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EsaC substrate for the ESAT-6 Secretion Pathway and its role in persistent infections of *S. aureus*

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Summary

Staphylococcus aureus encodes the specialized secretion system Ess (ESAT-6 secretion system). The *ess* locus is a cluster of eight genes (*esxA*, *essABC*, *esaABC*) of which *esxA* and *esxB* display homology to secreted ESAT-6 proteins of *Mycobacterium tuberculosis*. EsxA and EsxB require EssA, EssB and EssC for transport across the staphylococcal envelope. Herein, we examine the role of EsaB and EsaC and show that EsaB is a negative regulator of EsaC. Further, EsaC production is repressed when staphylococci are grown in broth and increased when staphylococci replicate in serum or infected hosts. EsaB is constitutively produced and remains in the cytoplasm whereas EsaC is secreted. This secretion requires an intact Ess pathway. Mutants lacking *esaB* or *esaC* display only a small defect in acute infection, but remarkably are unable to promote persistent abscesses during animal infection. Together, the data suggest a model whereby EsaB controls the production of effector molecules that are important for host pathogen interaction. One such effector, EsaC, is a secretion substrate of the Ess pathway and implements its pathogenic function during infection.

Keywords

WXG100 proteins; virulence; abscess formation

Introduction

S. aureus secretes a plethora of virulence factors into the extracellular milieu (Archer, 1998; Dinges *et al.*, 2000; Foster, 2005; Shaw *et al.*, 2004; Sibbald *et al.*, 2006). Like most secreted proteins, these virulence factors are translocated by the Sec machinery across the plasma membrane. Proteins secreted by the Sec machinery bear an N-terminal leader peptide that is removed by leader peptidase once the pre-protein is engaged in the Sec translocon (Dalbey and Wickner, 1985; van Wely *et al.*, 2001). Recent genome analysis suggests that Actinobacteria and members of the Firmicutes encode an additional secretion system that recognizes a subset of proteins in a Sec-independent manner (Pallen, 2002). Recently, this secretion system has been labeled type VII secretion system (Abdallah *et al.*, 2007). ESAT-6 (early secreted antigen target 6 kDa) and CFP-10 (culture filtrate antigen 10 kDa) of *Mycobacterium tuberculosis* represent the first substrates of this novel secretion system termed ESX-1 or *Snm* in *M. tuberculosis* (Andersen *et al.*, 1995; Hsu *et al.*, 2003; Pym *et al.*, 2003; Stanley *et al.*, 2003). In *S. aureus*, two ESAT-6 like factors designated EsxA and EsxB are secreted by the Ess pathway (ESAT-6 secretion system) (Burts *et al.*, 2005).

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M. tuberculosis variants lacking ESAT-6 (*esxA*) or CFP-10 (*esxB*) display severe defects in the establishment of tuberculosis (Guinn *et al.*, 2004; Hsu *et al.*, 2003; Sorensen *et al.*, 1995; Stanley *et al.*, 2003). In *S. aureus*, failure to produce EsxA and EsxB leads to decreased virulence in a murine abscess model of infection, suggesting that the Ess pathway is involved in the pathogenesis of acute staphylococcal infections (Burts *et al.*, 2005). Thus far, three genes, *essA*, *essB*, and *essC*, appear to be important for production of EsxA and EsxB and possibly secretion across the staphylococcal envelope. The genes are encoded within an eight gene cluster conserved in other Gram positive bacteria (Fig. 1). Of those only *esxA*, *esxB*, and *essC*, share homologs with genes of *M. tuberculosis* (Burts *et al.*, 2005; Pallen, 2002). The remaining genes in the cluster, *esaA*, *esaB*, and *esaC*, are dispensable for secretion of EsxA and EsxB and are referred as “accessory” factors for lack of attributable function (*esa*, ESAT-6 secretion accessory) (Burts *et al.*, 2005). The present study examines the role of *esaB* and *esaC* in this secretion system and their contribution to host pathogen interaction.

Results

Sequence analysis of *EsaB* and *EsaC*

esaB encodes an 80 amino acid protein that is conserved in the genome of many Gram-positive bacteria. Further, *esaB*-like genes are always found closely associated on the chromosome of Gram-positive bacteria with *esxA*- and *essC*-like genes (Fig. 1). The crystal structure of *B. subtilis* YukD (*EsaB* homologue) was recently solved and shown to adopt a fold that is closely related to ubiquitin. YukD lacks the C-terminal peptide that is crucial for the activity of ubiquitin, suggesting that YukD is unlikely to modify other polypeptides by covalent linkage (van den Ent and Lowe, 2005). *EsaB* is a predicted soluble protein without a canonical signal peptide. *esaC* encodes a predicted soluble 130 amino acid protein that is conserved in the genomes of staphylococci, but absent from the genomes of other bacteria. In all staphylococcal genomes sequenced thus far, *esaC* is located between *essC* and *esxB* on the staphylococcal chromosome, with the exception of USA200, a strain that harbors an inversion of *esaC* and *esxB*. An unrelated gene, also of unknown function, occupies the position analogous to that of *EsaC* in the genomes of other Gram-positive bacteria (Fig. 1). Although these genes share no homology with staphylococcal *EsaC*, they are of similar size and their individual products also lack amino acid sequence homology. Together these data suggest that a species specific gene occupies the position between *essC*- and *esxB*-homologues of Gram-positive Ess clusters, while *esaB* is conserved amongst these species (Fig. 1).

EsaC protein production is tightly controlled

Using *EsaC* specific rabbit antiserum for immunoblotting experiments, we failed to detect *EsaC* in total extracts of *S. aureus* strain Newman. We wondered whether *EsaC* may be produced in mutants of the Ess cluster and found that only the *esaB* mutant produced *EsaC*, whereas mutations in all other genes had no effect (Fig. 2A). The *esaB* phenotype was complemented by providing wild type *esaB* on a plasmid (Fig. 2B). *EsaB* was produced constitutively (Fig. 2A). We wished to examine whether the expression of *esaC* was negatively controlled in strain Newman. A quantitative RT-PCR analysis was used to compare *esaC* transcript levels in wild type Newman as well as an isogenic variant with transposon insertion in the *esaB* gene. This analysis revealed that *esaC* transcripts are increased 3-fold in an *esaB* mutant as compared to wild type *S. aureus* Newman (Fig. 2C). As a control, transcripts were analyzed from a strain lacking the complete open reading frame encoding *EsaC*. Neither the transposon insertion in *esaB* nor the deletion of *esaC* had polar effects on the expression of downstream genes *essB* and *esxB*, as verified by RT-PCR and immunoblot analyses (data not shown). Pulse labeling of staphylococci with [³⁵S]-

methionine was used to identify newly synthesized EsaC species via immunoprecipitation and autoradiography of proteins separated on SDS-PAGE. While *esaC* transcripts are observed both in wild type and isogenic *esaB* Newman strains, the EsaC polypeptide was only detected in a strain lacking *esaB*, but not in the wild type parent strain Newman (Fig. 2D). This result suggests that *esaC* regulation occurs by a post-transcriptional mechanism and can be relieved by mutations in *esaB*. When the minimal coding sequence of *esaC* was expressed under the control of the constitutive *hprK* gene promoter (pOS-*esaC*), a protein product could readily be detected by immunoblot with anti-EsaC antibodies (Fig. 3; TSB grown bacteria), implying that untranslated *esaC* sequences are required for EsaB-mediated regulation. An attempt to establish whether EsaB may interact with coding or untranslated *esaC* DNA and RNA sequences was unsuccessful. Further, purified EsaB was not found to interact with purified EsaC or stimulate EsaC hydrolysis when mixed with soluble crude extracts of staphylococci. Hence the mechanism whereby EsaB controls *esaC* expression or production remains unclear.

Serum grown staphylococci produce EsaC

We wondered whether EsaB-mediated repression of EsaC might be relieved when staphylococci are grown under conditions that mimic infection. Production of EsaC in *S. aureus* Newman was compared when bacteria were grown in tryptic soy broth (TSB) or serum by immunoblot analysis of whole culture lysates (Fig. 3). *S. aureus* Newman indeed produced EsaC when grown in human serum, suggesting that EsaB-mediated repression is reversible and may be modulated in response to host environmental factors. As noted above, when *esaC* was cloned on plasmid pOS1 and its expression driven by the *hprK* promoter (pOS-*esaC*), production of EsaC appeared to be constitutive (Fig. 3). Thus, production of EsaC is controlled by *cis* acting nucleic acid sequence elements, by EsaB and by host factors that must be present in human serum.

Clinical isolates grown in broth produce EsaC

We wondered whether EsaC production is also regulated in other staphylococcal strains and examined several isolates, including USA100, USA200, USA300, USA700, MW2, Mu50, and N315, all of which were grown to mid-log phase in TSB. Whole culture lysates (WC) were generated by lysostaphin digestion, normalized for total protein concentration, and examined by immunoblot using EsaC or ribosomal protein L6 specific antiserum. EsaC was readily detected in extracts of some staphylococcal strains, in particular strains USA300 and USA700 as shown in Fig. 4A (WC; left panel). Interestingly, DeLeo and colleagues reported that strain USA300 expressed greater amounts of various toxins and in particular exoproteins such as α -toxin, a phenomenon that could in part account for the increased virulence of the strain (Burlak *et al.*, 2007). Unlike *S. aureus* Newman, USA300 strain LAC produced EsaC under normal growth conditions in TSB. We therefore sought to determine whether EsaC production was regulated by EsaB in *S. aureus* USA300. The genome sequences for *S. aureus* Newman and USA300 have been determined, and are closely related in overall sequence and structure (Baba *et al.*, 2008; Diep *et al.*, 2006b). Hence, ϕ 85 was used to transduce the *esaB::erm* allele into strain USA300. USA300 carrying the *esaB::erm* allele and its isogenic parent were grown to mid-log phase in TSB. Whole bacterial culture extracts were generated with lysostaphin digestion, and examined by immunoblot with EsaC or L6 specific antisera. EsaC was detected with increased abundance in the *esaB* variant of *S. aureus* USA300 (Fig. 4A). Thus, even though the more virulent *S. aureus* USA300 can produce EsaC when grown in TSB, disruption of *esaB* causes a similar increase in EsaC production as observed for *S. aureus* Newman.

EsaC is a secreted factor

Cultures of wild type *S. aureus* strains USA300 and USA700 were grown to mid-log phase and proteins in the medium were separated from staphylococci by removing intact cells by centrifugation. Proteins in the supernatants were concentrated ~125 fold and separated on SDS-PAGE. The samples were subjected to immunoblotting and probed with anti-EsaC or anti-L6 (for cell lysis control) antibodies. Data in Fig. 4A (right panel) indicate that EsaC is indeed secreted into the medium of *S. aureus* strains USA300 and USA700. Since EsaC does not carry a canonical signal sequence, we wondered whether it may represent a substrate of the Ess pathway. To test this, plasmid pOS-*esaC* which leads to constitutive EsaC production in *S. aureus* Newman was electroporated in an isogenic variant that cannot express *essC*. EssC is an essential component of the ESAT-6 secretion system. Disruption of the *essC* gene indeed abolished secretion of EsaC and the protein accumulated in the cytoplasm of staphylococci (Fig. 4B). In sum, EsaC appears to be a novel substrate for the non-canonical Ess secretion pathway.

To examine the subcellular localization and efficiency of secretion of EsaC, we took advantage of strain Newman lacking *esaB* and strain USA300, both of which produce EsaC from the chromosomal locus. Cultures of *S. aureus* were separated into cytoplasm, membrane, cell wall, and medium (Fig. 4C; fractions C, M, W, MD, respectively). A whole culture extract was added as control (Fig. 4C; WC). Proteins in all fractions were revealed by immunoblotting with specific antibodies. As expected, strain Newman did not produce EsaC. However, EsaC was found in the culture medium of strains Newman lacking *esaB* and USA300 but not in the cytoplasm, membrane or cell wall, a distribution previously reported for EsxA and EsxB (Burts *et al.*, 2005). EsaC could not be detected in strain USA300 lacking *esxB* (Fig. 4C). Upon extended exposure of the immunoblot, a weak immuno-reactive EsaC species could be detected in the total culture sample but not in the conditioned medium (not shown), suggesting that EsxB is required for EsaC secretion (Fig. 4B). As a control, protein A (Spa) was detected in the cell wall fraction, whereas ribosomal protein L6 and membrane bound sortase A (SrtA) resided in the cytoplasm and the plasma membrane, as expected (Fig. 4C). Together, these results demonstrate that EsaC is secreted across the bacterial envelope into the culture medium in a manner requiring an intact type VII secretion system.

EsaC is produced during infection

Guided by our general hypothesis that the Ess pathway modulates host immune responses, we asked whether EsaC is produced during infection. Mice were infected with *S. aureus* Newman. Blood was collected from infected and control (mock infected) animals on days 0 and 30. The presence of anti-EsaC IgG in serum samples was tested in an ELISA using purified EsaC as immobilized antigen. Data in Fig. 5A show that animals infected with *S. aureus* Newman developed IgG type antibodies against EsaC, suggesting that the protein is synthesized by wild type Newman during infection and presented to the immune system. Further, human sera were collected from two patients that had been diagnosed with *S. aureus* infection and two healthy individuals. An ELISA revealed elevated anti-EsaC IgG in sera of acutely infected patients as compared to healthy individuals (Fig. 5B).

To further evaluate the ubiquitous nature of this host response, we asked whether EsaC antibodies were produced upon infection of mice with *S. aureus* USA100, USA200, USA300, USA700, MW2, Mu50, or N315. Staphylococci were grown to mid-log phase and ~ 10⁶ bacteria were used to infect groups of five three-week old mice. Blood was collected via retro-orbital bleeds on days 0 and 30. The presence of α -EsaC IgG was examined in an ELISA using purified EsaC as antigen (Fig. 5C; only data for day 30 are shown). Mice infected with *S. aureus* produced IgG antibodies against EsaC (Fig. 5C) but not against

SrtA, the transmembrane protein responsible for protein sorting in the bacterial envelope (Fig. 5D). In sum, EsaC is encoded by all staphylococcal strains examined thus far and appears to be produced during host infection. Further, infected hosts develop an antibody response toward EsaC but not SrtA, suggesting that the EsaC antigen must be presented to the host's immune system during infection and may be a secreted antigen in agreement with the general hypothesis that EsaC may be secreted during infection.

EsaB and EsaC are required for persistent infection

An intact type VII secretion system is required for host pathogen interaction both in staphylococci and pathogenic mycobacteria. We wondered whether the accessory factors EsaB and EsaC are also required for staphylococcal replication in infected hosts. To test this possibility, groups of 3-week old mice were challenged with 10^6 colony forming units (CFU) of wild type *S. aureus* Newman or isogenic variants lacking *esaB* or *esaC*. Animals (groups of 10-12) were killed five and thirty-six days after infection. Kidneys were removed *post mortem*. Tissue homogenate derived from the right kidney was spread on agar for colony formation and enumeration of staphylococcal load (Fig. 6), whereas the left kidney was fixed in formalin, thin sectioned and stained with hematoxylin and eosin for histopathology (Fig. 7). As compared to animals inoculated with wild-type *S. aureus* Newman, bacterial load five days following infection was reduced by 1.5 and 0.8 logs in abscesses of animals infected with *esaB* and *esaC* variants, respectively (Fig. 6). Histopathology of kidney tissue at the same time interval revealed that the total number of abscesses was reduced in organs from animals infected with *esaB* (2.1 ± 1.7) or *esaC* (1.5 ± 1.0) variants as compared to the wild-type parent (4.9 ± 2.2) (Fig. 7). Thus, although both *esaB* and *esaC* mutants appear to display virulence defects, these variants retain the ability of forming abscesses in infected host tissues.

Earlier work suggested that virulent *S. aureus* strains may persist in tissues of infected mice for a prolonged period of time (Xu *et al.*, 2004), similar to the clinicopathological features observed with human diseases caused by *S. aureus* (Musher *et al.*, 1994). If so, chronic-persistent features of staphylococcal infections may resemble those observed for tuberculosis, where ESAT-6 secretion is a reported virulence factor for acute infection (Pym *et al.*, 2003; Stanley *et al.*, 2003). To test whether the accessory genes of the staphylococcal Ess pathway contribute to pathogen persistence, animals were infected with wild-type *S. aureus* Newman; bacterial load as well as histopathology were examined 36 days following inoculation (Fig. 6 & 7). The average number of abscesses indeed increased from $4.9 (\pm 2.2)$ on day five to $6.9 (\pm 2.4)$ on day thirty-six, and the size of abscesses increased over time, whereas bacterial load remained persistently high at $2-3 \times 10^6$ cfu. In contrast to wild-type staphylococci, the bacterial load for animals infected with the *esaB* mutant dropped from 5×10^4 cfu on day five to 1.5×10^1 cfu on day thirty-six, while abscesses were either not detectable or were found to occur at reduced frequency and size. Similar to *esaB* variants, deletion of *esaC* also reduced the bacterial load from 2.5×10^5 cfu on day five to 1×10^3 cfu on day thirty-six, with a concomitant reduction in abscess number to $1.8 (\pm 1.5)$ and in abscess size. Of note, *esaC* mutants formed more abscesses and persisted at a higher bacterial load than *esaB* mutants. This observation is in agreement with the conjecture that EsaB may regulate not only *esaC* but also additional staphylococcal genes during infection.

Discussion

Research over the past several decades identified *S. aureus* exotoxins, surface proteins and regulatory molecules as important virulence factors (Foster, 2005; Mazmanian *et al.*, 2001; Novick, 2003). Much progress has been achieved regarding the regulation of these genes. For example, staphylococci perform a bacterial census via the secretion of auto-inducing peptides that bind to a cognate receptor at threshold concentration, thereby activating

phospho-relay reactions and transcriptional activation of many of exotoxin genes (Novick, 2003). During infection, this bacterial census termed Agr ensures massive secretion of virulence factors when staphylococcal counts are high, increasing the likelihood of bacterial spread in infected tissues and/or systemic dissemination (Novick, 2003). In this study, we report that staphylococci produce and secrete EsaC under conditions that occur when bacteria enter host tissues. Production of EsaC is regulated by EsaB, a cytoplasmic conserved protein also encoded within the Ess cluster. EsaB appears to repress EsaC production in a post-transcriptional manner. Bacteria lacking EsaB overproduce EsaC while wild type bacteria do not, unless they are replicating in host tissues. How EsaB mediates repression and senses environmental signals remains unknown. Genes in the *ess* pathway do not appear to be regulated by Agr or staphylococcal counts (data not shown).

The Ess pathway is an alternate secretion system reminiscent of alternate secretion systems of many pathogens (Pugsley, 1993) that transport polypeptides across the bacterial envelope. Like most alternate secretion systems, the Ess pathway appears to transport a limited set of substrates. In mycobacteria and staphylococci, the ESX-1 and Ess pathways transport proteins that belong to the WXG100 family such as ESAT-6, CFP-10, EsxA and EsxB (Burts *et al.*, 2005; Champion *et al.*, 2006; Stanley *et al.*, 2003). The genetic determinants of the ESX-1 and Ess pathways are clustered in discrete loci, dispensable for laboratory growth and essential for the pathogenesis of infectious diseases (Burts *et al.*, 2005; Hsu *et al.*, 2003; Pym *et al.*, 2002; Stanley *et al.*, 2003).

esaC is found only in the genomes of staphylococci, whereas other Gram positive bacteria encode a protein with similar predicted mass but unrelated sequence in the same genetic locus (Fig. 1). As EsaC is regulated and secreted by the Ess pathway, it may represent a unique effector of this secretion system that enables staphylococcal persistence in host tissues. Consistent with this conjecture is the finding that animals and humans mount a humoral immune response to EsaC during infection. During infection, all *S. aureus* strains secrete EsaC, and the more virulent clinical isolates have retained this activity even *in vitro*. EsaC does not bear any features of the WXG100 family of proteins and it is unclear how it is recognized by the Ess pathway. Secretion of non-WXG100 substrates by the ESX-1 pathway has also been reported for several antigens including EspA, EspB, Rv3483c, and Rv3615c (Fortune *et al.*, 2005; MacGurn *et al.*, 2005; McLaughlin *et al.*, 2007; Xu *et al.*, 2007). The alternate ESX-5 pathway has also been shown to secrete both WXG100 and non-WXG100 proteins in pathogenic mycobacteria (Abdallah *et al.*, 2006; Abdallah *et al.*, 2007). Tuberculosis is a chronic persistent bacterial infection that involves manipulation of host immune responses by the invading pathogen. *M. tuberculosis* *esxA* (ESAT-6) and *esxB* (CFP-10) are located within a segment of the genome named *region of difference 1* (RD1), which was identified by comparative genomics of virulent, avirulent and vaccine strains of mycobacteria (Cole *et al.*, 1998; Mahairas *et al.*, 1996; Pym *et al.*, 2002). Thus, RD1 (ESAT-6 secretion) is not only an important virulence pathway of mycobacteria, mutants in this pathway are also the single most prominent attenuating trait of vaccine strains such as BCG (Hsu *et al.*, 2003; Pym *et al.*, 2002; Pym *et al.*, 2003). ESX-1 appears to be important for escape of mycobacteria from the phagolysosome compartments of infected macrophages (Abdallah *et al.*, 2007). Exactly how ESX-1 secreted substrates contribute to escape is unknown.

S. aureus secretes two WXG100 proteins, EsxA and EsxB (Burts *et al.*, 2005) as well as a third polypeptide, EsaC. The contribution of EsaC production and secretion during *S. aureus* infection was revealed by analyzing *esaB* and *esaC* mutants in a murine model of abscess formation. Our observations suggest that the accessory factor EsaB may contribute to staphylococcal persistence. The experiments involved measurements of bacterial load and histopathological lesions in renal tissues. We observed that staphylococci persist within

abscesses of infected host tissues. Herein, we propose that the accessory factors EsaB and EsaC are critical for persistence in tissues. Indeed, bacteria lacking *esaB* or *esaC* are able to seed abscesses in tissues much like wild type staphylococci (Figs. 6 & 7, day 5). However the number of abscesses is reduced for both mutants and, in contrast to wild type infections, decreases over time, especially when bacteria lack *esaB* (Figs. 6 & 7, day 36). We hypothesize that during infection, EsaB-repression of EsaC is relieved. However, this cannot be the sole function of the EsaB protein since abscesses formed by *esaB* mutants fail to persist and are cleared more readily than abscesses resulting from infection with *esaC* variants. A molecular property for EsaB could not be deduced. Although the *B. subtilis* homologue YukD adopts a ubiquitin fold, it does not display ubiquitin-like activity (van den Ent and Lowe, 2005). Staphylococcal EsaB was not found to interact with, or modify EsaC or its corresponding coding sequence (data not shown). Although, a biochemical function for EsaB could not be described, EsaB must regulate not only EsaC but additional factors that also contribute to the establishment of persistent infections. Together, our observations point to the Ess locus as a novel secretion pathway of *S. aureus* that interferes with host immune responses and allows establishment of chronic persistent infections, similar to *M. tuberculosis*.

Experimental Procedures

Bacterial strains, plasmids and growth conditions

S. aureus cells were grown in tryptic soy broth at 37°C, respectively. Chloramphenicol and erythromycin were used at 10 mg l⁻¹, for plasmid and allele selection, respectively, when necessary. *S. aureus* strains MW2, Mu50, N315, USA100, USA200, USA300, and USA700, were obtained through the Network on Antimicrobial Resistance in *S. aureus* (NARSA, NIAID). All mutants used in this study with the exception of *esaC* were obtained from the Phoenix (ΦNΞ) library (Bae *et al.*, 2004). Each Phoenix isolate is a derivative of the clinical isolate Newman (Bae *et al.*, 2004; Duthie and Lorenz, 1952). All *bursa aurealis* insertions were transduced into wild-type *S. aureus* Newman or USA300 using bacteriophage φ85 and verified by immunoblot and PCR analysis. Most but not all USA300 isolates harbor three plasmids one of which encode the *ermC* gene (Diep *et al.*, 2006a). This plasmid can be lost readily (Diep *et al.*, 2006a), permitting the construction of mutants marked with the *ermC* allele.

For deletion of *esaC*, a 2-kbp DNA fragment flanking the *esaC* gene but carrying only the first and last four codons of *esaC* gene was amplified by PCR, with abutted BamHI-EcoRI restriction sites. The DNA fragment was cloned into pKOR1 for allelic replacement performed as described earlier (Bae and Schneewind, 2006). A second *esaC* allele was constructed by cloning a 2-kbp DNA fragment containing *esaC* and 1-kbp nucleotide sequence upstream and downstream of *esaC* respectively, into plasmid pTS1. In this case, a stop codon was introduced at position four of the *esaC* coding sequence. pTS1 carries a mutation that renders its DNA replication in staphylococci sensitive to temperature shift at 43°C. Allelic replacement was performed as described earlier (Burts *et al.*, 2005). Both *esaC* mutant alleles behaved identically and did not prevent production and secretion of EsxB encoded by the gene immediately downstream of *esaC*. All data shown in this study use the mutant carrying the entire deletion of the *esaC* gene.

The *E. coli* – *S. aureus* shuttle vector pOS1 that carries the *hprK* promoter and Shine Dalgarno sequence (275bp upstream of the *hprK lgt yvoF yvcD* translational start site) and three cloning sites NdeI, XhoI, BamHI, as described earlier (Bubeck Wardenburg *et al.*, 2006) was used for complementation studies. All cloning procedures were carried out in *E. coli* and ampicillin was used at 100 mg l⁻¹ for plasmid selection. The complementation plasmids pOS-*esaB* and pOS-*esaC* were generated by amplifying the minimal coding

sequence of each gene using primer pairs EsaB-XhoI-F aactcgagatgaatcagcacgtaaaagt and EsaB-BamHI-R aagatccctatagtaactcaaaatat for *esaB* and EsaC-NdeI-F aacatgaatttaattgatattga and EsaC-XhoI-R aactcgagtaattcattgctttattaaaat for *esaC*.

Culture Fractionation and Western blot experiments

Bacterial cells were grown at 37°C to an optical density of 0.8 at 660 nm (OD_{660nm}) in tryptic soy broth. 1.5 ml of culture was spun ($10,000 \times g$ for 4 min), and supernatants (1 ml) were transferred to a fresh tube. Proteins in the supernatant were precipitated with 7.5% trichloroacetic acid (TCA), and sedimented by centrifugation ($10,000 \times g$ for 10 min) (MD, medium fraction). For whole culture lysates (WC), cultures (1.5 ml) were incubated in the presence of lysostaphin (100 μ g/ml) for 30 min at 37°C and a 1-ml aliquot was precipitated with TCA.

For experiments using serum, colony forming units were counted and approximately 2×10^4 bacteria were added to 1.5 ml freshly drawn human blood placed in a sterile polystyrene round bottom tube. The samples were allowed to incubate with shaking at 37°C for 5 h and spun at $10,000 \times g$ for 4 min. Bacteria in the cell pellet were washed with TSM (Tris-HCl buffer 0.05 M, pH 8.0 containing 30% sucrose and 5 mM $MgCl_2$) to remove any proteins in the serum that would interfere with western blotting analysis and suspended in 1.5 ml Tris-HCl buffer 0.05 M, pH 8.0 containing 100 μ g/ml lysostaphin. 1 ml of the cell lysate was removed and precipitated with 7.5% TCA.

All TCA precipitates were washed with ice-cold acetone, solubilized in 50 μ l of 0.5 M Tris-HCl (pH 8.0)/4% SDS and heated at 90°C for 10 min. Proteins were separated on SDS/PAGE and transferred to poly(vinylidene difluoride) membrane for immunoblot analysis with appropriate polyclonal antibodies. Immunoreactive signals were revealed by using a secondary antibody coupled to horseradish peroxidase and chemiluminescence.

Staphylococcal fractionation

Cultures were centrifuged as described above and supernatants TCA precipitated in the presence of deoxycholic acid (MD, medium fraction of a 5 ml culture). Cell pellets of a 5 ml culture were washed with TSM buffer, suspended in 5 ml TSM buffer containing 100 μ g/ml lysostaphin and incubated at 37°C for 30 min. Protoplasts were collected by centrifugation at $10,000 \times g$ for 10 min, and the supernatant (W, cell wall fraction) was precipitated with TCA. The protoplasts were suspended in 5 ml membrane buffer (0.1 M Tris-HCl, pH 7.5/0.1 M NaCl/10 mM $MgCl_2$) and subjected to five rounds of freeze-thaw in a dry ice ethanol bath. Soluble proteins (C, cytoplasmic fraction) were separated from insoluble materials and membranes (M, membrane fraction) by centrifugation at $100,000 \times g$ for 30 min. All samples were TCA-precipitated before immunoblotting.

Labeling experiments and immunoprecipitation

Staphylococcal cultures were grown overnight in minimal medium, diluted 1:100 into minimal medium to OD_{660nm} 0.8 and metabolically labeled with 100 μ Ci [35 S]methionine for 2 min. TCA (5% final concentration) was added to stop all biological processes. All precipitates were washed with cold acetone and digested with lysostaphin in a 1 ml reaction volume of Tris-HCl buffer 0.5 M, pH 8.0 containing 100 μ g/ml of enzyme for 2 hours at 37°C. Digests were precipitated with TCA, washed with acetone and the samples were boiled in SDS (50 μ l 4% SDS, 0.5 M Tris-HCl, pH 8.0). Insoluble materials were removed by sedimentation. Total radioactive counts were measured using 5 μ l of each sample in a scintillation counter. The incorporation of radiolabeled amino acids was found to be similar between all the samples examined (~ 20 cpm/ μ l). Twenty μ l of each sample were immunoprecipitated with protein-specific antiserum and protein A beads. The beads were washed

five times in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, pH 7.5) and boiled in sample buffer prior to separation on SDS-PAGE. The gels were dried for visualization of radiolabeled polypeptides by autoradiography.

Transcriptional analysis of *esaC*

RNA from approximately 5×10^7 cells grown in tryptic soy broth was isolated using the RNeasy Midi Kit (Qiagen). The RNA was used to generate cDNA with random oligos (Promega). The relative abundance of *esaC* transcripts detected in Newman, *esaB* and *esaC* strains was measured qualitatively by PCR, using *Taq*DNA polymerase (Promega) with primers *EsaC-NdeI-F* and *EsaC-XhoI-R* and *sdrE130F* (tcgattttagtaggtacgac) and *sdrE640R* (tctacttttgaaggcgttg) for amplification of *esaC* and *sdrE* specific DNA fragments, respectively. Real-time PCR (RT-PCR) was performed using the 7300 Real time PCR System (Applied Biosystems) and data analyzed and interpreted using Relative quantification study (Sequence Detection 1.3.1).

Renal abscess

Overnight cultures of *S. aureus* strains were diluted 1:100 into fresh tryptic soy broth and grown for 3 h at 37°C. Staphylococci were centrifuged, washed twice, and diluted in PBS to yield an OD_{660nm} of 0.4 ($3-5 \times 10^7$ cfu per ml). Viable staphylococci were enumerated by colony formation on tryptic soy agar plates to quantify the infectious dose. Mice were anesthetized by intraperitoneal injection of 80–120 mg of ketamine and 3–6 mg of xylazine per kilogram of body weight. One hundred µl of bacterial suspension (0.5×10^6 colony forming units) was administered intravenously *via* retro-orbital injection into BALB/c mice (24-day-old female, 10 mice per group, Charles River Laboratories, Wilmington, MA). On days 5 and 36, groups of ten mice were euthanized by compressed CO₂ inhalation. Kidneys were removed and homogenized in PBS containing 1% Triton X-100. Aliquots of homogenates were diluted and plated on agar medium for triplicate determination of CFU. Student's t-test was performed for statistical analysis using the software Analyse-it™. For histology, kidney tissue was incubated at room temperature in 10% formalin for 24 h. Tissues were embedded in paraffin, thin-sectioned, stained with hematoxylin/eosin, and examined by microscopy.

ELISA

Sera from infected individuals were obtained from the University of Chicago Hospitals Clinical Laboratory. These studies were carried out in accordance with an IRB protocol approved for the collection of sera from infected and healthy individuals. BALB/c mice were infected with one hundred µl of bacterial suspension (0.5×10^6 colony forming units) as described above. Blood samples were drawn by retro-orbital bleeding on days 0 and 30. Sera were examined by ELISA for IgG titers with specific antigen-binding activity. Animal experiments were performed in accordance with institutional guidelines following experimental protocol review and approval by the Institutional Animal Care and Use Committee.

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Fig. 1. Schematic drawing of the *ess* cluster found in various Gram-positive bacteria as well as *M. tuberculosis*. Color of genes and proteins indicates: FtsK-SpoIIIE ATPases (FSD factors), yellow color; ESAT-6 like protein, red color; conserved proteins, grey shades. Genes located at the position of *esaC* are depicted in various colors.

Fig. 2.

EsaB regulates EsaC production. (A-B) Total cell cultures of strain Newman and variants were examined for production of EsaC. Staphylococci were grown in tryptic soy broth. Proteins in whole culture lysates were precipitated with TCA, separated by SDS-PAGE and detected by immunoblotting with specific antibodies [α -EsaC, α -EsaB and α -SrtA as a loading control]. Panel A shows extracts of wild type Newman and isogenic mutants as indicated. Complementation analysis of *esaB* mutant is shown in panel B. Immunoblot analysis of total cell extracts of Newman, *esaB*⁻ with no vector (-), vector alone (pOS), vector carrying *esaB* (pOS-*esaB*). (C) Quantitative RT-PCR analysis of *esaC* transcripts was performed by isolating RNA from *S. aureus* isogenic strains Newman, *esaC*, and *esaB*. Reverse transcriptional polymerase chain reaction (RT-PCR) was carried out using oligos specific for *sdrE* and *esaC* transcripts. *sdrE* transcript levels did not change in all three backgrounds (not shown). The ratio of *sdrE/esaC* transcripts in Newman was 3/1. (D) Cultures of wild type (Newman) and *esaB* or *esaC* mutant cells were radiolabeled with [³⁵S]-methionine for 2 min. Labeling was quenched by addition of trichloroacetic acid, staphylococci were lysed with lysostaphin and extracts solubilized in hot SDS. Total radioactive counts were measured using 5 μ l of each sample in a scintillation counter. Total cell extracts were subjected to immunoprecipitation with anti-EsaC antibodies. Samples were separated on SDS-PAGE and analyzed by autoradiography using a PhosphorImager.

Fig. 3. Staphylococci grown in serum produce EsaC. Staphylococci, Newman, *esaC* mutant with no vector (-), vector alone (pOS), vector carrying *esaC* (pOS-*esaC*), were grown in TSB or serum to the same density, washed and lysed with lysostaphin. Proteins in these extracts were precipitated with TCA, separated by SDS-PAGE and detected by immunoblotting with specific antibodies [α -EsaC, and α -SrtA as a loading control].

Fig. 4.

EsaC is a ubiquitous secreted antigen of the *S. aureus* Ess pathway. (A) *S. aureus* USA300 and USA700 secrete EsaC into the extracellular medium (MD). As control, regulation of EsaC expression in *S. aureus* Newman as well as USA300 is dependent on *esaB* as measured in whole culture lysates (WC). Antibodies against ribosomal protein L6 were used as a control for proper fractionation. (B) *EssC* is required for secretion of EsaC. Immunoblot analysis of total cell extracts of Newman or isogenic *essC* mutant, with vector alone (pOS) or vector carrying *esaC* (pOS-*esaC*). Production and secretion of EsaC was measured in whole culture lysates (WC) and culture supernatants (MD). Antibodies against ribosomal protein L6 were used as a control for loading and fractionation. (C) Subcellular location of EsaC. *S. aureus* cultures of strains Newman, Newman *esaB*, USA300 and USA300 *esxB* were grown to OD_{660nm} 0.8. Equal volumes of cultures were removed for preparation of whole cell lysates (WC) and fractionation of staphylococci into cytoplasm (C), membrane (M), cell wall (W) and medium (MD) fractions. Hence each cellular compartment is kept equimolar to the WC fraction. Proteins were precipitated with TCA, separated on SDS/PAGE, and detected by immunoblotting with specific antibodies [α -EsaC, α -ribosomal protein L6, α -SrtA, α -Spa (protein A)].

Fig. 5.

Mice and humans infected with *S. aureus* generate EsaC IgG specific antibodies. (A) Three-week-old BALB/c mice were injected retro-orbitally with $\sim 10^6$ CFU of strain Newman. Sera were collected on day 0 and 30 days post infection and analyzed for the presence of EsaC reactive antibodies. (B) Quantification of EsaC IgG levels in human sera obtained from patients infected or not with *S. aureus* (two sera each, respectively). (C-D) Three-week-old BALB/c mice were injected as in panel A with clinical strains as indicated on the figure. Sera were collected 0 and 30 days post infection (the 30-day data set is shown). IgG titers to EsaC and Sortase A are shown in panels C and D, respectively. In panel D, a rabbit polyclonal antibody raised against recombinant SrtA was used as a control. All IgG titers were determined in triplicate by ELISA and reported as an absorbance at 405 nm.

Fig. 6. Virulence of *S. aureus* *esaB* and *esaC* mutants. BALB/c mice were infected retro-orbitally with $\sim 10^6$ CFU for each strain. Both kidneys were harvested from mock (PBS) infected animals or mice infected with Newman, *esaB* or *esaC* isogenic variants, for 5 and 36 days and the right kidney for each animal was homogenized. Viable bacteria were counted after dilution and colony formation on tryptic soy agar. Statistical significance was examined with Student's *t* test, and averages and *P* values are indicated. The limit of detection was determined to be 10 CFU.

Fig. 7. Pathological substrate of infection caused by *S. aureus* wild type and *esaB* or *esaC* mutants. Kidneys of mice infected as described in figure 6 were removed 5 and 36 days post infection. The right kidney was used for CFU counts and the left was fixed with formalin. Formalin-fixed tissues were embedded, sectioned, and stained with hematoxylin/eosin. Microscopic images of whole kidneys (x10, top panels) or organ tissue at higher magnification (x100, lower panels) revealed fewer and less persistent abscesses in *esaB* or *esaC* infected animals. White arrows point to abscesses with a central concentration of staphylococci and PMN infiltrate. Numbers under each panel indicate the average number of abscesses per kidney with standard deviation, between 8 and 12 kidneys were examined per group. Statistical significance was examined with the Student *t* test, and *P* values were recorded.