EsxA and EsxB are secreted by an ESAT-6-like system that is required for the pathogenesis of *Staphylococcus aureus* infections

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Mycobacterium tuberculosis secretes ESAT-6, a virulence factor that triggers cell-mediated immune responses and IFN-γ production during tuberculosis. ESAT-6 is transported across the bacterial envelope by a specialized secretion system with a FSD (*F*tsK-*S*poIIIE *do*main) membrane protein. Although the presence of ESAT-6-like genes has been identified in the genomes of other microbes, the possibility that they may encode general virulence functions has hitherto not been addressed. Herein we show that the human pathogen *Staphylococcus aureus* secretes EsxA and EsxB, ESAT-6-like proteins, across the bacterial envelope. Staphylococcal *esxA* and *esxB* are clustered with six other genes and some of these are required for synthesis or secretion of EsxA and EsxB. Mutants that failed to secrete EsxA and EsxB displayed defects in the pathogenesis of *S. aureus* murine abscesses, suggesting that this specialized secretion system may be a general strategy of human bacterial pathogenesis.

specialized secretion | Gram-positive | exoprotein | *ess*

Staphylococcus aureus and many other Gram-positive bacteria are known to secrete virulence factors or exotoxins into the extracellular milieu. These factors are secreted via the Sec translocon across the membrane by a mechanism that requires N-terminal signal peptides for recognition. Recent advances in *Streptococcus pyogenes* and *Bacillus subtilis* revealed that the translocon may be part of a larger complex (ExPortal), that organizes transport of signal peptide-bearing precursor proteins to dedicated sites within the bacterial cell envelope (1, 2). Sec-independent translocation of virulence factors has been observed in group A streptococci and other Gram-positive species (3). The mechanism of substrate recognition and transport for these "signal peptide less" polypeptides has thus far not been revealed (4). In group A streptococci, secretion across the plasma membrane and injection of polypeptides into the cytosol of host cells uses a cytolysin-mediated transport mechanism (5), that functionally resembles type III secretion of Gram-negative bacteria (6).

Mycobacteria are equipped with the typical Sec machinery. However, much attention has been drawn to the secretion of two virulence factors, ESAT-6 (early secreted antigen target 6 kDa) and CFP-10 (culture filtrate antigen 10 kDa), encoded by *Mycobacterium tuberculosis* H37Rv *esxA* (Rv3875) and *esxB* (Rv3874). *M. tuberculosis* variants lacking *esxA* display defects in the establishment of tuberculosis and EsxA induces a strong T-cell response during infection (7). Comparative genomics of mycobacteria related to *M. tuberculosis* H37Rv identified multiple regions of difference that have been deleted from both virulent and avirulent species (8–10). *esxA* (Rv3875) is located within region of difference 1 (RD1). RD1 is the only locus specifically deleted in the attenuated *Mycobacterium bovis* bacillus Calmette–Guérin (bacille Calmette-Guérin) vaccine strain and avirulent *Mycobacterium microii* strain (8, 10–13). Although RD1 is certainly not the only virulence trait of mycobacteria, it appears to be a prominent factor in attenuating vaccine strains (10, 14, 15).

A survey of *M. tuberculosis* H37Rv genome sequence revealed at least 23 ESAT-6 homologues (9), 10 of which are encoded within five gene clusters that specify large soluble and membrane-bound ATPases with two or more FtsK/SpoIIIE-like domains (FSDs) (16, 17). Pallen (17) discovered ESAT-6 homologues in the sequenced genomes of other Gram-positive bacteria including *B. subtilis*, *Bacillus anthracis*, *Clostridium acetobutylicum*, *Listeria monocytogenes*, and *S. aureus*. The genes for the ESAT-6-like proteins are clustered with at least one gene encoding an FSD-type membrane protein (Fig. 1*B*), leading Pallen to propose that FSD ATPases may represent a universal portal for excretion of ESAT-6-like proteins (17).

Experimental support for this hypothesis was garnered when deletions of FSD-like genes (Rv3870, Rv3871) prevented the secretion of *M. tuberculosis* ESAT-6 and CFP-10 (14, 15, 18, 19). A third membrane protein encoded by Rv3877/snm4 was found to be required for secretion of ESAT-6 and CFP-10 (18). Therefore, in mycobacteria secretion of ESAT-6 and CFP-10 requires a specialized mechanism encoded by genes flanking *esxA* and *esxB* on the chromosome (Fig. 1*B*). This specialized secretion system has been referred to as Snm for *secretion* in *m*ycobacteria (18). Most importantly, this dedicated secretion pathway may explain the absence of processing of ESAT-6 or CFP-10 during secretion (7).

Although it has been surmised that FSD proteins may represent a general Gram-positive secretion system (17), this compelling hypothesis is still in need of experimental verification. Here, the putative ESAT-6 secretion system (Ess) encoded by a cluster of eight genes in the Gram-positive pathogen *S. aureus* has been examined (Fig. 1*B*). Two ESAT-6-like proteins, Ess extracellular A (EsxA) and EsxB, were found to be transported across the bacterial envelope. Furthermore, the requirement of the FSD protein, EssC, as well as two other membrane proteins, EssA and EssB, for transport of EsxA and EsxB in *S. aureus* was demonstrated. The importance of EsxA and EsxB secretion during *S. aureus* infection was revealed by analyzing *esxA*, *essC*, and *esxB* mutants in a murine model of abscess formation.

Materials and Methods

Strains, Media, and Growth Conditions. *Escherichia coli* and *S. aureus* were grown in Luria–Bertani broth and tryptic soy broth at 37°C, respectively. Ampicillin and erythromycin were used at 100 mg·liter^{-1} and 10 mg·liter^{-1}, respectively. *essABC*, *esaAB*, and *esxB* mutants were obtained from the Phoenix (ΦNΞ) library (20) (Table 1). Each Phoenix isolate is a derivative of the clinical isolate Newman (21) and carries the transposon *bursa aurealis* at a site on the chromosome that has been determined by DNA sequencing (20) and compared to *S. aureus* Mu50
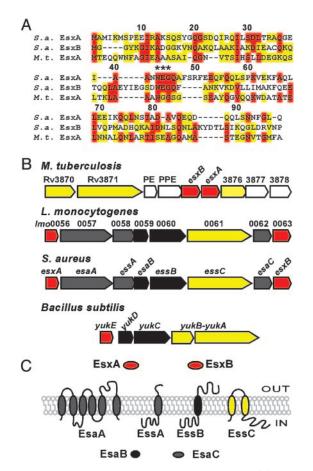
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Fig. 1. *S. aureus ess* locus encoding ESAT-6-like proteins. (*A*) Protein sequence alignment of *S. aureus* (S.a.) EsxA and EsxB with *M. tuberculosis* (M.t.) EsxA. *M. tuberculosis* and *S. aureus* EsxA display 20.8% identity and 25% similarity, whereas *S. aureus* EsxB and *M. tuberculosis* EsxA are 17.8% identical and 35% similar. All three proteins contain the WXG motif. (*B*) Comparison of the *M. tuberculosis* H37Rv ESAT-6 locus with the *S. aureus*, L. monocytogenes *ess* loci and the *B. subtilis yuk* locus. Color of genes and proteins indicates FSD factors (yellow), ESAT-6-like (red), mycobacterial genes (light gray), and staphylococcal (also in listeria or bacilli, dark gray). (*C*) Membrane topology or soluble character of proteins encoded by the *S. aureus ess* locus.

strains overproducing EsxA and EsxB used for antibody production are described in the supporting information, which is published on the PNAS web site.

Culture Fractionation. Cultures were grown to an optical density of 0.8 at 660 nm (OD$_{660}$), and 1.5 ml of culture was centrifuged at 10,000 × *g* for 4 min. Proteins in 1 ml of supernatant were precipitated with 7.5% trichloroacetic acid (TCA), using deoxycholic acid (0.2%) as a carrier, and sedimented by centrifugation 10,000 × *g* for 10 min (medium fraction). Culture (1.5 ml) was incubated in the presence of lysostaphin (100 μg/ml) for 30 min at 37°C. A 1-ml aliquot was precipitated with TCA (total culture). For extraction of proteins that are associated with the cell wall (loosely associated fraction), the cells of 1.5 ml of culture were washed three times with an equal volume of TSM buffer (50 mM Tris·HCl, pH 7.5/0.5 M sucrose/10 mM MgCl$_2$). A 1-ml cell suspension was transferred to a fresh tube and precipitated with TCA. All TCA precipitates were washed with ice-cold acetone, solubilized in 50 μl 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 (medium). Cell pellets were suspended in 1 ml TSM buffer containing 100 μg/ml lysostaphin and incubated at 37°C for 30 min. Protoplasts were collected by centrifugation at 10,000 × *g* for 10 min, and the supernatant (cell wall fraction) was precipitated with TCA. The protoplasts were suspended in 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 (cytoplasmic fraction) were separated from insoluble materials and membranes (membrane fraction) by centrifugation at 100,000 × *g* for 30 min. All samples were TCA-precipitated before immunoblotting.

Murine Abscess Model. *S. aureus* strains were grown at 37°C overnight in tryptic soy broth, diluted 100-fold in fresh broth and incubated at 37°C until an OD$_{660}$ of 0.4 was reached. Cells were washed and suspended in PBS, and 100 μl of bacterial suspension was injected intravenously into 10 6- to 8-week-old female BALB/c mice anesthetized by intraperitoneal injection of 100 mg/ml ketamine and 2 mg/ml xylazine. Viable staphylococci

genomic sequence (22). All *bursa aurealis* insertions were transduced into wild-type *S. aureus* Newman by using bacteriophage φ85 (23). Strain *esxA24* lacking the EsxA gene product and

Table 1. *S. aureus* strains used in this study

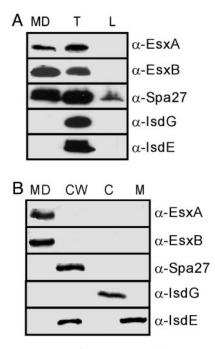| Strain	Genotype	*bursa aurealis* insertion/mutation			Phenotype	
		nt position	AA position	Orientation	EsxA secretion	EsxB secretion
Newman	Clinical isolate				Yes	Yes
ΦNΞ187–01	*esxA24*	24	8	NA	No	No
ΦNΞ09349	*esaA*::erm	1110	370	(+)	Yes	Yes
ΦNΞ03742	*essA*::erm	121	41	(−)	No	No
ΦNΞ 13152	*esaB*::erm	43	15	(+)	Yes	Yes
ΦNΞ 02411	*essB*::erm	199	67	(+)	No	No
ΦNΞ04464	*essC*::erm	792	264	(−)	No	No
ΦNΞ02191	*essC*::erm	1854	618	(−)	No	No
ΦNΞ02832	*essC*::erm	2161	721	(+)	Low	Low
ΦNΞ13038	*essC*::erm	3835	1,279	(+)	Yes	Yes
ΦNΞ07912	*esxB*::erm	2	1	(+)	No	No

All Phoenix strains (ΦNΞ) are isogenic to *S. aureus* Newman (21) and are part of our laboratory collection (20). In strain *esxA24,* a stop codon replaces the coding codon at position 8. AA, amino acid; erm, erythromycin resistance gene; nt, nucleotide.

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were enumerated by colony formation on tryptic soy agar to quantify the infection dose [$\approx 10^6$ colony forming units (cfu)/ ml]. Four days after challenge, mice were killed by CO_2 asphyxiation. Spleen, kidneys, and liver were removed, and organs were homogenized in 1 ml of 1% Triton X-100 in PBS. Dilutions of the homogenates were plated on agar for enumeration of viable staphylococci. The statistical analysis of the data were performed with Student's t test using ANALYZE-IT (Analyze-It Software, Leeds, England).

Results

A Cluster of *S. aureus* Genes Encoding ESAT-6-Like Proteins. *S. aureus* EsxA and EsxB correspond to the Mu50 ORFs SAV0282 and SAV0290 (22) and display 20.8% and 17.8% identity as well as 25% and 35% similarity to *M. tuberculosis* EsxA, respectively (Fig. 1*A*). The peptide sequences of *S. aureus* EsxA and EsxB encompass the WXG motif, a signature sequence of ESAT-6-like proteins (17) (Fig. 1*A*). EsxA and EsxB are encoded within a cluster comprised of eight predicted ORFs (*esxA*, *esaA*, *essA*, *esaB*, *essB*, *essC*, *esxY*, and *esxB*) as shown in Fig. 1*B* (*esa*, ESAT-6 secretion accessory gene). Beside *esxA* and *esxB*, the other genes in this cluster appear to be unique in the chromosome. The cluster is referred to as the Ess cluster. Genes located immediately downstream of *esxB* are not depicted in Fig. 1*B*. However, their conservation in other related clusters from low G+C organisms suggest that they may also be functionally associated with the Ess cluster. The deduced peptide sequence of one of these genes, *essC*, displays similarity with FSD proteins of the *M. tuberculosis* RD1 cluster (Fig. 1*B*). Because of a sequencing error, *essC* entry in the Mu50 genome appears as two ORFs (SAV0287–SAV0288) when it is, in fact, a single combined ORF (data not shown). Fig. 1*C* shows the membrane topology of four proteins, EsaA, EssA, EssB, and EssC, as predicted by the TMHMM algorithm (www.cbs.dtu.dk/services/TMHMM-2.0) and PSORT-B (24). Four other factors (EsxA, EsxB, EsaB, and EsaC) are predicted by the same computational methods to be soluble, and not membrane associated. A summary of the relevant features of proteins encoded by the *ess* locus is presented in Table 2, which is published as supporting information on the PNAS web site.

EsxA and EsxB Are Secreted into the Culture Medium of *S. aureus*. To study the location of EsxA and EsxB, cultures of *S. aureus* strain Newman were centrifuged and bacterial cells in the sediment were separated from the supernatant containing conditioned culture medium. Staphylococci were suspended in buffer and the cell wall was digested with lysostaphin, an endopeptidase that cleaves staphylococcal peptidoglycan (25). Proteins in the total lysate of staphylococci (T) or the culture medium (MD) were precipitated with TCA before separation on SDS/PAGE and immunoblotting. EsxA and EsxB were detected with specific rabbit antisera and were found in the total lysate and in the culture medium, indicating that *S. aureus* strain Newman secretes both polypeptides across the bacterial envelope (Fig. 2*A*). As a control, *S. aureus* IsdG, a cytoplasmic protein, and IsdE, a membrane lipoprotein, were found in total lysates but not in the bacterial culture medium (Fig. 2*A*) (26). As a control for polypeptides that are covalently anchored to the cell wall, we also examined staphylococcal protein A (27). Cell wall degradation products, i.e., peptidoglycan fragments with linked polypeptide, are being released from the staphylococcal surface, and some protein A can therefore be found in both the total lysate and culture medium (28) (Fig. 2*A*). Staphylococci were harvested from cultures by centrifugation and were boiled in hot SDS to release polypeptides that are only loosely associated with the cell wall (L). Boiling in hot SDS released small amounts of protein A, but not IsdE and IsdG. This is an expected result as the cell wall of staphylococci cannot be disintegrated by boiling in ionic
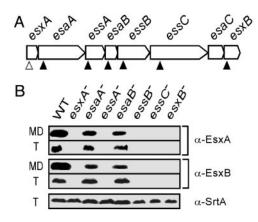

Fig. 2. Subcellular location of EsxA and EsxB. (*A*) *S. aureus* strain Newman cultures were separated into medium (MD), total (T), and loosely wall-associated (L) fractions. Proteins were precipitated with TCA, separated on SDS/PAGE, and detected by immunoblotting with specific antibodies [α-EsxA, α-EsxB, α-Spa (protein A), α-IsdG, and α-IsdE]. (*B*) *S. aureus* strain Newman cultures were fractionated into medium (MD), cell wall (CW), cytoplasm (C), and membrane (M) compartments. TCA-precipitated proteins in each compartment were revealed by SDS/PAGE and immunoblotting.

detergents (29). EsxA and EsxB were not found to be associated with the staphylococcal surface after boiling bacteria in hot SDS, suggesting that staphylococci ESAT-6-like proteins are soluble in the extracellular milieu.

Subcellular Localization of EsxA and EsxB. To examine the subcellular localization of EsxA and EsxB in staphylococci, cultures of *S. aureus* strain Newman were separated into cytoplasm, membrane, cell wall, and medium fractions (Fig. 2*B*). Proteins in all fractions were revealed by immunoblotting with specific antibodies. EsxA and EsxB were found exclusively in the culture medium. As a control, protein A was detected in the cell wall fraction, whereas IsdG and IsdE resided in the cytoplasm and the plasma membrane or cell wall of staphylococci, respectively (Fig. 2*B*). Together, these results demonstrate that *S. aureus* strain Newman secretes EsxA and EsxB across the bacterial envelope into the culture medium.

Factors Affecting the Production and Secretion of EsxA and EsxB. We wondered whether the factors encoded by the *S. aureus ess* cluster regulate production or promote secretion of EsxA and EsxB. Previous work from our laboratory generated the Phoenix (ΦNΞ) library, a collection of *bursa aurealis* insertion mutants in *S. aureus* strain Newman that have been mapped by inverted PCR and DNA sequencing (20). *S. aureus* strain Newman variants ΦNΞ09349, ΦNΞ03742, ΦNΞ13152, ΦNΞ02411, ΦNΞ04464, and ΦNΞ07912 carry *bursa aurealis* insertions in *esaA*, *essA*, *esaB*, *essB*, *essC*, or *esxB*, respectively (Table 1 and Fig. 3*A*). *S. aureus* Newman variant ΦNΞ187-01 carries a stop codon at position 8 of *esxA* coding sequence. The ability of these mutant strains to synthesize and secrete EsxA and EsxB was examined by immunoblotting of culture medium and total lysostaphin lysate samples as described in the legend of Fig. 2*A*.
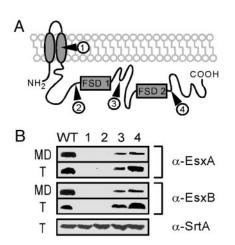
Fig. 3. Factors affecting the production and secretion of EsxA and EsxB. (*A*) Schematic drawing of the *ess* cluster with the position of *bursa aurealis* insertions (filled triangles) and *esxA24* mutation (open triangles). (*B*) Medium (MD) and total protein fractions (T) were separated on SDS/15% PAGE and immunoblotted with α-EsxA and α-EsxB. α-SrtA is used as a control for total protein loaded.

Bursa aurealis insertions at nucleotide positions 1,110 (codon 370) of *esaA*, or 43 (codon 15) of *esaB* did not affect the production or secretion of EsxA and EsxB when examined by immunoblot analysis (Fig. 3*B*). However, *bursa aurealis* insertions at nucleotide positions 121 (codon 41) of *esaA*, 328 (codon 110) of *essB*, or 792 (codon 264) of *essC* abolished production and thus secretion of both EsxA and EsxB polypeptides as immunoreactive species could not be detected in either the medium or total culture lysate of strains ΦNΞ03742, ΦNΞ02411, and ΦNΞ04464, respectively (Fig. 3*B*). One *bursa aurealis* insertion in the Phoenix library mapped to nucleotide 2 (codon 1) of *esxB* (mutant ΦNΞ07912), a mutation that abolished the expression of all EsxB immunoreactive species (Fig. 3*B*). The inability of strain ΦNΞ07912 (*esxB::erm*) to produce EsxB abolished all EsxA production and, therefore, secretion. Conversely, the inability of strain ΦNΞ187–01 (*esxA24*) to produce EsxA abolished all EsxB production as well (Fig. 3*B*). None of the mutants used here abolished the transcription of either *esxA* or *esxB* as judged by RT-PCR (see *Supporting Text*, which is published as supporting information on the PNAS web site), suggesting that the phenotypes observed were not caused by polar effects of mutations or to transcriptional down-regulation.

EsaA is a 1,009-aa polytopic membrane protein with six predicted transmembrane helices that is conserved in staphylococcal and listerial species but absent from mycobacteria (Fig. 1*B*). *essA* and *esaB* specify 152- and 80-aa long protein products, respectively. Bioinformatic prediction of subcellular localization suggests that EssA contains one transmembrane domain, whereas EsaB may be soluble as it lacks a canonical signal peptide (Table 1). EssB and EsaB share sequence homology with listerial genes located in a similar *ess* cluster, and with *B. subtilis* YukC and YukD, respectively (30). The function of YukC and YukD is unknown, but their corresponding genes map to a chromosomal locus with *yukA*, *yukB*, and *yukE* (30) (Fig. 1*B*). YukE bears the WXG motif typical of ESAT-6-like proteins.

EssC is homologous to two genes of *M. tuberculosis*, Rv3870 (snm1) and Rv3871 (snm2), and to *B. subtilis* YukA–YukB. Recent sequence analysis revealed that *yukA–yukB* represents a single ORF (31). EssC, Rv3870, Rv3871, and YukA sequences encompass one or more FSDs (17). Gene SA0276 (*essC*, in the N315 genome) is annotated as "conserved hypothetical protein, similar to diarrheal toxin." The annotation derives from similarity to the *Bacillus cereus* gene *bceT* and appears to be erroneous (32). Bioinformatic analysis predicted two transmembrane helices at the N terminus of the 1,479-aa EssC polypeptide
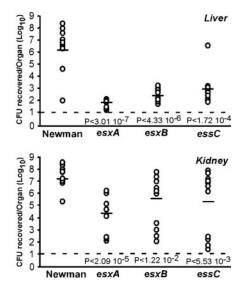

Fig. 4. Role of EssC in EsxA and EsxB secretion. (*A*) Predicted topology of EssC with insertion sites of *bursa aurealis* in each strain (filled triangles). EssC contains two FstK/SpoIIIE-like domains (FSD). (*B*) Immunoblotting of total cellular protein (T) or culture medium (MD) with α-EsxA and α-EsxB antibodies. Insertional mutations at codon 264 (mutant 1) and codon 618 (mutant 2) prevented production/secretion of EsxA and EsxB. Mutants 1–4 are strains ΦNΞ 04464 (insertion at codon 264), ΦNΞ 02191 (insertion at codon 618), ΦNΞ 02832 (insertion at codon 721), and ΦNΞ 13038 (insertion at codon 1279), respectively. α-SrtA was used as a control for the total protein loaded.

(Table 1 and Fig. 1*C*). The N and C termini of EssC are presumed to reside in the staphylococcal cytoplasm based on the assumption that the FSDs hydrolyze ATP in this compartment.

Phenotypic Analysis of Various Insertion Mutants in *essC*. The Phoenix library contains several strains carrying *bursa aurealis* insertions in the *essC* gene (Table 1 and Fig. 4*A*). Four *essC* mutants were used to analyze the effect of EssC truncations on the secretion of EsxA and EsxB (Fig. 4*B*). As shown in Fig. 3*B*, strain ΦNΞ 04464 carries a transposon insertion at nucleotide 792 (codon 264, mutant 1) (Fig. 4*A*), whereas strains ΦNΞ 02191, ΦNΞ 02832, and ΦNΞ 13038 carry *bursa aurealis* insertions at nucleotide positions 1854 (codon 618, mutant 2), 2161 (codon 721, mutant 3), and 3835 (codon 1,279, mutant 4), respectively (Fig. 4*A*). None of these transposon insertions affected the transcription of the two genes downstream (data not shown). Transposon insertion at codons 264 and 618 of *essC* abolished all production and secretion of EsxA and EsxB. In contrast, the synthesis and secretion of EsxB or EsxA occurred when *bursa aurealis* inserted at codons 721 and 1279 (Fig. 4*B*). These results imply that a single FSD domain is sufficient for EssC activity, and suggest that EsxA and EsxB secretion may be fueled by EssC-mediated ATP hydrolysis.

esxA, esxB, and essC Mutants Are Defective in the Pathogenesis of Staphylococcal Abscesses. *S. aureus* host infection and dissemination within organ tissues depends on staphylococcal synthesis and secretion of a wide variety of virulence factors (33). Because of the astonishing armament of staphylococci with virulence factors, the pathogenesis of *S. aureus* infections is considered multifactorial (33). Thus, with the exception of α-hemolysin (*hla*) mutants (34), strains carrying mutations that abrogate the expression of individual exoproteins typically do not display significant defects in the pathogenesis of *S. aureus* infections (35–37). We wondered whether the Ess secretion pathway might be involved in the establishment of staphylococcal disease. *S. aureus* Newman variants ΦNΞ187–01 (*esxA24*), ΦNΞ07912 (*esxB::erm*), and ΦNΞ04464 (*essC::erm*) do not synthesize EsxA and EsxB. These strains were chosen for experimental measurement of bacterial virulence. Viable staphylococci of mutant or

Fig. 5. *S. aureus esxA, esxB,* and *essC* mutants are defective in the pathogenesis of murine abscesses. Ten 6-week-old BALB/c mice were injected retro-orbitally with 10^6 cfu for each strain. Mice were killed 4 days after infection. Kidneys and liver were removed and homogenized. Viable bacteria were counted after dilution and colony formation on tryptic soy agar. Statistical significance was examined with the Student *t* test, and *P* values were recorded. The limit of detection (solid line) was determined to be 10 cfu (10^1).

wild-type Newman strains ($\approx 10^6$ staphylococci) were administered intravenously via retro-orbital injection into mice. The animals were killed 4 days after infection. Internal organs were removed, inspected for abscess formation, and then homogenized and spread on agar medium to quantify staphylococcal replication in host tissues by colony formation (38). Mutations in *esxA, esxB,* and *essC* genes caused a significant reduction in the ability of *S. aureus* Newman to establish kidney or liver abscesses in infected mice (Fig. 5 and data not shown). Kidneys of mice infected with Newman carried a mean value of 3.89×10^7 cfu per organ, whereas those of mice infected with strains lacking *esxA, esxB,* or *essC* harbored a mean value of 3.00×10^4 cfu per organ ($P < 2.09 \times 10^{-5}$), 6.17×10^5 cfu per organ ($P < 1.2 \times 10^{-2}$), and 2.51×10^5 cfu per organ ($P < 5.53 \times 10^{-3}$), respectively. It should be noted that half of the animals infected with mutant bacteria had cleared most staphylococci from the kidneys after 4 days, suggesting that complete clearing may occur by day 5 after infection with the mutant strains. The formation of liver abscesses in infected animals was even more affected when staphylococci did not produce EsxA and EsxB. Homogenized livers of mice infected with Newman were found to contain a mean value of 2.09×10^6 cfu per organ of staphylococci, whereas the livers of mutants lacking *esxA, esxB,* or *essC* contained a mean value of 9×10^1 cfu per organ ($P < 3.01 \times 10^{-7}$), 4.17×10^2 cfu per organ ($P < 4.33 \times 10^{-6}$), and 1.35×10^3 cfu per organ ($P < 1.72 \times 10^{-4}$), respectively.

Discussion

S. aureus as well as several other staphylococcal species are physiological commensals of the human skin and nares (39). Breaches in local defense, for example, a skin cut or hair follicle trauma, provide a bacterial opportunity for replication and spread in underlying tissues (39). Successful implementation of this strategy requires *S. aureus* secretion of exoproteins, factors that are transported across the bacterial envelope via the Sec secretion system (40). The establishment of staphylococcal abscesses with liquefaction necrosis can thus be viewed as the sum of all pathogenic events implemented by

exoproteins and the corresponding host responses (41). These infectious lesions provide further opportunity for invading pathogens to enter deeper tissues or to spread via blood circulation, whereupon a reiterative cycle of secretion and destruction will be initiated (33, 41).

Previous work showed that *S. aureus* secretes 30 or more exoproteins (42) and mutations that abrogate the expression and secretion of only one of these factors typically do not cause significant defects in pathogenesis when measured in animal models of disease (35, 37). Nevertheless, some exceptions to this unifying view have been revealed. *S. aureus* mutants with *hla* deletions display defects in the pathogenesis of skin or mammary abscess formation in a murine model (42). Panton–Valentine leucocidin (PVL), a bacteriophage-encoded heterodimeric toxin, is believed to inflict immune cell necrosis by selective interaction with host membranes (43). *S. aureus* strains lysogenic for PVL bacteriophage are known to cause necrotizing pneumonia in children and healthy adults, a frequently lethal disease (44). Staphylococcal toxinoses depend on the secretion and functional role of specific toxins, but not on staphylococcal tissue invasion as the deciding parameter of pathological outcome (41).

Herein we examined the ESAT-6 system of *S. aureus*, a specialized secretion pathway that contributes to the pathogenesis of blood borne infections. EsxA and EsxB, two proteins with sequence identity to *M. tuberculosis* ESAT-6 (EsxA), are secreted by *S. aureus* Newman and are expressed by other staphylococcal strains (data not shown). EsxA and EsxB do not contain a classical N-terminal signal sequence and the polypeptides do not appear to be processed upon secretion (data not shown). Furthermore, EsxB is required for EsxA synthesis as well as secretion and vice versa. In *M. tuberculosis*, EsxA physically interacts with EsxB both *in vitro* and *in vivo* (45, 46). The data obtained herein support the notion that staphylococcal EsxA and EsxB could also form a complex. Although molecular determinants for the presumed dimerization and secretion EsxA and EsxB are not yet known, the WXG motif or the predicted coiled-coil structure of ESAT-6-like proteins represent obvious candidates for such functions. Knowledge of the presumed EsxA/EsxB dimerization will be important for future design of experiments that seek to examine these factors in pathogenesis studies.

Mutations in *essA, essB,* or *essC* abolished synthesis and therefore secretion of EsxA and EsxB without affecting transcription of *esxA* and *esxB*. Because EssA, EssB, and EssC are predicted transmembrane proteins, it is tempting to speculate that these proteins may represent a secretion apparatus. Without secretion, EsxA and EsxB may be rapidly degraded in the bacterial cytoplasm or posttranscriptional feedback inhibition may reduce *esxA* and *esxB* expression. Although experimental distinction between these possibilities has not yet been achieved, it seems noteworthy that many secretion systems, including type III and sec pathways, regulate secretion substrate gene expression via posttranscriptional mechanisms until the machinery is fully assembled and functional (47).

ESAT-6 (EsxA) and CFP-10 (EsxB) of *M. tuberculosis* are known to play an important role in virulence (10), causing macrophage subversion (18) and host cell lysis (15). To study the phenotype of *esxA, esxB* and *essC* mutations in *S. aureus*, a murine model of staphylococcal blood-borne dissemination and abscess formation was chosen (38). In these experiments, inoculated staphylococci must resist phagocytic killing, adhere to organ tissues, and mount an invasive strategy that leads to bacterial replication and abscess formation. Our results indicate that *bursa aurealis* insertion in *esxA, esxB,* or *essC* caused a 2- to 4-log reduction in the ability of mutant staphylococci to establish abscesses in infected mice, depending on the organ system examined (Fig. 5). Therefore, secretion of EsxA and EsxB represents an important virulence strategy. In fact, only *S. aureus*

sortase A mutants seem to display a similarly strong phenotype with 2- to 3-log reduction in virulence in this model (23, 48).

It is still unclear whether *S. aureus* EsxA and EsxB function as toxins. For example, type III pathways of Gram-negative microbes are characterized by a conserved secretion machinery and chaperone genes; however, genes encoding effectors, i.e., proteins that implement virulence strategies by interacting with the host, are typically not conserved beyond species boundaries. Considering the conservation of *esxAB* and *essC* in genomes of several different bacteria, one could argue that the effectors of staphylococcal ESAT-6 may not yet have been identified. Indeed, preliminary analysis of the spectrum of secreted polypeptides in *esxA* and *esxB* mutant cultures suggests that the ESAT-6 pathway may transport or somehow affect secretion of several exoproteins (data not shown). Therefore, it seems plausible that ESAT-6 effectors may have evolved to perform specific functions in different microorganism. One could ask further whether immune responses to ESAT-6 factors can protect hosts against staphylococcal disease or whether mutant variants lacking ESAT-6 determinants can be exploited for live vaccine design with attenuated *S. aureus* strains. Future work in this area promises to be fertile, revealing EsxA/EsxB secretion mechanisms and a comprehensive view of EssABC substrates, or perhaps pointing to novel strategies that may prevent the plethora of human diseases caused by *S. aureus*, in particular drug-resistant strains with resistance to all known antibiotics (49).

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