Secretion of atypical protein subtrates by the ESAT-6 Secretion System of *Staphylococcus aureus*

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SUMMARY

Staphylococcus aureus encodes the specialized ESAT-6 Secretion System (ESS). EsxA and EsxB are secreted by the ESS pathway, and share sequence features of ESAT-6 and CFP-10 of the Type VII Secretion System (T7SS) of *Mycobacterium tuberculosis.* Unlike ESAT-6 and CFP-10, EsxA and EsxB do not interact. Instead, EsxB associates with a novel substrate, EsxD, and EsxA dimerizes with itself or EsxC (EsaC). Unlike EsxA and EsxB, EsxC and EsxD do not share obvious sequence features of WXG100 proteins nor PE/PPE and Esp families of proteins, all of which belong to the pfam EsxAB clan of mycobacterial T7SS. EsxD carries the C terminal motif YxxxD/E that has been proposed to target T7 substrates for secretion in mycobacteria. Here, we find that deletion, but not amino acid substitutions, in this motif prevent secretion of EsxA and EsxC but not EsxB or EsxD. This is unlike the genetic inactivation of *esxA, esxB, esxC* or *esxD* that leads to loss of secretion of all four substrates. Thus, substrate secretion can be uncoupled by deleting the last six amino acids of EsxD. The physical association of EsxC and EsxD with canonical WXG100 proteins suggests that these proteins belong to the EsxAB clan.

INTRODUCTION

Staphylococcal EsxA and EsxB are small, secreted proteins, that lack a canonical topogenic sequence. Typically, secreted proteins are synthesized as precursor proteins with an N-terminal type I or II leader sequence and are directed to the general Sec machinery. Leader sequences are cleaved following translocation of precursor proteins across the plasma membrane (Schneewind & Missiakas, 2012, Driessen & Nouwen, 2008). EsxA and EsxB belong to the WXG100 family of proteins named after the amino acid sequence motif WXG lying roughly in the center of a 100-amino acid long domain (Pallen, 2002). Genetic disruption of several genes has been shown to affect the synthesis and secretion of EsxA and EsxB. These genes are found in the so-called ESS cluster along with *esxA* and *esxB* and define the ESAT-6 Secretion System (ESS) of *S. aureus* (Burts *et al.*, 2005, Pallen, 2002, Burts *et al.*, 2008, Anderson *et al.*, 2011). This nomenclature is based on sequence features shared with *Mycobacterium tuberculosis* Mt-EsxA also known as Early Secreted Antigen 6 kDa, ESAT-6 and secreted by the ESX-1 pathway (Abdallah *et al.*, 2007). *M. tuberculosis* encodes five clusters, ESX-1 through ESX-5 specifying WXG100 proteins and their cognate secretion apparatus (Stoop *et al.*, 2012). ESAT-6 and CFP-10 form a tight dimer (ESAT-6•CFP-10) and CFP-10 has been shown to carry a C-terminal topogenic sequence that marks ESAT-6•CFP-10 for secretion (Champion *et al.*, 2006). The secretion signal of staphylococcal EsxA and EsxB is unknown but genetic deletion of one impairs the secretion of both gene products, suggesting that perhaps the proteins assemble into a complex (Burts et al., 2005). However, biochemical reconstitution of such a complex could not be achieved. Instead, an EsxA•EsxA homodimer was crystallized and the structure solved by X-ray

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(Sundaramoorthy *et al.*, 2008). Each subunit folds into an elongated cylinder of two helices bent by a hairpin carrying the WXG motif. This structure is reminiscent of other WXG100 protein pairs including ESAT-6•CFP-10 and EsxG•EsxH of *M. tuberculosis* ESX-1 and ESX-3 secretion systems, respectively (Renshaw *et al.*, 2005, Ilghari *et al.*, 2011) and the more unusual EsxR•EsxS pair composed of two four-helix bundles that assemble like two linked ESAT-6•CFP-10 structures (Arbing *et al.*, 2010). The notion that recombinant EsxA from *S. aureus* forms a homodimer leaves unresolved several genetic observations, including its requirement for secretion of EsxB. To address this problem, we sought to examine interacting partners of EsxA and EsxB in *S. aureus.* To this end, ESS-dependent secretion was examined in the clinical strain USA300, the current community acquired Methicillin Resistant *Staphylococcus aureus* isolate (MRSA) for which we previously reported increased ESS activity as compared to strain Newman. In this study, we resolve a previous conundrum by showing that the low ESS activity in strain Newman can be explained by the constitutive allele of the SaeR signaling kinase. Using strain USA300, we identify a new factor that interacts with EsxB, designated herein as EsxD. We find that EsxD is encoded within the ESS cluster and is secreted in an ESS-dependent manner although it lacks the WXG100 signature motif. Deletion of *esxD* abrogated the production of EsxB altogether and affected the secretion, but not the production, of EsxA and EsxC (formerly EsaC (Burts et al., 2008)). Our data suggest that EsxA and EsxC form both homo- and heterodimers whereas EsxB and EsxD appear to function as a heterodimer exclusively. Interestingly, EsxD carries the C-terminal sequence motif previously proposed to represent the universal secretion signal for mycobacterial T7 substrates (Daleke *et al.*, 2012). Alteration of this motif however did not prevent substrate secretion whereas deletion of the last six amino acids blocked secretion of EsxA and EsxC but not EsxB and EsxD. Together, the data suggest that staphylococci encode at least four secreted substrates that can assemble in four pairs containing canonical and non-canonical WXG100 partners and each pair plays an intricate contribution to the integrity of the ESS pathway.

RESULTS

The two-component system SaeRS represses expression to the *ess* gene cluster

The *S. aureus* ESS secretion cluster has been described previously using strain Newman (Burts et al., 2005). However, EsxA and EsxB are produced and secreted at a low level in this strain, as compared to strains USA300 and USA400 (Burts et al., 2008). In strain Newman but not USA300, EsxC (EsaC) is produced only when *esaB* is deleted (Burts et al., 2008). Further, EsxA and EsxB proteins are unstable making it difficult to assign a regulatory or translocation function to specific genes in the ESS cluster (Burts et al., 2008). The secretome of *S. aureus* is controlled at the transcriptional level by several regulators to coordinate environmental cues and virulence (Novick & Geisinger, 2008, Cheung *et al.*, 2004). In particular, the two-component system SaeRS of which SaeS is the signaling kinase and SaeR the response regulator (Giraudo *et al.*, 1994, Giraudo *et al.*, 1997) controls a large set of secreted virulence factors. Strain Newman carries an SaeS allele (L18P) with altered signaling property that renders SaeS constitutively active (Adhikari & Novick, 2008). We hypothesized that perhaps SaeRS is a negative regulator of the Ess cluster, and thus, the cluster would be largely repressed in strain Newman carrying the constitutive SaeS L18P allele. To test this hypothesis, levels of EsxA protein were compared in *S. aureus* strain Newman either wild-type or with *bursa aurealis* transposon insertions in the *saeR* or *saeS* genes (Fig 1A). EsxA production was increased in these mutants as compared to Newman. Increased EsxA as well as EssB and EssD production was also observed in strain USA300 carrying *saeRS bursa aurealis* alleles (Figure 1B). Thus, this finding indicates that the SaeRS locus negatively regulates the expression of the *ess* cluster. For this reason, we selected strain USA300 for subsequent studies on the Ess pathway.

EsxB interacts with EsxD (SAUSA300_0287)

In *S. aureus*, EsxA and EsxB are the only two proteins with an obvious WXG100 domain. Genetic disruption of either gene disrupted the production of both gene products in strain Newman leading to the hypothesis that perhaps the proteins interact physically (Burts et al., 2005). Sundaramoorthy et al. (Sundaramoorthy et al., 2008) reported that EsxA and EsxB purified from *E. coli* do not form a complex, an observation that we were able to confirm independently (Suppl. Figure 1S). These authors obtained crystals of EsxA (but not EsxB) and solved the structure of this recombinant EsxA homodimer (Sundaramoorthy et al., 2008). We wondered whether EsxA and EsxB interact in their natural environment. To this effect, we developed a strain of USA300 that produces histidine-tagged EsxB from a plasmid and purified the tagged protein from culture supernatants using Ni-NTA affinity chromatography. Candidate interacting proteins were eluted in buffer containing 8 M urea. The samples were desalted and subjected to mass spectrometry analysis. This approach consistently yielded a peak with mass 11,442.848, however tryptic digestion did not provide additional information toward the identification of this polypeptide (Figure 2A). A close look at the ESS cluster suggested a possible candidate, SAUSA300_0287, located two genes downstream of *esxB*. SAUSA300_0287 is predicted to encode a polypeptide of 105 amino acids with a calculated mass of 11,414.79, and a high α-helical content. The SAUSA300_0287 protein contains nine lysine residues, thus it would be poorly amenable to mass spectrometry analyses of tryptic digests. The difference in measured and predicted mass could be explained by formylation of the initiator methionine. To test our hypothesis, SAUSA300_0287 was cloned and expressed in strain USA300 with a histidine tag. The corresponding polypeptide was purified over Ni-NTA from the spent medium of bacterial cultures and bound proteins were eluted with imidazole, separated by SDS-PAGE and transferred to PVDF membrane for western blot analyses (Figure 2B). A strain, carrying vector alone, was used for control experiments. Immunoblot analyses revealed the presence of EsxB but not EsxA or EsxC in eluted fractions [Note that EsxC is a new name to designate EsaC, a protein that we described earlier as a non canonical ESS-dependent secreted substrate (Burts et al., 2008)]. Further, histidine-tagged SAUSA300_0287 was identified with a polyclonal antibody raised against the recombinant protein and tentatively designated as EsxD (Figure 2B). To confirm this interaction, recombinant histidine-tagged EsxD (His-EsxD) purified from *E. coli* was applied over GST-glutathione beads preloaded with GST-EsxA, GST-EsxB or GST-EsxC. Aliquots of unbound (U) and bound (B) proteins were separated by SDS-PAGE for Coomassie staining (Figure 2C). Most of His-EsxD eluted in the flow through (U) of the GST-EsxA and GST-EsxC columns, whereas most of it remained bound (B) to GST-EsxB. Interactions between the four proteins were also examined by using a bacterial two-hybrid system that monitored reconstitution of adenylate cyclase activity as increased expression of LacZ β-galactosidase. Candidate interacting pairs yielding active adenylate cyclase were monitored on agar plates containing the chromogenic substrate X-gal (Karimova *et al.*, 1998). As expected, when both fragments of adenylate cyclase were fused to EsxA, bacteria colonies (A:A) turned dark blue however candidate pairs EsxA/EsxB (A:B) or EsxB/EsxB (B:B) did not yield a significant change in color, suggesting preferred self-association for EsxA (Figure 2D; top row). Adenylate cyclase activity was also achieved upon expression of both *esxB* and *esxD* (D:B) but not when *esxD* was expressed in combination with *esxA* (D:A) or with itself (D:D) (Figure 2D; middle row). EsxC was also included in this analysis and the results indicate that like EsxA, EsxC is capable of self-association leading to active adenylate cyclase. Functional reconstitution also occurred for the EsxC and EsxA pair but not when EsxC was paired with EsxB or EsxD (Figure 2D; lower row). Together, the data suggest that EsxB associates preferentially with EsxD while EsxA and EsxC interact as homo- or hetero-oligomers.

EsxD is secreted by the ESS pathway

An immunoblotting approach was used to identify EsxD in bacterial extracts. A culture of *S. aureus* USA300 was grown to mid-logarithmic phase and fractionated by sedimentation to separate intact cells from medium. Cells were lysed with lysostaphin. Trichloracetic acid was added to both fractions (cell and medium) to precipitate proteins for subsequent separation and visualization of sample aliquots by SDS-PAGE and immunoblotting. EsxD was found in both cell and medium fractions of wild type bacteria (Figure 3A). Next, we examined whether the presence of EsxD in these fractions required an intact ESS gene cluster (Figure 3B). Previous studies indicated that at least four proteins EssA, EssB, EssC and EssD (formerly EsaD) are required for secretion of EsxA and EsxB. EssB and EssD have been shown to localize to the membrane (Burts et al., 2005, Anderson et al., 2011, Chen *et al.*, 2012). However, most analyses of the ESS gene cluster were performed in strain Newman using transposon disruption of individual genes, and we deemed it necessary to reassess the function of these genes in strain USA300. Thus, in-frame deletions were generated for the following genes, *essA, essC, esxA, esxB* and *esxC* as well as the newly identified *esxD* gene. Isogenic USA300 mutants in *essB* and *essD* (*esaD*) have been described (Chen et al., 2012, Anderson et al., 2011). The fractionation pattern of EsxD was examined systematically and compared to that of the other known secreted substrates, EsxA, EsxB and EsxC (Figure 3A). Cytosolic ribosomal protein L6 and secreted α-hemolysin (Hla) served as loading and fractionation controls, respectively (Figure 3A). Interestingly, EsxB and EsxD seemed to be unstable in mutants lacking *esxC, esxB, esxD* or *essD* and secretion was abolished in all the mutants (Figure 3A). Loss of stability is in agreement with the notion that EsxB and EsxD form a tight complex. Similarly, secretion of EsxA and EsxC was also abolished in all the mutants (*essABCD, esxABCD*). But unlike EsxB and EsxD, EsxA and EsxC accumulated in the cell pellets when not secreted and were stable in the absence of other substrates. This observation is in good agreement with the notion that EsxA and EsxC may engage in both homo- and hetero-complex formation unlike EsxB and EsxD. In light of these new data, we think it logical to rename EsaC and EsaD, to reflect their function as secreted substrate, EsxC (EsaC), and membrane-embedded component of the secretion machine, EssD (EsaD).

EssD is unstable in a strain lacking *esxD*

We wondered how loss of EsxD could affect the secretion of EsxA and EsxC. First, we asked if secretion of substrates could be restored, by expressing *esxD* on a plasmid (*esxD*/ p*esxD*$_{WT}$). Fractionation of cell cultures (wild type, *esxD* and *esxD*/p*esxD*$_{WT}$) followed by immunoblotting showed that production and secretion of all four substrates was restored, suggesting that deletion of *esxD* did not have a polar effect on the downstream gene *essD* (Figure 4). We wondered whether loss of *esxD* might affect components of the secretion machine. We surmise that EssA, EssB, EssC and EssD are part of this machine and the fates of EssB and EssD for which we have polyclonal antibodies could be easily addressed (Chen et al., 2012, Anderson et al., 2011). Immunoblotting of cellular extracts suggested that EssD, but not EssB, was clearly affected by the deletion of *esxD*. This phenotype could be complemented by plasmid-encoded *esxD* (Figure 4). It is interesting that the three proteins EsxB, EsxD and EssD, all encoded at the end of the ESS cluster have a triangular relationship in that deleting any one of the cognate genes affects the production or stability of all three proteins as judged by immunoblot (Figs. 3A; 4). Thus, the data suggest that the secretion defect observed in the *esxD* mutant could be attributed to a reduction in the levels of EssD.

Contribution of the YxxxE motif at the C-terminus of EsxD

Previously, Champion and colleagues showed that the last 7 amino acids of CFP-10 (Mt-EsxB) are necessary and sufficient for secretion of ESAT-6•CFP-10 (Champion et al., 2006). However, this study did not define a consensus sequence. Recently, Daleke and colleagues showed that C-terminal truncations of PE25 encoded within the ESX-5 cluster of *Mycobacterium marinum* abolished secretion of PE25 and its interacting partner PPE41 (Daleke et al., 2012). In this study, alanine mutagenesis identified tyrosine and glutamic acid at positions 87 and 91 of the PE25 polypeptide, as key residues for secretion. A more extensive analysis identified the YxxxD/E motif in several ESX-1 and ESX-5 substrates of *M. tuberculosis* and *M. marinum,* including the WXG100 CFP-10 protein and EspB and in some instances an experimental validation was also provided (Daleke et al., 2012). Interestingly, the last 6 amino acids of EsxD, $Y_{100}yniE_{104}G$, also encompass this motif. This prompted us to evaluate the universality of the sequence feature for secretion in Firmicutes. Briefly, the complementing plasmid ($pesxD_{WT}$) was modified to encode truncated EsxD lacking the last 6 amino acids ($pesxD_{1-99}$), or a variant with two alanine substitutions at tyrosine 100 and glutamic acid 104 ($pesxD_{Y100A, E104A}$). The plasmids were transferred in the mutant strain lacking chromosomal *esxD* and cultures were fractionated to evaluate the synthesis and localization of secreted substrates by immunoblot. In this assay, $EsxD_{Y100A, E104A}$ behaved similarly to full length $EsxD_{WT}$. However, deletion of the last 6 amino acids, $EsxD_{1-99}$, failed to restore secretion of EsxA and EsxC but surprisingly was dispensable for secretion of EsxB and EsxD (Figure 5). Examination of the machine components EssB and EssD by immunoblot revealed that plasmid encoded $EsxD_{1-99}$, could not restore wild type levels of EssD. In this strain ($esxD/pesxD_{1-99}$), EssD accumulated at about 20% of the level obtained with the complementing plasmid $pesxD_{WT}$. For comparison, expression of $EsxD_{Y100A, E104A}$ restored levels of EssD to about 75% ($pesxD_{Y100A, E104A}$ as compared to $pesxD_{WT}$) (Figure 5). As before, levels of EssB were not altered (Figure 5). The data suggest that EssD is a limiting factor for secretion of EsxA and EsxC. Thus, the last 6 amino acids of EsxD contribute to the stability of EssD and hence influence secretion of other substrates, suggesting perhaps an ordered sequence of secretion events or the existence of discrete sub-translocons for the secretion of substrates.

DISCUSSION

Sec-dependent secretion is a pathway that accounts for the translocation or insertion of proteins across the plasma membrane of bacteria. The genetic requirements for this pathway and the molecular intricacies leading to substrate recognition have been examined extensively (Schneewind & Missiakas, 2012, Chatzi *et al.*, 2013). The Sec pathway is thought to be absolutely essential for all bacterial growth. Many bacterial pathogens have evolved specialized secretion systems for the regulated or coordinated secretion of subsets of proteins that are generally dispensable for their growth in the laboratory, but critical for virulence and replication in infected hosts (Lee & Schneewind, 2001, Thanassi *et al.*, 2012). Gram-positive bacteria in the phylum Firmicutes encode a genetic cluster that bears some similarities with the well-characterized T7SS of acid-fast bacteria *M. tuberculosis, M. smegmatis* and *M. marinum* (Abdallah et al., 2007, Bitter *et al.*, 2009, Stoop et al., 2012). The salient feature of T7-like secretion systems includes the presence of small proteins of the WXG100 and SpoIIIE-FtsK-like ATPase family and was first noticed using a bioinformatic approach (Pallen, 2002). Shortly following this observation, experimental validations provided confirmation that proteins with a WXG100 domain are secreted with the help of several gene products that represent novel secretion systems (Pym *et al.*, 2003, Stanley *et al.*, 2003, Burts et al., 2005). *S. aureus* encodes the ESS cluster that includes two WXG100 proteins, EsxA and EsxB, and one protein with two FtsK-SpoIIIE domains, EssC (Burts et al., 2005). Several proteins in the ESS cluster, including EssC, are required for

secretion of EsxA and EsxB but none, other than these three, shares homology with proteins of T7SS in mycobacteria. Further, the protein EsaC, whose sequence features are only conserved in staphylococci, is also secreted in an ESS-dependent manner (Burts et al., 2008). Here, we show that a fourth protein encoded by SAUSA300_0287 immediately downstream of *esxB* is secreted in the culture medium. Importantly, this protein was identified through its association with EsxB. We hypothesize that such an association occurs in a structural manner that is reminiscent of well-characterized WXG100 homo- and hetero-dimers and thus, we have named this protein EsxD for ESAT-6 secreted extracellular protein D. Further experimentations prompted us to rename EsaC as EsxC and EsaD as EssD (Figure 3B). This rationale is based on the following observations. First, the genetic analysis of the *ess* gene cluster in strain USA300 clearly demonstrates that the capacity to produce EsxB and EsxD, but not EsxA and EsxC, is tightly linked to production of all four proteins. Second, deletion of any one of the four *esx* genes prevents the secretion of all four substrates. Third, ESS-dependent secretion is not observed in mutants carrying in-frame deletion in the *essA, essB, essC* or *essD* genes. Fourth, biochemical and bacterial two hybrid studies demonstrate the existence of the following pairs EsxB•EsxD, EsxA•EsxC, EsxA•EsxA and EsxC•EsxC. Finally, unlike EsxA and EsxC, the EsxB and EsxD proteins appear to be unstable as reflected by the loss of all immune reactive signals when bacteria lack *esxC, esxB, esxC* or *essD*. Thus, the data suggest that in *S. aureus*, the WXG100 protein EsxB is unstable and cannot be secreted unless it is interacting with the non-WXG100 protein EsxD.

Mycobacteria secrete several substrates that have been classified into the EsxAB clan CL0352 (pfam.janelia.org/clan/EsxAB) and encompass pfam06013 (WXG100), pfam00934 (PE), pfam00823 (PPE) and pfam10824 (Esp). While not found in firmicutes, PE and PPE proteins are named after the Pro-Glu (PE) and Pro-Pro-Glu (PPE) motifs located in conserved domains of approximately 110 (PE) and 180 (PPE) amino acids and their function is unknown (Cole *et al.*, 1998). A variable domain is sometimes fused at the C-terminus of PE or PPE proteins (Cole et al., 1998). Experimental evidence suggests that pairs of PE and PPE proteins interact as heterodimers and individual proteins may not be stable when expressed in isolation (Strong *et al.*, 2006). The X-ray structure of one such pair, *M. tuberculosis* Rv2431c (PE) and Rv2430c (PPE) shows that Rv2431c is made of two α-helices that run antiparallel to each other and cradle against two antiparallel α-helices of the PPE protein Rv2430c. This structural organization is reminiscent of WXG100 dimers and may represent a structure uniquely compatible for the T7 translocon. The pfam10824 group includes the ESX secretion-associated proteins, EspA, EspC, EspD encoded in an operon. Any genetic disruption in this operon affects the secretion of ESAT-6 and CFP-10 (Fortune *et al.*, 2005, MacGurn *et al.*, 2005, DiGiuseppe Champion *et al.*, 2009). It has been shown that EspC carries a C-terminal secretion signal that is interchangeable with that of CFP-10 and that it pairs with EspA (DiGiuseppe Champion et al., 2009). A structure for EspA, EspC or EspD is not yet available and homologues are not present in the genomes of Firmicutes. When the primary sequence of EsxD was queried using the Protein Model Portal (www.proteinmodelportal.org), a structural match with 3gwkC was retrieved from the Protein Data Bank (PDB). Although the two proteins, query and structural model, are only 14% identical at the amino acid level, 3gwkC is the crystal structure of the uncharacterized protein SAG1039 of *Streptococcus agalactiae* 2603 V/R that forms a homodimer and bears a canonical WXG100 domain signature. A similar structure has been deduced for the same protein from another strain, GBS1074 from *Streptococcus agalactiae* NEM316 (Shukla *et al.*, 2010).

Like EsxC, EsxD appears to be species specific and homologues of these proteins are not encountered outside of the genus *Staphylococcus*. Nonetheless, the genetic organization shown in Figure 3B is conserved in Firmicutes and members of the genera *Bacillus*,

Streptococcus and *Listeria*, which carry genes of similar size at the locations of *esxC* and *esxD*. Recently, it has been proposed that substrates of T7SS share the C-terminal motif YxxxE/D for secretion in mycobacteria (Daleke et al., 2012). This motif may only be carried by one of the partners in heterodimeric pairs. It was also speculated that the sequence motif could be FxxxD/E to include the CFP-10 protein of other high GC bacteria such as *Nocardia farcinica, Corynebacterium diphtheriae* and *Streptomyces coelicolor* (Daleke et al., 2012). An examination of staphylococcal substrates revealed the presence of two such motifs in the sequence of EsxD, $F_{85}teaD_{89}$ and $Y_{100}yniE_{104}$. $Y_{100}yniE_{104}$ represented the experimentally validated motif of Mycobacteriaceae and was further investigated. While, alanine substitutions in this motif did not alter substrate secretion, deletion of the last six amino acids (YnieEg) affected the stability of EssD, a candidate component of the ESS translocon and impaired the secretion of EsxA and EsxC. It is tempting to speculate that perhaps EsxD is a morphogenic substrate of the ESS translocon and may govern selective or sequential secretion of substrates.

In this work, we also provide some preliminary evidence for a contribution of the SaeRS two-component regulatory system in controlling expression of the ESS pathway. While, we have not performed an in-depth analysis of this regulation, this information has been useful to rule out the use of strain Newman for the study of ESS secretion. The regulation of the *ess* cluster is likely to be complex as is the case for many virulence factors in *S. aureus* (Cheung et al., 2004, Felden *et al.*, 2011, Novick, 2003). A recent analysis suggested that the monocistronic *esxA* transcript is driven by a sigma A (σ^A) promoter, and a putative and inactive σ^B promoter in strain Newman (Schulthess *et al.*, 2012). This analysis also revealed that *esxA* transcripts accumulate in *sarA* mutants, but are decreased in *agr*, *arlR* and *spoVG* mutants. Thus, *esxA* expression is regulated by several transcription factors known to fine-tune the secretome, in order to coordinate pathogenic strategies with bacterial cell density and environmental cues.

It is unclear why some Esx proteins self associate and others interact as heterodimers. Obviously, association may be a feature of secretion and subunits may not remain associated in the host. For example, lowering the pH results in dissociation of the ESAT-6•CFP-10 complex (de Jonge *et al.*, 2007). In *M. tuberculosis* and *M. marium* ESX-1-secreted ESAT-6 may insert into host membranes and form pores to escape intracellular compartments (Smith *et al.*, 2008, De Leon *et al.*, 2012, Hsu *et al.*, 2003). Gram-positive bacteria and acid fast staining bacteria appear to have championed Sec-independent secretion through the evolution of several modules. The fact that these proteins share very little sequence identity, both between and within species, but seem to share the same fold, is interesting. It seems reasonable to infer that both the WXG100 fold and dimerization may be pre-requisites for secretion. Sequence diversity may represent an immune escape mechanism reminiscent of antigenic variation. Further, the structural fold may be uniquely refractory to elicitation of broadly neutralizing antibodies. In *S. aureus*, the ESS pathway is required for persistence of deep-seated abscesses in infected animals (Anderson et al., 2011, Burts et al., 2008). Four secreted proteins must contribute to this process and their exact function and host targets remain to be identified.

EXPERIMENTAL PROCEDURES

Growth conditions

Staphylococci were grown in Tryptic Soy Broth (TSB). Chloramphenicol (Cm) was added to a final concentration of 20 µg/ml for plasmid selection and anhydrotetracycline was used at 50 ng/ml for allelic replacements of target genes (Bae & Schneewind, 2006). To assay for protein production and secretion, cultures were grown with vigorous shaking at 37°C for 2.5 h and the medium was supplemented with naïve horse serum to a final concentration of

0.2%. *E. coli* was grown in Luria Bertani medium at 37° C. When necessary, ampicillin (Amp) and kanamycin (Kan) were used at 100 μg/ml and 50 μg/ml, respectively. Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-indolyl-β-D- galactopyranoside (X-gal) were used at 0.5 mM and 40 μg/ml, respectively.

Cloning and strains

In frame deletion mutants were generated in *essA, essB, essC, esxC, esxB, essE* and *esxD* using the pKOR1 recombination plasmid as described (Bae & Schneewind, 2006). Briefly, 1kbp-flanking regions of DNA upstream and downstream of the target gene were ligated together using a *Bgl*II restriction enzyme site and the resulting 2kbp fragment of DNA was cloned into the pKOR1 plasmid vector using the Gateway BP Clonase II enzyme from Invitrogen (Cat #11789-020). For each construct, the upstream and downstream pieces of DNA contain the first and last 15 codons, respectively, of the gene targeted for deletion. Thus, the two DNA fragments are fused together to create a pseudogene of 30 codons. All cloning steps were performed in *E. coli* and plasmid DNA was first electroporated into strain *S. aureus* RN4220 prior to its introduction into strain USA300 at 30°C in the presence of chloramphenicol. pKOR1 does not replicate at temperatures above 30°C and integrates into the chromosome when cells are grown at 42°C. Selection for homologous recombination, and thus allelic replacement, was performed upon induction of the counter-selection system with anhydrotetracyline as described (Bae & Schneewind, 2006). Strains used in this study are listed in Table S1. For complementation studies, the minimal coding sequence of the *esxD* gene was cloned in plasmid pWWW412 derived from plasmid pOS1 (Bubeck Wardenburg *et al.*, 2006). In this construct, transcription of *esxD* is driven by the constitutive promoter of *hprK* (Bubeck Wardenburg et al., 2006). This plasmid was also used to clone *esxB* and *esxD* with a six-histidine repeat at the N-terminal for purification from *S. aureus* extracts. For protein production and purification in *E. coli*, the minimal coding sequence for EsxA and EsxB were cloned into plasmid pGEX-2TK (GE Healthcare) with N-terminal fusions to Glutathione S-transferase (GST) and *esxD* was cloned into plasmid pET15b (Novagen) resulting in a protein with an N-terminal six-histidine. For bacterial two-hybrid analyses, the minimal coding sequences of *esxA*, *esxB*, *esxC*, and *esxD* were cloned into the pCR®II-Blunt-TOPO® vector (Invitrogen). DNA fragments were excised using *Xba*I and *Kpn*I followed by subsequent ligation into pT18 or pT25 vectors (Euromedex, France) pre-cut with *Xba*I and *Kpn*I, resulting in plasmids pT18*esxA*, pT25*esxA*, pT18*esxB*, pT25*esxB*, pT18*esxC*, pT25*esxC*, pT18*esxD* and pT25*esxD*, respectively. All primers required for cloning are listed in Table S2.

Protein purification and mass measurement

Recombinant proteins were produced in *E. coli* BL21 (DE3). Overnight *E. coli* cultures were refreshed 1:100 in LB and incubated at 30°C until they reached an optical density at 600 nm (OD_{600nm}) of 0.5 at which time IPTG was added to the culture to a final concentration of 1 mM and incubated for 4 h longer. Cells were sedimented by centrifugation, suspended in 50 mM Tris-HCl pH7 buffer A containing 150 mM NaCl, 5% glycerol and frozen at −20°C overnight. Cell pellets were then thawed and lysed in a French pressure cell (Thermo). Crude lysates were centrifuged at 100,000 ×*g* and the filtered supernatant of extracts producing GST-EsxA or GST-EsxB was subjected to affinity purification using glutathione S-transferase affinity chromatography as described by the manufacturer (GE Healthcare). Proteins were either eluted with 10 mM glutatione in buffer A. When necessary, the N-terminal GST tag was cleaved with thrombin and thrombin removed by incubation with benzamadine sepharose beads per manufacturer's recommendations (GE Healthcare). His-tagged EsxD was found in the pellet following sedimentation of crude *E. coli* lysates at 100,000 ×*g* suggesting that recombinant EsxD is prone to aggregation. The pellets were suspended in buffer A containing 8 M urea and the samples were centrifuged once more at

100,000 ×*g* for 40 min. Solubilized His-EsxD was applied to a gravity flow column packed with Ni-NTA agarose beads. The column was washed with 2 column volumes of buffer A containing 8 M urea followed by elution with an imidazole gradient. All recombinant proteins were dialyzed in buffer A and stored at −80°C with the exception of denatured His-EsxD that was desalted in buffer A containing 8 M urea and used immediately to assess its ability to interact with GST-EsxA or GST-EsxB. For this assay, His-EsxD was diluted 10-fold in buffer A to bring the concentration of urea to 0.8M, a non-chaotropic concentration that favored refolding of His-EsxD. The renatured protein was subjected to centrifugation at 100,000 × *g* to remove any insoluble protein and a 1-ml volume was applied to gravity flow columns packed with glutatione beads preincubated with GST-EsxA or GST-EsxB. The 1-ml flow through samples were collected for analysis of unbound proteins whereas the beads were washed with 20 column volumes of buffer A and bound proteins eluted in 1-ml buffer A containing 10 mM glutathione. Proteins in the bound and unbound fractions were subjected to SDS-PAGE and western blot analysis as described below. For purification of His-EsxB and His-EsxD from *S. aureus*, exponentially growing cultures (twice 5 L and 100 ml, for His-EsxB and His-EsxD respectively) were spun and the filtered supernatant was applied to gravity flow columns packed with Ni-NTA agarose beads. The columns were washed with 10-column volumes of buffer A and interacting proteins eluted in buffer A containing 8 M urea. The material was desalted using Sephadex G-25 (GE Healthcare) in buffer A prior to analyses by mass spectrometry and SDS-PAGE followed by immunoblotting.

Animals for the generation of antisera

Experiments involving the generation of polyclonal antibodies using New Zealand white rabbits purchased from Harlan Sprague Dawley, followed protocols that were reviewed, approved and performed under the regulatory supervision of The University of Chicago's Institutional Animal Care and Use Committee (IACUC). Animals were managed by the University of Chicago Animal Resource Center, which is accredited by the American Association for Accreditation of Laboratory Animal Care and the Department of Health and Human Services (DHHS number A3523-01). Animals were maintained in accordance with the applicable portions of the Animal Welfare Act and the DHHS "Guide for the Care and Use of Laboratory Animals". Veterinary Care was under the direction of full-time resident veterinarians boarded by the American College of Laboratory Animal Medicine. Specific antisera were generated by emulsifying 50 μg of purified, renatured His-EsxD and dissolved in 500 μL of PBS with 500 μL of complete Freund's adjuvant (Difco) by sonication and subcutaneous inoculation of female New Zealand white rabbits. Two subsequent boosts were performed by emulsifying protein with incomplete Freund's adjuvant. Serum was harvested by cardiac puncture and stored at −80°C with 0.02% sodium azide.

Fractionation of bacterial cultures and immunoblotting

To assess production of proteins in *S. aureus*, aliquots of cultures were lyzed directly with lysostaphin. To assess secretion, culture aliquots were spun to separate proteins in the medium and the cells. Cells from bacterial pellets were washed and lyzed using lysostaphin. Proteins in these suspensions (lysed cultures or cells and medium) were precipitated by addition of 10 % final concentration trichloroacetic acid, washed in cold acetone, solubilized in 100 μl of buffer B (0.5 M Tris-HCl (pH 8.0), 4% SDS) and heated at 90 °C for 10 min. To assess proteins following purification over Ni-NTA or glutathione agarose beads, 100 μl sample aliquots were mixed with 100 μl of buffer B twice concentrated (2X). Proteins were separated on SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane for immunoblot analysis. Proteins were detected with specific rabbit polyclonal antibodies raised against purified protein at a dilution of 1:5,000 (α-EsxA, α-EsxB, α-EsxC, α-EsxD, α-EssB, α-EssD,) or 1:20,000 (α-L6, α-Hla). Immunoreactive products were revealed by

chemiluminescent detection after incubation with an anti-rabbit HRP-conjugate secondary antibody (1:20,000, Cell Signaling Technology).

Bacterial two-hybrid analyses

Genes *esxA, esxB, esxC* or *esxD* cloned in pT18 or pT25 were transformed into *E. coli* strain BTH101 (Euromedex, France) on agar containing kanamycin and ampicillin. Isolated colonies were used to inoculate LB medium supplemented with kanamycin and ampicillin and grown for 16 h at 37°C. Cultures were diluted to a final OD_{600} 0.5 and 10 µl plated on solid medium supplemented with kanamycin, ampicillin, IPTG and X-Gal. The plates were incubated at 37°C for 24 h to visualize any color change from white to blue as a reflection of positive interaction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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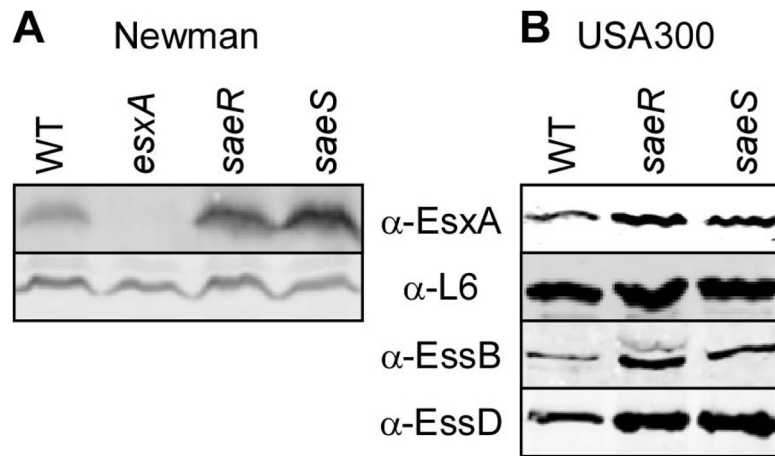
Figure 1. Mutations in the SaeRS two component system alter production of EsxA, EssB and EssD

Bacterial cultures of wild type and isogenic variants of strain Newman (**A**) or USA300 (**B**) grown overnight were diluted 1:100 in fresh medium supplemented with naïve horse serum to a final concentration of 0.2% and cultures were incubated at 37°C for 2.5 h. Proteins in lyzed culture extracts were precipitated with trichloroacetic acid, separated by SDS-PAGE and detected by immunoblotting with specific antibodies (α-EsxA, α-EssB, α-EssD or α-L6 as a loading control).
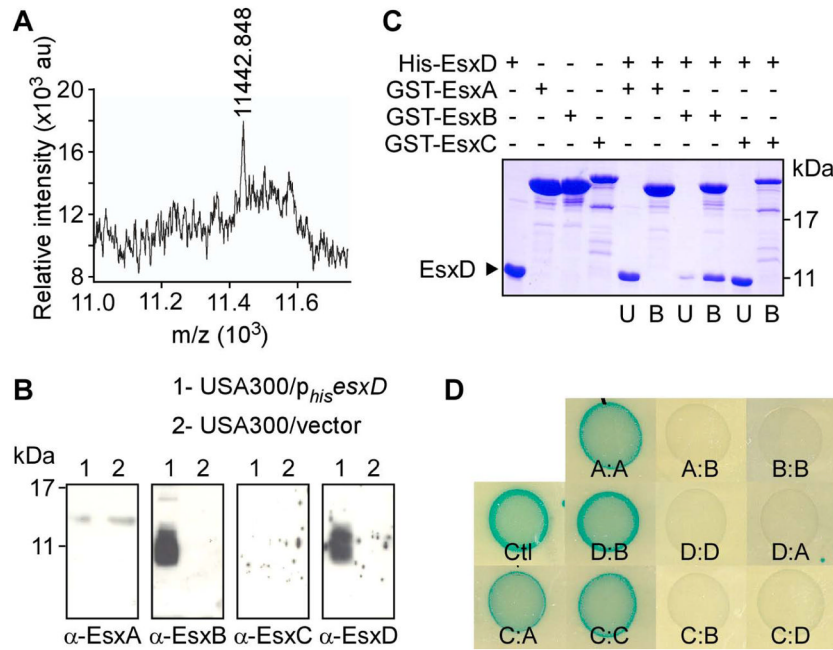
Figure 2. Identification of EsxD as an interaction partner of EsxB

(**A**) Mass spectrometry analysis of sample enriched for a protein of 11442.848 mass that co-purifies with EsxB in *S. aureus* USA300. (**B**) EsxB co-purifies with EsxD in *S. aureus* USA300. Wild type USA300 cultures carrying a plasmid expressing histidine tagged *esxD* (p$_{his}$*esxD*) or control vector were spun and secreted proteins purified over Ni-NTA agarose. Bound proteins were eluted with imidazole, separated on SDS-PAGE and detected by immunoblotting with specific antibodies (α-EsxA, α-EsxB, α-EsxC, α-EsxD). (**C**) EsxD interacts with EsxB *in vitro* but not with EsxA or EsxC. Purified GST-EsxA, GST-EsxB and GST-EsxC were loaded on glutathione sepharose and incubated with purified His-EsxD. U and B denote unbound proteins in the flow through and bound proteins interacting with GST hybrids, respectively. Samples were separated by SDS-PAGE and stained with coomassie. (**D**) Protein interactions examined with the bacterial two-hybrid system. Bacterial cultures were spotted on solid medium containing Xgal, and the blue color indicates a positive interaction as seen for the control (Ctl, middle row left). Interactions were tested for the following pairs: (1) top row, EsxA:EsxA (A:A), EsxA:EsxB (A:B) and EsxB:EsxB (B:B); (2) middle row: positive control (Ctl), EsxD:EsxB (D:B), EsxD:EsxD (D:D), EsxD:EsxA (D:A); (3) lower row: EsxC:EsxA (C:A), EsxC:EsxC (C:C), EsxC:EsxB (C:B), EsxC:EsxD (C:D).
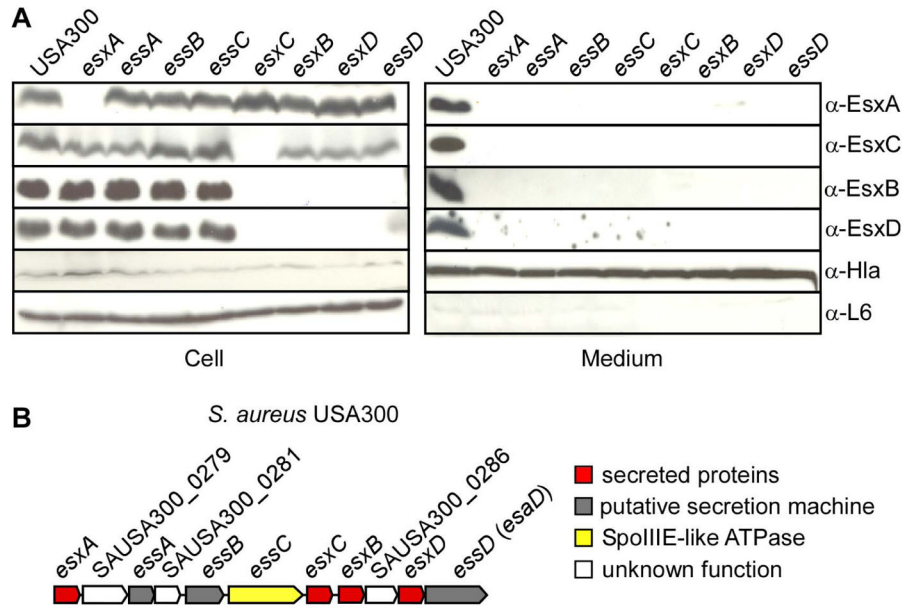
Anderson et al.

Figure 3. Genetic requirements for secretion of Esx proteins
(**A**) Schematic representation of the ESS gene cluster. Proteins with defined functions are indicated as follows: secreted proteins (red), FtsK SpoIIIE-like ATPase (yellow), proposed secretion machinery (grey), not known (white). The locus is annotated for strain USA300. Acronyms Esx and Ess refer to ESAT-6 like secretion extracellular and ESAT-6 like secretion system. Genes previously referred as ESAT-6 secretion accessory (EsaC, EsaD) have been renamed to better reflect their contribution to the secretion system. (**B**) Bacterial cultures of *S. aureus* USA300 (WT) or isogenic mutants, *esxA, esxB, esxC, esxD* and *essA, essB, essB, essC* were fractionated into cells and medium. Proteins in all fractions were precipitated with trichloroacetic acid, separated by SDS-PAGE and detected by immunoblotting with antibodies specific for secreted substrates (α-EsxA, α-EsxB, α-EsxC, α-EsxD) and cytosolic and secreted protein controls (α-L6, α-Hla).
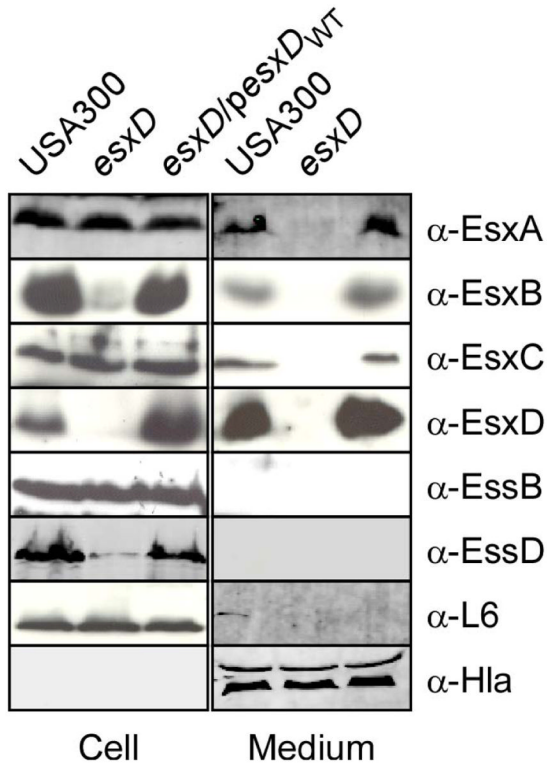
Figure 4. Expression of *esxD* in trans is sufficient to restore the secretion defect of the *esxD* mutant strain

Bacterial cultures of *S. aureus* USA300 or *esxD* mutants harboring or not a complementing plasmid (p*esxD*) were grown to OD_{600nm} of 1.0 and separated into cell and medium fractions. Proteins were precipitated with trichloroacetic acid, separated by SDS-PAGE and detected by immunoblotting with specific antibodies (α-EsxA, α-EsxB, α-EsxC, α-EsxD, α-EssB, α-EssD, and α-Hla or α-L6 for fractionation and loading controls).
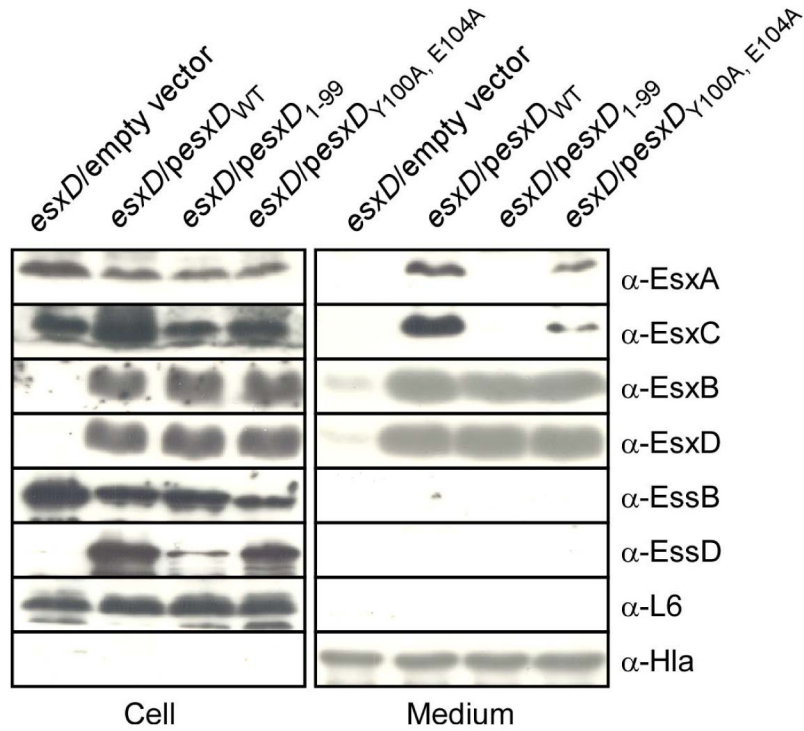
Figure 5. The last six amino acids of EsxD are important for secretion of EsxA and EsxC
Bacterial cultures of the *S. aureus esxD* mutant harboring either the empty vector or vector
with wild type *esxD* (p*esxD*$_{WT}$), truncated *esxD* (p*esxD*$_{1-99}$) or the *esxD* variant with double
alanine substitutions (p*esxD*$_{Y100A, E104A}$) were grown to OD$_{600nm}$ of 1.0 and separated into
cell and medium fractions. Proteins were precipitated with trichloroacetic acid, separated by
SDS-PAGE and detected by immunoblotting with specific antibodies (α-EsxA, α-EsxC, α-
EsxB, α-EsxD, α-EssB, and α-EssD, and α-Hla or α-L6 for fractionation and loading controls).