complex at some point during secretion and remains in the cytoplasm. Thus EsaD is released from the cell in a form that is immediately active, and the EsaG

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immunity protein remains in the producing cell where it may potentially serve further protective functions.

Secreted EsaD inhibits the growth of sensitive strains of *S. aureus*

We next addressed potential roles for EsaD. Gram-negative bacteria utilise a subset of their protein secretion systems to target toxins at bacterial competitors as well as at eukaryotic cells34–38. It was noted previously that although approximately 50% of *S. aureus* strains do not carry *esaD*, they encode at least two homologues of *esaG* close to their T7SS gene clusters18 (Fig 5a) suggesting they may produce EsaG-type proteins as a protective mechanism to prevent killing by EsaD-producing strains. To probe this, we assessed whether EsaG homologues from non *esaD*-containing strains MRSA252, ST398 and EMRSA15 could interact with EsaD(H528A) using two hybrid analysis. At least one EsaG homologue from each strain was able to interact with EsaD (Fig 5b), supporting the idea that orphan EsaG proteins serve to protect *S. aureus* from EsaD nuclease toxins.

A common feature of bacterial toxins, particularly those involved in interspecies competition, is that that they are polymorphic39,40. Comparison of EsaD sequences across *esaD*-encoding *S. aureus* strains shows extensive sequence variability within the nuclease domain but away from the predicted catalytic site (centred around H528; Fig S13). A likely explanation is that substitutions in this region of EsaD alter affinity for a cognate EsaG antitoxin and render normally resistant strains susceptible to attack by EsaD sequence variants. In this context it is interesting to note a cluster of EsaG homologues are encoded directly downstream of *esaDG* in *S. aureus esaD*-containing strains that is highly variable in number18. For example NCTC8325 encodes five of these (Fig 5a), whereas COL encodes eleven. These genes are not co-transcribed with *esaDG* in strain RN6390 (Fig S14), however analysis of RNA-seq data from18 indicated that transcripts from these genes are present under laboratory growth conditions.

We could readily delete the cluster of *esaG*-like genes from RN6390 indicating they are not required to neutralise EsaD, consistent with our prior conclusion that EsaG itself is the cognate EsaD antitoxin. Loss of this *esaG*-like gene cluster, or indeed absence of all EsaG-encoding proteins (including EsaG) also did not affect the secretion of EsxA or EsxC (Fig S9) confirming these proteins are not essential components of the T7SS machinery. However, two hybrid assay shows that they are able to interact with EsaD (Fig 5c), but that the interaction is not as strong as that seen for EsaD-EsaG, raising the possibility that they serve to protect RN6390 from EsaD sequence variants produced by other strains of *S. aureus*.

Together, the results presented above strongly suggest that *S. aureus* uses its T7SS to secrete a nuclease toxin that targets rival bacteria. To confirm this, we used *S. aureus* strain COL, which shows the highest level of T7S activity in laboratory growth media16 producing plasmid-encoded EsaDG as attacker, and incubated it with variants of strain RN6390. Fig 5d shows that there is an approximate two-log decrease in recovered prey cells when they are co-cultured with a T7SS^{+} strain of COL compared with a T7SS mutant strain, demonstrating there is T7-dependent growth inhibition. Importantly, this is completely dependent upon the toxic activity of EsaD as COL producing the H528A variant of EsaD no longer exhibited

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detectable growth inhibition (Fig 5d). Finally, as expected, EsaG offered some protection against the inhibitory effect of secreted EsaD as the RN6390 wild type tended to be less susceptible to EsaD-dependent growth inhibition than a strain lacking EsaG homologues, and the protective effect was enhanced by overproduction of plasmid-encoded EsaG in the prey cells. We conclude that *S. aureus* can use its T7SS to target bacterial competitors.

Discussion

It is well established that T7SSs play critical roles in mammalian infection and virulence. Here we demonstrate an important novel role for the *S. aureus* T7SS in the secretion of a nuclease toxin, EsaD, involved in interspecies competition. Thus, akin to the Gram-negative Type VI secretion system34,41, the T7SS appears to target eukaryotes and rival bacteria.

EsaD is the largest known substrate of the T7b system and the only one for which a function has been identified. We have shown that two T7 accessory factors are essential for the biogenesis of EsaD - EsaG that binds to and neutralises the toxic activity of the nuclease domain and EsaE that interacts with, and potentially stabilises, the N-terminal region. In this context it is interesting to note that large substrates of the Mycobacterial T7a system such as the PE/PPE proteins interact with specific chaperones of the EspG family that keep them in a secretion-competent state32 and deliver them to the cognate secretion machinery31. Although there is no detectable sequence similarity between EspG proteins and EsaE, it is possible that the proteins have analogous functions in the two distantly related secretion machineries.

The presence of T7SSs in non-pathogenic organisms such as *Streptomyces coelicolor*42 and *Bacillus subtilis*8,9 has previously been noted. Here we offer a likely explanation for the presence of this secretion system in environmental strains. We have demonstrated that secreted EsaD inhibits the growth of sensitive strains of *S. aureus*, indicating this nuclease toxin is used to target competitor bacteria. In support, strains of *S. aureus* lacking *esaD* encode at least two copies of the EsaG-like antitoxin, presumably as a protective mechanism. It is interesting to note that an EsaD homologue has been reported in *B. subtilis*23 that likely has toxic activity43, inferring that modulating bacterial competition is a conserved role for T7SSs.

It is currently not known how EsaD accesses the cytoplasm of target cells. There is no evidence that T7SSs form large extracellular needle-like structures that could deliver toxins directly into target cells, like to those seen for the Gram-negative Type III, IV and VI secretion systems1. Instead we suggest that EsaD is released into the environment where it binds to receptors on sensitive cells in a similar manner as bacteriocins and contact-dependent growth inhibition (CDI) toxins, as the first step in a cell entry pathway44,45. Further work will be required to dissect out the mechanism by which EsaD interacts with and traverses the cell envelope of *S. aureus*.

S. aureus is an important pathogen in polymicrobial human infections, for example infections of the skin and lung. Whether EsaD-mediated interspecies competition is critical

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for the establishment of virulence in animal and human infections and whether there are additional antibacterial toxins secreted by the *S. aureus* T7SS remain to be established.

Methods

Bacterial strains and growth conditions

Strains and plasmids used in this study are listed in Tables S1 and S2. *S. aureus* strains were grown in TSB medium at 37°C under vigorous agitation. Where required, chloramphenicol (Cm) at a final concentration of 10 μg/ml was added for plasmid selection. Anhydrotetracycline (ATC) was used as a selection during allelic gene replacement using the pIMAY system (1 μg/ml46) or for induction of target gene expression from the pRAB11 plasmid47; the concentrations used in each experiment are listed in the appropriate figure legends). *Escherichia coli* was grown aerobically in Lysogeny broth (LB) at 37°C. If required, cultures were supplemented with ampicillin (Amp, 100 μg/ml), Kanamycin (Kan, 50μg/ml) or Cm (15 μg/ml) for plasmid selection. Induction of plasmid-encoded gene expression was achieved by addition of isopropyl-β-D-galactopyranoside (IPTG), as indicated in the text. Light microscopy was carried out using a Zeiss light/fluorescence microscope with a 100x oil objective and images captured using an AXIO camera (Zeiss). The light microscopy images in Fig S3a were performed twice using different biological samples, representative images are shown. Bacterial two-hybrid analyses were performed as described48; quantitative assessment of protein interactions was undertaken by plating onto MacConkey medium49 containing 0.4% maltose as carbon source, and quantified by β-galactosidase assays (according to the method of50) on strains grown to exponential phase at 30°C and permeabilized with toluene. For all of the bacterial two hybrid experiments reported (Figs 2b, 3a, 3c, 5b, 5c, S10a) each interaction pair was scored on MacConkey maltose on at least four different occasions and β-galactosidase assays were performed at least twice, and representative results are presented. RT-PCR was undertaken on RNA prepared from *S. aureus* strain RN6390 grown aerobically in TSB to an OD_{600} of 2, as described previously16 using primer pairs listed in Table S3, and was performed twice (on the same biological sample – results presented in Fig S14b are representative).

Strain and plasmid construction

All oligonucleotide primers used in this study and cloning strategies to generate the strains and plasmids are outlined in Table S3. In-frame deletions of *S. aureus* genes were performed by allelic exchange using pIMAY46. For each gene, the upstream and downstream regions including at least the first three and last three codons were amplified from RN6390 genomic DNA using primers listed in Table S3. Clones were selected in *E. coli*, verified by DNA sequencing and introduced into *S. aureus* RN6390 strains by electroporation. Chromosomal deletions were verified by amplification of the genomic region from isolated genomic DNA (GeneElute Bacterial Genomic DNA Kit, Sigma Aldrich) and DNA sequencing of the amplified products.

To construct strains specifying chromosomally-encoded erythromycin resistance, the erythromycin resistance gene, *ermC*, was integrated into the RN6390 genome after base pair 14208 as this region was found to be devoid of transcriptional activity in the closely related

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strain NCTC 8325-451. A synthetic construct comprising *ermC* from *Staphylococcus lentus* plasmid pSTE2 under control of the *rpsF* promoter from *Bacillus subtilis* (purchased from Biomatik; sequence given in Fig S16) was cloned into pIMAY and integrated into the chromosome giving RN6390::*ermC*. Integration of the resistance gene was confirmed by sequencing and by testing for growth in the presence of 5 μg/ml erythromycin. Subsequently the *ermC* cassette was transduced from RN6390::*ermC* into other strains using phage ϕ11 as described52. Transduction was confirmed by PCR amplification using oligonucleotides Intctrl1 and Intctrl2 (Table S3).

Bacterial competition experiments

Overnight cultures of the indicated strains were subcultured in TSB (supplemented with 2μM hemin for attacker strains) and antibiotics as required and cultured with shaking at 37°C. Once OD_{600} of 0.5 was reached, induction of EsaD-HA and EsaG-His production in the attacker strains was initiated by the addition of 500 ng/ml of ATC. When cells reached OD_{600} of 2, 20ml of attacker strain, and 1ml of prey were separately harvested and resuspended in 1ml of TSB. 100ul of resuspended attacker cells were mixed with the same volume of prey cells (giving a 20:1 ratio) and incubated at 37°C with shaking for 16 h in sterile Eppendorf tubes. Co-cultures were then serially diluted in TSB and plated on selective agar (LB + 5 μg/ml erythromycin as all prey strains carried chromosomally-integrated *ermC* conferring resistance to erythromycin) for colony-forming unit determination. For experiments where plasmid-encoded EsaG-His was produced in prey cells, once strain RN6390 pEsaG-His reached an OD_{600} of 0.5, EsaG-His production was initiated by the addition of 250 ng/ml of ATC and cultured until an OD_{600} of 2 was reached after which they were used as prey as described above.

TUNEL assay for DNA fragmentation

DNA fragmentation was detected in fixed cells of *E. coli* BL21(DE3) harbouring pT7.5esaD and pT7.5esaDG using the Deadend fluorometric TUNEL system kit (Promega). This was undertaken on two biological replicates and representative results are shown in FigS3 c and d. Following induction of EsaD production by treating cell cultures with 1mM IPTG for 3 hours, cells were pelleted, washed twice with PBS and fixed with 4% formaldehyde in PBS for 30 minutes on ice. Following a further wash with PBS, cells were permeabilized with 1.5% triton X-100 solution in PBS for 1 hour on ice and stored in 70% ice cold ethanol at -20 °C overnight. The following day cells were spun down, washed and resuspended in equilibration buffer for 1 hour at 37°C. The cells were incubated in the dark for 2 hours with fluorescein 12-dUTP and recombinant terminal deoxynucleotidyl transferase (rTdT), after which the reaction was quenched by addition of 2x SCC buffer, and the cells washed with PBS. Following this fluorescein-labelled cells were spotted onto poly-D-lysine-treated slides and analysed by fluorescence microscopy using a Zeiss fluorescence microscope with a 100x oil objective and images captured using an AXIO camera (Zeiss), or detected and quantitated directly by flow cytometry (Flow cytometry Facility, Dundee University). Negative control samples were treated identically except that no rTdT was added.

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Purification of the EsaD nuclease domain

E. coli strain M15[prep4] harbouring pQE70-EsaG-EsaG-EsaD$_{421-614}$-His or pQE70-EsaG-EsaG-EsaD$_{421-614}$(H528A)-His was cultured to OD$_{600}$ of 0.5 at 37ºC, after which 1mM IPTG was added to each culture. An aliquot of the cells were harvested after 4 hours induction and resuspended in lysis buffer and the remainder of the cells were pelleted and frozen at -80°C for 30 mins. The nuclease domain of EsaD was subsequently purified following a protocol based on that described at http://openwetware.org/wiki/Knight:Purification_of_His-tagged_proteins/Denaturing_with_refolding with some modifications, and was undertaken once for each variant (Fig 2e). Briefly, the thawed pellets were resupended in lysis buffer (8 M Urea, 100 mM NaH_2PO_4, 10mM Tris·HCl, 10mM imidazole, 1 mM PMSF, pH 8.0) and sonicated for 2 mins on ice. The cell lysate was centrifuged at 13,200 × *g* for 30 min at 4 °C. Nickel affinity resin (Biorad) was equilibrated with lysis buffer and added to the cleared lysate and gently mixed on a rotary shaker for 2 h at 4 °C. The resin was washed five times with denaturing wash buffer (8 M Urea, 100 mM NaH_2PO_4, 150mM NaCl, 20mM imidazole, 1 mM PMSF, pH 8.0) followed by five washes with native wash buffer (50 mM NaH_2PO_4, 500mM NaCl, 20mM imidazole, 1 mM PMSF, pH 8.0). Finally, the bound protein was eluted into 200 µl of elution buffer (50 mM NaH_2PO_4, 500mM NaCl, 250mM imidazole, 1 mM PMSF, pH 8.0). For nuclease assays, 800 ng of plasmid pT18 was incubated with 0.4 µg of purified EsaD$_{421-614}$-His, purified EsaD$_{421-614}$(H528A)-His or an equivalent volume of elution buffer at 37°C for 1 hour in a final volume of 20µl the presence of either 50mM $MgCl_2$ or $ZnCl_2$ as indicated, after which the DNA was analysed by agarose gel electrophoresis. These assays were each performed three times and representative results are presented in Figs 2f and S5.

Cell fractionation, crosslinking and western blotting

For the isolation of cell and supernatant fractions to assess secretion activity, *S. aureus* strains were subcultured at 1/100 from an overnight grown pre-culture into fresh TSB medium. At OD$_{600nm}$ of 2, cells were harvested and the supernatant samples precipitated with trichloroacetic acid in the presence of deoxycholate, as described previously16. Harvested cell samples were washed once with PBS buffer, normalized to an OD$_{600}$ of 2 in PBS and lysed by addition of 50 µg/ml lysostaphin with incubation at 37°C for 30 min. All samples were mixed with an equal volume of LDS buffer and boiled for 10 min prior to analysis. For the secretion experiments shown in Fig 1b, 1c, 1e, S9a, S9b and S14 representative images are shown from at least two biological replicates, and in Figs 1d, 3d, 3e, S7 and S8 representative images are shown from at least six biological replicates. The fractionation of cells to give cell wall, membrane and cytoplasmic fractions was undertaken as described by16. Carbonate-washing of membranes was undertaken according to53. All fractionation experiments (Figs 1f, 3a and S6) were undertaken at least twice on separate biological samples, with representative results presented.

Formaldehyde crosslinking of cells was undertaken as described previously17 and crosslinking experiments were performed twice on separate biological samples – the results presented in Fig 3b and c are representative results. Western blotting was performed according to standard protocols using the following antibody dilutions: α-EsxA16 1:2500, α-EsxC16 1:2000, α-EssC16 1:10000, α-TrxA54 1:25000, α-SrtA (Abcam, catalogue

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number ab13959) 1:3000, α-HA (HRP-conjugate, Sigma catalogue number H6533) 1:10000, α-His (HRP-conjugate, Abcam catalogue number ab184607) 1:10000, α-Myc (HRP-conjugate, Invitrogen catalogue number R951-25) 1:5000, and goat anti Rabbit IgG HRP conjugate (Bio-Rad, catalogue number 170-6515) 1:10000.

Protein purification by nickel affinity isolation or immunoprecipitation

Cells of *E. coli* or *S. aureus*, grown as described in the figure legends, were harvested and resuspended in either ice cold (i) resuspension buffer (200 mM NaCl, 20 mM HEPES, pH 7.2) for Ni affinity purification or (ii) phosphate-buffered saline (PBS) for immunoprecipitation. Samples were then supplemented with a few flakes of DNase I, 1 mM PMSF and either lysozyme (for *E. coli*) or lysostaphin (for *S. aureus*). The samples were incubated at 37°C for 0.5-1 hour with gentle mixing on a rotating wheel after which cells were lysed by French press (for *E. coli*) or sonication (*S. aureus*). Unbroken cells and cellular debris was pelleted by centrifugation at 17,000 *g* and 4°C for 30 min and the supernatant was retained as the cell lysate. All protein purification experiments shown in Figs 2c, 2d, 3b, 3f, 3g, S4, S10b, S11 and S12 were performed at least twice with different biological replicates.

For Ni-affinity purification, 100 μl Ni-NTA resin (Biorad, catalogue number 156-0131) was equilibrated by washing twice in 1 ml ice cold wash buffer (200 mM NaCl, 15 mM imidazole, 20 mM HEPES, pH 7.2). Cell lysate was diluted to 5 μg/μl protein in ice cold wash buffer (200 mM NaCl, 15 mM imidazole, 20 mM HEPES, pH 7.2) in a final volume of 1.2 ml, added to the equilibrated Ni-NTA resin and gently agitated for 1 hour at 4°C. The Ni-NTA resin was pelleted by centrifugation, washed four times with 1 ml ice cold wash buffer and finally resuspended in 100 μl elution buffer (200 mM NaCl, 300 mM imidazole, 20 mM HEPES, pH 7.2), mixing at 4°C for 1 hour. The Ni-NTA resin was pelleted, the supernatant carefully removed and retained as the eluted fraction.

For immunoprecipitation, 40-100 μl of Anti-HA Agarose bead suspension (Sigma, catalogue number A2095) were pelleted, washed twice with PBS and mixed with cell lysate which was diluted to 5 μg/μl in 200 μl PBS. The suspension was incubated with agitation for at least 1 hour at 4°C after which the beads were pelleted and washed four times with 1 ml ice cold wash PBS. After the final wash the supernatant was aspirated to leave ~30 μl of PBS above the beads for final resuspension.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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References

1. Costa TR, et al. Secretion systems in Gram-negative bacteria: structural and mechanistic insights. Nat Rev Microbiol. 2015; 13:343–359. DOI: 10.1038/nrmicro3456 [PubMed: 25978706]
2. Pym AS, et al. Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. Nature medicine. 2003; 9:533–539.
3. Hsu T, et al. The primary mechanism of attenuation of bacillus Calmette-Guerin is a loss of secreted lytic function required for invasion of lung interstitial tissue. Proceedings of the National Academy of Sciences of the United States of America. 2003; 100:12420–12425. [PubMed: 14557547]
4. Stanley SA, Raghavan S, Hwang WW, Cox JS. Acute infection and macrophage subversion by Mycobacterium tuberculosis require a specialized secretion system. Proceedings of the National Academy of Sciences of the United States of America. 2003; 100:13001–13006. [PubMed: 14557536]
5. Abdallah AM, et al. A specific secretion system mediates PPE41 transport in pathogenic mycobacteria. Molecular microbiology. 2006; 62:667–679. [PubMed: 17076665]
6. Cole ST, et al. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature. 1998; 393:537–544. DOI: 10.1038/31159 [PubMed: 9634230]
7. Abdallah AM, et al. Type VII secretion--mycobacteria show the way. Nat Rev Microbiol. 2007; 5:883–891. [PubMed: 17922044]
8. Baptista C, Barreto HC, Sao-Jose C. High levels of DegU-P activate an Esat-6-like secretion system in *Bacillus subtilis*. PLoS ONE. 2013; 8:e67840.doi: 10.1371/journal.pone.0067840 [PubMed: 23861817]
9. Huppert LA, et al. The ESX system in *Bacillus subtilis* mediates protein secretion. PLoS ONE. 2014; 9:e96267.doi: 10.1371/journal.pone.0096267 [PubMed: 24798022]
10. Burts ML, Williams WA, DeBord K, Missiakas DM. EsxA and EsxB are secreted by an ESAT-6-like system that is required for the pathogenesis of *Staphylococcus aureus* infections. Proceedings of the National Academy of Sciences of the United States of America. 2005; 102:1169–1174. [PubMed: 15657139]
11. Pallen MJ. The ESAT-6/WXG100 superfamily -- and a new Gram-positive secretion system? Trends in microbiology. 2002; 10:209–212. [PubMed: 11973144]
12. Rosenberg OS, et al. Substrates Control Multimerization and Activation of the Multi-Domain ATPase Motor of Type VII Secretion. Cell. 2015; 161:501–512. DOI: 10.1016/j.cell.2015.03.040 [PubMed: 25865481]
13. Renshaw PS, et al. Structure and function of the complex formed by the tuberculosis virulence factors CFP-10 and ESAT-6. The EMBO journal. 2005; 24:2491–2498. DOI: 10.1038/sj.emboj.7600732 [PubMed: 15973432]
14. Sundaramoorthy R, Fyfe PK, Hunter WN. Structure of *Staphylococcus aureus* EsxA suggests a contribution to virulence by action as a transport chaperone and/or adaptor protein. Journal of molecular biology. 2008; 383:603–614. [PubMed: 18773907]
15. Sysoeva TA, Zepeda-Rivera MA, Huppert LA, Burton BM. Dimer recognition and secretion by the ESX secretion system in *Bacillus subtilis*. Proceedings of the National Academy of Sciences of the United States of America. 2014; 111:7653–7658. DOI: 10.1073/pnas.1322200111 [PubMed: 24828531]
16. Kneuper H, et al. Heterogeneity in ess transcriptional organization and variable contribution of the Ess/Type VII protein secretion system to virulence across closely related *Staphylocccus aureus* strains. Molecular microbiology. 2014; 93:928–943. DOI: 10.1111/mmi.12707 [PubMed: 25040609]
17. Jäger F, Zoltner M, Kneuper H, Hunter WN, Palmer T. Membrane interactions and self-association of components of the Ess/Type VII secretion system of *Staphylococcus aureus*. FEBS letters. 2016; 590:349–357. DOI: 10.1002/1873-3468.12065 [PubMed: 26785823]

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18. Warne B, et al. The Ess/Type VII secretion system of *Staphylococcus aureus* shows unexpected genetic diversity. BMC genomics. 2016; 17:222.doi: 10.1186/s12864-016-2426-7 [PubMed: 26969225]
19. Burts ML, DeDent AC, Missiakas DM. EsaC substrate for the ESAT-6 secretion pathway and its role in persistent infections of *Staphylococcus aureus*. Molecular microbiology. 2008; 69:736–746. DOI: 10.1111/j.1365-2958.2008.06324.x [PubMed: 18554323]
20. Anderson M, Aly KA, Chen YH, Missiakas D. Secretion of atypical protein substrates by the ESAT-6 secretion system of *Staphylococcus aureus*. Molecular microbiology. 2013; 90:734–743. DOI: 10.1111/mmi.12395 [PubMed: 24033479]
21. Wang Y, et al. Role of the ESAT-6 secretion system in virulence of the emerging community-associated *Staphylococcus aureus* lineage ST398. Scientific reports. 2016; 6:25163.doi: 10.1038/srep25163 [PubMed: 27112266]
22. Korea CG, et al. Staphylococcal Esx proteins modulate apoptosis and release of intracellular *Staphylococcus aureus* during infection in epithelial cells. Infection and immunity. 2014; 82:4144–4153. DOI: 10.1128/IAI.01576-14 [PubMed: 25047846]
23. Anderson M, Chen YH, Butler EK, Missiakas DM. EsaD, a Secretion Factor for the Ess Pathway in *Staphylococcus aureus*. Journal of bacteriology. 2011; 193:1583–1589. DOI: 10.1128/Jb.01096-10 [PubMed: 21278286]
24. Tabor S, Richardson CC. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proceedings of the National Academy of Sciences of the United States of America. 1985; 82:1074–1078. [PubMed: 3156376]
25. Butala M, Zgur-Bertok D, Busby SJ. The bacterial LexA transcriptional repressor. Cell Mol Life Sci. 2009; 66:82–93. DOI: 10.1007/s00018-008-8378-6 [PubMed: 18726173]
26. Hill TM, Sharma B, Valjavec-Gratian M, Smith J. *sfi*-independent filamentation in *Escherichia coli* Is *lexA* dependent and requires DNA damage for induction. Journal of bacteriology. 1997; 179:1931–1939. [PubMed: 9068638]
27. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. The Journal of cell biology. 1992; 119:493–501. [PubMed: 1400587]
28. Bos J, Yakhnina AA, Gitai Z. BapE DNA endonuclease induces an apoptotic-like response to DNA damage in *Caulobacter*. Proceedings of the National Academy of Sciences of the United States of America. 2012; 109:18096–18101. DOI: 10.1073/pnas.1213332109 [PubMed: 23074244]
29. Blower TR, Salmond GP, Luisi BF. Balancing at survival's edge: the structure and adaptive benefits of prokaryotic toxin-antitoxin partners. Curr Opin Struct Biol. 2011; 21:109–118. DOI: 10.1016/j.sbi.2010.10.009 [PubMed: 21315267]
30. Daleke MH, et al. Specific chaperones for the type VII protein secretion pathway. The Journal of biological chemistry. 2012; 287:31939–31947. DOI: 10.1074/jbc.M112.397596 [PubMed: 22843727]
31. Ekiert DC, Cox JS. Structure of a PE-PPE-EspG complex from *Mycobacterium tuberculosis* reveals molecular specificity of ESX protein secretion. Proceedings of the National Academy of Sciences of the United States of America. 2014; 111:14758–14763. DOI: 10.1073/pnas.1409345111 [PubMed: 25275011]
32. Korotkova N, et al. Structure of the *Mycobacterium tuberculosis* type VII secretion system chaperone EspG5 in complex with PE25-PPE41 dimer. Molecular microbiology. 2014; 94:367–382. DOI: 10.1111/mmi.12770 [PubMed: 25155747]
33. Zoltner M, et al. EssC: domain structures inform on the elusive translocation channel in the Type VII secretion system. The Biochemical journal. 2016; doi: 10.1042/BCJ20160257
34. Russell AB, et al. Type VI secretion delivers bacteriolytic effectors to target cells. Nature. 2011; 475:343–347. DOI: 10.1038/nature10244 [PubMed: 21776080]
35. Murdoch SL, et al. The opportunistic pathogen *Serratia marcescens* utilizes type VI secretion to target bacterial competitors. Journal of bacteriology. 2011; 193:6057–6069. DOI: 10.1128/JB.05671-11 [PubMed: 21890705]
36. Souza DP, et al. Bacterial killing via a type IV secretion system. Nature communications. 2015; 6:6453.doi: 10.1038/ncomms7453

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37. Hood RD, et al. A type VI secretion system of *Pseudomonas aeruginosa* targets a toxin to bacteria. Cell Host Microbe. 2010; 7:25–37. DOI: 10.1016/j.chom.2009.12.007 [PubMed: 20114026]
38. Aoki SK, et al. Contact-dependent inhibition of growth in *Escherichia coli*. Science (New York, NY). 2005; 309:1245–1248. DOI: 10.1126/science.1115109
39. Zhang D, de Souza RF, Anantharaman V, Iyer LM, Aravind L. Polymorphic toxin systems: Comprehensive characterization of trafficking modes, processing, mechanisms of action, immunity and ecology using comparative genomics. Biology direct. 2012; 7:18.doi: 10.1186/1745-6150-7-18 [PubMed: 22731697]
40. Jamet A, Nassif X. New players in the toxin field: polymorphic toxin systems in bacteria. MBio. 2015; 6:e00285–00215. DOI: 10.1128/mBio.00285-15 [PubMed: 25944858]
41. Ma AT, McAuley S, Pukatzki S, Mekalanos JJ. Translocation of a *Vibrio cholerae* type VI secretion effector requires bacterial endocytosis by host cells. Cell Host Microbe. 2009; 5:234–243. DOI: 10.1016/j.chom.2009.02.005 [PubMed: 19286133]
42. Akpe San Roman S, et al. A heterodimer of EsxA and EsxB is involved in sporulation and is secreted by a type VII secretion system in *Streptomyces coelicolor*. Microbiology (Reading, England). 2010; 156:1719–1729. DOI: 10.1099/mic.0.037069-0
43. Holberger LE, Garza-Sanchez F, Lamoureux J, Low DA, Hayes CS. A novel family of toxin/antitoxin proteins in *Bacillus* species. FEBS letters. 2012; 586:132–136. DOI: 10.1016/j.febslet.2011.12.020 [PubMed: 22200572]
44. Housden NG, Kleanthous C. Colicin translocation across the *Escherichia coli* outer membrane. Biochemical Society Transactions. 2012; 40:1475–1479. DOI: 10.1042/BST20120255 [PubMed: 23176501]
45. Willett JL, Gucinski GC, Fatherree JP, Low DA, Hayes CS. Contact-dependent growth inhibition toxins exploit multiple independent cell-entry pathways. Proceedings of the National Academy of Sciences of the United States of America. 2015; 112:11341–11346. [PubMed: 26305955]
46. Monk IR, Shah IM, Xu M, Tan MW, Foster TJ. Transforming the untransformable: application of direct transformation to manipulate genetically *Staphylococcus aureus* and *Staphylococcus epidermidis*. MBio. 2012; 3doi: 10.1128/mBio.00277-11
47. Helle L, et al. Vectors for improved Tet repressor-dependent gradual gene induction or silencing in Staphylococcus aureus. Microbiology (Reading, England). 2011; 157:3314–3323. DOI: 10.1099/mic.0.052548-0
48. Karimova G, Pidoux J, Ullmann A, Ladant D. A bacterial two-hybrid system based on a reconstituted signal transduction pathway. Proceedings of the National Academy of Sciences of the United States of America. 1998; 95:5752–5756. [PubMed: 9576956]
49. Sambrook, J., Russell, DW. Molecular cloning : a laboratory manual. 3rd edn. Cold Spring Harbor Laboratory Press; 2001.
50. Miller, JH. A short course in bacterial genetics: A laboratory manual and handbook for Escherichia coli and related bacteria. Cold Spring Harbor Laboratory Press; 1992.
51. Lei MG, et al. A single copy integration vector that integrates at an engineered site on the *Staphylococcus aureus* chromosome. BMC Res Notes. 2012; 5:5.doi: 10.1186/1756-0500-5-5
52. Novick RP. Genetic systems in staphylococci. Methods Enzymol. 1991; 204:587–636. [PubMed: 1658572]
53. Keller R, de Keyzer J, Driessen AJM, Palmer T. Co-operation between different targeting pathways during integration of a membrane protein. J of Cell Biol. 2012; 199:303–315. [PubMed: 23045547]
54. Miller M, et al. Staphylococcal PknB as the first prokaryotic representative of the proline-directed kinases. PLoS ONE. 2010; 5:e9057.doi: 10.1371/journal.pone.0009057 [PubMed: 20140229]

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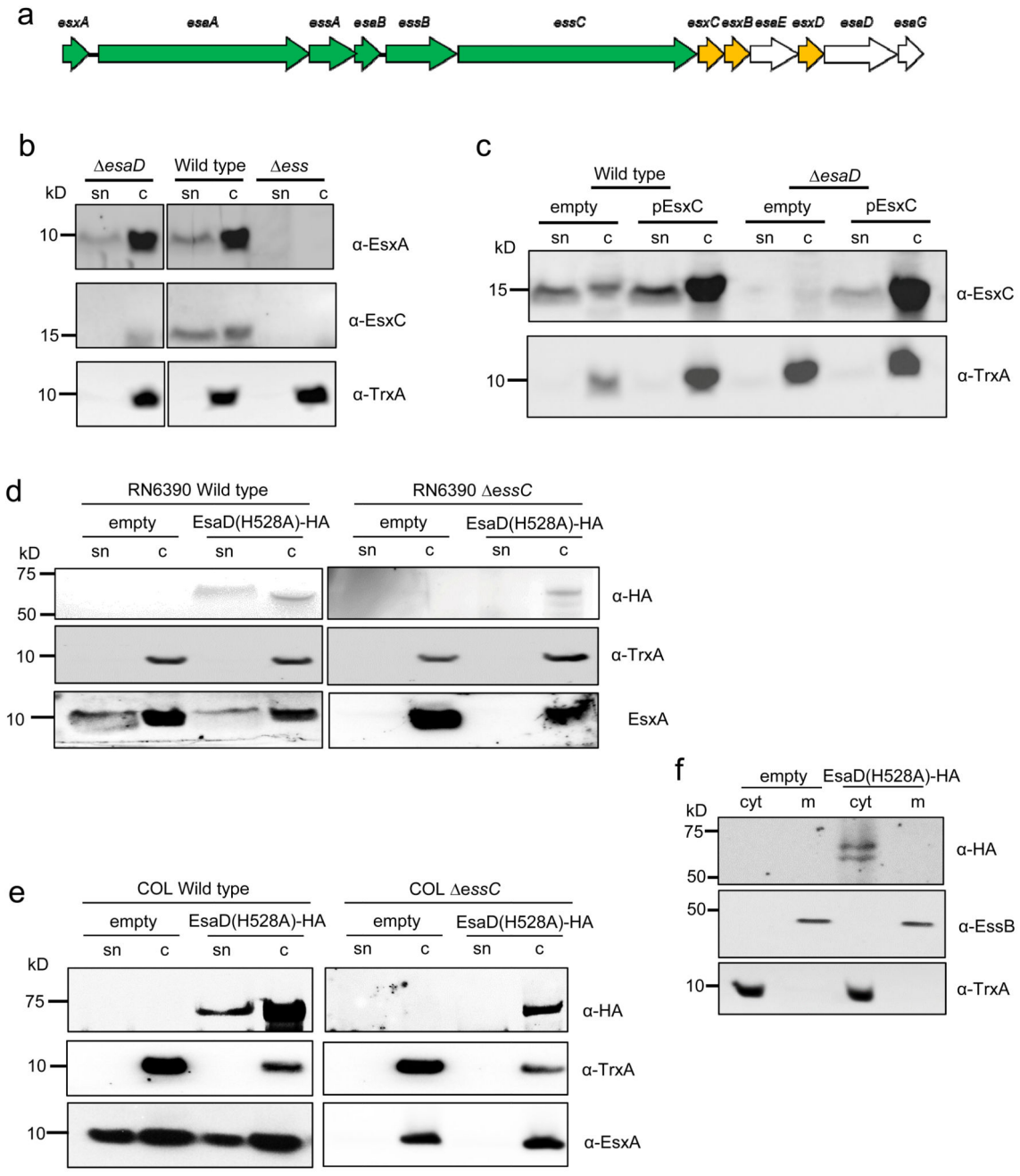

Fig 1. EsaD is a substrate of the T7SS.

a. The *ess* locus – genes coding for core components of the secretion machinery are in green, secreted components yellow and proteins investigated as part of this study in white. b. and c. EsaD is not required for secretion of EsxA and EsxC – (b) the RN6390 wild-type or isogenic deletion strains, as indicated, were cultured in TSB medium to OD_{600} of 2 or (c) the indicated strains harbouring pRAB11 (empty) or pRAB11-EsxC were cultured in TSB medium to OD_{600} of 0.5, then supplemented with ATC (50ng/ml; to induce plasmid-encoded gene expression) until OD_{600} of 2. Cells were pelleted and the supernatant (sn) was retained

as the secreted protein fraction. Samples of the supernatant and whole cells (an equivalent of 200μl of culture supernatant and 10μl of cells adjusted to OD1) were separated on 12 % bis-Tris gels and immunoblotted with the indicated antisera (with TrxA serving as a cytoplasmic control). Note that the samples were run on the same gel but intervening lanes have been spliced out (unspliced version is shown in Fig S9). (d) and (e). EsaD is secreted in an *essC*-dependent manner. The indicated *S. aureus* strains harbouring pRAB11 (empty) or pRAB11-EsaD(H528A)-HA were treated as described in (c) except that 250ng/ml ATC was used to induce EsaD(H528A)-HA production and an equivalent of 250μl of supernatant and 10μl of cells adjusted to OD_{600} of 1 were loaded f. Cells of the wild type *S. aureus* strain, RN6390, harbouring pRAB11 or pRAB11-EsaD(H528A)-HA from (d) were fractionated into cytoplasmic (cyt) and membrane (m) fractions. Samples of each fraction (20μl aliquot of cyt, and 2mg of membrane) were separated on 12% bis-Tris gels and immunoblotted using either anti-HA, anti-EssB (membrane protein control) or anti-TrxA (cytoplasmic control) antisera.

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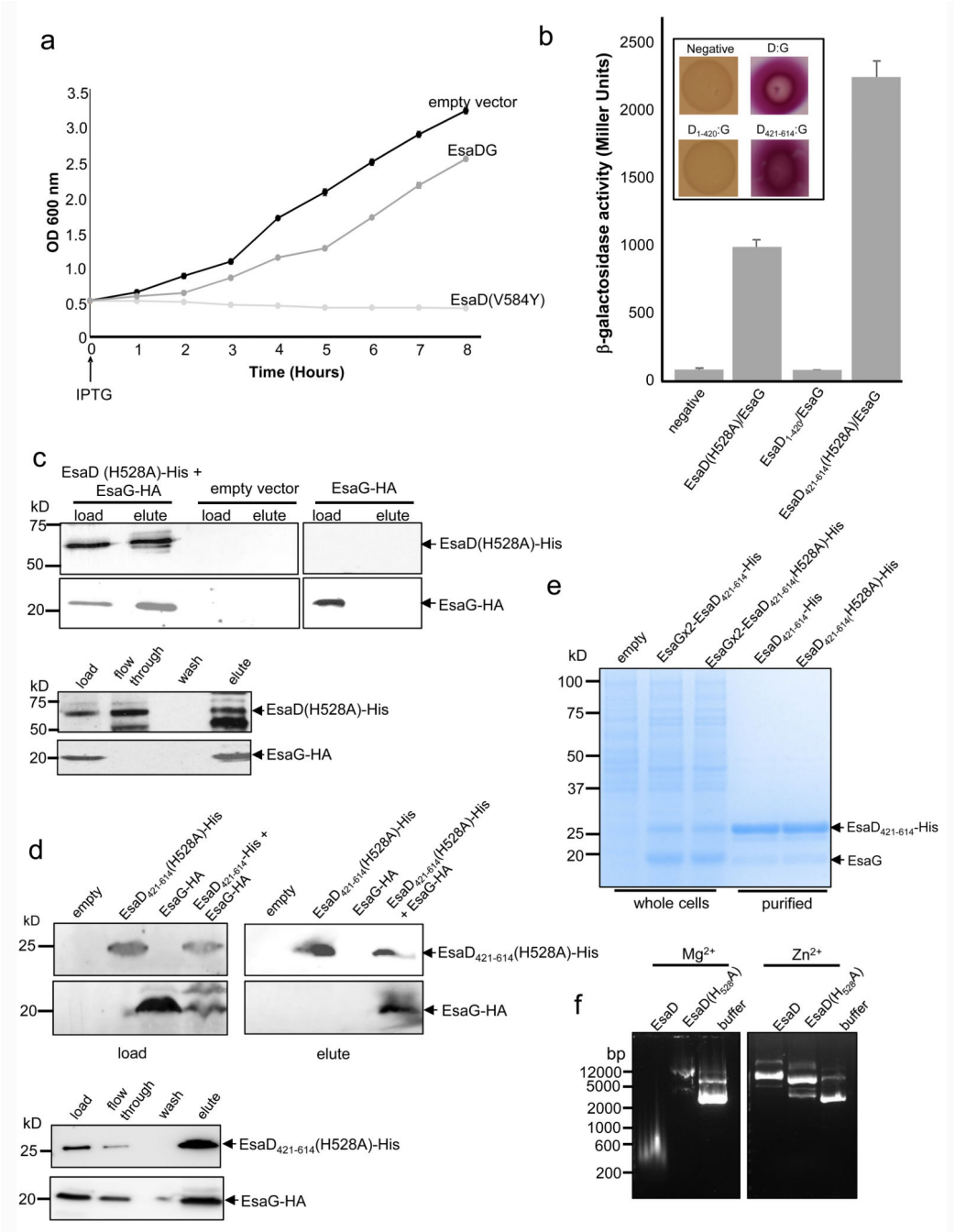

Fig 2. EsaDG form a nuclease toxin-antitoxin pair.

a. EsaD is toxic to *E. coli*. *E. coli* BL21(DE3) harbouring pT7.5 (empty vector), pT7.5-esaD(V584Y) or pT7.5-esaDG was cultured to OD_{600} 0.5, supplemented with 1 mM IPTG (time zero) and OD_{600} measured at 1 hr intervals (n=3 biological replicates, error bars are ± SD). b. – d. EsaG interacts with the nuclease domain of EsaD. b. Interactions between pT25-EsaG and EsaD variants fused to pT18 assessed by β-galactosidase activity assay in *E. coli* BTH101. BTH101 harbouring pT25 and pT18 was the negative control. Error bars are ± SD (n=3 biological replicates). Student's t-test gives p values < 0.00001 for EsaD/EsaG and

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$EsaD_{421-614}$/EsaG relative to the negative control. Inset shows the same strain/plasmid combinations on MacConkey maltose plates. c. and d. Top two panels: *S. aureus* RN6390 carrying c. pRAB11 (empty vector), pRAB11-EsaG-HA or pRAB11-EsaD(H528A)-His-EsaG-HA, or e. pRAB11 (empty), pRAB11-EsaG-HA, pRAB11-$EsaD_{421-614}$(H528A)-His or pRAB11-$EsaD_{421-614}$(H528A)-His-EsaG-HA was cultured to OD_{600} of 0.5, then supplemented with ATC (500ng/ml). Cells were harvested at OD_{600} 3, lysed and histidine-tagged EsaD purified. Cell lysate (load) and eluted fractions (20μl of each) were analysed by western blot with anti-His and anti-HA antisera. Bottom two panels show repeat experiments of: c. the EsaD(H528A)-His-EsaG-HA co-purification or d. the $EsaD_{421-614}$(H528A)-His-EsaG-HA co-purification. Samples of load (10μl), flow through (20μl), final wash (30μl) and elution fraction (30μl) were analysed using the same antisera. Coomassie-stained samples of the load and elute fractions are shown in Fig S4. e. *E. coli* M15[prep4] harbouring pQE70 alone (empty) pQE70-EsaG-EsaG-$EsaD_{421-614}$-His or pQE70-EsaG-EsaG-$EsaD_{421-614}$(H528A)-His were cultured to OD_{600} of 0.5 and supplemented with 1mM IPTG. An aliquot was harvested after 4 hours' induction and resuspended in lysis buffer. His-tagged EsaD nuclease domain (wild type or H528A variant) was purified in the presence of 8M urea, refolded and eluted as described in Methods. 10μl of each sample were separated (12% bis-Tris gel) and stained using coomassie instant blue. f. EsaD is a Mg^{2+}-dependent DNase. Plasmid DNA was incubated with purified $EsaD_{421-614}$-His, $EsaD_{421-614}$(H528A)-His or buffer alone with either 50mM $MgCl_2$ or $ZnCl_2$, after which the DNA was analysed by agarose gel electrophoresis.

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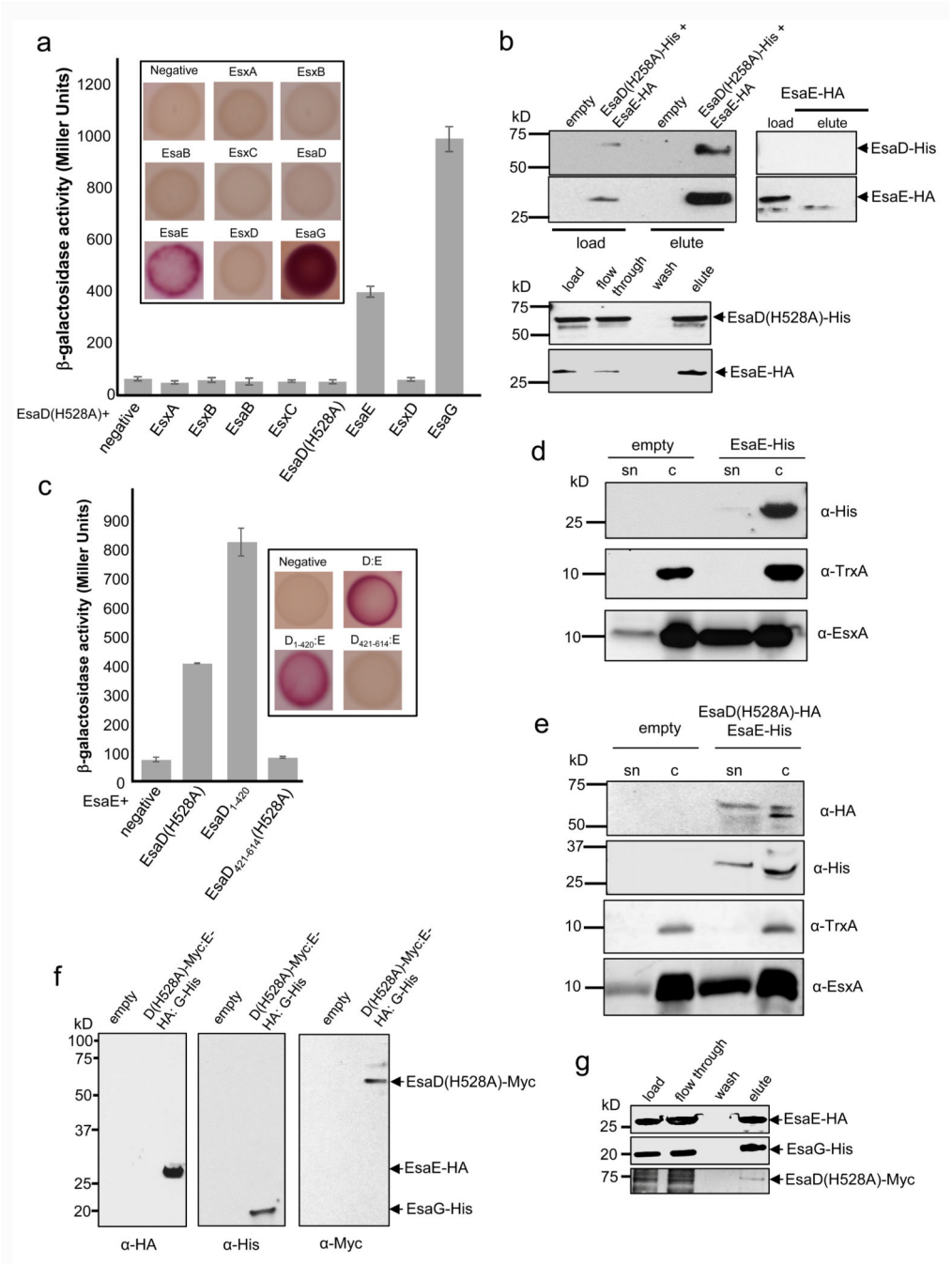

Fig 3. EsaE is co-secreted with EsaD and together with EsaG they form a ternary complex.

a – c. EsaE interacts with EsaD. Interactions between a. pT25-EsaD(H528A) and the indicated fusions to pT18 or c. pT25-EsaE and $EsaD_{1-420}$ or $EsaD_{421-614}$(H528A) fused to pT18 as well as pT18-EsaE and full length EsaD(H528A) fused to pT25 assessed by β-galactosidase activity assay in BTH101. BTH101 harbouring pT25 and pT18 was the negative control. Error bars are ± SD (n=3 biological replicates). Student's t-test gives p values < 0.00001 for EsaD(H528A)/EsaE, EsaD(H528A)/EsaG, EsaD/EsaE, and $EsaD_{1-420}$/EsaE relative to the negative control. Inset shows the same strain and plasmid combinations

on MacConkey maltose plates. b. Top two panels - *S. aureus* RN6390 carrying pRAB11 (empty), pRAB11-EsaE-HA or pRAB11-EsaD(H528A)-His-EsaE-HA was cultured to OD_{600} of 0.5 supplemented with 500ng/ml ATC and harvested at OD_{600} of 3. Cells were lysed and histidine-tagged EsaD(H528A) was purified. Cell lysate (load) and eluted fractions (20μl of each) were analysed by western blot with anti-His and anti-HA antisera. Coomassie-stained samples of these fractions are shown in Fig S4. Bottom two panels show repeats of EsaD(H528A)-His-EsaE-HA co-purification. Samples of load (10μl), flow through (20μl), final wash (30μl) and elution fraction (30μl) were analysed using the same antisera. d and e. EsaE is co-secreted with EsaD. *S. aureus* RN6390 harbouring pRAB11 (empty) and either d. pRAB11-EsaE-His or e. pRAB11-EsaD-His-EsaE-HA was cultured to OD_{600} of 0.5 supplemented with 250ng/ml ATC and harvested at OD_{600} of 3. Samples of supernatant and cells (equivalent to 250μl supernatant and 10μl cells adjusted to OD_{600} of 1) were separated on 12% bis-Tris gels and immunoblotted with the indicated antisera. f. EsaE, EsaD and EsaG form a ternary complex. *E. coli* M15[pRep4] carrying pQE70 (empty) or pQE70- EsaE-HA-EsaD(H528A)-Myc-EsaG-His was cultured to OD_{600} of 0.5, supplemented with 2 mM IPTG for 4 hours, harvested and lysed. HA-tagged EsaE was purified and 10μl, 25μl and 35μl of the elution fractions were analysed by western blot with anti-HA, anti-His and anti-myc antibodies, respectively. Coomassie-stained samples of these fractions are shown in Fig S10. g. Samples of load (10μl), flow through (20μl), final wash (30μl) and elution fraction (30μl) from the EsaE-HA-EsaD(H528A)-Myc-EsaG-His co-purification experiment shown in f. were analysed by western blotting with the same antisera.

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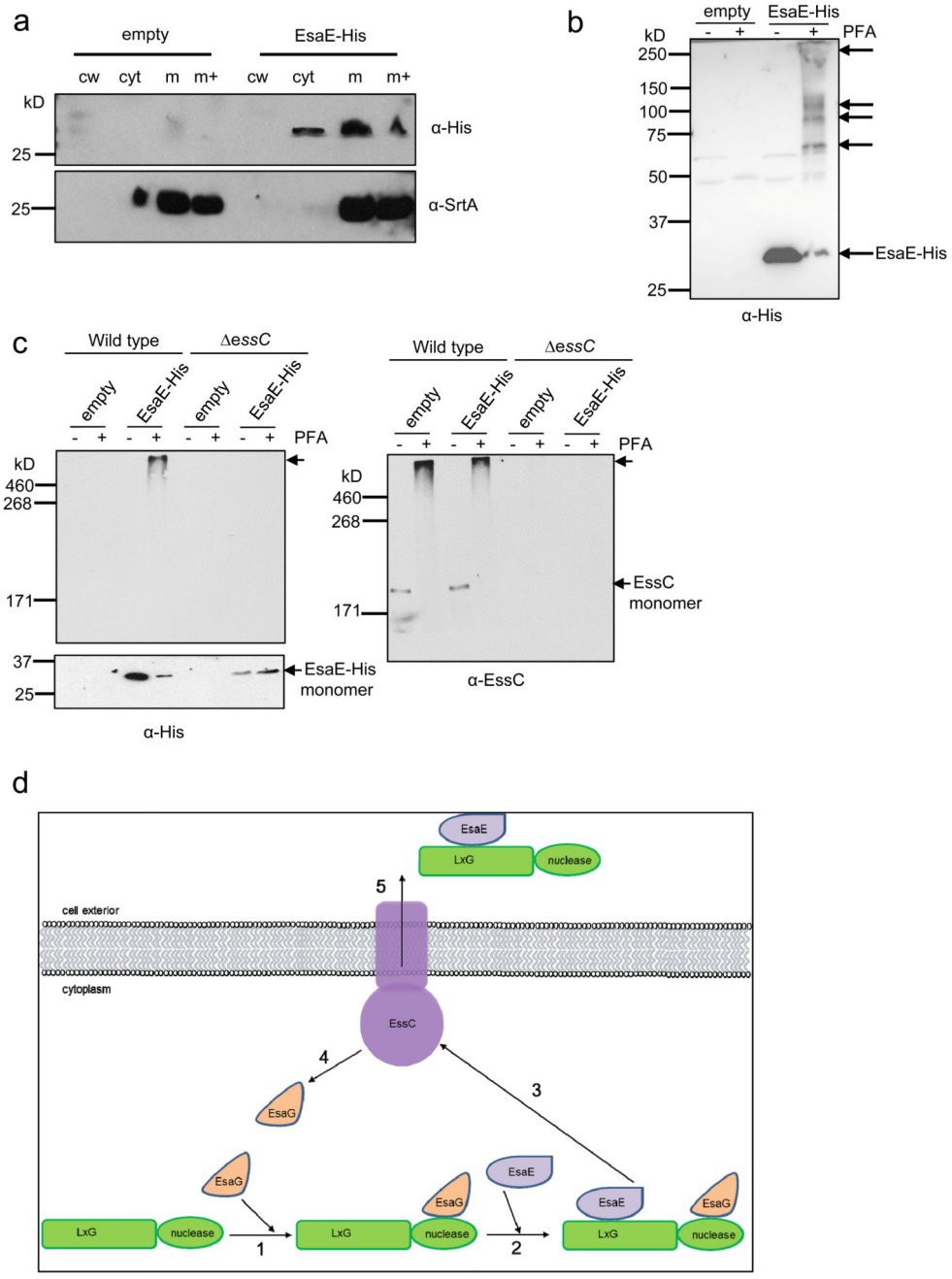

Fig 4. EsaE is a membrane-associated protein that interacts with multimeric EssC.

a. A proportion of EsaE is bound to the membrane. The *S. aureus* wild type strain, RN6390, harbouring pRAB11 (empty) or pRAB11-EsaE-His was cultured in TSB medium to OD_{600} of 0.5, supplemented with ATC (250ng/ml) and harvested at OD_{600} of 2. Cells were fractionated into cell wall (cw), cytoplasmic (cyt) and membrane (m) fractions. An aliquot of the membrane fraction was washed with 0.2 M Na_2CO_3 (m+). Samples of each fraction (20μl aliquot of cw and cyt; 2mg of membrane) were separated on 12% bis-Tris gels and immunoblotted using either anti-His or anti-sortase A (SrtA) antisera. b and c. EsaE

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crosslinks to a multimeric form of EssC. b. Whole cells of the *S. aureus* wild type (RN6390), or c. the wild type and the isogenic *essC* deletion strain, as indicated, harbouring pRAB11 (empty) or pRAB11-EsaE-His were cultured in TSB medium to OD_{600} of 0.5 supplemented with ATC (250ng/ml) and at OD_{600} of 2, cells were incubated with paraformaldehyde (PFA) as described under Methods. Following quenching, cells were lysed and membrane fractions prepared, and membrane protein (1mg for samples from the wild type strain, 10mg for samples from the *essC* strain) loaded on b. a bis-Tris gel containing 12% acrylamide or c. SDS-gels containing 5 % acrylamide (bottom panel in part C showing EsaE-His monomer is 12% bis-Tris gel) and analysed by western blot with the indicated antisera. d. Model for EsaD synthesis and secretion. Following synthesis of EsaD (shown in green), ***1*** EsaE binds to the N terminal region of the protein and ***2*** EsaG binds to the nuclease domain to prevent activity against the DNA of the producing cell. ***3*** the ternary complex is targeted to the secretion machinery facilitated by the interaction of EsaE with multimeric EssC. ***4*** EsaG is released from EsaD during the transport step and ***5*** the EsaD-EsaE complex is secreted out of the cell via the T7SS.

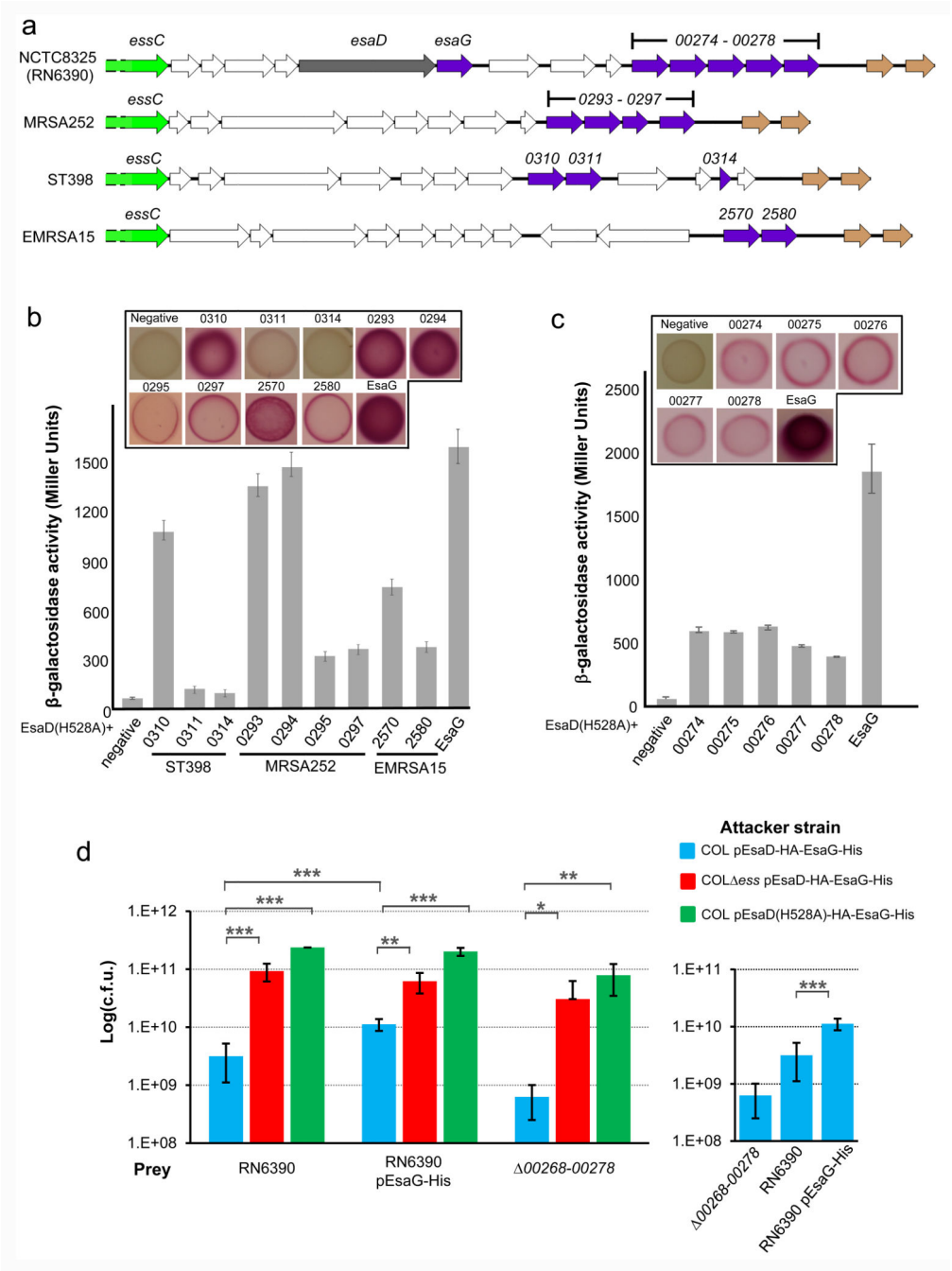

Fig 5. Secreted EsaD kills sensitive strains of *S. aureus*.

a. EsaG homologues are encoded in *S. aureus* strains that lack *esaD*. DUF600-family proteins encoded at the *ess* loci in *S. aureus* strains NCTC8325 (parental strain of RN6390), MRSA252, ST398 and EMRSA15. Genes encoding DUF600 proteins are shaded in purple, *essC* is shaded green and *esaD* grey. The two genes shaded in brown are highly conserved across all strains and define the 3' boundary of the *ess* locus18. b. and c. Interactions between pT25-EsaD(H528A) and DUF600 proteins; b. from strains ST398, MRSA252 and EMRSA15, and c. from strain NCTC8325. In each case the DUF600 reading frame was

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fused to pT18 and interaction with full length EsaD fused to pT25 assessed by β-galactosidase activity assay in *E. coli* BTH101. BTH101 harbouring pT25 and pT18 was the negative control. Error bars are ± SD (n=3 biological replicates). Student's t-test gives p values < 0.00001 relative to the negative control. Insets shows the same strain and plasmid combinations on MacConkey maltose plates. d. *In vitro* growth competition assays between the indicated attacker and prey strains in liquid medium. In each case the attacker strain (COL or COLΔ*ess*) overproduced EsaD-HA or EsaD(H528A)-HA along with EsaG-His as described in Methods, and was incubated with either RN6390, RN6390Δ*00268-00278* or RN6390 pEsaG-His as prey, as indicated. To the right, the three prey strains incubated with COL pEsaD-HA-EsaG-His as attacker are replotted next to each other to allow a more direct comparison. In all experiments five biological replicates of each attacking strain was used against a single culture of prey. Bars represent the average value of c.f.u. of prey bacteria at the end of the experiment. Asterisks indicate significant differences in c.f.u. * p value < 0.05; ** p value < 0.005, *** p value < 0.0005. Comparison of RN6390 with RN6390Δ*00268-00278* survival when COL pEsaD-HA-EsaG-His was used as attacker was not significant (p = 0.069). Error bars are ± SD (n = 5 biological replicates).