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CHARACTERIZATION OF EssD, A SECRETED NUCLEASE OF THE ESAT-6-LIKE SECRETION SYSTEM OF *STAPHYLOCOCCUS AUREUS*

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Introduction

Staphylococcus aureus, a commensal bacterium found to colonize the anterior nares, skin, and gastrointestinal tract of humans and domesticated animals, can cause highly invasive disease through a variety of manifestations based around the construction of abscess lesions at sites of infection (Crossley and Solliday, 1980; Lowy, 1998; Wertheim *et al.*, 2004, 2005; David and Daum, 2010; Fitzgerald, 2012; Peters *et al.*, 2013). Contained within the Gram-positive low G+C phylum of Firmicutes, the genus of *Staphylococcus* is made up of catalase positive, non-motile, non-spore forming cocci whose thick peptidoglycan cell walls confer resistance to high salt concentrations and temperatures (De Jonges *et al.*, 1992; Giesbrecht *et al.*, 1998; Götz, Bannerman and Schleifer, 2006). The genus derives its name from its grape-like cluster appearance when visualized under the microscope, and specifically the Greek word *Staphyle* which means "cluster of grapes" (Ogston, 1880). Colonies of *S. aureus* display golden-yellow coloration, and for this reason the species name stems from aurum, the Latin word for gold (Götz, Bannerman and Schleifer, 2006). Clinically, *S. aureus* identification and diagnosis require culturing from the site of infection (David and Daum, 2010). In order to differentiate *S. aureus* from other staphylococcal commensals, two diagnostic tests are used: the coagulase test and DNase test. Although there are greater than 40 species of staphylococci, many of which are known to colonize humans and/or other mammals, *S. aureus* has continuously adapted to take advantage of its various hosts and elicit aggressive disease in the healthiest of individuals (Otto, 2010).

S. aureus clinical relevance

In the United States alone, it is estimated that *S. aureus* is carried asymptomatically by 20-50% of the adult population, colonizing both the skin and nares of these individuals, and that these carriers can transfer *S. aureus* to uncolonized targets (Kluytmans, van Belkum and Verbrugh, 1997; Lowy, 1998; Kluytmans and Wertheim, 2005; Gorwitz *et al.*, 2008). Colonized individuals are at an increased risk of *S. aureus* infection, which can manifest in any tissue of the infected host, leading to pneumonia, bacteremia/sepsis, osteomyelitis, endocarditis, urinary tract infection, and the most common manifestation, skin and soft tissue infection (SSTI) (Kluytmans, van Belkum and Verbrugh, 1997; Lowy, 1998; Wertheim *et al.*, 2005; van Belkum, 2016). SSTIs are classically characterized as a painful, pus-filled lesion, also referred to as an abscess, beneath the skin's surface, and the surrounding tissue exhibits swelling and redness (Lowy, 1998; Morgan, 2011). In general, the infected host will require antibiotic treatment and/or surgical drainage/removal of the abscess in order to contain and clear the infection (Morgan, 2011). Annually, staphylococcal SSTIs alone affect greater than 10 million people, leading to millions of urgent care and emergency room visits and upwards of 500,000 hospitalizations (McCaig *et al.*, 2006; Hersh *et al.*, 2008). Additionally, in a study examining the overall number of staphylococcal SSTIs and their economic burden, it was determined that the national cost had reached $4.22 billion in 2009, a 26% increase from 2001 (Suaya *et al.*, 2014). Further adding to this burden is the ability of *S. aureus* to persist within the host and cause recurrent infections (Morgan, 2011). Indeed, recurrence of *S. aureus* infections is quite high (8 %) in those individuals with documented successful clearance of prior infection using surgical or antibiotic means (Fowler *et al.*, 1999).

S. aureus is also responsible for invasive and life-threatening infections (Lowy, 1998). When *S. aureus* invades deeper tissues, patients can become septic and staphylococci are able to spread throughout the infected host and seed abscesses in any tissue it can reach (Lowy, 1998; Watkins, David and Salata, 2012). Introduction of staphylococci into deeper tissues can be caused by a variety of patient injuries or even surgical procedures (Lowy, 1998). In fact, ever since its initial discovery *S. aureus* has been known as a leading cause of nosocomial infections due to its innate and acquired resistances to antiseptic and antimicrobial agents (Ogston, 1880). Additionally, *S. aureus* is able to adhere to surgical devices leading to catheter-related urinary tract infections and ventilator-associated pneumonia (Diekema *et al.*, 2004; National Nosocomial Infections Surveillance System, 2004; Shorr *et al.*, 2006; Pasquale *et al.*, 2013). Furthermore, simply being admitted to a hospital yields a 1% chance of incurring a *S. aureus* infection, and patients that acquire a *S. aureus* infection after an invasive medical procedure are estimated to be 5 times more likely to die during their hospital stay (Noskin *et al.*, 2005; Klein, Smith and Laxminarayan, 2007).

Antibiotic resistance

Though the blame cannot be placed solely on the misuse of antibiotics throughout the world, it cannot be ignored that the increasing burden of *S. aureus* infections in both the hospital and community would be less if *S. aureus* had not acquired resistance to the vast majority of antibiotics over the last 70 years (Chambers, 2001; Chambers and Deleo, 2009). Upon first being introduced into a clinical setting in the early 1940s, penicillin, a β-lactam based antibiotic, showed remarkable success improving the control and clearance of staphylococcal infections (Chambers, 2001; Chambers and Deleo, 2009). This improvement was short-lived

however, and by 1950, approximately 40% of *S. aureus* infections were caused by penicillin-resistant strains, and just 10 years later this number would increase to 80% (Chambers, 2001; Chambers and Deleo, 2009). This initial resistance was caused by the acquisition of a plasmid-based penicillinase encoding gene responsible for the cleavage and inactivation of penicillin (Chambers and Deleo, 2009). In order to counteract this form of resistance, chemists synthesized the drug variant methicillin, a β-lactam based compound that could not be degraded by penicillinase (Chambers and Deleo, 2009). The success of methicillin, like penicillin, was brief and soon, methicillin-resistant isolates of *S. aureus* were identified and found to have acquired the *mecA* gene which allows for broad resistance toward the entire class of β-lactam based drugs (Chambers and Deleo, 2009). These isolates are generally referred to as Methicillin-Resistant *S. aureus* (MRSA), and are highly resistant to a variety of drugs including all β-lactams. As a consequence, a limited number of drugs can be used as a last line of defense against MRSA strains (Chambers and Deleo, 2009).

In the United States, 40-50% of *S. aureus* infections are caused by MRSA isolates (Klein, Smith and Laxminarayan, 2007; Ray, Suaya and Baxter, 2013). Of even greater concern than simply the abundance of MRSA isolates, is the increase in healthy individuals presenting with MRSA infections without ever being hospitalized prior to the initial infection (Nimmo, 2012). This phenomenon is referred to as community acquired MRSA (CA-MRSA) infection, and in the context of the American epidemic, the leading causative agent is the clonal isolate USA300 (Tenover and Goering, 2009). To make matters worse, USA300 and many MRSA isolates exhibit harsher and more aggressive disease manifestations than their methicillin-sensitive

counterparts, including presentation with necrotizing fasciitis and necrotizing pneumonia (Boyle-Vavra and Daum, 2007; Montgomery *et al.*, 2008; Li *et al.*, 2009; Otto *et al.*, 2010).

The last 70 years indicate that antibiotic resistance is here to stay, and that the number of *S. aureus* infections and in turn the overall financial burden of these infections will continue to rise unless a different approach is taken. Hospital acquired MRSA infections are the cause of an estimated 11,000 plus deaths every year in the United States, and an annual economic burden of greater than 10 billion USD (Noskin *et al.*, 2005). Additionally, there are no successful vaccines that produce a protective response to *S. aureus* infections, regardless of antibiotic-resistance. For these reasons, now, more than ever, it is the time to rigorously pursue basic scientific research of these bacteria in hopes of discovering novel vaccine targets, new preventative measures, and better therapeutic treatments to defend ourselves against infection.

S. aureus pathogenesis

The focal point of *S. aureus* pathogenesis is the development and maturation of the canonical staphylococcal abscess lesion. The process by which an abscess is established and its overall cyclic nature is thought to involve four stages; where each stage's success is dependent on multiple characteristics of *S. aureus* (Cheng *et al.*, 2009, 2011). The first stage involves the initial inoculation of the host, whether it be a cut on the skin or a deep surgical wound, and the subsequent survival of staphylococci during this first contact against the host's innate immune response (Cheng *et al.*, 2011). Upon entry into the host, staphylococci may gain access to the

blood stream and disseminate to other tissues far away from the primary site of injury and inoculation (Cheng *et al.*, 2011; Edwards and Massey, 2011).

Regardless of whether *S. aureus* can disseminate or not, the second stage involves the initial seeding of what will eventually become a classical staphylococcal abscess in the infected organ tissue (Cheng *et al.*, 2011). This event involves a small focus of staphylococci surrounded by immune cell infiltrates within the infected tissue. Neutrophils are the principal immune cell found to be present during this step (Cheng *et al.*, 2011). As the staphylococci of this central nidus begin to replicate, more and more damage is caused to the neighboring organ tissue by both the bacteria themselves and the host immune response trying to control the infection (Cheng *et al.*, 2011).

The third stage is characterized by a densely populated staphylococcal abscess community (SAC) at the center of a massive infiltration of immune cells (Cheng *et al.*, 2011; Thammavongsa, Missiakas and Schneewind, 2013). This SAC is contained within a fibrin cuff, creating a capsule-like structure that protects the SAC from the increasing threat of immune cell infiltrates (Cheng *et al.*, 2011; Thammavongsa, Missiakas and Schneewind, 2013). Tissue destruction within the area around the SAC is due to lysis and propagation of the large number of lymphocytes and neutrophils that were recruited to the staphylococcal lesion (Cheng *et al.*, 2011; Thammavongsa, Missiakas and Schneewind, 2013). Specifically, a layer of necrotic immune cells, mostly dead neutrophils, is found directly adjacent to the fibrin cuff of the SAC, followed by a shell of otherwise healthy immune cells, and finally another layer of dead leukocytes (Cheng *et al.*, 2011; Thammavongsa, Missiakas and Schneewind, 2013). This outermost layer of dead cells is further segregated from the healthy tissue of the infected organ

by an eosinophilic shell composed of fibrin and/or extracellular matrix (Cheng *et al.*, 2011; Thammavongsa, Missiakas and Schneewind, 2013). This entire structure represents the third stage's final form, and a fully matured staphylococcal abscess.

During the fourth stage, the abscess is forced to the cortex of the infected organ as the host attempts to heal the tissue around the encapsulated staphylococcal lesion (Cheng *et al.*, 2011). Upon reaching the periphery of the infected tissue, the abscess is able to lyse and release the previously contained SAC into the surrounding area, thus completing the fourth stage (Cheng *et al.*, 2011). The fourth stage will then lead to dissemination and a new cycle of abscess formation, that unless contained through antibiotic or surgical treatment will lead to the host's eventual death (Cheng *et al.*, 2009, 2011; Liu *et al.*, 2011; Ziegler *et al.*, 2011).

The *S. aureus* arsenal of virulence factors

In order to accomplish the complex pathogenic processes described above, *S. aureus* utilizes a large arsenal of multifunctional virulence factors. These virulence factors play crucial roles at each stage of the pathogenesis of *S. aureus* infection including, but not limited to, tissue adhesion, anti-phagocytic functions, coagulation, host cell toxicity, and modulation of inflammation and the adaptive immune response.

S. aureus avoids bacterial clearance through a variety of immune evasive mechanisms (Nizet, 2007). One such mechanism is via the cell-associated factor staphyloxanthin, which is a carotenoid pigment responsible for the golden coloration of *S. aureus* colonies and involved with staphylococcal survival in the host (Nizet, 2007). Staphyloxanthin exhibits powerful antioxidant attributes that grants *S. aureus* resistance to the oxidative burst of neutrophils and

other phagocytic cells (Clauditz *et al.*, 2006; Nizet, 2007; Leejae, Hasap and Voravuthikunchai, 2013). Other anti-phagocytic virulence factors are manifested through secreted proteins that can either prevent recruitment of leukocytes or inhibit opsonophagocytic functions of the host (Nizet, 2007). The chemotaxis inhibitory protein of staphylococci (CHIPS) is one such factor. CHIPS, which is produced by many clinical isolates of *S. aureus*, works by blocking the cellular receptors for N-formyl peptides and the complement-associated protein C5a, and thus limiting leukocyte chemotaxis to the site of infection (de Haas *et al.*, 2004; Postma *et al.*, 2004; Nizet, 2007). In addition to CHIPS, *S. aureus* also secretes the staphylococcal complement inhibitor (SCIN) which inhibits the activity of C3 convertase on the bacterial surface (Nizet, 2007). In doing so, SCIN blocks C3b opsonization of staphylococci and decreases the phagocytic capabilities of innate immune cells to take up and kill *S. aureus* (Rooijakkers *et al.*, 2005; van Wamel *et al.*, 2006; Nizet, 2007). Additionally, CHIPS and SCIN have been shown to be secreted upon initial infection owing to their importance in disrupting the innate immune response of the host (Rooijakkers *et al.*, 2006).

It has been recognized that neutrophils are a crucial element of host control of staphylococcal infections, and *S. aureus* has adapted specific strategies for counteracting these cells in addition to the above-mentioned virulence factors (Rigby and DeLeo, 2012). One of the unique antimicrobial activities that neutrophils are endowed with is the ability to expel their genomic content and produce DNA-based neutrophil extracellular traps (NETS) (Brinkmann *et al.*, 2004; Nizet, 2007). These NETS are capable of constraining bacteria and eliciting bactericidal activity (Brinkmann *et al.*, 2004; Nizet, 2007). *S. aureus* bypasses these NETS by degrading them with their secreted staphylococcal nuclease (Thammavongsa *et al.*, 2009; Berends *et al.*, 2010;

Thammavongsa, Missiakas and Schneewind, 2013). Furthermore, *S. aureus* produces a cell wall-anchored protein, adenosine synthase A (AdsA), a 5'-nucleotidase that produces adenosine through the consecutive hydrolysis of ATP, ADP and AMP (Thammavongsa *et al.*, 2009; Thammavongsa, Missiakas and Schneewind, 2013). The production of adenosine resulting from the combined activities of secreted nuclease and AdsA during infection, suppresses the immune response by inducing apoptosis of local macrophages through caspase-3 activation (Thammavongsa *et al.*, 2009; Thammavongsa, Missiakas and Schneewind, 2013).

An additional, although debatable, mechanism involved with staphylococcal survival in the host is the production of a polysaccharide capsule. The majority of *S. aureus* isolates produce capsules that confer some anti-phagocytic function, yielding staphylococci that are more resistant to phagocytic killing (Lee *et al.*, 1987; Nizet, 2007). Loss of capsular polysaccharide production is dispensable for invasive disease, and thus it is considered a less prominent virulence factor (Cheng *et al.*, 2009).

If phagocytic cell recruitment and killing cannot be prevented by the previously described means, *S. aureus* is more than prepared to use deadly force through secreted toxins (DuMont *et al.*, 2013; Surewaard *et al.*, 2013). *S. aureus* produces an array of leukotoxins that cause damage and/or death to macrophages, neutrophils, and T cells (DuMont *et al.*, 2013; Surewaard *et al.*, 2013; Yoong and Torres, 2013). Specifically, this family of bi-component leukotoxins consists of Leukocidin AB/GH (LukAB/GH), Leukocidin ED (LukED), γ-hemolysin (Hlg), and Panton-Valentine Leukocidin (PVL) (DuMont *et al.*, 2013; Surewaard *et al.*, 2013; Yoong and Torres, 2013). Mutants lacking any one of the genes for these toxins have been shown to be defective in their ability to cause disease, further underlining the importance in

manipulating host inflammation and preventing bacterial control by killing phagocytic cells (Wang *et al.*, 2007; Alonzo III *et al.*, 2012; Powers *et al.*, 2012; DuMont *et al.*, 2013; Surewaard *et al.*, 2013; Yoong and Torres, 2013). In addition to these secreted leukotoxins, *S. aureus* secretes δ- toxin which is the founding member of a family of small helical peptides with amphipathic properties called phenol soluble modulins (PSMs) (Bernheimer and Schwartz, 1964; Alouf *et al.*, 1989). These PSMs, using their amphipathic helices, have been shown to induce membrane lysis and neutrophil killing which in turn enhance staphylococcal intracellular survival (Bernheimer and Schwartz, 1964; Alouf *et al.*, 1989; Surewaard *et al.*, 2013).

Despite the number of toxins described above and their importance to staphylococcal virulence, the most well-known toxin produced by *S. aureus* is the β-barrel, pore-forming toxin, α-hemolysin (Hla) (Berube and Bubeck Wardenburg, 2013). Virtually conserved in all clinical isolates of *S. aureus*, α-hemolysin is secreted as a monomeric protein and upon binding to its receptor, undergoes oligomerization into a heptameric structure which allows for pore formation and host cell damage (Gouaux *et al.*, 1994; Song *et al.*, 1996; Berube and Bubeck Wardenburg, 2013). The host receptor for Hla was identified as A Disintegrin and Metalloprotease 10 (ADAM-10), which proves to play an important role in staphylococcal pathogenesis other than simply being a receptor for a toxin (Wilke and Wardenburg, 2010). Upon binding to Hla, the zinc-dependent metalloprotease activity of ADAM-10 is upregulated leading to epithelial and endothelial barrier disruption via E-cadherin cleavage (Wilke and Wardenburg, 2010; Inoshima *et al.*, 2011; Powers *et al.*, 2012). Furthermore, like leukotoxins and PSMs, Hla has also been shown to manipulate the inflammatory response of the host as well as inhibit the ability of phagocytic cells to take up and kill staphylococci (Gemmell *et al.*,

1982; Hruz *et al.*, 2009; Becker *et al.*, 2014). Due to the variety of affects Hla can elicit during infection, it is no surprise that it has been shown to be a key factor in the disease progression of *S. aureus* endocarditis, sepsis, pneumonia, and skin and soft tissue infections (Bayer *et al.*, 1997; Nilsson *et al.*, 1999; Bubeck Wardenburg, Patel and Schneewind, 2007; Kennedy *et al.*, 2010; Powers *et al.*, 2015). Furthermore, and of great concern, it has been shown that more virulent MRSA isolates exhibit altered genetic control of Hla, PVL, and PSMs leading to increased production of these toxins and more severe disease manifestations within individuals infected with these strains (Li *et al.*, 2009; Chua *et al.*, 2011; DeLeo *et al.*, 2011).

One *S. aureus* protein that truly works to disrupt the function and response of both the innate and adaptive immune systems is staphylococcal protein A (SpA). SpA is a cell wall-anchored protein that is comprised of five IgG binding domains (IgBDs) (Dossett *et al.*, 1969; Lindmark, Thorén-Tolling and Sjöquist, 1983; Kim *et al.*, 2012). These IgBDs are capable of binding IgG through both their Fcγ and Fab portions. Fcγ binding reverses the orientation of IgG molecules on the staphylococcal surface preventing IgGs from working as opsonins (Dossett *et al.*, 1969; Lindmark, Thorén-Tolling and Sjöquist, 1983; Kim *et al.*, 2012, 2016). This mechanism works two-fold, by preventing both the opsonophagocytic nature of IgGs and ablating any downstream Fc-mediated effector functions of immune cells that could recognize IgG-bound staphylococci (Dossett *et al.*, 1969; Lindmark, Thorén-Tolling and Sjöquist, 1983; Kim *et al.*, 2012). These functions are associated with the cell wall-anchored nature of SpA, but through natural turnover of the staphylococcal cell-wall, SpA can be released into the extracellular milieu (Sakane and Green, 1978; Hillson *et al.*, 1993; Goodyear and Silverman, 2004; Kim *et al.*, 2012, 2016). This cell-free form of SpA can then act as a B cell superantigen by binding to B cell

receptors directly and triggering activation of apoptosis (Sakane and Green, 1978; Hillson *et al.*, 1993; Goodyear and Silverman, 2004; Kim *et al.*, 2012, 2016). In doing so, SpA essentially prevents the host from producing an effective adaptive immune response by both destroying the immune cells necessary for antibody production, and preventing any antibodies that are produced from recognizing staphylococci in a protective manner. In fact, organisms that survive *S. aureus* infections do not produce protective immunity, at least partially due to the lack of neutralizing antibody production (Kim *et al.*, 2010, 2012, 2016). Furthermore, immunization of mice with a "non-toxinogenic" SpA lacking the functionality of its five IgBDs conferred protection when challenged with a lethal dose of *S. aureus*, and these challenged mice elicited a greater antibody response against a range of staphylococcal antigens while also increasing opsonophagocytic killing of *S. aureus* (Kim *et al.*, 2010).

S. aureus encodes many virulence factors accounting for many pathogenic attributes, and targeted deletion of such genes does not attenuate this bacterium significantly (Cheng *et al.*, 2009). That being said, *S. aureus* utilizes enzymes called sortases that can interact with an LPXTG motif within the C-terminus of secreted proteins in order to catalyze the covalent attachment of these proteins to the cell wall of staphylococci (Mazmanian *et al.*, 1999; Ton-That *et al.*, 1999). The *S. aureus* sortase has been determined to attach upwards of 20 proteins to the staphylococcal cell wall, and of these sortase-anchored proteins many are known virulence factors (Mazmanian, Ton-That and Schneewind, 2001). Mutations in any one of these virulence factors, such as *adsA* or *spa*, will cause some defect in pathogenesis; however, the deletion of sortase produces a strain of *S. aureus* with a dramatic loss in its ability to cause disease across all stages of infection due to the mislocalization of multiple virulence factors

(Mazmanian *et al.*, 2000; Cheng *et al.*, 2009). Even then, these mutants are not completely defunct in their ability to cause infection; and for this reason, and the overall immune evasive nature of *S. aureus* described above, a successful and efficacious vaccine strategy has yet to be developed.

Protein secretion

Protein secretion into or across the cell membrane is an essential characteristic of all bacterial life (Freudl, 2013). Cell division, membrane stability, and nutrient acquisition, among a variety of other requirements for survival, all depend on an intact secretion machine, and bacteria have evolved conserved mechanisms to ensure adequate transport and secretion of proteins (Freudl, 2013). The canonical secretory pathway consists of the SecY, SecE, and SecG proteins that together for the transmembrane translocon; the SecA ATPase facilitates protein translocation across the SecYEG translocon (Cabelli *et al.*, 1988; Young Jae, Rajapandi and Oliver, 1994; Economou *et al.*, 1995; Freudl, 2013). Targeting of proteins for secretion relies on an N-terminal leader sequence that is present in the newly synthesized precursor protein (Izard and Kendall, 1994; Driessen and Nouwen, 2008; Schneewind and Missiakas, 2012; Freudl, 2013). The leader sequence, consisting of a positively charged amino-terminus followed by a helical stretch of hydrophobic amino acids, is recognized by the SecYEG secretion machine and mediates efficient secretion (Izard and Kendall, 1994; Driessen and Nouwen, 2008; Schneewind and Missiakas, 2012; Freudl, 2013). Upon translocation to the extracellular space, the leader sequence is proteolytically cleaved via a signal peptidase, and the mature protein is released from the translocon (Dalbey and Wickner, 1985; Driessen and Nouwen, 2008; Schneewind and Missiakas, 2012).

Bacterial pathogens commonly utilize secretion systems beyond that of the classical secretory pathway in order to secrete virulence factors into the extracellular milieu or to inject them directly into host cells (Freudl, 2013; Cianfanelli, Monlezun and Coulthurst, 2016; Green and Mecsas, 2016; Byndloss *et al.*, 2017; Ratner *et al.*, 2017). These specialized pathogenic secretion systems are in addition to the canonical pathway, and therefore are often dispensable for bacterial growth in laboratory media. Often, their specific functions are involved with virulence, and deletion of these secretion systems causes defects in pathogenesis, and often can even lead to the generation of attenuated or avirulent variants. A variety of secretion systems have been identified in pathogenic bacteria and the following section will give a brief overview on the type III (T3SS), type IV (T4SS), type VI (T6SS), and type VII (T7SS) secretion systems.

Secretion systems of bacterial pathogens

Gram-negative bacteria can utilize type III secretion systems (T3SS) to elaborate needle-like "injectisomes" on the bacterial surface which allows for delivery of effector proteins directly from the bacterial cytoplasm into the cytoplasm of a suitable host cell (Cornelis, 2010; Buttner, 2012; Dewoody, Merritt and Marketon, 2013; Byndloss *et al.*, 2017; Deng *et al.*, 2017). Pathogenic bacteria such as *Yersinia sp.*, *Salmonella sp.*, *Shigella sp.*, *Escherichia coli*, *Pseudomonas sp.*, *Chlamydia sp.*, and countless others employ their specific T3SS to modulate the host's defenses to promote disease. These T3SSs have been shown to be precisely controlled at various levels, from transcriptional to post-translational regulation, in order to guarantee the injection of the effectors at the right time and place upon sensing the correct target cell (Wiley, Rosqvist and Schesser, 2007; Hayes, Aoki and Low, 2010; Dewoody, Merritt

and Marketon, 2013; Deng *et al.*, 2017). Some bacteria, such as *Salmonella* Typhimurium, harbor two T3SSs for distinct processes during infection (Galán, Curtiss and 3rd, 1989; Hensel *et al.*, 1995; Tsolis *et al.*, 1999). During *Salmonella* Typhimurium infection, T3SS-1 allows for infiltration into the cells of the gastrointestinal epithelium, while T3SS-2 is required for bacterial survival within phagocytic host cells (Galán, Curtiss and 3rd, 1989; Hensel *et al.*, 1995; Tsolis *et al.*, 1999).

Other Gram-negative bacteria including *Legionella sp.*, *Agrobacterium sp.*, *Brucella sp.*, and *Helicobacter sp.* utilize a type IV secretion system to promote pathogenesis. The T4SS is a distantly related to bacterial DNA conjugation systems; however, T4SSs have evolved to export effector proteins and/or effector DNA directly into host cells via a pilus-like translocation system (Zambryski *et al.*, 1980; Cascales and Christie, 2003; Voth, Broederdorf and Graham, 2012). Two broad classes of T4SSs have been identified and studied: type IVA (T4ASS) and type IVB (T4BSS) (Christie and Vogel, 2000; Sexton and Vogel, 2002; Nagai and Kubori, 2011). T4ASSs, also referred to as VirB systems, were initially characterized within *Agrobacterium tumefaciens*, a plant pathogen that causes crown gall disease. *A*. *tumefaciens* can high jack the plant cell biosynthesis pathway by injecting DNA, termed transferred DNA (T-DNA), via its T4ASS (Yajko and Hegeman, 1971; Schell and Van Montagu, 1977; Schell *et al.*, 1979; Shirasu and Kado, 1993; Fullner, Lara and Nester, 1996). T-DNA reprograms the host cell toward the production of a specific opine, that can differ depending on the infectious strain, but ultimately serves as carbon and nitrogen sources for the invading bacteria (Schell and Van Montagu, 1977; Schell *et al.*, 1979; Escobar and Dandekar, 2003). T4BSSs, also referred to as Dot/Icm systems, are based on the well described T4SS of *Legionella pneumophila*, the causative agent of Legionnaires'

disease. The intracellular pathogen, *L. pneumophila* is taken up by amoebae or phagocytic host cells and utilizes its T4BSS to subvert the phagocytic compartment and host cell machinery, yielding the so-named *Legionella*-containing vacuole (LCV) (Rowbotham, 1980; Horwitz and Maxfield, 1984; Abu Kwaik, 1996; Segal and Shuman, 1999). Although, the type IVB machine and secreted effectors are essential for the creation of the LCV and survival of the bacterium, it has been recognized that no one effector alone is responsible for this process (Bardill, Miller and Vogel, 2005; Campodonico, Chesnel and Roy, 2005; Laguna *et al.*, 2006; VanRheenen *et al.*, 2006). In fact, it is estimated that over 300 effector proteins are secreted via the *Legionella* T4BSS and together contribute to LCV formation and intracellular replication (Ninio and Roy, 2007; Heidtman *et al.*, 2009; Gomez-Valero *et al.*, 2011).

The most recently characterized specialized secretion system is the type VI secretion system (T6SS) identified in 2006 (Mougous *et al.*, 2006; Pukatzki *et al.*, 2006). It has been recognized that T6SSs are elaborated by the majority of Gram-negative bacteria, including *Vibrio sp.*, *Pseudomonas sp.*, *Salmonella sp.*, *Burkholderia sp.*, *Proteus sp*., *Escherichia coli*, *Yersinia sp.*, and *Bacteroides sp*. (Cascales, 2008; Mulder, Cooper and Coombes, 2012; Weyrich *et al.*, 2012; Liu *et al.*, 2013; Cianfanelli, Monlezun and Coulthurst, 2016; Hecht *et al.*, 2016). Similar to T3SSs and T4SSs, the T6SS is a contact dependent secretion system that translocates effector proteins directly into the target cell (Pukatzki *et al.*, 2007; Hood *et al.*, 2010; Zoued *et al.*, 2014). The structure of the secretion apparatus is shaped like a syringe and is reminiscent of the contractile tail of bacteriophage T4; injection of effector proteins requires the repetitive assembly, contraction/injection, and disassembly of this apparatus (Pukatzki *et al.*, 2006; Leiman *et al.*, 2009; Shneider *et al.*, 2013; Kudryashev *et al.*, 2015). The syringe-like appendage

is capped by VgrG proteins and PAAR (Proline-Alanine-Alanine-Arginine) motif-containing proteins, which are believed to create the piercing tip that facilitates penetration through target cell membranes and subsequent injection of T6SS effector molecules (Pukatzki *et al.*, 2006; Leiman *et al.*, 2009; Shneider *et al.*, 2013; Kudryashev *et al.*, 2015; Hachani, Wood and Filloux, 2016). Since its discovery, T6SSs have been implicated in both eukaryotic infection and interbacterial competition, and to this extent bacteria such as *Burkholderia thailandensis* and *Pseudomonas aeruginosa* have been characterized as having multiple non-redundant T6SSs necessary for these specific functions (Filloux, Hachani and Bleves, 2008; Hood *et al.*, 2010; Schwarz *et al.*, 2010; Russell *et al.*, 2013; Jiang *et al.*, 2014). In addition, it has been shown that the T6SS in *Bacteroides fragilis* plays a role in strain-specific selection that mediates colonization of the mouse gut with a non-toxigenic strain and promotes intestinal health (Hecht *et al.*, 2016).

The seventh secretion system, although more rare and often considered a Gram-positive/monoderm-associated secretion system, was originally discovered and characterized in *Mycobacterium tuberculosis* (Mtb). Historically, scientists first reported a 6-kDa protein in the extracellular milieu of mycobacterial cultures with a strong antigenic T cell response “activity”, and in 1995, this protein was named the “6-kDa early secretory antigenic target” (ESAT-6) (Sorensen *et al.*, 1995; Ravn *et al.*, 1999; Arend *et al.*, 2000; Mustafa *et al.*, 2000; Abdallah *et al.*, 2007). In addition to ESAT-6, a 10-kDa protein that is encoded by the gene immediately upstream of the gene for ESAT-6 was found in the culture filtrates of Mtb and shown to interact with ESAT-6 (Berthet *et al.*, 1998; Skjøt *et al.*, 2000; Okkels *et al.*, 2004). It was determined that both ESAT-6 and this “10 kDa culture filtrate protein” (CFP-10) were present as full length

proteins without a recognizable signal peptide or cleavage site, and due to this it was assumed that ESAT-6/CFP-10 were released into the extracellular milieu following cell lysis (Sorensen *et al.*, 1995; Berthet *et al.*, 1998; Skjøt *et al.*, 2000; Okkels *et al.*, 2004).

ESAT-6 and CFP-10, also referred to as EsxA and EsxB, have since been recognized as the founding members of the family of WXG100 proteins and are one of the staples of what would eventually be termed the type VII secretion system (T7SS) (Skjøt *et al.*, 2000; Pallen, 2002; Okkels *et al.*, 2004). WXG100 proteins are ~100 amino acids long with a Tryptophan-X-Glycine motif at the center of the protein (Skjøt *et al.*, 2000; Pallen, 2002; Okkels *et al.*, 2004). NMR analyses revealed that in the ESAT-6/CFP-10 complex, both proteins exhibit helix-turn-helix structures and are oriented in an inversely parallel manner (Renshaw *et al.*, 2005; Sundaramoorthy, Fyfe and Hunter, 2008).

Upon further analysis of the mycobacterial genome, it was recognized that genes encoding FtsK/SpoIIIE-like ATPase proteins were always found near ESAT-6/CFP-10 (Pallen, 2002). It was hypothesized that these conserved ATPase-encoding genes and other nearby genes may form a novel secretion system for secretion of ESAT-6/CFP-10, and multiple publications confirmed these assumptions by demonstrating that these are indeed required for secretion of ESAT-6/CFP-10 (Pallen, 2002; Hsu *et al.*, 2003; Pym *et al.*, 2003; Stanley *et al.*, 2003; Guinn *et al.*, 2004) It was also determined that CFP-10 and the FtsK/SpoIIIE-like ATPase, later termed EccC, interact through a pseudo-signal sequence within the C-terminus of CFP-10 that allows EccC to recognize it (Stanley *et al.*, 2003; Champion *et al.*, 2006; Rosenberg *et al.*, 2015). A model has been proposed whereby binding of CFP-10 triggers oligomerization of EccC and

stimulates its ATPase activity thus promoting secretion of ESAT-6/CFP-10 (Rosenberg *et al.*, 2015).

Along with ESAT-6/CFP-10 and FtsK/SpoIIIE-like ATPases, mycobacterial T7SSs harbor one or more conserved subtilisin-like serine proteases named mycosins (MycP), which play non-redundant functions and have been shown to contribute to mycobacterial growth and ESX secretion (Brown *et al.*, 2000; Sassetti, Boyd and Rubin, 2003; Griffin *et al.*, 2011). The exact activity contributed by T7SS-associated mycosins is however not resolved; while mutants lacking *mycP$_1$* cannot secrete effectors, strains expressing a protease inactive form of MycP$_1$ continue to secrete effectors with a yield greater than that of wild-type Mtb (Ohol *et al.*, 2010; Wagner *et al.*, 2013; van Winden *et al.*, 2016).

The importance of this novel secretion system, which has since been named as the ESX-1 type VII secretion system, was made evident when it was recognized that the mycobacterial vaccine strain, Bacillus Calmette-Guèrin (BCG), had a genetic lesion, termed Region of Difference 1 (RD1), that removed this *esx-1* gene cluster (Harboe *et al.*, 1996; Pym *et al.*, 2002; DiGiuseppe Champion *et al.*, 2009). Loss of ESAT-6 (EsxA) secretion has been associated with the reduced granuloma formation and chronic infection associated with BCG infections in animal models (REF). Further, recombinant ESAT-6 (EsxA) has been shown to elicit protection in the form of a vaccine antigen (Brandt *et al.*, 2000; Cambier *et al.*, 2014).

The role of type I interferon (IFN) in tuberculosis and its overall importance for mycobacterial infections have been well documented. One mechanism for IFN activation involves ESX-1 mediated leakage of the phagolysosome (Gao *et al.*, 2013; Dey *et al.*, 2015;

Wassermann *et al.*, 2015; Watson *et al.*, 2015). Following uptake by phagocytic cells, intracellular mycobacteria secrete ESAT-6 (EsxA) which is thought to form pores in the phagosomal membrane and to facilitate mixing of the phagosomal compartment, containing mycobacterial virulence factors, with the cytoplasm (Houben *et al.*, 2012; Dey *et al.*, 2015; Wassermann *et al.*, 2015; Watson *et al.*, 2015). It has been proposed that mycobacterial DNA and cyclic-di-nucleotides also leak during this process and are sensed in the host's cytoplasm by cognate receptors, including cyclic GMP-AMP (cGAMP) synthase (cGAS) and the Stimulator of Interferon Genes (STING) (Gao *et al.*, 2013; Dey *et al.*, 2015; Wassermann *et al.*, 2015; Watson *et al.*, 2015). cGAS converts double-stranded DNA into cGAMP which can then bind STING and facilitate the transcriptional stimulation of IFN (Gao *et al.*, 2013; Dey *et al.*, 2015; Wassermann *et al.*, 2015; Watson *et al.*, 2015). Additionally, ESX-1 secretion has been shown to trigger inflammasome activation leading to the maturation of interleukin-1β (IL-1β) and its subsequent signaling cascade, and that recognition of mycobacterial DNA by another cytoplasmic DNA sensor, AIM2, is required for this IL-1β response (Hornung *et al.*, 2009; Manzanillo *et al.*, 2012; Watson *et al.*, 2012, 2015; Gao *et al.*, 2013; Dey *et al.*, 2015; Wassermann *et al.*, 2015). Although the actual formation of pores leading to phagosomal leakage has been attributed to insertion of ESAT-6 into membranes, recently the pore-forming capabilities of recombinant ESAT-6 has been disputed, yet the field still agrees that an intact ESX-1 T7SS and ESAT-6 secretion are required for successful mycobacterial infections (Conrad *et al.*, 2016).

In addition to ESX-1, Mtb encodes four additional T7SSs designated ESX-2, -3, -4, and -5 (Abdallah *et al.*, 2007; DiGiuseppe Champion and Cox, 2007; Esther J.M. Stoop, Bitter and van der Sar, 2012; Newton-Foot *et al.*, 2016). Clusters of gene encoding ESX-2, -3, -4, and -5 are

found throughout the Mtb genome, and all harbor genes that encode homologs of ESAT-6/CFP-10 as well as FtsK/SpoIIIE-like ATPases (Abdallah *et al.*, 2007; DiGiuseppe Champion and Cox, 2007; Esther J.M. Stoop, Bitter and van der Sar, 2012; Newton-Foot *et al.*, 2016). Through the process of identifying these novel type VII secretion clusters, it was also recognized that WXG100 proteins such as ESAT-6/CFP-10 are not the only secreted substrates (Abdallah *et al.*, 2006; Daleke *et al.*, 2012; Esther J.M. Stoop, Bitter and van der Sar, 2012; Newton-Foot *et al.*, 2016). Two novel families of proteins called PE and PPE for the presence of Proline-Glutamic acid or Proline-Proline-Glutamic acid motifs, respectively, generally found in the N-terminal portion of the protein, were also found to be secreted in a T7-dependent manner (Abdallah *et al.*, 2006; Daleke *et al.*, 2012; Esther J.M. Stoop, Bitter and van der Sar, 2012; Newton-Foot *et al.*, 2016). Due to the limited sequence homology between non-WXG100 proteins, experimental approaches are necessary to identify and validate true T7 substrates.

The ESX-3 T7SS was originally characterized as an iron and zinc scavenging system essential for uptake of metal-binding proteins and growth of Mtb in metal-depleted conditions (Serafini *et al.*, 2009, 2013, Siegrist *et al.*, 2009, 2014; Tufariello *et al.*, 2016). Although, EsxG and EsxH are classical WXG100-like proteins of the ESX-3 secretion system, PE5 and PPE4 are thought to be the actual secreted effectors of metal homeostasis (Tufariello *et al.*, 2016). On the other hand, EsxG and EsxH play important roles in mycobacterial pathogenesis independently of iron acquisition, specifically a role in phagosomal survival (Portal-Celhay *et al.*, 2016; Tinaztepe *et al.*, 2016; Tufariello *et al.*, 2016). Furthermore, EsxH has been shown to interfere with the endosomal sorting complex required for transport (ESCRT), and in doing so,

prevents Mtb-infected macrophages and DCs from properly presenting antigen and activating $CD4^+$ T cell (Portal-Celhay *et al.*, 2016).

The ESX-5 T7SS is a phosphate-regulated secretion system found in only slow-growing mycobacterial species, including Mtb, and is activated under phosphate depleted conditions (Gey van Pittius *et al.*, 2006; Abdallah *et al.*, 2008; Elliott and Tischler, 2016). Although the *esx-5* gene cluster harbors a pair of WXG100-like proteins, it has been best characterized for the secretion of PE and PPE proteins (Shah and Briken, 2016). PE and PPE effectors of the ESX-5 system contribute to the reduction of certain pro-inflammatory cytokines during infection and promote inflammasome maturation and IL-1β secretion (Abdallah *et al.*, 2006, 2008, 2011). The ESX-5 system has also been implicated in triggering macrophage cell death and release of intracellular mycobacteria (Abdallah *et al.*, 2011). In addition to their roles in subverting the immune system, some ESX-5 secreted effectors may insert into the mycobacterial envelope and affect the stability and permeability of this structure (Bottai *et al.*, 2012; Ates *et al.*, 2015).

The ESX-2 and ESX-4 secretion systems remain poorly characterized. There is currently no experimental evidence to indicate that ESX-2 or ESX-4 are involved in pathogenesis or actively secrete effector proteins. However, ESX-4 has been implicated in a unique horizontal gene transfer mechanism called distributive conjugal transfer (DCT) (Parsons, Jankowski and Derbyshire, 1998; Flint *et al.*, 2004; Coros, DeConno and Derbyshire, 2008; Gray *et al.*, 2013). It was shown that the ESX-1 T7SS of *Mycobacterium smegmatis* is required in both the donor and recipient strains during DCT; however, the ESX-4 system is only a required element of the recipient and thus plays an important role in accepting and integrating the transferred genetic element (Gray *et al.*, 2016).

Pallen was the first to note the genetic clustering of ESAT-6/CFP-10 and FtsK/SpoIIIE-like ATPases encoding genes in Mtb and to propose that these clusters may represent novel secretion systems (Pallen, 2002). This initial *in silico* analysis also revealed homologous genes in the genomes of some Gram-positive bacteria (Pallen, 2002). Using the unique WXG100 motif, other pathogenic organisms like *Bacillus anthracis*, *Corynebacterium diphtheriae*, *Listeria monocytogenes*, and *Staphylococcus aureus* were identified as harboring their own distantly-related versions of the canonical mycobacterial T7SS (Pallen, 2002). Since the identification of a T7SS-like gene cluster in *S. aureus*, efforts have been made to determine its role in pathogenesis.

The ESAT-6-like/Type VIIb Secretion System of *S. aureus*

The staphylococcal type VII secretion systems have been referred to as ESAT-6-like Secretion Systems (ESS) or type VIIb secretion systems (T7bSSs) because of their distinct differences from the classical ESX-1 T7SS of Mtb or type VIIa secretion system (T7aSS) (Burts *et al.*, 2005; Abdallah *et al.*, 2007). Sequence similarity is extremely limited between the staphylococcal *ess* and mycobacterial *esx* gene clusters and protein machines (Pallen, 2002; Burts *et al.*, 2005; Abdallah *et al.*, 2007). That being said, two WXG100-like genes were identified in *S. aureus* strain Newman and named *esxA* and *esxB* (Pallen, 2002; Burts *et al.*, 2005; Abdallah *et al.*, 2007). Unlike their mycobacterial equivalents these genes were not located next to one another, but separated by six genes that have since been determined as required components of the staphylococcal *ess* gene cluster (Burts *et al.*, 2005; Burts, DeDent and Missiakas, 2008; Chen *et al.*, 2012; Anderson *et al.*, 2013; Kneuper *et al.*, 2014). Additionally, it was determined that EsxA and EsxB do not interact to form heterodimeric

structures like ESAT-6/CFP-10, and that EsxA forms a homodimer instead (Sundaramoorthy, Fyfe and Hunter, 2008).

Two other ESS-secreted proteins, EsxC and EsxD, were eventually identified and characterized in the MRSA clinical isolate, USA300 (Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2013). *esxC* is found immediately upstream of *esxB*, while *esxD* is located two genes downstream of *esxB* in the genomes of *S. aureus* Newman and USA300 (Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2013). Neither EsxC nor EsxD harbor a WXG100 motif or exhibit sequence similarity to any other ESAT-6/CFP-10 proteins; however, EsxC forms heterodimers with EsxA, as well as homodimers with itself, and EsxD interacts with EsxB (Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2013). Interestingly, deletion of *esxD* produced a strain that also lacked any immunoreactive signal for EsxB, although production of EsxA and EsxC was unaffected (Anderson *et al.*, 2013). It is assumed that EsxB/EsxD heterodimerization is required for stability of the two proteins, and that EsxB is unstable and rapidly degraded in the absence of EsxD. Of note, EsxD harbors a proposed pseudo-signal sequence, a YxxxD/E motif within its C-terminus, that has been shown to be important in targeting CFP-10 to the mycobacterial ESX-1 secretion machine (Stanley *et al.*, 2003; Champion *et al.*, 2006; Daleke *et al.*, 2012; Rosenberg *et al.*, 2015). In USA300, mutational lesions in this pseudo-signal sequence did not alter secretion; however, deletion of the final six amino acids of EsxD prevented secretion of EsxA and EsxC while allowing secretion of EsxB and EsxD (Anderson *et al.*, 2013). Thus, the staphylococcal ESS pathway secretes at least four small substrates, and their ability to be stably produced and secreted may depend on dimerization and interactions between these different dimers, specifically through the C-terminus of EsxD.

Within the six genes that separate *esxA* and *esxB* is a single gene called *essC*, which encodes a transmembrane protein harboring two FtsK/SpoIIIE-like ATPase domains in its C-terminus that is oriented on the cytoplasmic side of the membrane (Burts *et al.*, 2005; Tanaka *et al.*, 2007). Furthermore, it was shown that at least one of these ATPase domains was required for secretion of EsxA and EsxB (Burts *et al.*, 2005). In addition to *essC*, three other membrane proteins are encoded by the genes between *esxA* and *esxB*: EsaA, EssA, and EssB (Burts *et al.*, 2005; Chen *et al.*, 2012; Kneuper *et al.*, 2014). Deletion of *esaA*, *essA*, *essB*, or *essC* ablates secretion of all of the known ESS-secreted substrates (Burts *et al.*, 2005; Burts, DeDent and Missiakas, 2008; Chen *et al.*, 2012; Anderson *et al.*, 2013; Kneuper *et al.*, 2014).

esaA is a conserved gene within Gram-positive *ess* gene clusters that encodes a large membrane protein predicted to harbor 6 transmembrane domains (Burts *et al.*, 2005; Kneuper *et al.*, 2014). The membrane-embedded EsaA protein has been shown to possess a large unstructured extracellular loop, and the *Bacillus subtilis* homologue, YueB, has been identified as a phage receptor (São-José, Baptista and Santos, 2004; São-José *et al.*, 2006; Dreisbach *et al.*, 2008). Although no specific functions for EsaA have been determined in *S. aureus*, it is possible that EsaA is a key component in the ESS translocation pore due to its numerous transmembrane domains. *essA* is conserved amongst the *ess* gene clusters of Firmicutes, and other than encoding a membrane protein required for ESS secretion there is little known about its exact function (Burts *et al.*, 2005; Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2013).

EssB is a member of the Cluster of Orthologous Groups 4499 (COG4499) which is encoded by *essB*, a closely conserved gene within *ess* clusters of Gram-positive bacteria (Burts *et al.*, 2005; Chen *et al.*, 2012). *essB* produces a monotopic membrane protein that harbors a

kinase-like fold within its cytoplasmic domain, and has been shown to bind analogues of ATP (Zoltner *et al.*, 2013). Like *esaA* and *essA*, deletion of *essB* abolishes secretion of ESS substrates (Chen *et al.*, 2012). Interestingly, expression of EssB variants, in a wild-type USA300 background, lacking either the extracellular N-terminus or intracellular C-terminus yielded a dominant-negative affect on ESS secretion (Chen *et al.*, 2012). Furthermore, recombinant EssB expressed and purified from *E. coli* oligomerized into rod-shaped structures as visualized via transmission electron microscopy (Chen *et al.*, 2012). Based on this data, it has been proposed that EssB oligomerizes in *S. aureus* to facilitate ESS secretion, and that truncated versions of EssB sequester away full-length EssB, preventing secretion and resulting in a dominant-negative phenotype (Chen *et al.*, 2012).

Immediately upstream of *essB*, and partially overlapping with *essA*, is the *esaB* gene which encodes a small cytoplasmic protein with a ubiquitin-like fold (Burts *et al.*, 2005; van den Ent and Löwe, 2005; Burts, DeDent and Missiakas, 2008). In *S. aureus* strain Newman, production of ESS proteins, specifically EsxC, was low compared to the USA300 clinical isolate; however, upon deletion of *esaB* in the Newman background, production and secretion of EsxC increased to levels similar to those observed with strain USA300 (Burts, DeDent and Missiakas, 2008). Based on these observations, it is assumed that EsaB plays some role in controlling expression or production of ESS proteins via a negative regulatory mechanism (Burts, DeDent and Missiakas, 2008). In *S. aureus* strain, RN6390, deletion of *esaB* was found to abrogate ESS secretion, indicating that EsaB may play strain-specific roles (Kneuper *et al.*, 2014). Additionally, it was determined that the innately low levels of ESS protein expression in strain Newman is due to the presence of an allele that produces the SaeR signaling kinase in a constitutively

active state, which ultimately inhibits ESS protein production (Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2013). For this reason, and the importance of understanding the more clinically relevant MRSA strains of *S. aureus*, USA300 has since been used as the model for ESS research in the Missiakas laboratory.

Several publications from our laboratory have highlighted the contribution of *ess* genes toward virulence of *S. aureus* (Burts *et al.*, 2005; Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2011). Growth of *S. aureus* in media supplemented with mammalian blood, serum, or tissue homogenates induces expression and the ultimate secretion of the ESS (Burts *et al.*, 2005; Chen *et al.*, 2012; Anderson *et al.*, 2013). Insertional or clean deletions of *ess* genes in Newman and USA300 yield mutants that are defective in their ability to form abscess lesions, both in the overall number and the structural integrity of the lesions, during a sub-lethal murine renal abscess model (Burts *et al.*, 2005; Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2011). Additionally, the bacterial burden recovered from kidneys infected with *ess* deficient mutants is diminished compared to wild-type infected kidneys (Burts *et al.*, 2005; Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2011). Finally, animals infected with *ess* mutants are able to clear these bacteria and it has been proposed that the ESS pathway contributes to the large persistence observed by wild *type S. aureus* presumable by subverting or inactivating host immune defenses (Burts *et al.*, 2005; Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2011).

In an alternative infection model of pneumonia, it was shown that approximately 60-85% of mice infected with an *ess* mutant survived the infection, whereas only 0-20% of those animals survived the wild-type infection (Ishii *et al.*, 2014; Kneuper *et al.*, 2014). Furthermore, EsxA and EsxB have been implicated in an anti-apoptotic capacity when staphylococci are taken

up by A549 epithelial cells, and have been shown to be important for the release of bacteria from this intracellular environment (Korea *et al.*, 2014).

Recently it has been recognized that the staphylococcal *ess* gene cluster can be broken up into four modules (Warne *et al.*, 2016). The first module, module 1, consists of *esxA*, *esaA*, *essA*, *esaB*, and *essB*, and is found in essentially all staphylococcal isolates endowed with an *ess* cluster (Warne *et al.*, 2016). Module 2 begins with one of four *essC* alleles, *essC1*, *-2*, *-3*, or *-4*, and is followed by downstream genetic elements that are allele specific to the upstream *essC* gene (Warne *et al.*, 2016). It is assumed that the genes downstream of *essC* are accessory proteins for ESS secretion and possible secreted substrates as well (Warne *et al.*, 2016). This model is based on the findings that three of the four genes located immediately downstream of *essC1* in strains USA300 and Newman indeed encode ESS-secreted substrates: *esxC*, *esxB*, and *esxD* (Burts *et al.*, 2005; Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2013; Warne *et al.*, 2016). In its entirety, module 2 of USA300 is comprised of *essC1*, *esxC*, *esxB*, *essE*, *esxD*, and *essD*. *essE* was a heretofore uncharacterized genetic element of the USA300 *ess* gene cluster. Initial characterization of *essD* (*esaD*) showed that it is required for secretion of ESS substrates and the protein accumulates at the cell membrane (Anderson *et al.*, 2011).

Module 3 begins directly downstream of *essD* in USA300 with a gene that encodes a protein containing a Domain of Unknown Function 600 (DUF600) (Anderson *et al.*, 2011; Warne *et al.*, 2016). Along with this first DUF600-encoding gene, USA300 harbors 9 more *duf600*-like genes interspersed between other genes within its third module (Anderson *et al.*, 2011; Warne *et al.*, 2016). DUF600-encoding genes were identified within the genomic region of module 3 for all *S. aureus* isolates containing an ESS, and the number of *duf600*-like genes ranged from 2

to 13 (Warne *et al.*, 2016). The genetic elements that break up the *duf600* gene repeats are primarily putative membrane proteins, and these genes are sometimes present both within module 3 as well as upstream of *esxA* (Warne *et al.*, 2016). Module 2 and 3 are the most diverse modules, and the *essC* allele within module 2 may direct what genetic elements are contained within module 3, similar to how *essC* alleles seem to dictate the downstream genes within module 2 (Warne *et al.*, 2016). In contrast to modules 2 and 3, module 4 consists of only two highly conserved protein coding sequences that were shown to be present in all isolates identified (Warne *et al.*, 2016). The first gene within module 4 is proposed to encode a cystatin-like protein harboring a Domain of Unknown Function 4467 (DUF4467) as well as a predicted N-terminal lipobox sequence (Warne *et al.*, 2016). The second gene of module 4 encodes a protein that is predicted to have 3 transmembrane helices consistent with Domain of Unknown Function 4064 (DUF4064) proteins (Warne *et al.*, 2016). There is little to no experimental evidence thus far confirming any role in ESS secretion for any of the genes within modules 3 and 4.

Thesis objectives

One of the greatest challenges in studying the *esx* and *ess* gene clusters of mycobacterial and non-mycobacterial species is the ability to assign phenotypes to specific genes and their protein products. Intuitively, genes encoding membrane proteins of T7SSs are good candidates for the secretion machinery or translocon and their loss should abolish secretion of effectors. However, it has been observed that the deletion of genes that encode *bona fide* secreted effectors also prevents secretion of other effectors (Fortune *et al.*, 2005; Xu *et al.*, 2007; Burts, DeDent and Missiakas, 2008; DiGiuseppe Champion *et al.*, 2009; Anderson *et*

al., 2013). Thus, it is very difficult to deduce the effector function of distinct secreted proteins when deletion of one substrate prevents the secretion of all the substrates of the secretion system. The ideal experimental model requires the identification of discrete alleles for each effector with mutations that impair effector function without affecting secretion. During the course of this work, *essD* was identified as the most suitable candidate for such studies.

essD was originally proposed to act as a machine component of a putative ESS translocon because the corresponding protein accumulated at the membrane and its loss impaired the secretion of EsxA, EsxB, EsxC and EsxD (Anderson *et al.*, 2011). *essD* encodes a 617 amino acid long protein that falls under the Cluster of Orthologous Groups 5444 (COG5444), a conserved component of Gram-positive T7bSSs (Garufi, Butler and Missiakas, 2008; Anderson *et al.*, 2011). A bioinformatic analysis revealed that the C-terminal portion of COG5444 proteins are often interchangeable regions that harbor toxin-like catalytic domains, and in the case of staphylococcal EssD, the C-terminus was annotated as a distinct domain and putative endonuclease_NS_2 (Garufi, Butler and Missiakas, 2008; Anderson *et al.*, 2011). This bioinformatic analysis also identified a conserved genetic association with *duf600* genes. In fact, several DUF600-encoding genes are always found in the vicinity of one endonuclease_NS_2-encoding gene (Zhang, Iyer and Aravind, 2011; Holberger *et al.*, 2012). In some organisms, the two genes appear to be fused. For example, gene *yeeE* of *Bacillus subtilis* encodes a single protein that harbors an endonuclease_NS_2 domain at its N-terminus and a DUF600 domain at its C-terminus (Zhang, Iyer and Aravind, 2011; Holberger *et al.*, 2012). During the course of this work, it was demonstrated that EssD is a nuclease and catalytically-inactive variants that did not affect secretion were obtained. The second chapter of this thesis sets out to elucidate the

nuclease activity of EssD and define the relationship of DUF600 proteins with EssD and the staphylococcal ESS pathway.

Another challenging aspect within the field of staphylococcal ESS research is elucidation of the molecular mechanisms by which the ESS manipulates the host to promote abscess formation and persistence. In order to accomplish this, a USA300 mutant lacking the module 2-associated genetic element, *essE*, was used, and subjected to extensive characterization to determine its role in ESS secretion. The third chapter in this thesis describes a series of experiments that attempt to identify how ESS influences the recruitment of immune cells and sets up an inflammatory cytokine response that insure the successful outcome for the bacterium during the infection. The fourth chapter in this thesis describes the preliminary characterization of ESS module 4-associated genetic elements and is followed by a conclusion chapter that highlights new findings and presents a model to account for the contribution of the ESS cluster during infection.

Chapter II

EssD, a Nuclease Effector of the *Staphylococcus aureus* ESS Pathway

Except for text referring to Figures 7, 9, and 10, the work presented in this chapter has been published as a manuscript in The Journal of Bacteriology: EssD, a Nuclease Effector of the *Staphylococcus aureus* ESS Pathway (https://doi.org/10.1128/JB.00528-16). The authors of that manuscript were Ryan Jay Ohr, Mark Anderson, Miaomiao Shi, Olaf Schneewind, and Dominique Missiakas.

Abstract

Specialized secretion systems of bacteria evolved for selective advantage, either killing microbial competitors or implementing effector functions during parasitism. Earlier work characterized the ESAT-6 secretion system (ESS) of *Staphylococcus aureus* and demonstrated its contribution to persistent staphylococcal infection of vertebrate hosts. Here, we identify a novel secreted effector of the ESS pathway, EssD, that functions as a nuclease and cleaves DNA but not RNA. EssI, a protein of the DUF600 family, binds EssD to block its nuclease activity in the staphylococcal cytoplasm. An essD knockout mutant or a variant lacking nuclease activity, *essD*^{L546P}, elicited a diminished interleukin-12 (IL-12) cytokine response following bloodstream infection of mice, suggesting that the effector function of EssD stimulates immune signaling to support the pathogenesis of *S. aureus* infections.

Importance

Bacterial type VII or ESAT-6-like secretion systems (ESS) may have evolved to modulate host immune responses during infection, thereby contributing to the pathogenesis of important diseases such as tuberculosis and methicillin-resistant *S. aureus* (MRSA) infection. The molecular mechanisms whereby type VII secretion systems achieve their goals are not fully elucidated as secreted effectors with biochemical functions have heretofore not been identified. We show here that MRSA infection relies on the secretion of a nuclease effector that cleaves DNA and contributes to the stimulation of IL-12 signaling during infection. These results identify a biological mechanism for the contribution of the ESS pathway toward the establishment of MRSA disease.

Introduction

The ESAT-6-like secretion system (ESS) of *Staphylococcus aureus* is conserved in the phylum *Firmicutes* and bears similarities with the well-characterized type 7 secretion system (T7SS) of *Actinobacteria*, including *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*, and *Mycobacterium marinum* (Abdallah *et al.*, 2007; Bitter *et al.*, 2009; Esther J M Stoop, Bitter and van der Sar, 2012). As with many other specialized secretion systems, genes encoding ESS and the T7SS are present within clusters (Abdallah *et al.*, 2007). Genetic analyses have been used to identify the genes that specify the machinery for substrate secretion while secreted factors have been identified as immune reactive species in the extracellular medium of bacterial cultures (Pym *et al.*, 2002; Stanley *et al.*, 2003; Burts *et al.*, 2005; Burts, DeDent and Missiakas, 2008; Garufi, Butler and Missiakas, 2008; Anderson *et al.*, 2013). Candidate factors

for secretion machines share little to no sequence similarity between ESS and T7SS clusters (Abdallah *et al.*, 2007). However, both ESS and T7SS clusters encode secreted and conserved substrates that belong to the protein family pfam06013 (WXG100) within the EsxAB clan CL0352 (pfam.janelia.org/clan/EsxAB) (Pallen, 2002; Abdallah *et al.*, 2007). In the *Actinobacteria* phylum but not *Firmicutes*, the EsxAB clan CL0352 also includes the protein families pfam00934 (PE), pfam00823 (PPE), and pfam10824 (Esp) (Cole *et al.*, 1998; Strong *et al.*, 2006). Sequence identity between protein members of the pfam06013 group (WXG100) is low, often less than 15%, but all of these proteins share a small domain of approximately 100 amino acids with the distinctive WXG motif in the center (Pallen, 2002). The atomic structures of several pfam06013 proteins revealed a conserved fold with two side-by-side α-helices linked with a hairpin bend formed by the WXG motif (Renshaw *et al.*, 2005; Sundaramoorthy, Fyfe and Hunter, 2008; Ilghari *et al.*, 2011; Poulsen *et al.*, 2014). *S. aureus* secretes EsxA and EsxB, two canonical WXG100 proteins (Burts *et al.*, 2005). EsxA forms a dimer with itself and with EsxC, whereas EsxB forms a dimer with EsxD (Anderson *et al.*, 2013). While EsxC and EsxD associate tightly with WXG100 proteins, they do not share the typical sequence features of the pfam06013 family or any other member of the EsxAB clan CL0352 (Anderson *et al.*, 2013).

In virulent strains of *M. tuberculosis*, EsxA (Mt-EsxA) is secreted by intracellular bacteria and has been proposed to form discrete pores that allow mixing of vacuolar and cytosolic contents during infection (Houben *et al.*, 2012). In a model involving bacterial DNA release, the accessibility to cytosolic molecules in turn leads to AIM2 and cyclic GMP-AMP synthase (cGAS) activation (Manzanillo *et al.*, 2012; Watson, Manzanillo and Cox, 2012; Wassermann *et al.*, 2015). Cytosolic DNA sensing by AIM2 leads to activation of the inflammasome interleukin-1β

(IL-1β) pathway (Hornung *et al.*, 2009). Cyclic GMP-AMP (cGAMP) signaling is mediated by STING, the mammalian sensor for cytoplasmic DNA and bacterial cyclic dinucleotides, and drives type I interferon (IFN) activation (Gao *et al.*, 2013). Thus, ESX-1 activity accounts for both the stimulation of IFN and IL-1β responses that contribute to disease progression in tuberculosis (TB) (Dey *et al.*, 2015; Wassermann *et al.*, 2015; Watson *et al.*, 2015).

In *S. aureus*, the ESS pathway contributes to the stimulation of IL-12 (p40) and IL-12 (p35) production and secretion during mouse infection (Anderson *et al.*, 2016). This immunomodulatory activity is thought to contribute to *S. aureus* virulence and persistence within deep-seated abscess lesions (Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2011). Here, we demonstrate that *S. aureus* ESS secretes EssD, which functions as a nuclease to cleave DNA. EssD nuclease activity in the bacterial cytoplasm is blocked by EssI, a small cytoplasmic protein that binds to the nuclease domain of EssD and whose gene is located adjacent to *essD*. Because the EssD-EssI module is reminiscent of toxin-antitoxin modules, we examine whether ESS may contribute a competitive advantage over other staphylococcal species and skin commensals but find no evidence of such antimicrobial activity *in vitro*. Instead, we observe reduced IL-12 production in mice infected with *S. aureus essD* mutants. We therefore propose that EssD represents an effector of the staphylococcal ESS pathway.

Results

***essI* suppresses *essD* induced toxicity.** *essD* encodes a large polypeptide with mosaic structure (Fig. 1A), reminiscent of effectors in specialized secretion systems (Galán *et al.*, 2009). Initial attempts to express *essD* in *Escherichia coli* resulted in the selection of mutants with

single nucleotide transversions and transitions in the 3' portion of the gene, causing either codon substitutions (Asp^{544}Gly, Leu^{546}Pro, and Trp^{563}Arg) or nonsense mutations (Lys^{584}*). Plasmid-borne expression of *essD* in cells that also expressed *essI1*, a gene of unknown function located immediately downstream on the staphylococcal chromosome (Fig. 1B), alleviated the selection for mutational lesions. Of note, the *essI1* (SAUSA300_0289) product is a member of the DUF600 protein family (domain of unknown function 600); nine genes (*essI2* to *essI10*) encoding additional DUF600 family members are located downstream of *essD* (Fig. 1B). DUF600 proteins vary in length from 128 to 166 amino acids and share 66 to 93% sequence identity (Fig. 1C).

Expression of *essD* from the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *lac* promoter (pSRK-Kan^r vector) and *essI1* or *essI6* to *essI10* (*essI6-10*) from the arabinose-inducible promoter (pBAD-Amp^r vector) in *E. coli* (Fig. 1C) revealed that growth of the strain carrying plasmids expressing P_{lac}-*essD* and P_{ara}-*essI1* in Luria broth supplemented with IPTG was strictly dependent on arabinose induction of *essI1* (Fig. 2B). Additionally, *E. coli* P_{lac}-*essD* P_{ara}-*essI6-10* also displayed a requirement for arabinose induction of DUF600 proteins for growth in the presence of IPTG (Fig. 2A and B). Growth inhibition in the presence of IPTG is caused by *essD* expression as *E. coli* P_{lac}- P_{ara}-*essI1*, lacking the *essD* gene, did not display a growth impediment in the presence of IPTG (Fig. 2B).

E. coli P_{lac}-*essD* P_{ara}-*essI6-10* cultures were grown for 3 h, followed by induction with IPTG. Culture aliquots were withdrawn at 0 (T0), 1 (T1), and 2 (T2) h following addition of IPTG to the growth medium and examined with fluorescence microscopy of 4',6'- diamidino-2-phenylindole (DAPI)-stained samples (Fig. 2B and C). Samples were also diluted and plated on

agar with arabinose as well as kanamycin and ampicillin for selection of *E. coli* carrying P_{lac}-*essD* (Kan^r) and P_{ara}-*essI6-10* (Amp^r) (Fig. 2D). Following IPTG induction, *E. coli* P_{lac}-*essD* P_{ara}-*essI6-10* cells lost DAPI fluorescence staining of chromosomal DNA in cultures that had not received the arabinose inducer of DUF600 proteins (Fig. 2C). CFU counts of *E. coli* P_{lac}-*essD* P_{ara}-*essI6-10* were diminished at T1 and T2 after IPTG addition but not at T0 when IPTG was just added to *E. coli* P_{lac}-*essD* P_{ara}-*essI6-10* cultures without arabinose supplement (Fig. 2D). These data support a model whereby *essD* expression is detrimental for *E. coli* growth and causes loss of cell viability, presumably due to the degradation of chromosomal DNA. Coexpression of *essI* prevents the bactericidal activity of EssD.

Association of EssD and EssI. Expression of wild-type *essD* along with *essI1* from the constitutive *hrpK* promoter of plasmid pWWW412 (p*essD-essI1*) did not affect growth of the *S. aureus essD* mutant (*essD::erm* strain). Further, pWWW412 expression of *essD** (p*essD**) in *E. coli* or *S. aureus* did not impair bacterial growth, suggesting that the Leu^{546}Pro substitution abolishes the toxicity of the *essD* product. We generated p*essD**$_{HIS}$, which carries the coding sequence for 10 histidyl residues inserted immediately upstream of the stop codon. Bacteria from 8-liter cultures of *S. aureus* USA300 *essD::erm* harboring plasmid p*essD-essI1* or p*essD**$_{HIS}$ were lysed, and cleared lysates were subjected to immobilized metal affinity chromatography (IMAC) on Ni-nitrilotriacetic acid (Ni-NTA)–Sepharose (Fig. 3). Eluates were analyzed by immunoblotting with rabbit antiserum raised against purified recombinant $EssD^N$ (residues 1 to 200; anti-$EssD^N$), $EssD^C$ (residues 434 to 617; anti-$EssD^C$), or EssI1 (anti-EssI). The data demonstrate that EssD*$_{HIS}$ (predicted M_r of 70,073) elutes as a 72-kDa anti-$EssD^N$ immune-reactive species following IMAC (Fig. 3, lanes 2) whereas untagged EssD does not (Fig. 3, lanes

1). Immunoblotting with anti-EssD^{C} and Coomassie-stained SDS-PAGE analysis demonstrated that EssD*$_{HIS}$ elutes predominantly as C-terminal cleavage products of the full-length polypeptide (Fig. 3). Immunoblotting with anti-EssI revealed the coelution of EssI1 and EssD*$_{HIS}$ during IMAC (Fig. 3). Edman degradation identified SEGKH (EssD*$_{HIS}$ residues 434 to 438) and MTFEE (EssI1 residues 1 to 5) as phenylhydantoin-released residues in five subsequent Edman cycles. These data indicate that EssD*$_{HIS}$ (residues 434 to 617) and EssI (residues 1 to 163) were copurified from *S. aureus* USA300 *essD::erm* (p*essD**$_{HIS}$) during IMAC. Further, *S. aureus* cleaves EssD*$_{HIS}$ and wild-type EssD (see below) to generate C-terminal fragments that bind EssI1.

EssD nuclease activity is inhibited by EssI. EssD harbors a 133-residue domain (residues 475 to 607) with homology to pfam13930 (predicted DNA/RNA nonspecific nuclease from various bacterial species; E value, 6.3×10^{-48}) (Fig. 1A). We hypothesized that EssD consisting of residues 475 to 607 (EssD$_{475–607}$) may function as a nuclease and that the Leu^{546}Pro substitution abolishes its nuclease activity (Fig. 1A). To test this, primer pairs were designed to amplify DNA fragments from *essD* and *essD** encoding EssD$_{434–617}$, EssD$_{456–617}$, EssD$_{476–617}$, EssD$_{486–617}$, and EssD$_{496–617}$ (Fig. 4A). *essD* alleles were cloned into the pSRK vector and transformed into *E. coli* P$_{ara}$-*essI6-10*, and transformants were plated on Amp/Kan agar supplemented with arabinose. Plasmids expressing P$_{lac}$-*essD*$_{434–617}$, P$_{lac}$-*essD*$_{456–617}$, P$_{lac}$-*essD*$_{476–617}$, and P$_{lac}$-*essD*$_{486–617}$ supported growth of *E. coli* P$_{ara}$-*essI6-10* on Amp/Kan plates with arabinose, whereas the plasmid expressing P$_{lac}$-*essD*$_{496–617}$ did not (Fig. 4A). As a control, P$_{lac}$-*essD**$_{434–617}$ and P$_{lac}$-*essD**$_{496–617}$ supported growth of *E. coli* P$_{ara}$-*essI6-10* plated on Amp/Kan agar supplemented with arabinose. These data suggest that EssI requires EssD residues 486 to 496 to neutralize the cytotoxicity associated with the nuclease activity encoded by P$_{lac}$-*essD*$_{496–617}$ (Fig. 4A). In

agreement with this conjecture, growth of *E. coli* P_{lac}-*essD*$_{434–617/HIS}$ P_{ara}-*essI6-10* but not of *E. coli* P_{lac}-*essD**$_{434–617/HIS}$ P_{ara}-*essI6-10* in LB medium supplemented with IPTG was dependent on arabinose supplement for the induced expression of DUF600 proteins (Fig. 4BC).

The cleared lysate of *E. coli* P_{lac}-*essD*$_{434–617/HIS}$ P_{ara}-*essI6-10* was subjected to affinity chromatography on Ni-NTA–Sepharose, and samples were analyzed by immunoblotting (Fig. 4D, lanes 1 to 4). EssI coeluted with EssD$_{434–617/HIS}$ when 0.5 M imidazole was flowed over Ni-NTA–Sepharose (Fig. 4D, lanes 1 to 4). When 8 M urea was flowed over Ni-NTA– Sepharose loaded with *E. coli* P_{lac}-*essD*$_{434–617/HIS}$ P_{ara}-*essI6-10* lysate, EssI was displaced from the column and did not elute with 0.5 M imidazole buffer, unlike EssD$_{434–617/HIS}$ (Fig. 4D, lanes 5 to 8). Purified, refolded EssD$_{434–617/HIS}$ and EssD$_{434–617/HIS}$-EssI complex were added to plasmid DNA, to annealed oligonucleotides (double-stranded DNA [dsDNA]), or to RNA, and reaction products were analyzed by agarose gel electrophoresis (Fig. 4E). Both plasmid DNA and dsDNA, but not RNA, were degraded by EssD$_{434–617/HIS}$ (Fig. 4E). In contrast, EssD$_{434–617/HIS}$-EssI did not degrade plasmid DNA, dsDNA, or RNA (Fig. 4E). Mock-treated (control) or EssD$_{434–617/HIS}$- or EssD$_{434–617/HIS}$-EssI-treated dsDNA was also analyzed by reverse-phase high-performance liquid chromatography (rpHPLC), and eluate was analyzed by absorbance at 260 nm (A_{260}). Data in Fig. 4F demonstrate that EssD$_{434–617/HIS}$ degrades dsDNA, whereas EssD$_{434–617/HIS}$-EssI did not significantly impact the integrity of dsDNA. Together, these data suggest that EssD$_{434–617}$ functions as a nuclease that cleaves dsDNA and that this enzymatic activity is inhibited by binding of EssI. We think it is likely that other members of the DUF600 protein family of *S. aureus* USA300, i.e., EssI1 to EssI10, may also bind EssD and block its nuclease activity.

EssD is secreted in an ESS-dependent manner. Earlier work characterized the ESS pathway in *S. aureus* strain Newman (Burts *et al.*, 2005; Burts, DeDent and Missiakas, 2008) and reported that the N-terminal part of EssD is exposed on the bacterial surface (Anderson *et al.*, 2011). *S. aureus* Newman carries an L-to-A change at position 18 encoded by *saeS* (*saeS*-L18P), which encodes the sensory kinase of the two-component virulence regulator of staphylococci (Steinhuber *et al.*, 2003; Schäfer *et al.*, 2009). Because of the *saeS*-L18P mutation, the ESS pathway cannot be fully activated in *S. aureus* Newman (Chen *et al.*, 2012; Anderson *et al.*, 2013). To analyze the expression and subcellular localization of EssD in cultures of the American MRSA epidemic clone, USA300 harboring wild-type *saeS* (Diep *et al.*, 2006), staphylococci were grown in serum-conditioned medium to an A_{600} of 1.0. Lysostaphin, a staphylococcal murein hydrolase (Schindler and Schuhardt, 1964), was added to lyse staphylococci in culture aliquots; proteins were precipitated with TCA and analyzed by immunoblotting with anti-EssD^{N}, anti-EssD^{C}, anti-EssI, and anti-SrtA (Fig. 5A). Wild-type USA300 cultures produced full-length 72-kDa EssD and its cleavage products of 45 (anti-EssD^{N}), 66, 63, and 25 (anti-EssD^{C}) kDa as well as EssI and SrtA, whereas the *essD::erm* mutant produced only EssI and SrtA (Fig. 5A). USA300 cultures were centrifuged to separate the extracellular medium (Fig. 5B, MD) from the staphylococcal sediment (Fig. 5B, Cell). Staphylococci were lysed with lysostaphin, and bacterial extracts were centrifuged at 108,000 × g to separate cytoplasmic proteins from membrane-associated proteins in the sediment (Fig. 5B, Sol and Insol, respectively). Full-length EssD and its cleavage products were secreted into the extracellular medium, and bacterial EssD was found predominantly associated with bacterial membranes (Fig. 5B). In wild-type USA300, EssI was detected as a soluble component of the bacterial cytoplasm and associated with the membrane

sediment (Fig. 5B, α-EssI). Of note, membrane-associated EssI was not detected in *essD::erm* mutant staphylococci (Fig. 5B, α-EssI). These data suggest that EssI membrane association requires EssD. Although the abundance of EssI clearly exceeds that of EssD, EssI dissociates from EssD during its travels across the cell envelope as EssI is not found in the extracellular medium (Fig. 5B). As controls, α-hemolysin, a secreted product (anti-HLA), was found in the culture medium, whereas sortase A sedimented with the bacterial membranes (anti-SrtA) and ribosomal protein L6 remained in the cytoplasm (anti-L6).

To test whether EssD is secreted via the ESS pathway, we fractionated wild-type USA300 and *essD::erm*, Δ*essB*, and Δ*essB*/p*essB* cultures to separate the extracellular medium from bacterial lysates and analyzed samples by immunoblotting (Fig. 5C, MD and Cell, respectively). As a control, wild-type USA300 secreted EsxC into the extracellular medium, whereas *essD* and *essB* mutant strains did not (Fig. 5C). The EsxC secretion defect of the Δ*essB* mutant was complemented by plasmid-borne expression of wild-type *essB* (Δ*essB*/p*essB* strain) (Fig. 5C). Similarly, EssD was secreted by wild-type staphylococci and the p*essB*-complemented strain but not by the *essD* and *essB* mutants, suggesting that EssD is indeed a secretion substrate of the ESS pathway (Fig. 5C). As expected, all strains examined secreted α-hemolysin (HLA) into the medium and retained EssI in the bacterial cytoplasm (Fig. 5C).

The nuclease activity of EssD is not required for secretion. To examine whether the nuclease activity of EssD contributes to ESS secretion, the *essD*-L546P allele was recombined on the chromosome of *S. aureus* USA300, yielding the *essD*^{L546P} strain. As before, cultures of wild-type USA300 and isogenic *essD::erm* and *essD*^{L546P} strains were centrifuged to separate the extracellular medium from bacterial lysates to analyze the fate of EssD, and EsxC by

immunoblotting (Fig. 6A, MD and cell, respectively). Both EssD and EsxC were found in the extracellular medium of the USA300 and *essD*^{L546P} cultures but failed to be secreted by the *essD::erm* mutant strain (Fig. 6A). All strains secreted α-hemolysin (HLA) into the medium and retained the ribosomal protein L6 in the bacterial cytoplasm (Fig. 6A). Culture supernatants of the USA300, *essD::erm*, *essD*^{L546P}, Δ*essB*, and Δ*essB*/p*essB* strains were filtered, and nuclease activity was assessed by using plasmid DNA as a substrate (Fig. 6B). Degradation of DNA was observed only for culture filtrates of wild-type and complemented Δ*essB*/p*essB* strains, confirming that secreted *essD*^{L546P} is not active. Furthermore, complementation of the USA300 *essD::erm* variant with a plasmid encoding for only the N-terminal 475 amino acids of EssD, p*essD*^{N475}, rescued secretion of EsxA and EsxC as well as secretion of EssD itself (Fig. 7). This supports the hypothesis that the C-terminal domain of EssD-like genes is interchangeable, and that the N-terminal portion, containing the classical COG5444 motif), is the crucial part for secretion of ESS substrates.

***S. aureus* ESS does not impact microbial competition.** The EssD-EssI module is reminiscent of pairs of effector toxins and their cognate immunity proteins identified in the type VI secretion systems, which endow Gram-negative bacteria with competitive advantages over other microbial species (Russell, Peterson and Mougous, 2014). We therefore wondered whether *S. aureus* ESS may also provide a competitive advantage during the bacteria's encounters with other microbes. We first assessed whether EssD production affects *S. aureus* replication. When overnight cultures of wild-type strain USA300 or the *essD::erm* mutant strain were diluted into fresh medium and bacterial replication was monitored at 600 nm (A_{600}), the two strains multiplied at similar rates (Fig. 8A). Culture aliquots were collected at 3, 4, and 6 h

following dilution of overnight cultures and plated on agar medium for colony formation and enumeration. No significant differences in plating efficiencies were observed (Fig. 8B). Next, we asked whether secreted EssD might confer a growth advantage for *S. aureus* over bacteria that lack *essI*-like genes. To test this possibility, wild-type USA300 or the isogenic *essD::erm* variant was grown in the presence of *Staphylococcus simulans* MK148 or *Staphylococcus epidermidis* 12228, isolates which do not harbor genes for ESS clusters or EssI (DUF600) immunity proteins. To assess competitive advantage during prolonged coincubation of microbes, bacteria were enumerated by plating on agar and enumeration of colonies (Fig. 8C). During *S. simulans* MK148 coincubation with *S. aureus*, replication of *S. simulans* MK148 was diminished by ~1 log. However, this growth inhibition cannot be attributed to the ESS pathway or EssD secretion as deletion of *essD* in *S. aureus* USA300 did not alleviate the growth inhibition of *S. simulans* MK148 or *S. epidermidis* 12228 (Fig. 8C). Immunoblot analyses of lysates derived from *S. simulans* MK148 and *S. epidermidis* 12228 cultures confirmed that these isolates did not produce proteins immunoreactive with the anti-EssI polyclonal serum (Fig. 8D).

Recently, Christensen *et al.* demonstrated that *S. epidermidis* 14.1.R1, a skin isolate, effectively killed *Propionibacterium acnes* and *Propionibacterium granulosum*, commensals of the human skin (Christensen *et al.*, 2016). Genome sequencing of *S. epidermidis* 14.1.R1 revealed an ESS cluster with DUF600 proteins similar but not identical to the four different variants identified in the genome sequences of *S. aureus* isolates (Christensen *et al.*, 2016; Warne *et al.*, 2016). It is, however, not clear whether the ESS cluster of *S. epidermidis* 14.1.R1 is indeed responsible for implementation of competitive growth advantages over *P. acnes* and *P. granulosum* isolates. To test whether the *S. aureus* ESS cluster affects microbial competition, *P.*

acnes and *P. granulosum* were used as indicator strains on agar plates also inoculated with wild-type *S. aureus* USA300 and either its isogenic *essD::erm* or *essD*^{L546P} variant (Fig. 8E). Compared to the positive control, *S. epidermidis* 14.1.R1, the *S. aureus* isolates did not impose growth inhibition on either *P. acnes* or *P. granulosum* (Fig. 8E). Further, *S. epidermidis* 14.1.R1 did not kill *S. aureus* USA300 or its *essD* mutants, nor was it capable of killing three other staphylococcal strains, *S. epidermidis* 12228, *S. carnosus*, and *S. simulans* (Fig. 9A-G). Additionally, the only staphylococcal strain that exhibited intra-genus antagonism was *S. epidermidis* when co-cultured with the non-*S. aureus* species (Fig. 9A, C, and D).

***S. aureus* EssD nuclease activity affects IL-12 signaling during bloodstream infection.**

Anderson *et al*. reported that the surge of IL-12 cytokine signaling associated with *S. aureus* bloodstream infection in mice requires EssE-mediated secretion of ESS pathway effectors (Anderson *et al.*, 2016). We wondered whether the EssD nuclease activity of *S. aureus* USA300 is also required for the induction of IL-12 signaling. To address this question, cohorts (n = 5) of C57BL/6 mice were infected by intravenous inoculation with 5×10^7 CFU of *S. aureus* USA300 wild-type or its *essD::erm* and *essD*^{L546P} variants (Fig. 7F). IL-12 cytokine levels were determined in blood samples drawn 12 h after challenge and compared to those of mice mock infected with phosphate-buffered saline (PBS). As expected, *S. aureus* USA300 bloodstream infection caused a surge in IL-12 signaling (Ziegler *et al.*, 2011), which was diminished during infection with the *essD::erm* and *essD*^{L546P} variant strains (Fig. 7F). These data suggest that the nuclease activity of EssD exerts an effector function during *S. aureus* host infection, stimulating IL-12 cytokine signaling to modulate host immune responses.

***S. aureus*-infected BMDMs elicit *essD*-dependent secretion of IL-12.** Although IL-12 has been initially characterized as the potential reason for this immune response; IL-12 and IL-23 are heterodimeric cytokines that share the IL-12p40 subunit and thus it cannot be ruled out that the phenotype observed for IL-12 is in fact due to IL-23 production. To that extent *ex vivo* Bone-Marrow-Derived Macrophage experiments were performed using a Gentamicin-protection-like infection model. Interestingly, BMDMs infected with wild-type USA300 elicited IL-12 secretion similar to what is seen in the mouse, and this IL-12 secretion was similarly depreciated when BMDMs were infected with the *essD::erm* variant (Fig. 10A). ELISA analysis of IL-23p19, IL-6, and RANTES secretion under these conditions was also monitored, and both IL-23p19 and RANTES (Fig. 10B,D) do not show an *essD*-dependent secretion, whereas IL-6 exhibited a phenotype similar to the observed levels of IL-12 secretion produced by wild-type and the *essD::erm* mutant (Fig. 10C). Furthermore, examination of IL-23 production, via an IL-23p19-specific ELISA, indicate that IL-23 is produced at concentrations approximately 100 times lower than IL-12 and does not appear to be secreted in an EssD-dependent manner (Fig. 10B). Although macrophages cannot be considered the sole IL-12 or IL-6-producing cell, these data indicate that, in the context of a mouse infection, at least some of the cytokine secretion is being produced via macrophages through an *essD*-dependent mechanism.

Discussion

EssD belongs to the cluster of orthologous group 5444 (COG5444), members of which are widely distributed in Gram-positive bacteria. COG5444 genes are associated with ESS gene clusters (Garufi, Butler and Missiakas, 2008; Anderson *et al.*, 2011). However, the C-terminal domain of this gene is highly variable. In *S. aureus*, the C-terminal domain of COG5444 (EssD) is

annotated as endonuclease_NS_2 (protein family PF13930). In *Listeria monocytogenes*, COG5444 (Lmo0066) carries a VIP2 C-terminal domain, which is otherwise known to modify host actin via ADP-ribosylation (Anderson *et al.*, 2011). In *Bacillus anthracis*, COG5444 (EsxL) harbors a C-terminal domain of the nucleotide deaminase superfamily (Garufi, Butler and Missiakas, 2008). The C-terminal domains have been grouped into the novel SUKH superfamily that encompasses the nuclease and nucleic acid deaminase families (Zhang, Iyer and Aravind, 2011). As with the contact-dependent inhibition (CDI) systems (Aoki *et al.*, 2010) and colicin-like nuclease toxins of proteobacteria (Cascales *et al.*, 2007), SUKH superfamily members are found in close association with a distinct superfamily of proteins proposed to function as SUKH immunity factors (Zhang, Iyer and Aravind, 2011; Holberger *et al.*, 2012; Jamet *et al.*, 2015). Holberger *et al*. provided biochemical validation for this model by demonstrating that the C-terminal domains of three COG5444-like proteins (YobL, YxiD, and YqcG) of *B. subtilis* 168 display cytotoxic RNase activities that are neutralized by the binding of cognate antitoxin (immunity) proteins (Holberger *et al.*, 2012). However, the biological significance of *B. subtilis* cytotoxic RNase activities has not yet been elucidated (Holberger *et al.*, 2012). SUKH and immunity proteins appear to cluster with various known or predicted secretion systems and have been termed polymorphic toxin systems (Zhang, Iyer and Aravind, 2011; Zhang *et al.*, 2012). These secretion systems include filamentous surface proteins such as the rearrangement hot spot (Rhs) and related YD-peptide repeat proteins, contact-dependent inhibition (CDI), and T5SS, T6SS, and ESS secretion systems (Zhang, Iyer and Aravind, 2011; Zhang *et al.*, 2012). Similar to CDI and Rhs- and T6SS-mediated contact-dependent inhibition, toxins are injected into target cells presumably to help establish a niche and defend it against other bacteria

(Silverman *et al.*, 2012; Koskiniemi *et al.*, 2013). We demonstrate here that EssD is a secreted effector of the ESS pathway in *S. aureus* with broad nuclease activity toward double-stranded DNA. Intracellular EssD is highly cytotoxic, and its DNase activity is inhibited by binding to EssI. However, the EssD-EssI module does not appear to confer on *S. aureus* a growth advantage over other commensals of the human skin and nares, including *P. acnes*, *P. granulosum*, *S. epidermidis*, and *S. simulans*.

Bacterial pathogens evolved with their hosts and manipulate the recognition of their pathogen-associated molecular patterns by pattern recognition receptors or usurp vertebrate immune signaling schemes to establish an environment conducive to their lifestyle. *Escherichia coli*, *Shigella boydii*, *Haemophilus ducreyi*, *Actinobacillus actinomycetemcomitans*, *Helicobacter hepaticus*, and *Campylobacter jejuni* produce cytolethal distending toxin (CDT) (Lara-Tejero and Galán, 2000). CDT is comprised of three subunits (CdtA, CdtB, and CdtC), and its CdtB effector, a homologue of DNase I, cleaves double-stranded DNA *in vitro* and host cell chromatin *in vivo* (Lara-Tejero and Galán, 2002). Association of CdtA and CdtC with CdtB is necessary for CDT binding and uptake into eukaryotic cells (Lara-Tejero and Galán, 2001), where CdtB travels into the nucleus to generate double-strand breaks, arresting the cell cycle of intoxicated cells at G2M and activating DNA damage response pathways (Lara-Tejero and Galán, 2000). *Salmonella enterica* serovar Typhi typhoid toxin is assembled as an A_2B_5 molecule with two A subunits, PltA, a homologue of *Bordetella pertussis* pertussis toxin with ADP-ribosyltransferase activity, and CdtB, a homologue of *C. jejuni* CdtB (Song, Gao and Galán, 2013). When analyzed in a humanized mouse model, injection purified typhoid toxin (PltA-CdtB-PltB5) causes clinical signs of typhoid fever and eliminates bloodstream neutrophils in a manner that is dependent on CdtB

nuclease but not PltA activity (Song *et al.*, 2010; Song, Gao and Galán, 2013). Thus, nuclease effectors represent a key virulence strategy for bacterial pathogenesis. Here, we add further support to this model by demonstrating that *S. aureus* EssD, a nuclease effector, contributes to the pathogenesis of bloodstream infections. *S.* Typhi produces typhoid toxin only during infection, when it is released into the extracellular environment by a unique transport mechanism involving vesicle carrier intermediates (Spanò *et al.*, 2008). Similarly, EssD secretion also occurs during infection, and we presume that the ESS pathway may direct EssD into the nucleus of host cells but do not yet know whether this involves intracellular or extracellular staphylococci.

Earlier work identified inflammatory myeloid cells as sources of IL-12 secretion during bacterial infection (Gately *et al.*, 1998). In agreement with this conjecture, depletion of dendritic cells in mice abrogates IL-12 signaling during *S. aureus* infection (Schindler *et al.*, 2012). IL-12 production skews the immune system of infected hosts toward T_H1 responses and the activation of cellular immunity by promoting the differentiation of naive CD4 T cells into gamma interferon-producing T_H1 cells (Gately *et al.*, 1998). As *S. aureus* abscess lesions require persistent supplies of immune cells for the destruction of host tissues and the drainage of purulent material with staphylococci, it seems plausible that staphylococci activate IL-12 signaling to accomplish these goals. In addition, IL-12 skewing of gamma interferon-producing T_H1 cells also impedes T_H2 polarization (Zundler and Neurath, 2015), suggesting that the ESS pathway also interferes with the host's ability to produce antibodies. Thus, ESS secretion of EssD may contribute to both virulence and immune evasive strategies of *S. aureus*.

Materials and Methods

Bacterial growth conditions. *S. aureus*, *S. epidermidis*, *S. carnosus*, and *S. simulans* were grown in tryptic soy broth (TSB) or agar (TSA) at 37°C, supplemented with chloramphenicol (Cm; 20 µg/ml) for plasmid selection and 0.2% heat-inactivated horse serum (Gibco/Life Technologies) for induction of the ESS pathway (Chen *et al.*, 2012). For coculture assays between staphylococcal species, bacteria were grown in TSB supplemented with 0.2% heat-inactivated horse serum at 37°C to an absorbance at 600 nm (A_{600}) of 10.0. Ten microliters of bacterial suspensions was mixed in a 1:1 ratio and spotted on TSA supplemented with 0.2% heat-inactivated horse serum at 37°C for 9 h. Bacteria grown on these plates were transferred to PBS, serially diluted, and plated in triplicate for CFU enumeration on TSA containing chloramphenicol (Cm; 5 µg/ml) or kanamycin (Kan; 20 µg/ml) to enumerate *S. simulans* or *S. epidermidis* and *S. aureus* USA300. To assess whether USA300 displays a growth advantage over *Propionibacterium* strains, cultures of *P. acnes* and *P. granulosum* were incubated in reinforced clostridial medium (BD) at 37°C under anaerobic conditions (80% N_2, 10% CO_2, and 10% H_2) for 48 to 72 h and diluted to an A_{600} of 1.0, and 500 µl was plated on TSA. Cultures (3 µl) of test bacteria were added on top of *Propionibacterium* lawns, and plates were incubated at 37°C under anaerobic conditions for another 72 h. These same procedures were followed when intra-staphylococcal antagonism assays were performed with the modification of growth in aerobic conditions for 24-48 h. *E. coli* was grown in Luria-Bertani (LB) broth or agar at 30°C or 37°C. Where necessary, ampicillin (Amp; 100 µg/ml), Kan (50 µg/ml), arabinose (Ara; 0.2%), and isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.5 mM) were added to *E. coli* cultures. To examine growth of *E. coli* carrying various plasmids, overnight cultures grown at 30°C in LB

broth containing Amp, Kan, and arabinose were diluted 1:100 in 50 ml of fresh broth supplemented with both antibiotics and in the presence or absence of arabinose. IPTG was added 3 h after dilution of cultures. Bacterial growth in cultures was monitored by recording absorbance (A_{600}). To assess viability, culture aliquots were serially diluted, plated on agar in the presence of antibiotic and arabinose when needed, and grown overnight at 30°C.

Bacterial strains and plasmids. *S. aureus* RN4220 was used to passage plasmid DNA. USA300 LAC, a clone of the American community-acquired methicillin-resistant *S. aureus* (CA-MRSA) epidemic strain (Diep *et al.*, 2006), was used as the wild-type *S. aureus* strain for all other experiments. The *essD::erm*, Δ*essB*, and complemented Δ*essB*/p*essB* strains have been described earlier (Anderson *et al.*, 2011; Chen *et al.*, 2012). *S. epidermidis* ATCC 12228 (*S. epidermidis* 12228), *P. acnes* (ATCC 6919), and *P. granulosum* (ATCC 25564) were obtained from the American Type Culture Collection (ATCC.org). *S. simulans* MK148 (ATCC 27848) and *S. epidermidis* 14.1.R1 were gifts from Friedrich Götz and Holger Brüggemann, respectively. For the cloning of *essD*, three additional codons were included upstream of the start codon assigned in the referenced sequence (*Staphylococcus aureus* subsp. *aureus* USA300_FPR3757; NCBI RefSeq accession number NC_007793.1). Primers 1 (5'-ATAAGCTAGCAGGAGGTGCCAACATGACATTTG-3') and 2 (5'-TGGCGGTACCTTATTCTTCTAGCTCTTTAATATATTGCTCGAT-3') as well as primers 3 (5'-CGATGCTAGCTACTGAACCAAGCAGTGATGAA-3') and 4 (5'-GCCGGTACCCTACTCTTGCTCTTTAACATACTTCTCTAC-3') were used to amplify by PCR the gene SAUSA300_0289 or the last five contiguous DUF600-encoding genes, SAUSA300_0298 to SAUSA300_0302, using USA300 chromosomal DNA as the template. Inserts were cloned into

the NheI and KpnI sites of the pBAD24 vector carrying the arabinose promoter and ampicillin resistance cassette (pBAD-Amp^r). The resulting plasmids expressed P_{ara}-*essI1* and P_{ara}-*essI6-10*, respectively. Primers 5 (5'-GGCAAACCATATGACAAAAGATATTGAATATCTAACAGCTG-3') and 6 (5'-ATGTCTCGAGCTACTTATTTAATATTCTTCTAATATTTCTTTCACCATTAA-3') or primers 7 (5'-GGCAGGTCATATGTACACCAAGGTTGAATTCGG AGAACACTAT-3') and 8 (5'-ATATCTCGAGCTAATGGTGATGGTGATGGTGCTTATTTAATATTCTTCTAATATTTCTTTCACCATTAA-3') were used to amplify full-length *essD* or the nuclease-encoding fragment with six appended histidine codons at the 3' end, $essD_{434–617/HIS}$, respectively. Wild-type template DNA was used for PCR. Cloning of *essD* also resulted in the isolation of the *essD* Leu^{546}Pro allele where leucine at position 546 within the nuclease domain was changed to proline (*essD**). This plasmid was used as a template for amplification using primers 7 and 8. All *essD*-bearing fragments were cloned into the NdeI and XhoI sites of the *E. coli* pSRK vector carrying the lactose promoter and resistance marker for kanamycin (pSRK-Kan^r). The three resulting plasmids were named P_{lac}-*essD*, P_{lac}-$essD_{434–617/HIS}$, and P_{lac}-$essD^*_{434–617/HIS}$. For the cloning of fragments encompassing the last 162, 142, 132, and 122 amino acids of EssD, the following primers were used for 5' amplification: 9 (5'-GGCAGGTCATATGTACACCAAGGTTGAATTCGGAGAACACTAT-3'), 10 (5'-GGCACCGCATATGAATATTGAATACACAACACCTACTGGTCAC-3'), 11 (5'-GGCAGGGCATATGATATATCGAACCGATCATAAAGGTCGCATA-3'), and 12 (5'-CCCCGGCCATATGAAAGAAGTTTATGTAGACAATCTCTCTCT-3'). Cloning of these fragments was performed as described for wild-type *essD* using primer 6 for 3' amplification. For the purification of recombinant proteins used to generate antibodies, primers 13 (5'-ATCGGATCCATGCATGACATGACAAAAGATATTGAATATC-3') and 14 (5'-

GCAGAATTCCTAAGCTACTGTCATGTAATCACAAGAA-3') or 15 (5'-ATCGGATCCAGTGAAGGCAAACATAGTATAAGTAGC-3') and 16 (5'-TACCCGGGCTACTTATTTAATATTCTTCTAATATTTCTTTCACCATTAA-3') were used to amplify the coding region for the first 200 amino acids or last 185 amino acids of EssD, respectively, using template DNA from the Leu^{546}Pro variants. Primers 17 (5'-AAGGATCCATGACATTTGAAGAGAAG-3') and 18 (5'-AAGAATTCTTATTCTTCTAGCTCTTTA-3') were used to amplify the coding region of *essI1*, and the insert was subsequently cloned into BamHI and EcoRI or BamHI and SmaI sites of vector pGEX-2T, respectively. For complementation studies in *S. aureus*, primers 19 (5'-GGCAGGCCATATGCATGACATGACAAAAGATATTGAATATCTAACAGCTG-3') and 20 (5'-ACGGGATCCTTATTCTTCTAGCTCTTTAATATATTGCTCGAT-3') were used to amplify *essD-essI1* from wild-type DNA and for cloning into the NdeI and BamHI sites of pWWW412, resulting in the plasmid p*essD-essI1*. For cloning of *essI1* into pSRK, primers 21 (5'-GGCAAACCATATGACATTTGAAGAGAAGCTTAGCAAAATATACAAT-3') and 22 (5'-ATGTCTCGAGTTATTCTTCTAGCTCTTTAATATATTGCTCGAT-3') were used. Primers 5 and primers 25 (5'-TATTCTCGAGCTAATGGTGATGGTGATGGTGCGCCTTTAGTTTCTTAGGTCTAAGTCTTG-3') were used to amplify the coding sequence for generating the p*essD*^{N475} complementing strain from wild-type USA300 DNA. Primers 5 and 8 were used to amplify DNA from the template bearing the mutant allele for the Leu^{546}Pro substitution. The insert was also cloned in pWWW412, resulting in plasmid p*essD**$_{HIS}$. To construct the isogenic *essD*^{L546P} variant recombined on the chromosome of strain USA300, primer 21 (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGACAAAAGATATTGAATATCTAACAGCTG-3') and 22 (5'-GGTTATCAATGTCTTTTGAACCACCAAACATTCTAGCGAT-3') and primers 23 (5'-

CTGTGGCACAAAGTAAATTTATCAACCGTCCATTTAAGGA-3') and 24 (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTATTCTTCTAGCTCTTTAATATATTGCTC-3') were used to amplify two DNA fragments that were ligated together and reamplified using primers 21 and 24. The new product was cloned into the allelic replacement vector, pKOR1, as described previously (Bae and Schneewind, 2006). Whole-genome sequencing of the wild-type USA300 parent and two *essD*^{L546P} recombined isolates was performed using Truseq DNA-seq Library Preparation and Illumina MiSeq technology by the Next Generation Sequencing Core at Argonne National Laboratory. Raw sequence data were analyzed using the bioinformatics software Geneious.

Microscopy. Aliquots of culture samples were fixed with 4% formaldehyde for 10 min, washed three times with PBS, and transferred onto a microscope slide. One drop of SlowFade Gold solution with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies) was added to each slide. Specimens were overlaid with a coverslip and visualized with an Olympus AX70 microscope using a 100× objective and a UV filter, and images were captured with a digital camera.

Purification of proteins and biochemical assays. For the purification of glutathione S-transferase (GST)- or histidine-tagged proteins from *E. coli*, strains were grown at 37°C in LB broth with appropriate antibiotics and inducers in a 4-liter volume until they reached an A_{600} of 1.0. Spent culture medium was discarded following centrifugation at 9,000 × g for 10 min. Bacteria in cell pellets were suspended in 20 ml of buffer A (50 mM Tris-HCl, pH 7.5, 300 mM NaCl) and lysed by passage through a French pressure cell (twice at 15,000 lb/in^2). Lysates were cleared by centrifugation at 100,000 × g for 2 h at 4°C, and the supernatants were loaded by

gravity flow onto glutathione-Sepharose beads (GE Healthcare) or Ni-NTA beads (Qiagen) preequilibrated in buffer A. Beads were washed with 20 volumes of buffer A, and bound proteins were eluted with either 20 mM reduced glutathione or increasing concentrations of imidazole (up to 0.5 M). Eluted proteins were dialyzed into PBS or buffer B (see below), quantified by bicinchoninic acid assay (Pierce), and kept at 4°C for immediate use or stored frozen at -80°C. Proteins dialyzed in PBS were used for the generation of rabbit polyclonal antibodies as described earlier (Chan *et al.*, 2014). For purification of proteins from *S. aureus*, 8-liter cultures were grown in TSB with appropriate antibiotics at 37°C with shaking until they reached an A_{600} of 2.0. Samples were processed as described for the purification of histidine-tagged proteins from *E. coli* except that bacterial suspensions were first incubated with lysostaphin (100 µg/ml) at 37°C for 1 h prior to passage through the French pressure cell. To assess nuclease activity, proteins (5 µl; ~ 0.05 µg) or 5 µl of supernatant of cultures grown to an A_{600} of 3.0 was incubated with either 500 ng of pGEX-2T DNA or 100 ng of annealed complementary 48-nucleotide-long oligomers (double-stranded DNA substrate; dsDNA) or 100 ng of total RNA from *E. coli* purchased from Life Technologies. Reactions were performed in a 20-µl volume in buffer B (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, and 1 mM dithiothreitol [DTT], pH 7.8), and tubes were incubated at 37°C for 3 h. DNA loading buffer (6×; 30% [vol/vol] glycerol, 0.25% [wt/vol] bromophenol blue, 0.25% [wt/vol] xylene cyanol) was added to each tube prior to separation of samples on a 1% or 2% agarose gel. DNA in the gel was visualized with a UV transilluminator for image acquisition. For HPLC analysis, 7 µg of dsDNA was incubated with ~0.7 µg of proteins or buffer control in a total reaction volume of 700 µl in buffer B for 20 h at 37°C. Samples were heated at 95°C for 10 min,

centrifuged at 17,000 × g for 10 min, and loaded onto a C18 column (250 mm by 3 mm; 5-µm particle size [BDS Hypersil C18; Thermo Fisher Scientific]) maintained at 42°C and run at a flow rate of 0.5 ml/min. Buffer C (65 mM potassium phosphate, pH 6.0) was used as the mobile phase for 10 min, followed by a 50-min-long gradient of 1 to 100% buffer D (65 mM potassium phosphate, 25% methanol, pH 6.0) and a 5-min wash with 100% buffer D.

Protein secretion and subcellular fractionation. To examine the production and subcellular localization of EssD in *S. aureus*, overnight cultures were diluted 1:100 in 50 ml of TSB supplemented with appropriate antibiotics and heat-inactivated horse serum. Cultures were grown with shaking at 37°C until an A_{600} of 1.0. Lysostaphin was added to the whole culture (20 µg/ml) at 37°C for 1 h, yielding samples labeled as culture lysate. To identify proteins secreted in the medium, cultures were subjected to centrifugation (9,000 × g for 10 min), and the spent medium was filtered with a 0.22-µm-pore-size Millex-GP unit and transferred to a fresh tube (medium fraction [MD]). Cells in the pellet were washed and lysed with lysostaphin (20 µg/ml) at 37°C for 1 h, and cellular lysates were further subjected to centrifugation at 100,000 × g for 2 h at 4°C to separate soluble (Sol) from insoluble (Insol) proteins associated with the membrane. Proteins in all fractions were precipitated by adding 100% trichloroacetic acid (TCA) to a final concentration of 10% and left on ice for 1 h. Precipitated materials were sedimented by centrifugation (13,000 × g for 10 min at 4°C) and washed with cold acetone, and pellets were air dried before suspension in 160 µl of 0.5 M Tris-HCl, pH 8.0.

SDS-PAGE and immunoblotting. Bacterial and protein extracts were mixed with 1/5 volume of sample buffer (0.1 M Tris-HCl [pH 8.0], 4% SDS, 20% glycerol, 2 mM β-

mercaptoethanol, 0.04% bromophenol blue), heated at 90°C for 10 min, and subjected to 12% SDS-PAGE. Proteins were visualized either by direct staining of gels using Coomassie brilliant blue R-250 (Aldrich-Sigma) or by immunoblotting following electrotransfer to polyvinylidene difluoride (PVDF) membranes. For immunoblotting, membranes were blocked by incubation in PBS containing 0.1% Tween-20 (PBST) and 5% milk for 1 h at room temperature. To prevent binding of primary antibodies to protein A, 0.8 mg of human IgG was added to 10 ml of blocking buffer. After incubation (1 h at room temperature), immune serum (primary polyclonal antibodies) was added, and the membranes were incubated for up to 16 h at 4°C. Membranes were washed four times for 10 min in PBST and incubated with secondary antibody (goat anti-rabbit HRP-linked antibody; Cell Signaling Technology) in PBST with 5% milk for 1 h. Membranes were washed four times with PBST, incubated with SuperSignal West Pico Chemiluminescent Substrate (Life Technologies), and developed on Amersham Hyperfilm ECL (GE Healthcare Life Sciences).

Antisera. New Zealand White rabbits were purchased from Harlan Sprague Dawley and used to generate the polyclonal $EssD^{N}$ (first 200 amino acids of EssD), $EssD^{C}$ (last 185 amino acids of EssD), and EssI (EssI1 encoded by SAUSA300_0289) antisera. Purified antigens (100 μg) were emulsified with complete Freund's adjuvant (Difco), and emulsions were injected subcutaneously into the rabbits. At 21-day intervals, two booster immunizations with 100 μg of antigen emulsified with incomplete Freund's adjuvant were performed. Sera were obtained from blood samples, mixed with 0.02% sodium azide, aliquoted, and frozen for long-term storage at -80°C or stored at 4°C for reiterative use.

Animal infection. Cohorts of five 5-week-old C57BL/6 mice (Jackson Laboratory) were anesthetized with 65 mg/ml ketamine and 6 mg/ml xylazine per kilogram of body weight via intraperitoneal injection. Animals were infected via retro-orbital injection with 5×10^7 CFU of bacteria grown to an A_{600} of 0.4, washed, and suspended in 100 µl of PBS. Control animals were inoculated with 100 µl of PBS alone. At 12 h postinfection, mice were euthanized via CO_2 inhalation, and blood was collected by cardiac puncture for serum preparation and analysis. A Novex Mouse IL-12 Antibody Pair kit CMC0123 (Life Technologies) was used to measure IL-12 (p70) levels.

BMDM infection. The day prior to infection of Bone-Marrow-Derived Macrophages (BMDMs) complete RPMI-1640 medium (4.5 g/l glucose, 10 mm HEPES, 1 mm sodium pyruvate) supplemented with 20% FBS and Pen/Strep is prepared and pre-warmed to 37^{o}C. Previously frozen aliquots of BMDMs stored in liquid nitrogen are removed and thawed in a 37^{o}C water bath with gentle agitation. BMDMs are washed with warm complete RPMI-1640 medium once, suspended and counted with a hemocytometer to determine cell concentration before being seeded in 24-well plates at 3.5 x 10^5 cells per well, in 1 ml media. On the same day BMDMs are seeded, cultures of the staphylococcal strains to be examined are inoculated in TSB and incubated at 37^{o}C with shaking overnight. The next day, overnight cultures are refreshed at ~1:100 in 30 ml TSB containing 0.2% heat-inactivated horse serum and incubated at 37^{o}C with shaking until an A_{600} of 0.4-0.6 was reached. Cultures were pelleted and washed with PBS twice, before being diluted in pre-warmed complete RPMI-1640 medium without antibiotics. The final concentration was calculated to yield a Multiplicity of Infection of 1 (1 macrophage to 1 bacteria). Media was removed from the wells of the 24-well plate seeded with BMDMs, and the

bacterial-RPMI-1640 suspension was added to the wells. Plates were centrifuged at 150 x g for 5 minutes, followed by incubation at 37oC with 5% CO_2 for 1 hour. Subsequently, the media was removed, replaced with complete RPMI-1640 media with Gentamicin at a final concentration of 20 μg/ml, and allowed to incubate at 37^{o}C with 5% CO_2 for 20 hours. Media was removed and subjected to cytokine analysis with ELISA kits specific to IL-12p70, IL-23p19, IL-6, and RANTES.

Ethics statement. The preparation of rabbit antibodies and mouse challenge experiments were performed according to protocols that were reviewed, approved, and performed under the regulatory supervision of The University of Chicago's Institutional Animal Care and Use Committee (IACUC). Animal experiments were conducted in accordance with recommendations detailed in the Guide for the Care and Use of Laboratory Animals (National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals. and Institute for Laboratory Animal Research (U.S.), 2011). Animal care was managed by The University of Chicago Animal Resource Center, which is accredited by the American Association for Accreditation of Laboratory Animal Care and acts in compliance with the NIH guidelines on laboratory animal care and use.

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Chapter III

EssE Promotes *Staphylococcus aureus* ESS-Dependent Protein Secretion to Modify Host Immune Responses during Infection

The work presented in this chapter has been published as a manuscript in The Journal of Bacteriology: EssE Promotes *Staphylococcus aureus* ESS-Dependent Protein Secretion to Modify Host Immune Responses during Infection (https://doi.org/10.1128/JB.00527-16). The authors of that manuscript were Ryan Jay Ohr, Mark Anderson, Khaled A. Aly, Salvatore Nocadello, Hwan K. Kim, Chloe E. Schneewind, Olaf Schneewind, and Dominique Missiakas.

Abstract

Staphylococcus aureus, an invasive pathogen of humans and animals, requires a specialized ESS pathway to secrete proteins (EsxA, EsxB, EsxC, and EsxD) during infection. Expression of *ess* genes is required for *S. aureus* establishment of persistent abscess lesions following bloodstream infection; however, the mechanisms whereby effectors of the ESS pathway implement their virulence strategies were heretofore not known. Here, we show that EssE forms a complex with other members of the ESS secretion pathway and its substrates, promoting the secretion of EsxA, EsxB, EsxC, EsxD, and EssD. During bloodstream infection of mice, the *S. aureus essE* mutant displays defects in host cytokine responses, specifically in the production of interleukin-12 (IL-12) (p40/p70) and the suppression of RANTES (CCL5), activators of T_H1 T cell responses and immune cell chemotaxis, respectively. Thus, *essE*-mediated secretion of protein effectors via the ESS pathway may enable *S. aureus* to manipulate host immune responses by modifying the production of cytokines.

Importance

Staphylococcus aureus and other firmicutes evolved a specialized ESS (EsxA/ESAT-6-like secretion system) pathway for the secretion of small subsets of proteins lacking canonical signal peptides. The molecular mechanisms for ESS-dependent secretion and their functional purpose are still unknown. We demonstrate here that *S. aureus* EssE functions as a membrane assembly platform for elements of the secretion machinery and their substrates. Furthermore, *S. aureus* EssE-mediated secretion contributes to the production or the suppression of specific cytokines during host infection, thereby modifying immune responses toward this pathogen.

Introduction

Staphylococcus aureus, a commensal of humans and their domesticated animals (Wertheim *et al.*, 2004; Fitzgerald, 2012), is also an invasive pathogen that replicates via the formation of abscess lesions in tissues of infected hosts (Lowy, 1998; David and Daum, 2010). Abscess formation requires staphylococcal coagulases, secreted products associating with host prothrombin to generate a fibrin shield, thereby establishing a physical barrier between the pathogen and the host's immune defenses (Friedrich *et al.*, 2003; Cheng *et al.*, 2010; Thomer *et al.*, 2016). *S. aureus* lesions attract large numbers of immune cells, predominantly neutrophils and lymphocytes, whose lysis and proliferation in the vicinity of staphylococcal abscess communities is associated with tissue destruction (Thammavongsa, Missiakas and Schneewind, 2013). Drainage of the ensuing purulent exudate ensures the spread of *S. aureus* in infected individuals or transmission to new hosts (Cheng *et al.*, 2011). Without surgical intervention or implementation of effective antibiotic therapy, infected hosts cannot clear *S. aureus* from deep-

seated abscesses or from skeletal and internal organ lesions (Cheng *et al.*, 2009; Liu *et al.*, 2011; Ziegler *et al.*, 2011).

In addition to manipulating host hemostasis, *S. aureus* elaborates immune evasive strategies aimed at interfering with the chemotaxis of immune cells, the activation of complement, and opsonophagocytosis or the bactericidal activities of phagocytes (Spaan *et al.*, 2013). Earlier work identified the pathogen's ESS pathway (EsxA/ESAT-6-like secretion system), which is encoded by a cluster of contiguous genes on the staphylococcal chromosome (Fig. 11A) (Burts *et al.*, 2005; Burts, DeDent and Missiakas, 2008). When induced for *ess* expression during growth in vertebrate blood or serum, *S. aureus* ESS secretes four small proteins, designated EsxA, EsxB, EsxC, and EsxD (Burts *et al.*, 2005; Chen *et al.*, 2012; Anderson *et al.*, 2013). Mutations that abrogate *ess* expression diminish the abundance of abscess lesions and their bacterial load compared to levels of lesions seeded by wild-type *S. aureus* (Burts *et al.*, 2005; Anderson *et al.*, 2011). Further, lesions derived from mutants with defects in *ess* expression are formed more rarely and cleared more frequently than abscesses populated with wild-type *S. aureus* (Burts, DeDent and Missiakas, 2008).

The mechanisms whereby the *S. aureus* ESS pathway implements its immune evasive strategies in the host were heretofore not known. Here, we show that *essE*, encoding a membrane-associated protein, is required for *S. aureus* secretion of EsxA, EsxB, EsxC, and EsxD. EssE forms a complex with EsxC and with other components of the ESS pathway, including EssC, EssD, and EssI. In the accompanying paper (Ohr *et al.*, 2016), we report that EssD is also secreted by the ESS pathway and that the protein bears a C-terminal nuclease domain ($EssD^{C}$), whose activity is inhibited by EssI in the bacterial cytoplasm. Here, we report that interaction

with EssE in the cytosol of *S. aureus* is important for EssD stability. Unlike wild-type *S. aureus*, *essE* mutants display defects in host cytokine responses, specifically the production of interleukin-12 (IL-12) (p40/p70) and the suppression of RANTES (CCL5), activators of T_H1 T cell responses and T cell chemotaxis, respectively (Ziegler *et al.*, 2011; McNicholas *et al.*, 2014). We propose that *essE*-mediated secretion of protein effectors via the ESS pathway may enable *S. aureus* to manipulate host immune responses by modifying the production of specific cytokines.

Results

EssE copurifies with EssD. *S. aureus* expression of *essD* is required for Esx protein secretion by the ESS pathway (Anderson *et al.*, 2011; Ohr *et al.*, 2016). EssD is located in the bacterial membrane owing to the presence of a hydrophobic domain between residues 217 and 250 (Anderson *et al.*, 2011). Nevertheless, *S. aureus* also secretes EssD into the extracellular medium (Ohr *et al.*, 2016). We wondered whether retention of EssD at the plasma membrane could be caused by interaction with other protein ligands and used a biochemical approach to test this model. First, the *essD* open reading frame was extended at the 3' end with 10 codons for C-terminal histidine residues (His_{10}), enabling affinity chromatography purification on Ni-nitrilotriacetic acid (Ni-NTA)–Sepharose (Ohr *et al.*, 2016). Cloning of *essD* is associated with bacterial toxicity owing to the nuclease activity of EssD products (Ohr *et al.*, 2016). Fortuitously, we isolated *essD*$^*_{His}$, a mutant with a single codon substitution ($Leu^{546}Pro$), abolishing nuclease activity of EssD$^*_{His}$, while restoring secretion of Esx proteins and of EssD$^*_{His}$ (Ohr *et al.*, 2016). As a control for affinity chromatography, we expressed nontagged *essD* together with *essI1*, encoding a cytoplasmic polypeptide that binds to and inhibits the EssD nuclease (Ohr *et al.*,

2016). Ultracentrifuged lysates of *S. aureus essD::erm* carrying plasmids p*essD-essI1* and p*essD*$^{*}_{His}$ were subjected to solubilization with n-dodecyl-β-D-maltopyranoside (DDM) followed by Ni-NTA affinity chromatography to isolate products from plasmid-borne *essD* variants (Fig. 11B). Bound proteins were eluted with 10, 50, and 100 mM imidazole buffer, and aliquots of the eluate were analyzed by separation on SDS-PAGE gels and Coomassie blue staining (Fig. 11B). Compared to the EssD control, several proteins specifically eluted from Ni-NTA resin that had been charged with EssD$^{*}_{His}$ lysate (Fig. 11B). Gel slices with proteins marked by arrowheads in Fig. 11B were excised; proteins were cleaved with trypsin (C terminally of positively charged amino acids) and identified by mass spectrometry measurements of positively charged peptides, whose average masses were within the error rate for calculated masses assigned by in silico trypsin cleavage of translation products from the *S. aureus* genome (Shevchenko *et al.*, 2007). Mass spectrometry and bioinformatics analyses identified full-length EssD, its 24-kDa C-terminal nuclease domain (see below), EssE, and the EssD nuclease inhibitor EssI (20 kDa) (Fig. 11B). The EssD nuclease and EssI eluted in equimolar amounts, suggesting that the two proteins form an equimolar complex. The identity of EssD and EssI was also confirmed by Edman degradation (Ohr *et al.*, 2016). The 224-residue EssE is encoded by *essE* (SAUSA_0286), which is located between *esxB* and *esxD* within the ESS pathway gene cluster (Fig. 11A). Homology searches with EssE polypeptide sequence failed to identify domains with known or predicted biochemical functions. The *essE* open reading frame was cloned into pGEX-2TK to produce the translational hybrid glutathione S-transferase (GST)–EssE that was purified from cleared *Escherichia coli* lysates by affinity chromatography followed by thrombin treatment to remove GST. A rabbit was immunized with purified EssE to generate polyclonal antibodies specific for

the recombinant polypeptide (anti-EssE serum). Anti-EssE was used to confirm the copurification of EssE from *S. aureus* extracts containing EssD*$_{His}$ but not from lysate with untagged EssD (Fig. 11B).

EssE is required for *S. aureus* Esx protein secretion. To avoid polar effects of *essE* mutations on the expression of flanking genes in the ESS cluster (Fig. 11), pKOR1 technology was used to generate an in-frame deletion of 194 codons in the *essE* gene of methicillin-resistant *S. aureus* (MRSA) isolate USA300 LAC (SAUSA300_0286) (Bae and Schneewind, 2006). For plasmid complementation studies, we constructed p*essE* for plasmid-borne, constitutive P_{hrpK}-mediated expression of *essE*. Mid-log-phase cultures of the wild-type, Δ*essE*, and Δ*essE*/p*essE* variant *S. aureus* strains were fractionated to separate the extracellular medium and cell lysates (Fig. 12A, MD and cell, respectively). Proteins in each compartment were analyzed by SDS-PAGE and immunoblotting, which revealed that EssE was associated with *S. aureus* USA300 cells and was not secreted into the extracellular medium (Fig. 12A). The Δ*essE* mutant did not express the *essE* gene; however, EssE production was restored following transformation of Δ*essE* mutant cells with p*essE* (Δ*essE*/p*essE* strain) (Fig. 12A). As expected, *S. aureus* USA300 secreted EsxA, EsxB, EsxC, and EsxD into the extracellular medium (Fig. 12A). The Δ*essE* mutant did not secrete EsxB and EsxC and also displayed reduced secretion of EsxA (Fig. 12A). Additionally, the production of EsxB and EsxD was greatly diminished in Δ*essE* staphylococci (Fig. 12A). However, this was not due to loss of gene transcription, as shown by reverse transcription-PCR (RT-PCR) experiments; both *esxB* and *esxD* transcripts continued to be synthesized in the Δ*essE* mutant in a manner comparable to that of USA300 (Fig. 12B). All phenotypic defects of the Δ*essE* mutation on ESS secretion were ameliorated when mutant

staphylococci were transformed with p*essE* (Fig. 12A and B). As controls, both wild-type and Δ*essE* mutant staphylococci secreted α-hemolysin (Hla) into the extracellular medium, whereas ribosomal protein L6 (L6) remained associated with the bacterial cells (Fig. 12A). Thus, the 194-codon in-frame deletion abolishes *essE* expression, causing specific defects in the secretion of Esx proteins by the *S. aureus* ESS pathway without impacting bacterial lysis or the secretion of Hla via the canonical Sec secretion pathway.

EssE impacts *S. aureus* processing of EssD. Earlier work reported that mutations in genes for substrates of ESS secretion (*esxB*, *esxC*, or *esxD*) resulted in defects of *S. aureus essD* expression (Anderson *et al.*, 2011, 2013). The Esx secretion defect for mutants with defects in other *ess* genes had also been attributed to the loss of *essD* expression (Anderson *et al.*, 2013). To determine whether such a defect occurs in the *essE* mutant, we examined whole-cell lysates of wild-type *S. aureus* USA300 LAC as well as the Δ*essE*, Δ*essE*/p*essE*, and *essD::erm* mutants by immunoblotting with antibodies raised against amino acids 1 to 200 (anti-EssD^{N}) or 432 to 617 (anti-EssD^{C}) of the EssD polypeptide (617 residues). When samples were probed with anti-EssD^{N}, a 72-kDa immunoreactive species was detected in cell lysates of the wild-type but not of the *essD* mutant staphylococci (Fig. 13A, black arrow). In addition to cross-reactive species that were also identified in *essD* mutant lysate, anti-EssD^{N} detected immunoreactive species with ~43-kDa mobility on SDS-PAGE gels (Fig. 13A). Immunoblotting with anti-EssD^{C} identified a 72-kDa EssD (black arrow) as well as a smaller 24-kDa product (white arrow). These data suggest that the 72-kDa species represents full-length EssD, which is cleaved into the stable C-terminal fragment (24-kDa protein reactive with anti-EssD^{C}). The abundance of full-length EssD (72 kDa) was diminished in the *S. aureus* Δ*essE* mutant while that of the C-terminal 24-kDa fragment was

increased. Processing of the N-terminal EssD domain was not affected by the Δ*essE* mutation. Compared to wild-type staphylococci, the Δ*essE*/p*essE* variant produced similar amounts of full-length EssD and N- and C-terminal cleavage products, indicating that the observed defects on EssD processing are all attributable to *essE* expression.

To determine whether *essD* expression reciprocally affects the abundance of EssE, we analyzed *S. aureus* extracts by immunoblotting with antibodies specific for EssE (anti-EssE) (Fig. 13B). However, mutations in *essD* did not affect the abundance of EssE in staphylococcal cells. As a control, staphylococcal cell extracts were probed with antibodies specific for ribosomal protein L6 (anti-L6), validating that similar amounts of cell extracts had been analyzed in the immunoblot experiments.

Affinity chromatography of EssE. Several components of the ESS pathway encompass hydrophobic segments and are predicted to be localized in the bacterial membrane (Burts *et al.*, 2005). When subjected to the PSORT (psort.hgc.jp) and TMHMM (cbs.dtu.dk/services/TMHMM) algorithms, EssE, however, was not identified as a membrane protein; it lacks segments with ≥10 consecutive hydrophobic amino acids (Eisenberg *et al.*, 1984). To determine the subcellular localization of EssE, the cell walls of staphylococci were removed with lysostaphin, bacterial cells were lysed in a French press, and lysates were ultracentrifuged (100,000 × g for 2 h). Soluble proteins were separated, and the supernatant (cytosol) from insoluble proteins sedimented with the membrane fraction (membrane) (Fig. 14A). Proteins in both fractions were precipitated with trichloroacetic acid (TCA), solubilized in sample buffer, and analyzed by immunoblotting. As a control, sortase A (SrtA), a membrane protein, was found predominantly in the membrane fraction, while L6 remained soluble in the

cytosol (Fig. 14A). EssE was unambiguously identified by comparing cytosol and membrane fractions from the wild-type and Δ*essE* and Δ*essE*/p*essE* mutant strains (Fig. 14A). Further, EssE was observed with equal abundance in the cytosol and membrane fractions (Fig. 14A). Of note, the distribution of EssE between cytosol and membrane fractions was not affected by plasmid-borne expression of *essE* in Δ*essE*/p*essE* variant staphylococci (Fig. 14A).

To enable biochemical analyses of EssE, we generated $EssE_{STREP}$ with an 8-amino acid Strep-Tag extension at the C terminus of the polypeptide. Plasmid-borne expression of $essE_{STREP}$ by the Δ*essE*/p$essE_{STREP}$ variant restored the slightly reduced production of EsxC and the diminished abundance of EssD in the Δ*essE* mutant to a level observed in wild-type staphylococci (Fig. 14B). *S. aureus* Δ*essE*/p*essE* and Δ*essE*/p$essE_{STREP}$ strains were grown to an A_{600} of 2.0, and bacteria were sedimented by centrifugation. Following cell wall removal with lysostaphin, staphylococci were lysed using a French press, and membranes were sedimented by ultracentrifugation, incubated with 2% DDM on ice, and again subjected to ultracentrifugation. Proteins solubilized by 2% DDM were subjected to affinity chromatography on Strep-Tactin–Sepharose and eluted with desthiobiotin buffer. Immunoblot analysis of eluates revealed affinity chromatography purification of $EssE_{STREP}$ but not of EssE (Fig. 14C). Immunoblotting with a panel of rabbit antibodies raised against purified recombinant components of the *S. aureus* ESS pathway identified copurification of EsxC, EssC, EssD, and EssI during affinity chromatography of lysate from staphylococci with $EssE_{STREP}$ but not with EssE (Fig. 14C). Clearly, a protein complex can be isolated with tagged EssE. The secreted substrates EsxC and EssD are enriched in this complex, and at least one of four presumed secretion

machinery components of the ESS pathway, the EssC ATPase (Fig. 11), is also recruited to this complex.

Mouse cytokine responses during bloodstream infection with wild-type and Δ*essE* mutant *S. aureus*. Earlier work reported that during mouse lung infection, *S. aureus* secretion of α-hemolysin, a pore-forming toxin, promotes inflammasome activation by providing access to pathogen-associated molecular patterns (PAMPs), thereby activating proinflammatory responses via the secretion of IL-1β (Wardenburg and Schneewind, 2008; Kebaier *et al.*, 2012). We sought to study the impact of the ESS pathway on host cytokine responses in a mouse model of *S. aureus* bloodstream infection (Cheng *et al.*, 2009). Cohorts (n = 5) of C57BL/6 mice were infected by intravenous inoculation with 5×10^7 CFU of *S. aureus* USA300. Cytokine responses were analyzed in serum samples from cardiac blood drawn 12 h after infection. Compared to mock treatment in mice, *S. aureus* USA300 infection stimulated IL-1α, IL-1β, IL-6, IL-12 (p40/70), RANTES, keratinocyte-derived chemokine (KC), monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), granulocyte colony-stimulating factor (G-CSF), and gamma interferon (IFN-γ) signaling (Fig. 15). During infection with the Δ*essE* mutant, the serum concentrations of IL-1β and IL-12 (p40/70) were diminished, whereas bloodstream release of RANTES was increased (Fig. 15). These data suggest that the ESS pathway contributes to *S. aureus* activation of proinflammatory cytokine responses such as IL-1β secretion. As reported earlier, *S. aureus* bloodstream infection of mice promoted the secretion of IL-12 (p40/70) (Schindler *et al.*, 2012), a heterodimeric cytokine comprised of p40 and p35 (Kobayashi *et al.*, 1989; Gately *et al.*, 1998). IL-12 is produced in monocytes, macrophages, and dendritic cells to promote development and activation of cytotoxic T

lymphocytes, natural killer cells, lymphokine-activated killer cells, and macrophages (Schindler *et al.*, 2012). Thus, the *S. aureus* ESS pathway appears to activate immune cells for the secretion of IL-12 (p40/70) as the increased secretion of this cytokine was abolished during mouse infection with the Δ*essE* mutant (Fig. 15). Of note, secretion of RANTES (CCL5), a chemoattractant for memory T lymphocytes and monocytes secreted by T cells, was enhanced during bloodstream infection with the Δ*essE* mutant (Fig. 15).

Macrophage responses to infection with wild-type and Δ*essE* mutant *S. aureus*. Our data suggest that during bloodstream infection, *S. aureus* may employ the ESS pathway to simultaneously promote the activation of proinflammatory cytokines while also restricting the trafficking of immune cells to sites of staphylococcal infection. To examine the impact of altered cytokine responses on staphylococcal disease, cohorts (n = 8 to 10) of C57BL/6 mice were infected by intravenous injection with 5×10^6 CFU of *S. aureus* USA300 or the Δ*essE* mutant. Five days after challenge, mice were euthanized and necropsied, and renal tissues were analyzed for the enumeration of abscess lesions in histopathology slides and quantification of bacterial load in tissue homogenates (Fig. 16A and B). USA300 infection caused an average of 3.0 surface abscesses per kidney while Δ*essE* mutant staphylococci produced an average of 0.95 surface abscesses (Fig. 16A) (P = 0.036). The average load of *S. aureus* USA300 per gram of kidney tissues was 7.9 $\log_{10}$ CFU, and it was 7.4 $\log_{10}$ CFU for the isogenic Δ*essE* mutant (Fig. 16B) (P = 0.0024), a reduction similar to that observed for other mutant alleles of the ESS cluster (Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2011). Immunohistochemical analysis of thin-sectioned tissue was used to assess the abundance of anti-F4/80-positive macrophages and of RANTES production in renal abscess lesions. Compared to lesions elicited

by wild-type *S. aureus*, abscesses formed by the Δ*essE* mutant displayed increased staining with RANTES (Fig. 16C). Image analysis using Fiji software revealed that the average darkness staining for RANTES increased by 41% in the Δ*essE* mutant compared to that in USA300 (Fig. 16E) (P = 0.03). Immunohistochemistry using anti-F4/80 antibody revealed an infiltration of macrophages within abscess lesions formed by the Δ*essE* mutant (Fig. 16D). While macrophages were also recruited to lesions formed by USA300, the staining mostly surrounded abscesses (Fig. 16D). Thus, the Δ*essE* mutation not only affects Esx protein secretion and the production of cytokines during bloodstream infection but also impacts the ability of staphylococci to establish infectious lesions and to manipulate cellular immune responses of the host.

Discussion

Type VII secretion systems (T7SS) were discovered in *Mycobacterium tuberculosis* to promote the secretion of T cell antigens (ESAT-6 and CFP-10) (Abdallah *et al.*, 2007). Genes encoding ESAT-6/CFP-10 and their corresponding secretion machinery are located in a contiguous cluster now designated ESX-1 (Houben *et al.*, 2012). The ESX-1 T7SS enables translocation of proteins from the bacterial cytoplasm across the phagolysosomal membrane into the cytosol of infected macrophages. Mycobacterial EccC, a transmembrane protein with three ATPases of the FtsK/SpoIIIE-like family, is thought to be responsible for recognizing WXG100 proteins as substrates for T7SS (Rosenberg *et al.*, 2015). Recent work proposed that binding of a discrete peptide signal in CFP10 (*M. tuberculosis* EsxB [Mt-EsxB]), a mycobacterial member of the WXG100 family, promotes EccC oligomerization and ATPase activity for transport of folded WXG100 dimers across the bacterial envelope (Rosenberg *et al.*, 2015).

Homologs of the mycobacterial T7SS that transport WXG100 proteins have also been described in firmicutes, including *Bacillus anthracis* (Garufi, Butler and Missiakas, 2008), *Bacillus subtilis* (Sysoeva *et al.*, 2014), and *Staphylococcus aureus* (Burts *et al.*, 2005). Firmicutes carry some (EsxA, EsxB, and EccC), but not all, of the mycobacterial ESX-1 components required for secretion of ESAT-6 and CFP-10 (Pallen, 2002), and their pathways are designated type VIIb (Abdallah *et al.*, 2007). In *S. aureus*, the *ess* (type VIIb) locus has been divided into four modules (1 to 4) (Warne *et al.*, 2016). Modules 2 and 3 are variable among some of the different sequence type (ST) isolates of this pathogenic species (Warne *et al.*, 2016). Module 2 encodes EccC; i.e., it harbors *essC* of *S. aureus* (Burts *et al.*, 2005). Four *essC* alleles (*essC1* to *essC4*) carry sequence variation in the terminal FtsK/SpoIIIE-like ATPase domain and are associated with specific groups of downstream genes, presumably encoding specific secretion substrates (Warne *et al.*, 2016). The *essC1* variant has the highest frequency (90/153 isolates) and was found in strains belonging to *S. aureus* clonal complexes (CCs) that are frequently associated with human invasive disease (CCs 1, 5, 7, 8, 9, 25, 51, and 88) (Warne *et al.*, 2016). *essC3* is found in CC30 and ST239 isolates, whereas *essC4* and *essC2* are restricted to CC22 and to CC15 and ST398 isolates, respectively (Warne *et al.*, 2016). In the community acquired (CA)-MRSA isolate USA300 LAC (CC8), module 2 includes *essC1* as well as the secretion substrate genes *esxC*, *esxB*, *esxD*, and *essD* in addition to *essE* (*esaE*), a gene of heretofore unknown function. These genes are absent from *S. aureus* isolates with secretion pathways comprised of *essC2* to *essC4* (Warne *et al.*, 2016). Module 3 encompasses a complex arrangement of predicted genes, including members of the DUF600 family, which vary in number even for isolates from the same CC (Warne *et al.*, 2016). Finally, module 4 includes two genes for hypothetical transmembrane

proteins that are conserved among all *S. aureus* isolates. The requirements of the two module 4 genes for T7SS in *S. aureus* are, however, not known.

Here, we characterize EssE, which is located in the staphylococcal cytoplasm and forms a complex with EssD, EsxC, EssI (DUF600 protein), and EssC. EssE stabilizes EssD degradation and promotes the secretion of EsxA, EsxB, EsxC, EsxD, and EssD into the extracellular medium. Other components of the ESS secretion apparatus—EsaA, EssA, and EssB—were not observed in detergent-extracted EssE (Burts, DeDent and Missiakas, 2008). Based on these observations, we propose a model whereby EssE may act as an assembly platform for the secretion of EsxC and EssD.

When analyzed in mouse models of bloodstream infection or pneumonia, mutations in *ess* genes of CC8 isolates *S. aureus* Newman, USA300 LAC, RN6390, and COL result in reduced virulence and diminished staphylococcal persistence (Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2011; Kneuper *et al.*, 2014). Similar phenotypes have been observed for *essB* variants of *S. aureus* ST398 isolates in mouse models for bloodstream infection and skin abscess formation (Wang *et al.*, 2016). For ST398 isolates, expression of *ess* has been associated with neutrophil lysis and staphylococcal escape from phagocytic killing; however, it is not yet clear whether this phenotype is attributable to specific effectors secreted via the *essC2* T7SS (Wang *et al.*, 2016).

The molecular basis for *S. aureus ess* manipulation of host immune responses was heretofore not known. In *M. tuberculosis*, secretion of Mt-EsxA by the ESX-1 pathway into host cells activates cyclic GMP-AMP synthase (cGAS) and cyclic GMP-AMP (cGAMP) signaling via

STING, the mammalian sensor for cytoplasmic DNA and bacterial cyclic dinucleotides (Gao *et al.*, 2013), thereby stimulating type I interferon (IFN) responses (Dey *et al.*, 2015; Wassermann *et al.*, 2015; Watson *et al.*, 2015). IFN activation is associated with disease progression in tuberculosis (TB) and other infectious diseases. ESX-1 activity also leads to inflammasome activation and secretion of the proinflammatory cytokine IL-1β (Wassermann *et al.*, 2015). Finally, Mt-EsxA secretion has been shown to be required for formation of TB granulomas and the persistence of mycobacteria in these lesions (Cambier *et al.*, 2014). A recent model for cGAS and STING signaling postulates that Mt-EsxA may stimulate IFN signaling via pore formation and DNA transfer into the host cell cytosol (Wassermann *et al.*, 2015; Watson *et al.*, 2015).

We show here that the *S. aureus* ESS pathway of the CC8 isolate USA300 LAC stimulates IL-12 (p40) and IL-12 (p35) production during host infection while limiting the secretion of RANTES (CCL5). IL-12 (p40) is shared between IL-12 (p70) and IL-23 and activates T_H1 and T_H17 responses, respectively, as well as gamma interferon signaling during bacterial infection, through the activation of pattern recognition receptors recognizing pathogen-associated molecular patterns (O'Garra and Arai, 2000; van de Vosse *et al.*, 2009). Impaired T_H1 and T_H17 responses are associated with mucocutaneous *S. aureus* and *Candida* infections (Maródi *et al.*, 2012; O'Shea *et al.*, 2012). In mice and humans, the IL-12 heterodimer signals through the IL-12 receptor (IL-12R), which is comprised of IL-12Rβ1 and IL-12Rβ2 (Trinchieri, 2003). IL-23, a complex formed from IL-23 (p19) and IL-12 (p40), signals via IL-23R and IL-12Rβ1 (Oppmann *et al.*, 2000). IL-12 and IL-23 impact the development of T_H1 cell and IL-17-producing T helper (T_H17) cell responses, respectively (Teng *et al.*, 2015). In a model of IL-12-mediated activation and recruitment of myeloid cells and other immune cells for staphylococcal abscess

development, one would predict that mutational lesions abrogating IL-12 production or IL-12 receptor signaling may provide increased resistance toward *S. aureus* infection. The impact of IL-12/IL-23 signaling on the pathogenesis of *S. aureus* infection of mice has been studied in several challenge models even though the impact of IL-12 signaling on the outcome of staphylococcal diseases remained enigmatic. Following intravenous *S. aureus* challenge, IL-12p40$^{-/-}$ (IL-12/IL-23-deficient) mice displayed elevated mortality rates and increased staphylococcal loads in renal tissue 20 days after challenge (Dodson *et al.*, 2001). Intranasal challenge of *S. aureus* leads to lung infection; both the associated disease mortality and the bacterial burden are increased in IL-12p35$^{-/-}$ mice (Hilliard *et al.*, 2015). Following intracerebral inoculation of staphylococci, the initial phases of brain abscess formation were not impacted by IL-12 (p35) deficiency; however, IL-12p35$^{-/-}$ mice displayed fewer clinical disease symptoms and healed more rapidly than wild-type mice (Held *et al.*, 2013). In humans, *S. aureus* infections result in a transient increase in anti-staphylococcal antibody levels; but protective immunity is not observed, and recurrent infections occur frequently (Kim *et al.*, 2012). *S. aureus* secretes enterotoxins and staphylococcal protein A (SpA) that function as T cell (McCormick, Yarwood and Schlievert, 2001) and B cell superantigens (Silverman and Goodyear, 2006), respectively. IL-12 secretion mediated by the ESS pathway may further compromise the induction of a humoral immune response because IL-12 skewing of gamma interferon-producing T_H1 cells impedes T_H2 polarization and thereby the host's ability to produce antibodies (Zundler and Neurath, 2015).

RANTES (CCL5) is expressed by many cells, including T lymphocytes, macrophages, and platelets, and may be induced by NF-κB activation following stimulation by CD40L or IL-15 (Chenoweth *et al.*, 2012). Abscess lesions of *essE* strain-infected mice stained heavily for

RANTES in a manner that correlated with an increased recruitment of macrophages to these lesions. Since RANTES is upregulated when animals are infected with the *essE* variant, we postulate that during infection with wild-type *S. aureus*, the ESS pathway acts to limit macrophage trafficking to abscess lesions. In this regard, it seems noteworthy that *S. aureus* also secretes the LukED pore-forming toxins that specifically target myeloid cells expressing CCR5 (Alonzo III *et al.*, 2012). CCL5 is a ligand of CCR5, a seven-transmembrane G-protein-coupled receptor that modulates diverse signaling cascades upon ligand interaction. Thus, *S. aureus* may disable CCL5-CCR5 activity by both blocking RANTES production (ESS-dependent) and killing CCL5 target cells. Future work will need to address the contribution of ESS-mediated IL-12 production in inflammatory myeloid cells and inhibition of CCL5 production toward the establishment of persistent infection in mice.

Materials and Methods

Bacterial cultures. *S. aureus* cultures were grown in tryptic soy broth (TSB) or agar (TSA). Chloramphenicol was added to a final concentration of 20 μg/ml for plasmid selection. Anhydrotetracycline was used at 50 ng/ml for pKOR1-mediated allelic replacements of target genes (Bae and Schneewind, 2006). To assay for protein production and secretion, staphylococcal cultures were grown with vigorous shaking at 37°C for 2.5 h in TSB supplemented with 0.2% horse serum. *Escherichia coli* cultures were grown in Luria-Bertani medium at 37°C (Anderson *et al.*, 2013). Ampicillin was used at 100 μg/ml for plasmid selection in *E. coli*.

Bacterial strains and plasmids. USA300 LAC, a clone of the American community-acquired methicillin-resistant *S. aureus* (CA-MRSA) epidemic strain (Diep *et al.*, 2006), was used as the wild-type *S. aureus* strain. *E. coli* DH5α was used for cloning experiments. USA300 variant *essD::erm*, *essD::erm*/p*essD-essI1*, and *essD::erm*/p*essD**$_{His}$ strains are described elsewhere (Ohr *et al.*, 2016). The USA300 variant Δ*essE* strain was generated by allelic replacement with plasmid pKOR1 to fuse the first 15 and the last 15 codons of *essE*, resulting in a deletion of 194 codons. Briefly, two 1-kbp DNA sequences flanking *essE* upstream and downstream of the deletion were amplified from USA300 template DNA with primers EssE1 (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGAAGATGATGTTAAAAAGCTTATTAC-3') and EssE2 (5'-AAAGATCTTAATTCTTCGTAAGAAAAATAATC-3') as well as EssE3 (5'-AAAGATCTGGATTCGATACTGATGAAAATC-3') and EssE4 (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGGATTAATACAAGATAAAACTACC-3'). PCR products were cut with BglII and ligated at the restriction site. The ligation product was cloned into pKOR1 using the Gateway BP Clonase II enzyme from Invitrogen (catalog number 11789-020). Procedures for cloning and allelic replacement were performed as previously described (Anderson *et al.*, 2013). The complementing plasmid p*essE* carries the coding sequence of *essE* under transcriptional control of the constitutive *hprK* promoter in pWWW412 (Bubeck Wardenburg, Williams and Missiakas, 2006). p*essE* was assembled by PCR amplification of the *essE* coding sequence using USA300 template and primers EssE5 (5'-AACATATGAAAGATGTTAAGCGAATAG-3') and EssE6 (5'-AAGGATCCTTACTCCTCTGCTTTATTAATATG-3'). The PCR product was cut with NdeI and BamHI and cloned into the corresponding sites of pWWW412. p*essE*$_{STREP}$ expresses a variant of *essE*

from the *hprK* promoter of pWWW412. To assemble p*essE*$_{STREP}$, the *essE* gene was amplified with EssE5 and EssE7 (5'-AAGGATCCTTACTTCTCGAACTGTGGGTGGCTCCACTCCTCTGCTTTATTAATATG-3'), thereby extending the open reading frame by eight codons to yield EssE$_{STREP}$ with eight additional amino acids at the C terminus (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys). For protein production and purification from lysates of *E. coli*, the *essE* coding sequence was PCR amplified with the primers EssE8 (AACATATGAAAGATGTTAAGCGAATAG) and EssE9 (AAGGATCCTTACTCCTCTGCTTTATTAATATG), cut with BamHI and EcoRI, and ligated to pGEX-2TK (GE Healthcare) restricted with the same enzymes. Isopropyl-β-D-thiogalactopyranoside (IPTG)- inducible expression from the P_{tac} promoter of pGEX-2TK-EssE in *E. coli* generates a hybrid protein comprised of glutathione S-transferase (GST) linked via a thrombin cleavage site to EssE.

RNA extraction and qualitative cDNA PCR analysis. RNA was extracted from 10-ml cultures grown to an absorbance of 1.0 at 600 nm (A_{600}). Cultures were centrifuged (9,000 × g for 10 min), and the bacterial sediment was suspended in 1 ml of TRIzol containing 0.5 ml of glass beads (0.1-mm diameter). Samples were beat four times in a FastPrep-24 (MP Biomedicals) homogenizer, 200 µl of chloroform was added, and tubes were shaken for 30 s, incubated at 25°C for 3 min, and again centrifuged (12,000 × g for 15 min). Isopropanol (500 µl) was added to 500 µl of the carefully recovered upper phase and incubated at 25°C for 10 min, and samples were again centrifuged (12,000 × g for 10 min). Sediments were washed with 75% ethanol, dried, and rehydrated with 50 µl of diethylpyrocarbonate-treated water, and contaminating DNA was removed using a Turbo DNA-free kit (Ambion) to obtain 100 ng/µl RNA

per sample. An iScript cDNA synthesis kit (Bio-Rad) was used to generate cDNA. Reverse transcriptase (RT) was omitted in control experiments. cDNA and control samples were used for qualitative PCR analysis using GoTaq Flexi DNA polymerase (Promega). Primer pairs for amplification of *esxB*-, *esxD*-, and 16S-specific transcripts were CGCTGAGTATATCGAAGGTAGTG and CCATCGGTTGTACTA ATTCTTCTTG (*esxB*), CATTAGTGGTCTCAAAGGTCCA and GTAAAGCTTGGCAAATTCCGT (*esxD*), and GAAAGCCACGGCTAACTACG and CATTTCACCGCTACACATGG (16S), respectively.

Fractionation of bacterial cultures and immunoblot analysis. To assess protein secretion, *S. aureus* cultures were grown to an A_{600} of 1.0, and 50-ml aliquots were centrifuged (9,000 × g for 10 min). The extracellular medium was removed with the supernatant and separated from the bacterial sediment. Staphylococci were washed and suspended in 50 ml of 50 mM Tris-HCl (pH 7.0) and 10 mM $MgCl_2$ (TM medium), incubated with 20 μg/ml lysostaphin at 37°C for 60 min, and centrifuged (100,000 × g for 40 min at 4°C) to sediment membranes (Anderson *et al.*, 2013). Soluble proteins from the cytoplasm were collected with the supernatant and separated from insoluble membrane proteins. Membrane sediment was suspended in 50 ml of TM medium. Proteins in each fraction (extracellular medium, cytoplasm, or membrane) were precipitated with 10% trichloroacetic acid (TCA), incubated for 30 min on ice, and centrifuged (13,000 × g for 10 min). The supernatants were discarded, and protein precipitates were washed with 1 ml of ice-cold acetone and again sedimented by centrifugation (13,000 × g for 10 min). Acetone supernatants were discarded, and protein sediments were dried prior to solubilization in sample buffer (0.5 M Tris-HCl [pH 8.0], 4% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue) and heated at 90°C for 10 min.

In an alternate protocol, proteins from lysostaphin-digested staphylococci were subjected directly to TCA precipitation (without fractionation of cytoplasm and membrane), washed in acetone, and solubilized in sample buffer. Protein samples were separated on 15% SDS-PAGE gels, electrotransferred to polyvinylidene difluoride (PVDF) membrane, and analyzed by immunoblotting with rabbit polyclonal antibodies raised against purified proteins. Immunoreactive products were revealed by chemiluminescent detection after incubation with an anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology).

Protein purification from *S. aureus*. Cells from 8 liters of *S. aureus* culture that had grown to an A_{600} of 2.0 were sedimented by centrifugation (9,000 × g for 10 min). Cells were suspended in buffer A (50 mM Tris-HCl [pH 7.5], 150 mM NaCl), 20 μg/ml lysostaphin was added, and mixed samples were incubated for 1 h at 37°C. The resulting protoplasts were lysed in a French press at 14,000 lb/in^2 (ThermoSpectronic, Rochester, NY). Unbroken cells were removed by centrifugation (5,000 × g for 15 min). Crude lysates were separated with the supernatant from sedimented protoplasts and peptidoglycan and subjected to ultracentrifugation (100,000 × g for 2 h at 4°C). Soluble proteins in the staphylococcal cytoplasm were separated from the membrane sediment and subjected via gravity flow to chromatography on Strep-Tactin–Sepharose (IBA) or Ni-nitrilotriacetic acid (Ni-NTA)–Sepharose with a packed volume of 2 ml preequilibrated with buffer A. Columns were washed with 20× bed volumes of buffer A and eluted with either 4 ml of 2.5 mM desthiobiotin in buffer A (Strep-Tactin–Sepharose) or stepwise gradients of 50 to 500 mM imidazole in buffer A (Ni-NTA–Sepharose). For affinity chromatography of membrane-associated proteins, the sediment of

staphylococcal membranes (100,000 × g for 2 h) was extracted with 40 ml of 2% n-dodecyl-β-D-maltoside (DDM) in buffer A for 2 h at 4°C. Extracts were again subjected to ultracentrifugation (100,000 × g for 2 h at 4°C), and DDM-solubilized proteins were subjected via gravity flow to chromatography on Strep-Tactin–Sepharose (IBA) or Ni-nitrilotriacetic acid (Ni-NTA)–Sepharose with a packed volume of 2 ml preequilibrated with buffer A. Columns were washed with 20× bed volumes of buffer A and eluted with either 4 ml of 2.5 mM desthiobiotin in buffer A (Strep-Tactin–Sepharose) or stepwise gradients of 50 to 500 mM imidazole in buffer A (Ni-NTA–Sepharose). Aliquots of eluted fractions were mixed with an equal volume of sample buffer, separated on 15% SDS-PAGE gels, and analyzed by Coomassie blue staining or immunoblotting. The identification of proteins was performed by mass spectrometry of tryptic digests at the Taplin Mass Spectrometry Facility at Harvard Medical School.

EssE antiserum. A New Zealand White rabbit was purchased from Harlan Sprague Dawley and used to generate the polyclonal EssE antiserum. EssE antigen was purified via GST-EssE affinity chromatography and cleaved with thrombin, and EssE was isolated as described previously (Anderson *et al.*, 2011). Purified EssE (100 μg) was emulsified with complete Freund's adjuvant (Difco), and the emulsion was injected subcutaneously into the rabbit. At 21-day intervals, two booster immunizations with 100 μg of EssE emulsified with incomplete Freund's adjuvant were performed. Serum was obtained from blood samples, mixed with 0.02% sodium azide, aliquoted, and frozen for long-term storage at -80°C or stored at 4°C for reiterative use.

Animal challenge. Cohorts of female, 6-week-old C57BL/6 mice (Jackson Laboratory) were anesthetized by intraperitoneal injection with an aqueous solution containing 65 mg/ml

ketamine and 6 mg/ml xylazine per kg of body weight. Anesthetized mice were infected by intravenous injection with 0.5×10^7 to 5×10^7 CFU of staphylococci into the retro-orbital venous plexus (Cheng *et al.*, 2009). For inoculation, *S. aureus* cultures were grown in TSB with rotation at 37°C to an A600 of 0.4. Bacteria were sedimented by centrifugation (5,000 × g for 10 min), washed, and suspended in phosphate-buffered saline (PBS) in a volume of 100 l. Control animals were mock infected by injection with 100 l of PBS without bacteria. To analyze cytokines in blood at 12 h postinfection, cohorts of mice (n = 5) were euthanized by CO2 inhalation, blood was retrieved by cardiac puncture, and serum samples were prepared. A Bio-Plex Pro Mouse Cytokine 23-Plex assay kit (M60009RDPD; Bio-Rad) was used for the analysis of serum cytokines. The multiplex data were analyzed with one-way analysis of variance (ANOVA) using Bonferroni's multiple-comparison test. Five days after infection, cohorts of mice (n = 10) were euthanized and necropsied, and both kidneys were removed. For each animal, tissues from the right kidney were homogenized, and serial dilutions of homogenates were spread on agar plates for enumeration of the staphylococcal load. Bacterial loads were analyzed with a two-tailed Mann-Whitney test to measure statistical significance. The left kidneys were flash frozen in 22-oxacalcitriol (OCT) medium for cryosectioning in 12-m intervals using a Thermo Scientific Microm HM550 microtome. Sections were stained with hematoxylin and eosin (H&E) using a Rapid Chrome frozen section staining kit from Thermo Scientific, and samples were visualized on a dissecting microscope. An unpaired two-tailed Student t test with Welch's correction was used to evaluate differences in abscess enumeration. Sections directly above or below those used for abscess enumeration were subjected to immunohistochemistry (IHC) staining. Slides were fixed with acetone for 30 min at 20°C and blocked for 1 h with a solution

containing 1.5% bovine serum albumin (BSA) and human IgG. Slides were washed in PBS and incubated for 1 h with anti-F4/80 antibody (anti-F4/80), a marker specific for macrophages, or anti-RANTES antibody (anti-RANTES). Slides were washed in PBS and incubated for 1 h with an HRP-conjugated secondary antibody, washed again in PBS, and incubated for 1 min with diaminobenzidine-hydrogen peroxide solution to reveal areas of antibody binding. The reaction was quenched by plunging slides into PBS solutions. Slides were counterstained with hematoxylin and mounted, and images were analyzed using a Panoramic Viewer from 3D Histech, Ltd. Fiji image software was used to quantify staining of IHC images. Specifically, average darkness was determined by taking the entire image and separating the diaminobenzidine staining from the hematoxylin counterstain via color deconvolution. Statistical analysis was performed using a two-tailed Mann-Whitney test.

Ethics statement. The preparation of rabbit antibodies and mouse challenge experiments were performed according to protocols that were reviewed, approved, and performed under the regulatory supervision of The University of Chicago's Institutional Animal Care and Use Committee (IACUC). Animal experiments were conducted in accordance with recommendations detailed in the Guide for the Care and Use of Laboratory Animals (National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals. and Institute for Laboratory Animal Research (U.S.), 2011). Animal care was managed by The University of Chicago Animal Resource Center, which is accredited by the American Association for Accreditation of Laboratory Animal Care and acts in compliance with NIH guidelines of laboratory animal care and use.

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Chapter IV

Identification of a novel lipoprotein of the *Staphylococcus aureus* ESS pathway

Introduction

The ESAT-6-like Secretion System (ESS) of *Staphylococcus aureus* is encoded by a cluster of 11 contiguous genes that are required for assembly of the type VIIb secretion machine and secretion of its effector substrates (Burts *et al.*, 2005; Anderson *et al.*, 2011; Ohr *et al.*, 2016; Warne *et al.*, 2016). The importance of the ESS secretion pathway on *S. aureus* pathogenesis has been recognized via characterization of various *ess* mutants, all of which are incapable of secreting the five previously identified substrates of the ESS: EsxA, EsxB, EsxC, EsxD, and the newly identified secretion substrate, EssD (Burts *et al.*, 2005; Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2011, 2013; Chen *et al.*, 2012; Ohr *et al.*, 2016). These mutants are unable to form classical staphylococcal abscesses and are lacking in their ability to persist within the host (Burts *et al.*, 2005; Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2011, 2016).

The staphylococcal *ess* gene cluster was recently identified as harboring four genetic modules within the cluster that can differ on a strain to strain basis (Warne *et al.*, 2016). The highly conserved module 1 includes the genes *esxA*, *esaA*, *essA*, *esaB*, and *essB* and is presumed to encode the core proteins for the most basic staphylococcal ESS machine (Warne *et al.*, 2016). The second module is noted as one of two highly variable modules within the *ess* gene cluster, is found immediately downstream of *essB*, and starts with one of four alleles for the *essC* gene (*essC1-4*) (Warne *et al.*, 2016). Module 2 of the clinical MRSA isolate, USA300, consists of *essC1*, *esxC*, *esxB*, *essE*, *esxD*, and *essD* (Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2011,

2013, 2016; Ohr *et al.*, 2016; Warne *et al.*, 2016). The *essC* allele seems to dictate what downstream genes are found within module 2, and many of these genes may encode secretion substrates of the *essC*-specific allele (Warne *et al.*, 2016). Previously published data supports this assumption, as *esxC*, *esxB*, *esxD*, and *essD* have all been shown to encode secreted proteins of the USA300 ESS (Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2011, 2013, 2016; Ohr *et al.*, 2016; Warne *et al.*, 2016). Module 3 is the second highly variable region within the staphylococcal *ess* gene clusters and is denoted by containing genes that encode EssI-like proteins that contain the DUF600 motif (Anderson *et al.*, 2011; Ohr *et al.*, 2016; Warne *et al.*, 2016). *essI* genes have been recognized as being necessary for inhibiting the toxic nuclease activity of EssD, and the number of EssI-encoding genes within module 3 can vary from 2 to 13 copies (Ohr *et al.*, 2016; Warne *et al.*, 2016). In addition to *essI* genes, module 3 often contains genes which encode for putative membrane proteins (Warne *et al.*, 2016). In USA300, module 3 contains four additional genes intercalated between *essI* genes, SAUSA300_0290, _0291, _0292, and _0297. SAUSA300_0290 and SAUSA300_0297 are duplicate genes annotated as encoding DUF5079-containing proteins. SAUSA300_0291 encodes for a protein with a DUF5080 fold, and SAUSA300_0292 is simply annotated as encoding a hypothetical protein of 72 amino acids. None of these elements have been characterized yet, and their involvement in ESS secretion is unknown. Module 4 harbors only two genetic elements, and contrary to modules 2 and 3, these genes are conserved across all staphylococcal isolates possessing an *ess* gene cluster (Warne *et al.*, 2016).

In USA300, SAUSA300_0303 and _0304 are the representatives of the highly conserved module 4. SAUSA300_0303 is a 375 bp open reading frame that is designated as encoding a

putative 124 amino acid lipoprotein. Based on SignalP 4.1, TMHMM, and Phyre^2 Protein Fold Recognition Server analysis, the N-terminus of SAUSA300_303 is proposed to contain a signal peptide with a cleavage site between residues 24 and 25 and there are no evident transmembrane domains (Warne *et al.*, 2016). Additionally, SAUSA300_303 is proposed to have a cystatin-like fold present between the 25th and 118th amino acid, and the protein is a member of the DUF4467/pfam14729 superfamily of proteins (Warne *et al.*, 2016). Although cystatin-like proteins are primarily known as cysteine protease inhibitors, it has been recognized that the cystatin fold may be shared by other proteins that are not protease inhibitors. A search in the genome of USA300, identifies SAUSA300_2355, a protein homolog of SAUSA300_303. The two proteins share 31% identity at the amino acid level with 68% positives, and an E value of 4^{-15}. SAUSA300_0304 is a 399 bp genetic element that encodes a putative membrane protein of 132 amino acids. Based on SignalP 4.1, TMHMM, and Phyre^2 Protein Fold Recognition Server analysis, SAUSA300_0304 is not predicted to have a signal peptide cleavage site but should harbor 3 transmembrane helices which would place the N-terminus within the cell and the C-terminus on the extracellular side. SAUSA300_0304 is also a member of the DUF4064/pfam13273 superfamily, and the genome of strain USA300 encodes a second SAUSA300_0127, that shares 39% identity at the amino acid level with SAUSA300_0304 with 53% positives, and an E value of 0.005 (Warne *et al.*, 2016). For the purpose of this study, SAUSA300_0303 and SAUSA300_0304 will be referred to as *escA* (EscA) and *escB* (EscB) (ESAT-6 secretion conserved gene A and B).

In order to determine the potential importance of EscA and EscB on ESS secretion, a double knockout strain, Δ*escAB*, lacking both the *escA* and *escB*, was constructed, and the

contribution of these genes toward secretion was examined. The sequence of the STREP tag was appended at the C-terminus of EscA and EscB (EscA $_{STREP}$ and EscB$_{STREP}$) and used for immune detection in bacterial extracts and to establish the subcellular localization of EscA and EscB. Further, EsaA, EssB, EssC, and EssD were found to copurify with EscA$_{STREP}$. Together, the data indicates that module 4 genes belong to the *ess* cluster.

Results

EscA fractionates with the membrane of *S. aureus*. Using the methicillin-resistant *S. aureus* USA300 strain as the wild-type background, a Δ*escAB* mutant was generated through allelic replacement of the *escA* and *escB* coding sequences with the first 15 codons of *escA* and the last 15 codons of *escB*, as previously described (Bae and Schneewind, 2006). Four plasmids were generated expressing either wild type or STREP-tagged *escA* and *escB* variants yielding plasmids p*escA*, p*escB*, p*escA*$_{STREP}$, or p*escB*$_{STREP}$, respectively. One additional plasmid expressed both genes simultaneously, p*escAB*. Each plasmid, as well as an empty vector control, was subsequently transformed into wild-type strains USA300, Δ*essB*, or *ΔescAB*. To determine if the STREP-tagged constructs produced the expected products, whole cell lysate (WC) samples were generated from cells cultured to an A_{600} of 3.0 at 37^{o}C with vigorous shaking in TSB with 0.02% heat-inactivated horse serum at a pH of 5.5 (growth conditions referred to as ESS-inducing conditions). Briefly, cultures were pelleted, media removed, and cell were suspended in PBS with lysostaphin, incubated at 37^{o}C to lyse the staphylococci, and TCA precipitated. Samples were subjected to immunoblot analysis with a mouse monoclonal antibody against the STREP antigen (α-STREP). Regardless of the strain background, staphylococci that harbored the p*escA*$_{STREP}$ construct produced an immunoreactive band at approximately 18 kDa (Fig. 17A). No

immunoreactive signals could be detected in extracts from other strains including those carrying plasmid p*escB*$_{STREP}$ (Fig. 17A). For this reason, all subsequent studies focus on the characterization of EscA.

Using a modified version of the protocol described above, samples of the culture filtrate (CF), soluble cell fraction (Sol), and insoluble cell fraction (Insol) were recovered and examined for the subcellular location of EscA$_{STREP}$. Cultures were grown under ESS-inducing conditions and upon reaching an A_{600} of 3.0 were centrifuged. Culture supernatants were transferred to new tubes and subjected to 0.22 µm filtration prior to undergoing TCA-precipitation. Cells in the pellets were lysed with lysostaphin and French Press passaging, followed by ultracentrifugation to separate the soluble (Sol) and insoluble (Insol) fractions and TCA-precipitation. All samples were analyzed via immunoblot with polyclonal antibodies to the N-terminus of EssD (α-EssD^{N}), a previously described ESS-secreted protein that also fractionates with the cell, polyclonal antibodies to sortase A (α-SrtA), a known staphylococcal membrane protein, and α-STREP antibodies (Fig. 17B) (Ohr *et al.*, 2016). As expected, an immune reactive signal specific to EssD was present in the culture filtrate (CF) of the wild-type background strains, but not in the filtrate of strain Δ*essB*, consistent with EssD being an ESS substrate (Fig. 17B) (Ohr *et al.*, 2016). No signal was identified in the α-STREP immunoblot of the culture filtrate (CF) samples for any strain or plasmid construct combination, indicating that EscA$_{STREP}$ was not secreted into the extracellular milieu in an ESS-dependent or -independent manner (Fig. 17B). Additionally, all strain backgrounds harboring the p*escA*$_{STREP}$ construct showed an α-STREP immune reactive signal at ~18 kDa in the ultra-insoluble (Insol) but not in the ultra-soluble (Sol) fraction (Fig. 17C). This fractionation pattern was similar to that of the endogenous

SrtA protein indicating that $EscA_{STREP}$ localizes to the insoluble, membrane fraction of staphylococci, and that this subcellular localization is independent of an intact ESS machine (Fig. 17C).

EscA copurifies with other proteins of the ESS cluster. Even though subcellular localization of EscA was unaffected in strain Δ*essB*, it is possible that EscA may interact with other factors of the ESS cluster. To examine this possibility, EscA was purified from wild-type USA300 carrying either p*escA*$_{STREP}$ or p*escA*. 4 liters of both cultures were grown to an A_{600} of 2.0 under ESS-inducing conditions, and cells were pelleted and the spent culture medium was discarded. Cell pellets were lysed as described above and treated with 2% n-dodecyl-β-D-maltoside (DDM) to facilitate membrane solubilization before being subjected to Strep-Tactin–Sepharose affinity chromatography and elution with 5 mM desthiobiotin. SDS-PAGE examination of the elution samples involved both Coomassie staining and immunoblot analysis with ESS-specific antibodies (Fig. 18). Coomassie staining of eluted proteins revealed that samples harboring $EscA_{STREP}$ display a distinctive profile with a unique species at ~18 kDa; when probed by immunoblotting, this species was recognized by the α-STREP antibodies (Fig. 18A). Further immunoblot analyses identified EsaA, EssB, EssC, and EssD in the samples harboring $EscA_{STREP}$ but not in the control sample harboring untagged EscA (Fig. 18B). Neither EssA, EssE, EsxA, EsxB, EsxC, nor EsxD were unambiguously identified as co-eluting with $EscA_{STREP}$ in these experiments (Fig. 18B).

To confirm these observations, proteins were excised from slices of Coomassie-stained gel shown in Fig. 18A, subjected to trypsin degradation and analyzed by mass spectrometry at the Taplin Mass Spectrometry Facility at the Harvard Medical School. Observed masses were

examined for matches against an *in silico*-trypsin digest of translational products from strain USA300. 81 total tryptic peptide hits were observed in the $EscA_{STREP}$ elution sample, and only 2 in the untagged EscA sample that served as the negative control. MS analysis confirmed that the 18 kDa band is indeed $EscA_{STREP}$. The tryptic peptides obtained matched the entirety length of EscA with the exception of the first 24 amino acids. This result is in agreement with topology predictions indicating that the proteins bears a cleavable signal peptide between residues 24 and 25. EscA is a lipoprotein that remains tethered to the plasma membrane by acylation of the N-terminal cysteine following processing of the precursor. MS analysis also identified peptides matching the EsaA, EssB, EssC, and EssD proteins as well as EssI1, EssI4, and EssI6. Taken together, these data indicate that a complex of ESS proteins, including EscA, forms within staphylococcal membranes and that these interactions are strong enough that this ESS complex can be pulled down with $EscA_{STREP}$ when subjected to affinity chromatography.

Deletion of *escA* and *escB* does not abolish ESS secretion under inducing conditions.

Considering the interactions identified between $EscA_{STREP}$ and multiple ESS machine components, an extensive evaluation of ESS secretion in the Δ*escAB* background was performed. Following the same procedures performed to isolate the culture filtrate (CF) samples described in Fig. 17B, wild-type USA300 and its isogenic Δ*essB* mutant harboring empty vector (EV) controls and the Δ*escAB* mutant harboring either an EV control, p*escA*, p*escB*, or p*escAB* were subjected to ESS secretion analysis under ESS-inducing conditions. At an A_{600} of 3.0, culture filtrates (CF) and corresponding whole cell (WC) samples were taken and underwent preparation for immunoblot analysis. The CF and WC samples were analyzed with antibodies against EsxA, EsxB, EsxC, $EssD^{C}$, and L6 (a cytoplasmic control for spontaneous cell

lysis) (Fig. 19). All four secreted substrates, EsxA, EsxB, EsxC, and EssD were shown to be secreted to similar levels regardless of the presence or absence of *escA or escB.* As expected, ESS-dependent secretion was abolished in the control strain Δ*essB* (Fig. 19).

Secretion of EsxB is altered in strains lacking *escA*. ESS substrates often accumulate in the cytosol of *S. aureus* and the secretion yield can be improved when cultures are grown under inducing conditions *i.e.* when TSB is supplemented with serum and when the medium is slightly acidified. Nonetheless, a small amount of secretion can be observed when bacteria are grown in TSB only. To rule out the possibility that *escA* and *escB* may be dispensable when bacteria are grown under inducing conditions, the secretion assay was repeated using cultures grown in plain TSB. For an initial evaluation, culture filtrates (CF) from wild-type USA300 or its isogenic mutants, Δ*essB* and Δ*escAB*, were recovered and prepared for immunoblot analysis. Minimal secretion of EsxA, EsxC, and EssD was observed in these conditions and secretion was not altered in the absence of *escAB* (Fig. 20A). Surprisingly, secretion of EsxB was greatly reduced in the *escAB* mutant (Fig. 20A). Importantly, this phenotype could be complemented by transformation with plasmids encoding either *escA* alone or *escA-escB* (Fig. 20B). This data suggests that *escA* contributes to the secretion of EsxB.

Discussion

The type VIIb secretion system of *S. aureus*, also referred to as the ESAT-6-like secretion system (ESS), is a fairly novel secretion system found primarily in mycobacterial and Gram-positive organisms. Since its original identification, research directed toward understanding the ESS of staphylococci and its role in pathogenesis has been fruitful; however, there are still many

unknown elements of the secretion system. The identification of both conserved and variable modules within the *ess* gene cluster leads to several questions. What is the minimum genetic requirement for secretion of ESS substrates? Do variable genes encode strain-specific secreted effectors? Does the secretion machine select between canonical WXG100 and non-canonical effectors? Our preliminary analysis begins to address how module 4 contributes to the ESS pathway.

Module 4 is highly conserved amongst staphylococcal isolates and carries two genes of unknown function (Warne *et al.*, 2016). Here, we report that the first gene, *escA* (SAUSA300_0303 within the USA300 genome), encodes a protein that sediments with the membrane fraction of *S. aureus* in agreement with a predicted lipobox motif. We find that EscA purifies in a complex with EsaA, EssB, EssC, and EssD. Further, *escA* is required for secretion of EsxB when bacteria are grown in TSB, conditions that are sub-optimal for ESS-mediated secretion. The mechanism by which EsxB secretion is abolished in the absence of *escA* is unknown, but it can be theorized that the difference in phenotypes between ESS-inducing and non-inducing conditions could be due in part to the proposed cystatin-like fold of EscA as well as off-target effects of the ESS-inducing conditions themselves. When subjecting staphylococci to ESS-inducing conditions there is a notable decrease in classically secreted, or non-ESS-secreted, proteins due to the lower pH being an inhibitor of expression of these genes (Weinrick *et al.*, 2004). Previous studies have noted that the overall proteolytic activity of staphylococcal cultures is lower due to a lack of secreted proteases, like the serine protease SplF, when grown at pH 5.5 (Weinrick *et al.*, 2004). If the cystatin-like fold of EscA allows it to act as a protease inhibitor, it is possible that EscA could prevent certain ESS proteins, such as

EsxB, from being degraded by extracellular proteases present in the medium of cultures grown under normal laboratory conditions. However, upon growth in ESS-inducing conditions, the low pH blunts the expression and production of these proteases and thus render EscA dispensable (Weinrick *et al.*, 2004). Further research on EscA needs to be performed to confirm these hypotheses. Generation of recombinant EscA and *in vitro* testing for protease inhibitor capabilities, and identification of the protease(s) responsible for degrading EsxB in the absence of EscA will be necessary to confirm these suppositions.

Materials and Methods

Bacterial cultures. *S. aureus* was cultured in tryptic soy broth (TSB) or agar (TSA). When needed, chloramphenicol was added at a final concentration of 20 μg/ml for plasmid selection. For pKOR1-mediated allelic replacement of *escA* and *escB*, anhydrotetracycline was used at 50 ng/ml (Bae and Schneewind, 2006). ESS-inducing conditions were produced via vigorous shaking at 37°C in TSB supplemented with 0.2% horse serum at a pH of 5.5; whereas non-inducing conditions were produced via vigorous shaking at 37^{o}C in TSB alone. *Escherichia coli* was cultured in Luria-Bertani medium at 37°C, and ampicillin was added at a final concentration of 100 μg/ml for plasmid selection (Anderson *et al.*, 2013).

Bacterial strains and plasmids. The community-acquired, methicillin-resistant *S. aureus* (CA-MRSA) strain, USA300 LAC, was used as the wild-type *S. aureus* strain (Diep *et al.*, 2006). Initial cloning experiments were all performed in the *E. coli* DH5α background. The isogenic USA300 variant, Δ*essB*, is described elsewhere (Chen et al., 2012; Anderson et al., 2013). The USA300 variant Δ*escAB* strain was generated by pKOR1 allelic replacement by sewing the first

15 codons of *escA* and the last 15 codons of *escB* together, resulting in the deletion of these two coding sequences. Briefly, two 1-kbp DNA fragments, either upstream of *escA* or downstream *escB*, were amplified from USA300 template DNA with primers I (5'-GGGGACAAGTTTGTACAAAAAAGCCTGAAGGTGGAGAGGTTGTTTTTAATTATAC-3') and II (5'-GATAAGTCGCTCAATATTCTTTTCATCATTTTTCTCC-3') as well as III (5'-AGCGACTTATCATAAACATCGTATATTG-3') and IV (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGGGACGACTGGGTAAATAACGTAGTC-3'). PCR products were ligated together and cloned into pKOR1 using the Gateway BP Clonase II enzyme from Invitrogen (catalog number 11789-020). Subsequent cloning and allelic replacement was performed following protocols previously described (Anderson *et al.*, 2013). Complementing plasmids p*escA*, p*escA*$_{STREP}$, p*escB*, and p*escB*$_{STREP}$ carry the coding sequences of either *escA* or *escB*, with or without the addition of the coding sequence for a C-terminal STREP-tag (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys), under control of the constitutive *hprK* promoter in pWWW412 (Bubeck Wardenburg, Williams and Missiakas, 2006). p*escA* was constructed by PCR amplification of the *escA* coding sequence using USA300 template and primers V (5'-GATCGAGCTCATGAAAAGAATATTGGTAGTATTTTTAATG-3') and VI (5'-CTAGGGATCCTTAATCTTTTTCCACTTCTACATTTTC-3'), and p*escA*$_{STREP}$ was constructed using primers V and VII (5'-CTAGGGATCCTTATTTTTCGAATTGAGGATGTGACCAGTCGACATCTTTTTCCACTTCTACATTTTC -3'). p*escB* was constructed by PCR amplification of the *escB* coding sequence using USA300 template and primers VIII (5'-GTCTCATATGGAAAAATCGATCAAAATAATGACAATAATAGG-3') and IX (5'-CTAGGGATCCTTATGATAAGTCGCTTATTTCATCTTTA-3'), and p*escB*$_{STREP}$ was constructed

using primers VIII and X (5'-CTAGGTCGACTGATAAGTCGCTTATTTCATCTTTA-3'). The *escA* PCR products were cut with SacI and BamHI and cloned into the pWWW412 derivative, pSEW16, which has the NdeI restriction site replaced with a SacI site. The *escB* and $escB_{STREP}$ PCR products were cut with NdeI and BamHI or NdeI and SalI, respectively, and cloned into either pWWW412 or its derivative, pSEW5, which has the coding sequence for a STREP tag incorporated immediately downstream of BamHI followed by the addition of a SalI restriction site, respectively.

Protein purification from *S. aureus*. 4 liters of *S. aureus* USA300 harboring either the p*escA* or p$escA_{STREP}$ were cultured until an A_{600} of 2.0 was achieved, and cells were pelleted via centrifugation at 8,000 × g for 10 min. Sedimented cells were suspended in Phosphate Buffered Saline (PBS) (1.5 mM NaH_2PO_4, 8.5 mM Na_2HPO_4, 145 mM NaCl) with 20 μg/ml of lysostaphin, and incubated with vigorous shaking at 37°C for 1 hour. The resulting lysate was subsequently passaged twice through a French press at 14,000 lb/in^2 (ThermoSpectronic, Rochester, NY). Cells that were not lysed were removed by centrifugation at 3,000 × g for 10 min. In order to solubilize staphylococcal membranes, n-dodecyl-β-D-maltoside (DDM) was added to the whole cell lysates to a final concentration of 2% w/v and incubated at 4°C for 16 hours with light agitation. DDM-solubilized whole cell lysates were subjected via gravity flow to Strep-Tactin–Sepharose (IBA) chromatography with a packed bed volume of 2 ml pre-equilibrated with PBS with 2% DDM. Columns were washed with 40× bed volumes of PBS with 0.2% DDM and eluted with 2 ml of 5 mM desthiobiotin in PBS with 0.02% DDM. Samples of the eluate fractions were mixed at a 1:1 ratio with sample buffer, separated on 15% SDS-PAGE gels, and analyzed by Coomassie blue staining and immunoblotting. Further identification of co-purified proteins was

performed by cutting out bands of interest and submitting them for mass spectrometry analysis of tryptic digests at the Taplin Mass Spectrometry Facility at Harvard Medical School.

Protein secretion and subcellular fractionation. To examine ESS secretion in the Δ*escAB* strain, and determine the production and subcellular localization of $EscA_{STREP}$ and $EscB_{STREP}$ in *S. aureus*, overnight cultures were diluted 1:100 in 40 ml of TSB supplemented with appropriate antibiotics. For ESS-inducing conditions 0.02% heat-inactivated horse serum was added to the TSB media, and the pH adjusted to 5.5 with hydrochloric acid, whereas non-inducing conditions utilized TSB alone. Cultures were grown with shaking at 37°C until an A_{600} of 3.0. For whole cell lysates, 1 ml of cells was centrifuged at 8,000 x g for 10 minutes, suspended in PBS with 20 µg/ml of lysostaphin and incubated at 37°C for 1 h with vigorous shaking (whole cell [WC]). For identification of secreted proteins, the remaining 39 ml of culture was centrifuged at 8,000 × g for 10 min, and the supernatant was filtered with a 0.22-µm-pore-size Millex-GP unit and transferred to a fresh tube (culture filtrate [CF]). Cells in the pellet were washed, suspended in PBS with 20 µg/ml lysostaphin, and incubated with vigorous shaking for 1 hour at 37°C. The resulting lysate was passaged twice through a French press at 14,000 lb/in^2, and subsequently subjected to ultra-centrifugation at 100,000 × g for 2 h, at 4°C to separate soluble [Sol] from insoluble [Insol] proteins associated with the membrane. Proteins in all fractions, WC, CF, Sol, and Insol, were precipitated by adding 100% trichloroacetic acid (TCA) to a final concentration of 10% and left on ice for 1 h. TCA-precipitated samples were centrifuged at 15,000 × g for 30 minutes at 4°C, supernatant discarded, suspended in ice-cold acetone and left on ice for 1 h. Acetone-washed samples were centrifuged at 20,000 x g for 10 minutes, acetone discarded, and pellets allowed to air dry before being suspended in 150 µl of 0.5 M Tris-HCl, pH 8.0.

SDS-PAGE and immunoblotting. Resolubilized protein extracts were mixed at a 1:1 ratio with 2x sample buffer (0.1 M Tris-HCl [pH 8.0], 4% SDS, 20% glycerol, 2 mM β-mercaptoethanol, 0.04% bromophenol blue), heated at 90°C for 10 min, and subjected to 12-15% SDS-PAGE. Proteins were visualized by Coomassie brilliant blue R-250 (Aldrich-Sigma) staining of gels or by immunoblotting with previously generated polyclonal antibodies following electrotransfer to polyvinylidene difluoride (PVDF) membranes. Immunoblotting analysis was performed by blocking PVDF membranes with PBS containing 0.1% Tween-20 (PBST) and 5% milk for 1 hour at room temperature. The blocking buffer was supplemented with 1.0 mg of human IgG per 10 ml of buffer to prevent binding of primary antibodies to staphylococcal protein A. After incubation with the hIgG-containing blocking buffer, primary polyclonal antibodies were added, and the membranes were incubated overnight at 4°C. Membranes underwent four washes with PBST for 10 minutes per wash followed by incubation secondary antibody (goat anti-rabbit HRP-linked antibody; Cell Signaling Technology) in PBST with 5% milk for 1.5 hours. Membranes were washed four more times as described above and SuperSignal West Pico Chemiluminescent Substrate (Life Technologies) added before development of the membranes with Amersham Hyperfilm ECL (GE Healthcare Life Sciences).

Chapter V

Conclusions

Staphylococcus aureus is a Gram-positive coccus that is a part of the normal microflora of the human skin and nasal passages; however, the organism is also a pathogen endowed with a variety of virulence factors (Lowy, 1998). *S. aureus* can present as the causative agent of various invasive diseases such as skin and soft tissue infections, blood stream infections which can progress to sepsis and endocarditis, as well as osteomyelitis and pneumonia (Lowy, 1998; Kuehnert *et al.*, 2006; Klevens *et al.*, 2007; Deleo *et al.*, 2010; Lessa *et al.*, 2010). Further, the rapid emergence and prevalence of methicillin resistant strains make *S. aureus* an organism of chief concern for public health (Klevens *et al.*, 2006, 2007; Popovich, Weinstein and Hota, 2008). There is currently no vaccine against *S. aureus* and the plethora of disease manifestations and morbidity and mortality caused by *S. aureus*, make it an organism of paramount priority for continued research to discern new preventative and therapeutic measures.

The pathogenesis of *S. aureus* is an extremely complicated process that occurs through a variety of virulence factors, many with multifaceted functions, interacting with various host cells and secreted factors, allowing the bacterium to infect all tissues (Cheng *et al.*, 2009, 2011). Presumably, the initial infection requires some disruption in the protective barrier of the skin and inoculation of *S. aureus* (Lowy, 1998; Regev *et al.*, 1998; Tsokos and Püschel, 1999). Skin and soft tissue infections are the most prevalent and generally present as purulent lesions also termed staphylococcal abscesses (Lowy, 1998; Klevens *et al.*, 2007). Staphylococcal lesions are

not constrained to the skin however, and *S. aureus* can lead to invasive infections characterized by sepsis and formation of deep tissue abscesses (Cheng *et al.*, 2009, 2011). Persistent infections are thought to occur in part due to the difficulty of clearing these deep-seated abscesses, which can take multiple weeks (Cheng *et al.*, 2009, 2011). As the infection persists, the abscess that consists of a nidus of staphylococci surrounded by dead and living immune cells will migrate to the cortex of the infected organ and eventually burst, thereby releasing infectious staphylococci that may seed new abscesses and continue the cycle until the infection is cleared or the host dies (Cheng *et al.*, 2009, 2011). Abscess formation is a pathogen-driven process that requires several secreted bacterial factors (Cheng *et al.*, 2009, 2011).

One set of virulence factors that is important for abscess establishment and persistence is a cluster of genes that encode the ESAT-6-like Secretion System (ESS) (Burts *et al.*, 2005; Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2011, 2016). The staphylococcal ESS is a Sec-independent secretion system that was first identified as a distant homologue of the Type VII Secretion System (T7SS) of *Mycobacterium tuberculosis* (Pallen, 2002; Burts *et al.*, 2005). The importance of the ESS on staphylococcal pathogenesis has been established through characterization of *ess* mutants in mouse infection models. Such mutants fails to secrete the five known substrates of the ESS pathway: EsxA, EsxB, EsxC, EsxD, and EssD (Burts *et al.*, 2005; Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2013; Ohr *et al.*, 2016). Mice infected with *ess* mutants do not develop canonical staphylococcal abscess lesions and over time, these animals can clear the infection (Burts *et al.*, 2005; Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2011, 2016). Earlier work suggested that the *ess* cluster consists of eleven genes. Interestingly, deletion of any of the genes within the cluster, including substrates for the ESS,

will cause secretion to be abolished (Burts *et al.*, 2005; Burts, DeDent and Missiakas, 2008; Anderson *et al.*, 2013). One such gene essential for ESS secretion and abscess formation is *essD* (Anderson *et al.*, 2011).

essD is the eleventh gene in the *ess* gene cluster and encodes a 617 amino acid protein that fractionates with other membrane proteins in agreement with the presence of a hydrophobic sequence between amino acids 217 and 250 (Anderson *et al.*, 2011). Presumably, the protein adopts a topology whereby the N-terminus is outside the cell and the C-terminus remains in the cytoplasm (Anderson *et al.*, 2011). Animals infected with an *essD* mutant develop abscesses that are reduced in size as compared to abscesses formed by wild type bacteria (Anderson *et al.*, 2011). Histology analyses showed that the size difference is due to a reduction of immune cells recruited to these lesions (Anderson *et al.*, 2011). In turn, this failure to recruit immune cells leads to decreased persistence (Anderson *et al.*, 2011).

Efforts to further characterize *essD* were hindered due to various failed attempts in cloning *essD* in *E. coli* (Ohr *et al.*, 2016). A closer examination revealed the presence of premature stop codons or C-terminal missense mutations in the sequences of plasmid-encoded *essD* (Ohr *et al.*, 2016). A bioinformatics analyses suggested that the C-terminus contained a putative Endonuclease_NS_2 domain (Ohr *et al.*, 2016). Interestingly, genes harboring this specific nuclease domain are always found next to genes encoding proteins containing the Domain of Unknown Function 600 (DUF600), and the genome of *S. aureus* USA300 includes ten *duf600* genes downstream of *essD* (Ohr *et al.*, 2016). It was hypothesized that the toxicity of *essD* is due to the proposed nuclease domain, and that *duf600* genes, renamed here as *essI* genes, are necessary and sufficient to inhibit this toxicity. Furthermore, if EssD is indeed a

nuclease, what is it targeting and is this novel nuclease activity critical for successful abscess formation and staphylococcal pathogenesis?

During the course of this work, the toxicity of EssD and the ability of EssI to inhibit this toxicity within *E. coli* was determined via growth kinetics assays where expression of both genes was controlled by separate inducers. It was determined that the bactericidal effect exhibited by EssD corresponded with a loss of genetic material as visualized by microscopy imaging of DAPI-stained cultures expressing *essD* and not *essI*. Furthermore, the nuclease domain alone was sufficient to cause toxicity, as well as be inhibited by co-expression of *essI*. Additionally, a single nucleotide substitution yielding a leucine to proline mutation at residue 546 ablated the toxicity of EssD ($EssD^{L546P}$). Pull-down experiments were performed using an *E. coli* strain producing both EssI and the nuclease domain of EssD harboring a C-terminal histidine tag ($EssD_{434–617/HIS}$). From these experiments, it was determined that EssD and EssI form noncovalent interactions, and that EssD is a fully functional DNA nuclease when EssI is no longer bound.

The EssD/EssI interaction exhibited in *E. coli* can be recapitulated in pull-down experiments using *S. aureus* strains expressing His-tagged EssD. Furthermore, these pull-down experiments revealed a dominant C-terminal fragment containing the final 184 amino acids of EssD, residues 434-617. Using newly generated, affinity-purified antibodies against the first 200 amino acids of EssD, the final 184 amino acids of EssD, and EssI, fractionations of staphylococcal cultures were performed to determine EssD and EssI localization. EssD was found primarily in the insoluble membrane fraction, and yielded a variety of N- and C-terminal fragments including the C-terminal fragment identified in pull-down experiments. Interestingly, EssD was

also found in the culture medium and this was dependent on an intact *ess* cluster for secretion across the membrane. EssI, on the other hand, was found in the soluble fraction; however, in wild-type *S. aureus* producing EssD, EssI could also be found to a lesser degree in the insoluble membrane fraction indicating that the interaction between EssD and EssI can pull EssI to the membrane. At some point, this interaction is displaced, as EssI was never found in the culture medium.

It was also recognized that another protein encoded by a heretofore uncharacterized gene of the ESS gene cluster, EssE, interacted with EssD in the membrane of staphylococci. Characterization of EssE identified a novel component of the staphylococcal ESS pathway. EssE was assumed to be a soluble component of the ESS pathway; however, fractionation experiments determined that EssE was present equally in the soluble and insoluble cell fractions. Additionally, deletion of *essE* not only prevented secretion of the classical ESS substrates, but certain ESS proteins, such as EssD, were destabilized in the absence of EssE and the amount of these unstable proteins decreased or was abolished in *essE* mutants. It is presumed that EssE may act as a chaperone in some capacity, facilitating the shuttling of ESS substrates from the cytoplasm to the ESS machine components at the membrane and their eventual translocation.

Due to preliminary data generated during the characterization of the *essE* mutant, that indicated IL-12 secretion is upregulated during mouse infection in an ESS-dependent manner, cytokine analysis was performed on mouse blood 12 hours after infection with staphylococcal strains with either wild-type *essD*, the $essD^{L546P}$ allele, or the *essD::erm* insertional deletion (Anderson *et al.*, 2016; Ohr *et al.*, 2016). IL-12 levels were found to be significantly increased in

the serum of animals infected with the wild type strain USA300 as compared to animals infected with an *essD* mutant. Animals infected with strain expressing the *essD*^{L546P} allele also displayed lower levels of IL-12 in their sera suggesting that the nuclease activity is important to sustain a stronger IL-12 response during infection.

I hypothesize that the ESS-modulated immune response works through the Th1 arm of the immune system, and this is initiated via IL-12 secretion upon infection. In doing so, staphylococci skew the immune system away from both the Th2 and Th17 immune responses, allowing neutrophils and macrophages to be recruited to the budding staphylococcal lesions and facilitate formation of a persistent abscess, while inhibiting a proper adaptive and protective immune response from being mounted. Initial cytokine data show an increase in the IL-12p40 subunit and IL-6 which are associated with generating and stabilizing the Th1 and Th17 immune responses, and this occurs in an ESS-dependent manner. Additionally, these responses can promote recruitment of neutrophils via chemokines such as KC/CXCL1 which was also shown to be secreted in an ESS-dependent manner. The importance of T cells in controlling *S. aureus* infection has previously been shown during a murine model of persistent infection where RAG2$^{-/-}$ mice that received purified T cells, and not B cells, exhibited lower bacterial loads than control mice (Ziegler *et al.*, 2011; Schindler *et al.*, 2012; Tebartz *et al.*, 2015). However, T cells appear to also be important for a successful *S. aureus* infection, specifically abscess formation, as kidneys from RAG2$^{-/-}$ and CD3$^{-/-}$ mice infected with *S. aureus* display aberrant abscesses (Ziegler *et al.*, 2011; Schindler *et al.*, 2012; Tebartz *et al.*, 2015). Due to the ESS's already recognized role in staphylococcal persistence and abscess formation, as well as the newly identified cytokine profiles that may skew the immune response towards a specific T

helper cell subset, determination of whether the ESS preferentially polarizes T cells during a persistent mouse infection is of utmost importance.

The nuclease activity of EssD appears to be crucial for the immunomodulatory activity of the ESS pathway during infection, however the exact mechanism whereby this is accomplished remains unknown. The nuclease activity of EssD could modulate cytosolic immune sensing of DNA by host cells. Perhaps, hydrolysis by EssD gives rise to distinct nucleotides that can modulate the activity of dedicated receptors. The chemical identification of such nucleotides would be extremely valuable. Along these lines, it would be interesting to evaluate the cytokine response of host cells subjected to treatment with the products of EssD nuclease activity. Furthermore, if a cytokine response from EssD-degraded DNA can be elicited, it would be of great interest to determine how the host recognizes these products. STING and TLR9 are two major DNA sensors within the cytoplasm or phagosome of host cells, respectively, and a connection between these sensors and the nuclease of EssD may occur during *S. aureus* infection of phagocytic cells. Experimentation to determine if the EssD-dependent cytokine production occurs through either STING or TLR9 signaling axis should be performed. siRNA knockdown studies or generation of BMDMs from STING and TLR9 knockout mice would allow for the best examination of these questions.

EssD represents the first component of the ESS that bears a specific catalytic activity. One of the greatest challenges in elucidating the role of these secretion systems stems from the dominant effect exhibited by genetic mutations. Thus, it is very hard to deduce individual functions for effector proteins. Moving forward, the ability to use of the *essD*^{L546P} variant to parse out the roles the *ess* plays in infection should yield large steps for the field. Along with

the novel components that have begun preliminary characterization in Chapter 4 of this thesis, I believe the staphylococcal *ess* field will begin to recognize that the differences identified by Warne *et al*., 2016 are due to bacteria requiring specific *ess* clusters for specific niches. Similar to how the differences between ESX T7SSs of Mtb were initially shrouded in mystery and have since been characterized and recognized as very specialized T7SS variants, the staphylococcal field will also grow to understand this.

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Appendix

Figures

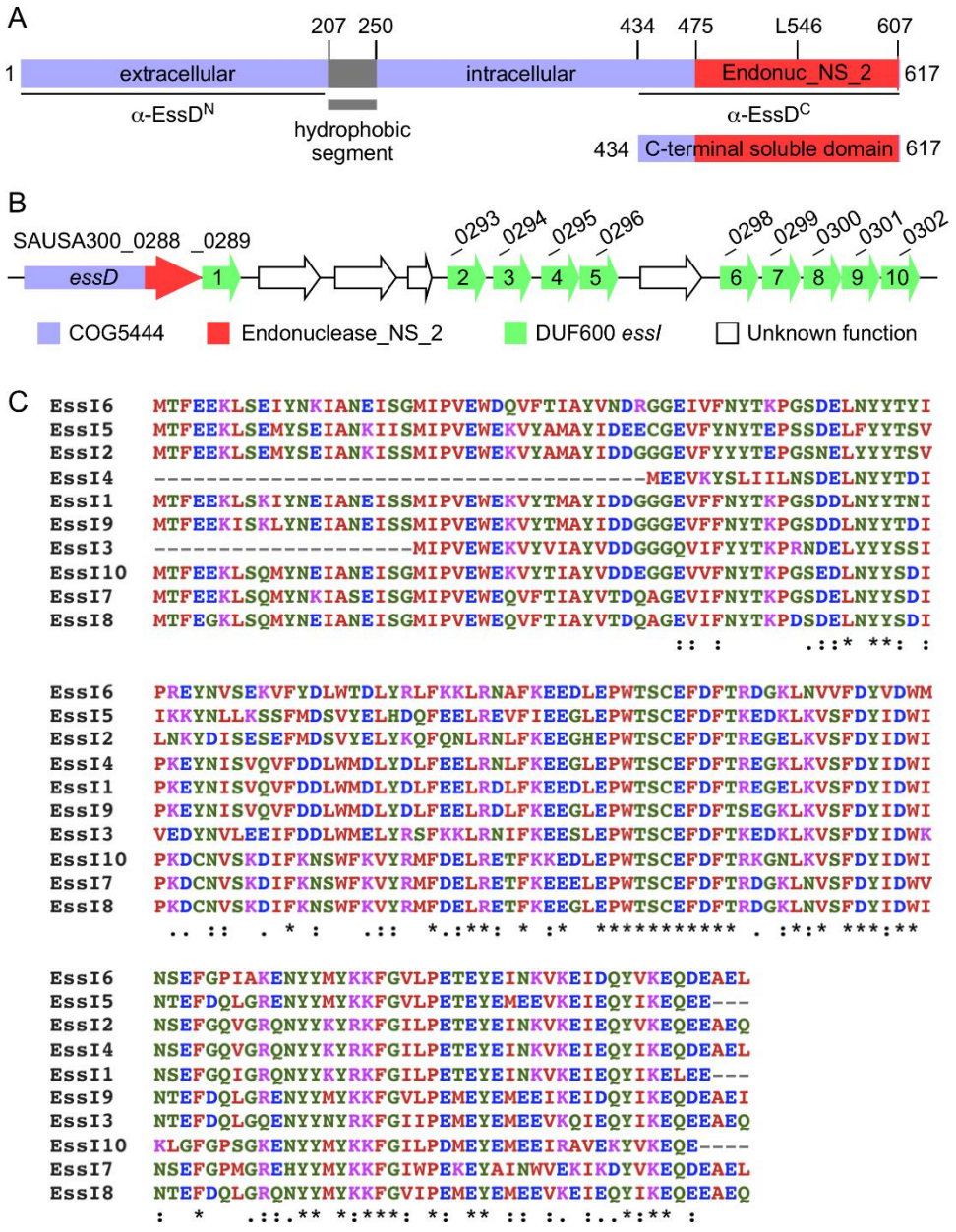

Figure 1. EssD and EssI proteins of *S. aureus* USA300. (A) Domain organization of EssD. EssD carries the COG5444 domain (blue) and a predicted nuclease domain (endonuclease_NS_2; red). The hydrophobic segment was identified with the TMHMM server, version 2.0. Numbers indicate positions of amino acid residues in the linear polypeptide. L546 indicates the position of Leu^{546}, a residue essential for cytotoxicity. (B) Diagram of the genomic region carrying the *essD* and *essI* genes in *S. aureus* subsp. *aureus* USA300_FPR3757 as annotated in NCBI RefSeq NC_007793.1. *essI* genes with DUF600 domains are shown in green. (C) Alignment of the 10 open reading frames bearing DUF600 in *S. aureus* subsp. aureus USA300_FPR3757 (NCBI RefSeq accession number NC_007793.1) using Clustal W. The symbols denote identical residues in all sequences (*), highly conserved columns (:), and weakly conserved columns (.).

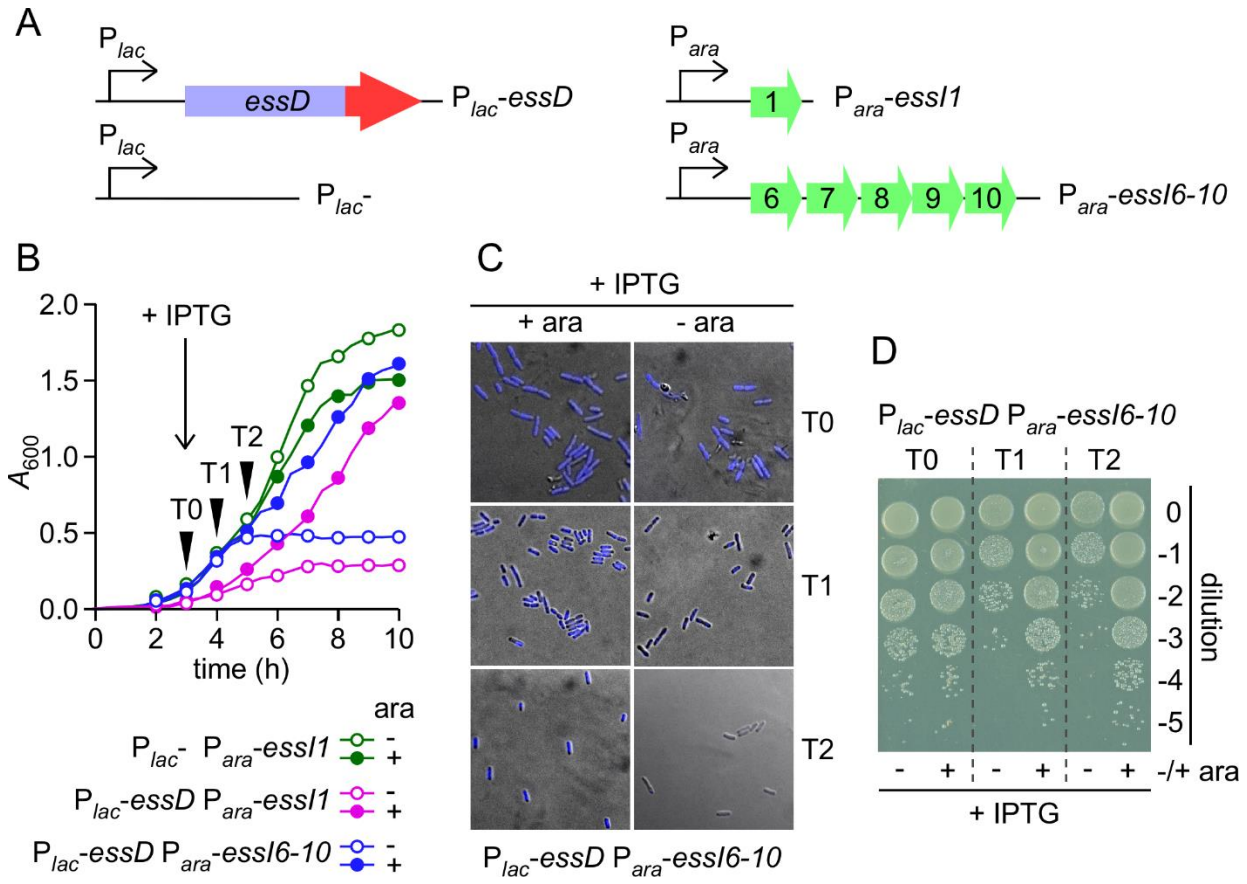

Figure 2. Cytotoxicity of EssD in *E. coli*. (A) Diagram depicting plasmid constructs with *ara* and *lac* promoters. (B to D) Growth and viability of *E. coli* variants harboring plasmids carrying the *essD* and *essI* genes. Overnight cultures were normalized to an A_{600} of 5, diluted 1:100 into fresh medium with (+) or without (-) arabinose (ara) and grown at 37°C. IPTG was added 3 h after dilution. Growth was monitored as increased absorbance (A_{600}) over 6 h (B). At timed intervals (0, 1, and 2 h) after IPTG addition (T0, T1, and T2), cultures of the P_{lac}-*essD* P_{ara}-*essI6-10* strain grown with or without arabinose and with IPTG were examined for integrity of genetic content (C) and for viability (D). Cells were stained with DAPI and visualized with an Olympus AX70 microscope and UV filter. Pseudocolor was used for image display in panel C. Cultures were serially diluted (0 to -5), and 10-µl aliquots of each dilution were spotted on agar medium containing ampicillin, kanamycin, and arabinose. An image of the plate after overnight incubation at 30°C is shown in panel D.

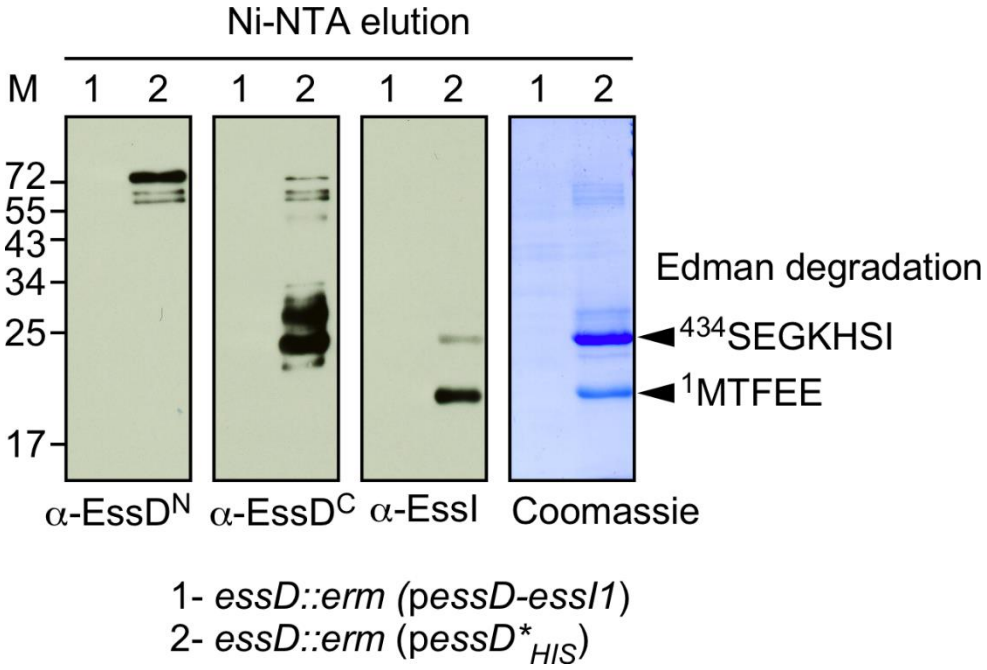

Figure 3. Purification of EssD from *S. aureus.* *S. aureus* strain USA300 *essD*::*erm* was transformed with plasmid p*essD-essI1* or p*essD*$_{HIS}$* to produce wild-type EssD or the nontoxic Leu^{546}Pro variant with a C-terminal histidine tag (lanes 1 and 2, respectively). Both strains were grown at 37°C in TSB and chloramphenicol. Cultures were centrifuged, sedimented bacteria were lysed in buffer A, and cleared bacterial lysates were subjected to Ni-NTA chromatography. Beads were washed twice with 20 volumes of buffer A containing 30 and 50 mM imidazole, and bound proteins were eluted with 0.5 M imidazole. Eluted samples were separated by SDS-PAGE and either stained with Coomassie or transferred to PVDF membrane for immunoblot analyses with anti-EssD^N, anti-EssD^C, and anti-EssI polyclonal sera. Numbers to the left indicate the mobility of molecular mass markers (lane M). Arrowheads point to the two bands subjected to Edman degradation with the resulting amino acid sequences identified during this analysis.

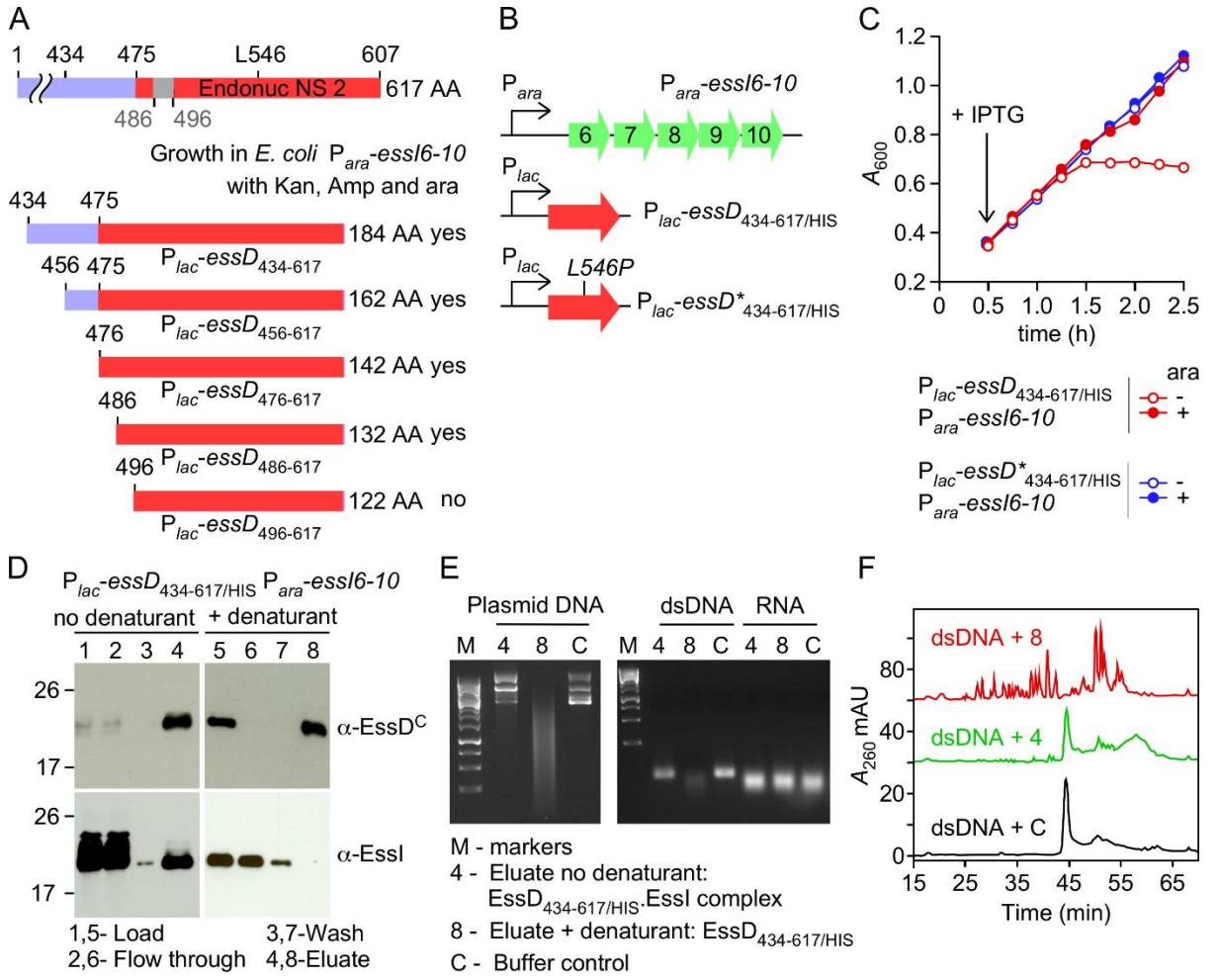

Figure 4. Biochemical characterization of EssD$_{434–617/HIS}$. (A) EssD sequence elements required for DNase activity and inhibition by EssI. Plasmids encoding N-terminal truncations of EssD were transformed in *E. coli* P$_{ara}$-*essI6-10* for the selection of viable clones in the presence of kanamycin, ampicillin, and arabinose. (B) Diagram depicting plasmid constructs with *lac* and *ara* promoters. (C) Growth of *E. coli* variants carrying P$_{ara}$-*essI6-10* with either P$_{lac}$-*essD*$_{434–617/HIS}$ or P$_{lac}$-*essD**$_{434–617/HIS}$ plasmids was monitored as described in the legend to Fig. 2B. (D) Wild-type EssD$_{434–617/HIS}$ was purified from cleared lysate of *E. coli* P$_{lac}$-*essD*$_{434–617/HIS}$ P$_{ara}$-*essI6-10*. Aliquots of samples prior to purification (Load), flowthrough, wash, and eluates were separated by SDS-PAGE and transferred to PVDF membrane for immunoblotting with anti-EssD^{C} and anti-EssI polyclonal sera. Lanes 1 to 4 and 5 to 8 were loaded with sample aliquots of preparations purified in buffer A without urea (no denaturant) or buffer A containing 8 M urea (+ denaturant), respectively. Bound proteins were eluted in buffer A containing 0.5 M imidazole. Numbers to the left indicate the mobility of molecular mass markers. (E) Plasmid DNA, dsDNA, or RNA was incubated with proteins eluted in fraction 4 or 8 or buffer control. Following incubation, reaction products were separated on an agarose gel and visualized with a UV transilluminator for image acquisition. (F) Reaction products following incubation of dsDNA with sample 4, sample 8, or buffer control C were separated by reverse-phase chromatography, and absorbance was recorded at 260 nm (A_{260}). AU, arbitrary units.

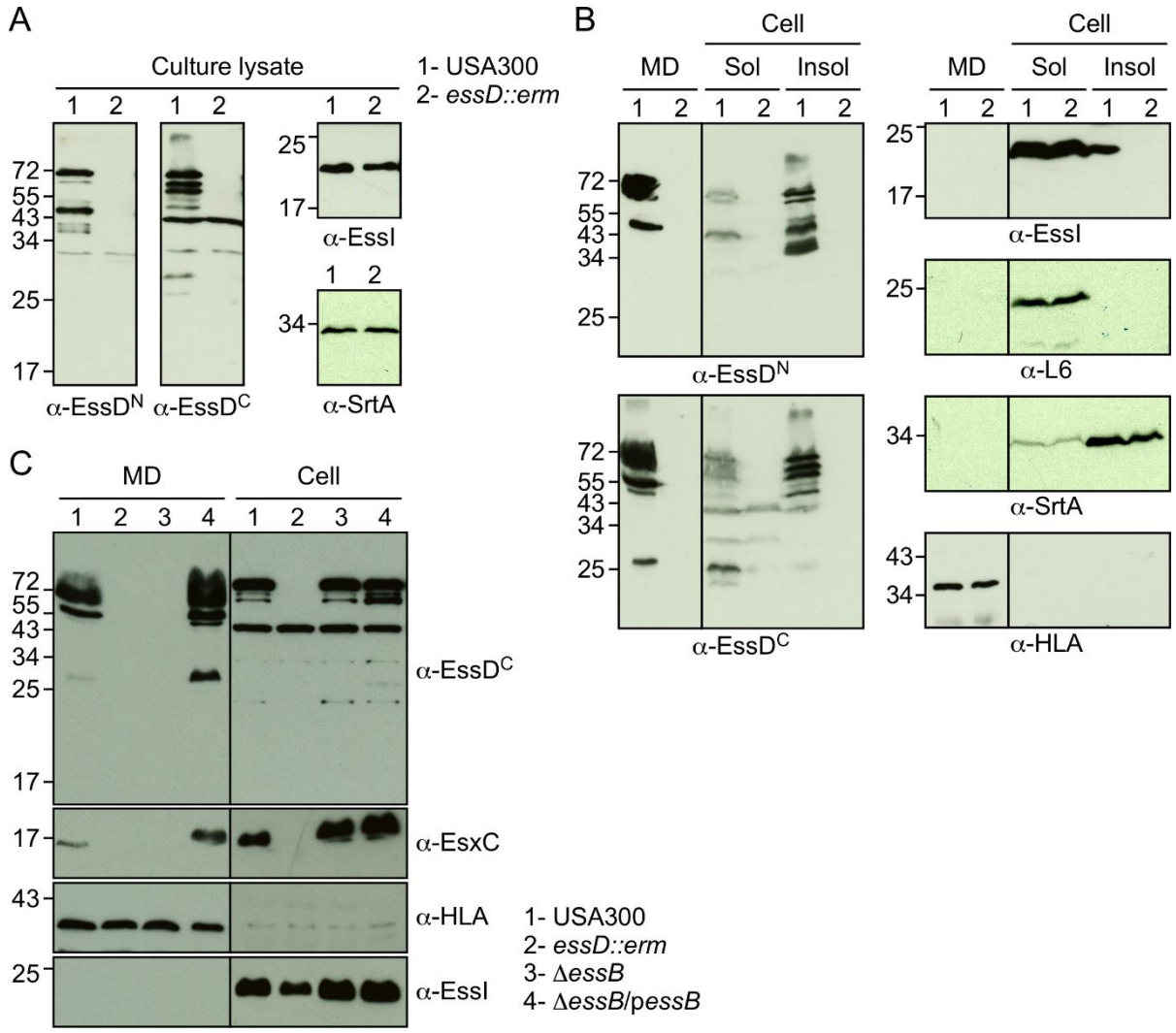

Figure 5. Subcellular localization of EssD and EssI proteins. *S. aureus* strain USA300 (lanes 1) and the isogenic *essD*::*erm* variant (lanes 2) were grown at 37°C in TSB supplemented with serum. Cultures were either incubated with lysostaphin yielding whole culture lysates (A) or fractionated into medium (MD), soluble (Sol), and insoluble (Insol) subcellular contents (B). Proteins were precipitated by the addition of TCA, suspended in sample buffer prior to separation by SDS-PAGE, and transferred to PVDF membranes for immunoblot analyses with anti-EssD^{N}, anti-EssD^{C}, anti-EssI, anti-SrtA, anti-L6, or anti-HLA polyclonal sera. Numbers to the left indicate the mobility of molecular mass markers. (C) EssD is secreted in an *essB*-dependent manner. *S. aureus* strain USA300 (lanes 1), the isogenic *essD*::*erm* strain (lanes 2), the Δ*essB* mutant (lanes 3), and Δ*essB*/p*essB* (lanes 4) complemented variant were grown at 37°C in TSB supplemented with serum and appropriate antibiotics. Cultures were centrifuged to separate medium (MD) from the bacterial sediment. Staphylococci were treated with lysostaphin for complete lysis (cell). Proteins were precipitated by the addition of TCA, suspended in sample buffer prior to separation by SDS-PAGE, and transferred to PVDF membranes for immunoblot analyses with anti-EssD^{C}, anti-EssI, anti-EsxC, or anti-HLA polyclonal sera. Numbers to the left indicate the mobility of molecular mass markers.

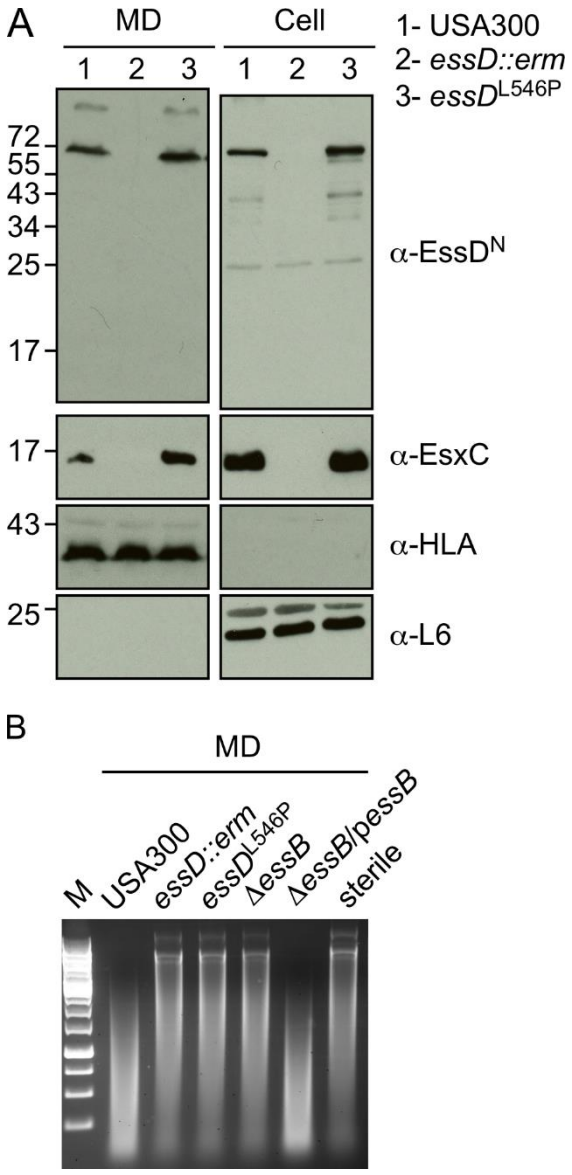

Figure 6. The nuclease activity of EssD is dispensable for secretion. (A) *S. aureus* strain USA300 (lanes 1) and the isogenic *essD*::*erm* (lanes 2) or *essD*^{L546P} (lanes 3) variant were grown at 37°C in TSB supplemented with serum. Cultures were spun to separate the medium (MD) from intact cells that were subsequently lysed with lysostaphin to release all cellular content (cell). Proteins were TCA precipitated, suspended in sample buffer prior to separation by SDS-PAGE, and transferred to PVDF membranes for immunoblot analyses with anti-EssD^{N}, anti-EsxC, anti-L6, or anti-HLA polyclonal sera. Numbers to the left indicate the mobility of molecular mass markers. (B) Cultures of *S. aureus* strain USA300 and the isogenic *essD*::*erm*, *essD*^{L546P}, Δ*essB*, and Δ*essB*/p*essB* variants were grown at 37°C in TSB supplemented with serum and appropriate antibiotics. The supernatant of these cultures was used to assay for nuclease activity using plasmid DNA. Sterile TSB was used as a control (sterile). Following incubation, reaction products were separated on an agarose gel and visualized with a UV transilluminator for image acquisition. Lane M, molecular mass markers.

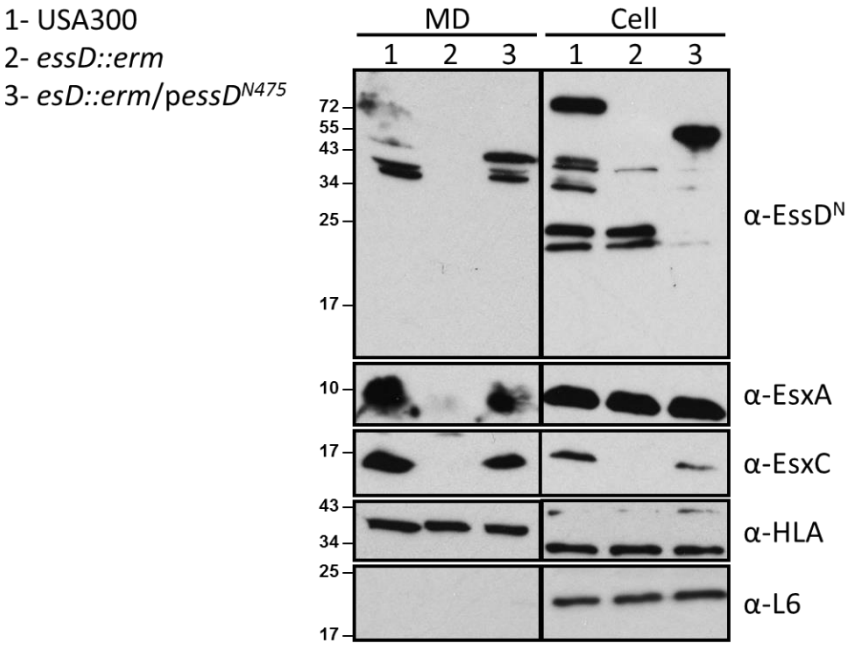

Figure 7. The first 475 amino acids of EssD are sufficient for secretion. *S. aureus* strain USA300 (lanes 1), the isogenic *essD*::*erm* variant (lanes 2), or *essD*::*erm* complemented with p*essD*^{N475} (lanes 3) were grown at 37°C in TSB supplemented with serum at pH 5.5. Complementation of *essD::erm* with a plasmid encoding a truncated EssD protein that consisted of only the N-terminal 475 amino acids restored secretion of EsxA and EsxC. Cultures were centrifuged to separate the medium (MD) from intact cells that were subsequently lysed with lysostaphin to release all cellular content (Cell). Proteins were TCA precipitated, suspended in sample buffer prior to separation by SDS-PAGE, and transferred to PVDF membranes for immunoblot analyses with anti-EssD^{N}, anti-EsxA, anti-EsxC, anti-HLA, or anti-L6 polyclonal sera. Numbers to the left indicate the mobility of molecular mass markers.

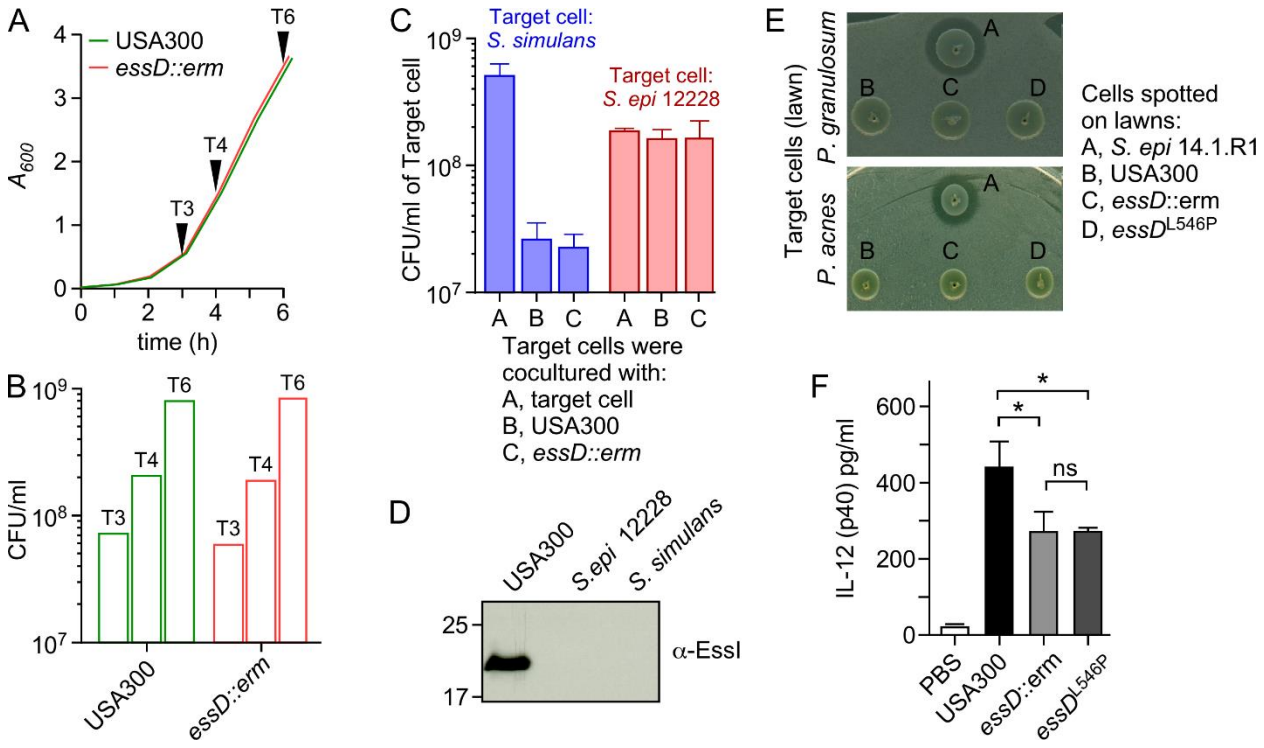

Figure 8. Expression of *essD* does not confer any growth advantage and elicits an IL-12 response during infection. (A) Overnight cultures of wild-type USA300 and the isogenic *essD*::*erm* variant were normalized to an A_{600} of 5, diluted 1:100 into fresh medium, and grown with shaking at 37°C. Growth was monitored by recording the A_{600}. (B) Aliquots of cultures shown in panel A were removed at 3, 4, and 6 h (T3, T4, and T6, respectively) and plated to assess viability. (C) *S. epidermidis* 12228 or *S. simulans* was cocultured with *S. aureus* USA300 or the isogenic *essD*::*erm* variant. Bacteria were mixed at a 1:1 ratio and plated on TSA. Immediately and 9 h following plating, cocultured bacteria were serially diluted and plated for enumeration of viable target cell counts. The data are presented as the average ± standard error of the mean from three independent competition experiments. (D) Identification of EssI in culture lysates of *S. epidermidis* (*S. epi*) 12228, *S. simulans*, and *S. aureus* USA300 using Western blotting. Samples were prepared and analyzed as described in the legend to Fig. 6A. (E) Cultures of *P. acnes* and *P. granulosum* grown to an A_{600} of 1.0 were plated on TSA, and the *S. epidermidis* 14.1.R1, USA300, *essD*::*erm*, or *essD*^{L546P} strain was spotted on top of *Propionibacterium* lawns. Pictures of plates incubated at 37°C under anaerobic conditions for 72 h are shown. (F) Cohorts of C57BL/6 mice (n = 5) were inoculated intravenously with PBS (control) or with 5×10^7 CFU USA300 or with the *essD*::*erm* and *essD*^{L546P} isogenic variants. Blood was sampled 12 h postinfection, and serum IL-12 (p70) was analyzed by enzyme-linked immunosorbent assay. Data were averaged, repeated for reproducibility, and analyzed by one-way ANOVA using Bonferroni's multiple-comparison test and with an unpaired t test in panel D (*, $P < 0.05$; ns, not significant).

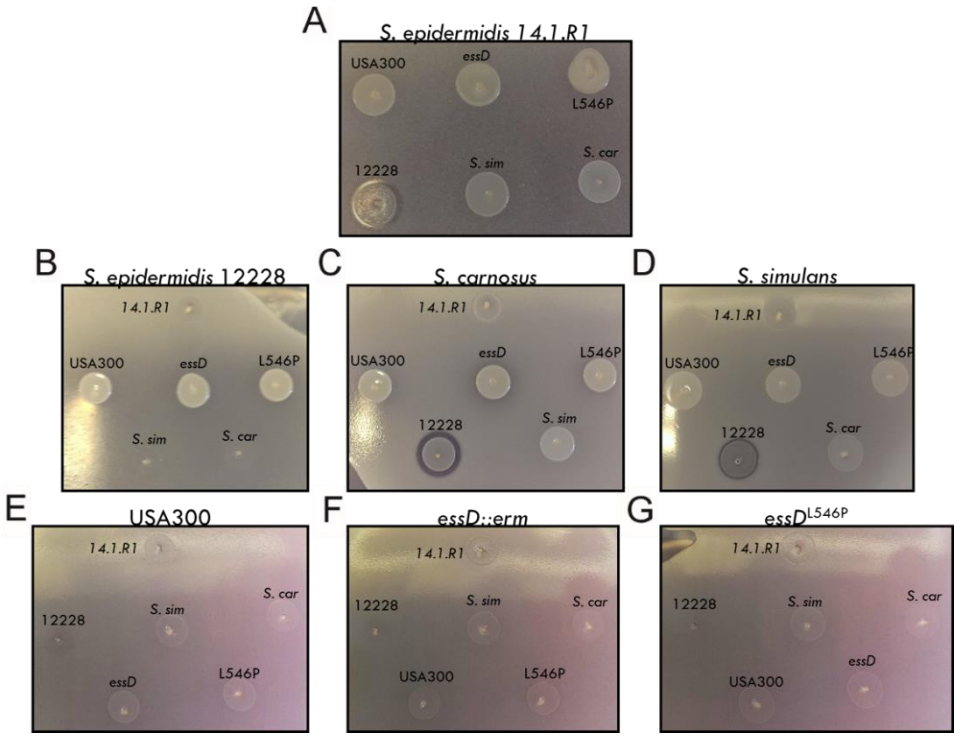

Figure 9. *S. epidermidis* 14.1.R1 does not antagonize growth of other staphylococci. (A-G) Overnight cultures of *S. epidermidis* 14.1.R1 (A), *S. epidermidis* 12228 (B), *S. carnosus* (C), *S. simulans* (D), and wild-type USA300 (E) or its isogenic variants, *essD*::*erm* (F) and *essD*^{L546P}(G), were standardized to an A_{600} of 1.0 and plated as a lawn on TSA. The six staphylococcal species that were not used as the lawn were then spotted on top of the lawns. Pictures of plates incubated at 37°C for 24-48 h are shown. *S. epidermidis* 14.1.R1 did not exhibit staphylococcal killing, indicating that the antagonism it exhibits with *Propionibacterium* species is through a genus specific mechanism.

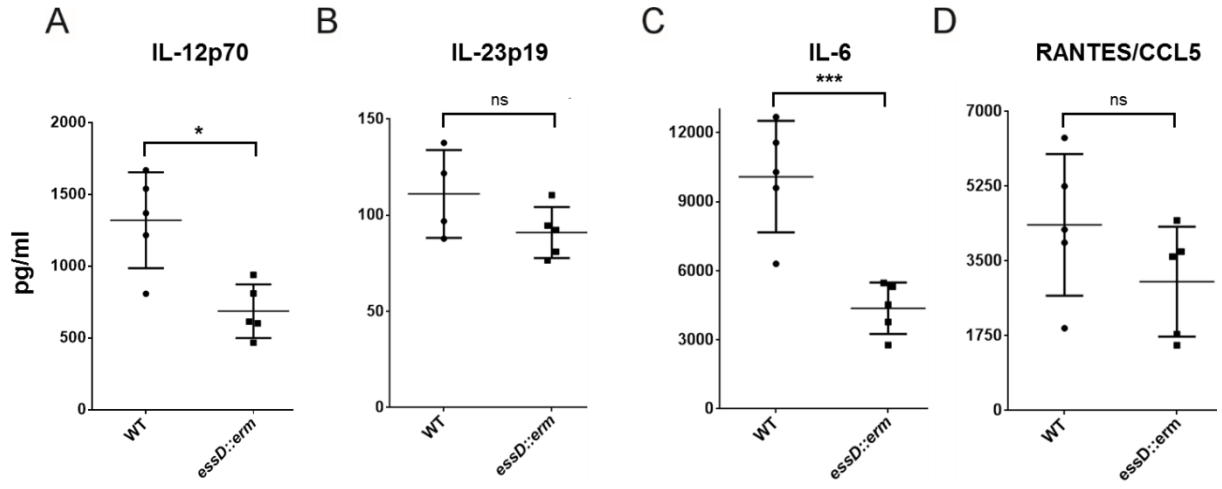

Figure 10. *S. aureus* infection of BMDMs elicits *essD*-dependent IL-12 secretion. (A-D) BMDMs were infected at an MOI of 1 for one hour at 37^{o}C with 5% CO_2. Media was removed, cells washed, and replaced with media containing Gentamycin at 20 µg/ml and incubated for an additional 19 hours. Media from the wells was taken and subjected to IL-12p70 (A), IL-23p19 (B), IL-6(C), and RANTES/CCL5(D) ELISA analysis. Wild-type USA300 was capable of inducing high levels of IL-12 and IL-6 secretion, whereas the *essD::erm* mutant was defective in this ability. Data were analyzed by comparison through an unpaired Students T-test (*, $P < 0.05$; ***, $P < 0.001$; ns, not significant).

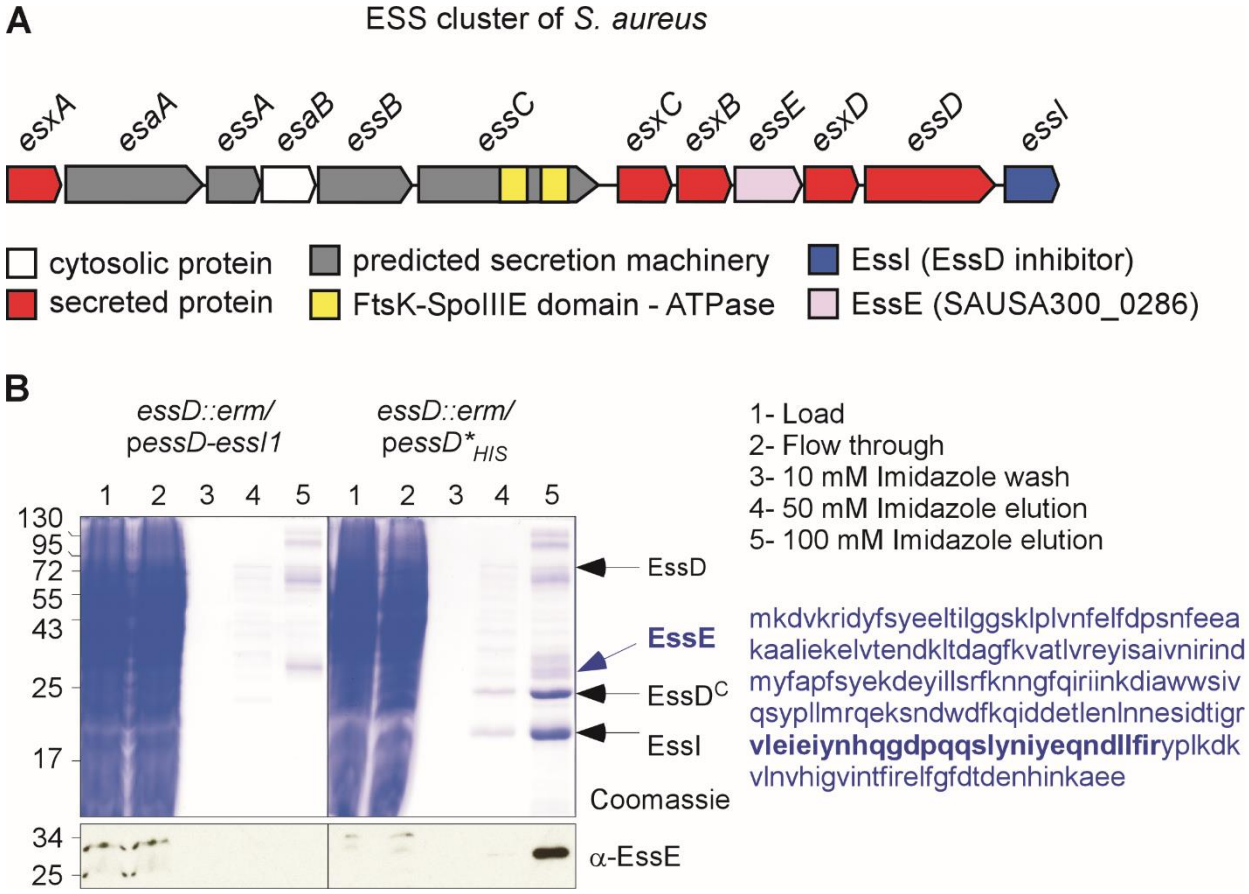

Figure 11. EssE is a ligand of EssD. (A) Schematic representation of the ESS cluster in *S. aureus*. (B) Cultures of *S. aureus* strain USA300 *essD*::*erm* carrying plasmid p*essD-essI1* or p*essD**$_{His}$ to produce wild-type EssD or the nontoxic Leu^{546}Pro variant with a C-terminal histidine tag were grown at 37°C and centrifuged, and sedimented bacteria were lysed to generate cleared lysates that were treated with DDM to solubilize membrane proteins for purification over Ni-NTA (lanes 1). The flow through containing unbound proteins (lanes 2), 10 mM imidazole wash (lanes 3), and the 50 and 100 mM imidazole elution fractions (lanes 4 and 5) were separated by SDS-PAGE and either stained with Coomassie blue or transferred to PVDF membrane for immunoblot analyses with the anti-EssE polyclonal serum. Numbers to the left indicate the mobility of molecular mass markers. Arrows point to bands corresponding to proteins identified by mass spectrometry. The sequence of EssE is shown in blue, and the region identified by mass spectrometry is in bold.

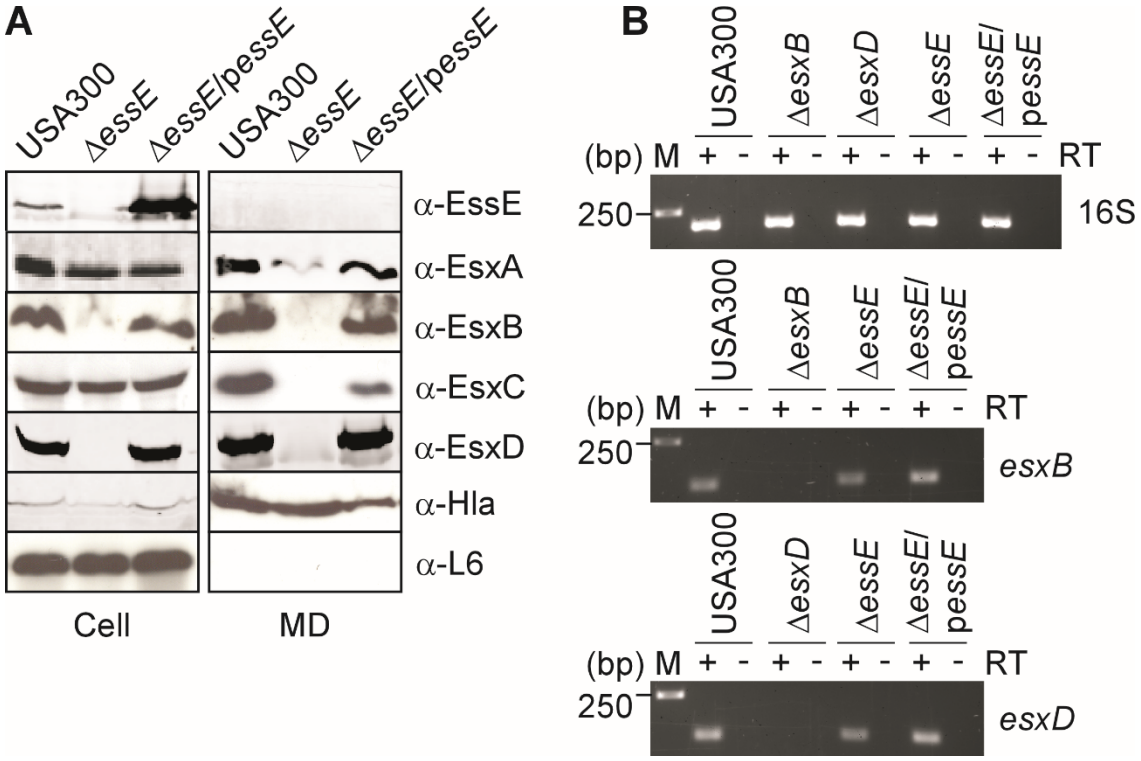

Figure 12. EssE is required for the secretion of Esx proteins. (A) To assess production and secretion of proteins encoded by the ESS cluster, *S. aureus* cultures of strain USA300 or its isogenic Δ*essE* mutant with or without complementing plasmid (Δ*essE*/p*essE* strain) were grown to an A_{600} of 1.0 and centrifuged to separate the medium (MD) fraction from intact cells in the sediment. Cells were suspended in PBS buffer and lysed with lysostaphin to release all cellular content (Cell). Proteins in the medium and cell extracts were precipitated by the addition of TCA to a final concentration of 10% prior to separation by SDS-PAGE, followed by transfer for immunoblot analyses. Immunoblotting was performed using rabbit polyclonal antibodies specific for EssE, EsxA, EsxB, EsxC, EsxD, alpha-toxin (Hla), and ribosomal protein L6 (L6) proteins. Hla is a well-characterized protein secreted in an Ess-independent manner. L6 is a cytoplasmic protein. (B) To assess whether expression of *esxB* and *esxD* was affected following deletion of *essE*, *S. aureus* cultures were grown as described for panel A for extraction and purification of total mRNA. Reverse transcriptase (RT) was used to generate cDNA of *esxB*, *esxD*, and 16S transcripts. Expression of *esxB* and *esxD* in the USA300, Δ*essE*, and Δ*essE*/p*essE* strains was qualitatively assessed by running RT-PCR products on agarose gels. Total mRNA was also extracted from strains lacking *esxB* or *esxD* (Δ*esxB* or Δ*esxD* strain) to unambiguously identify the cognate transcripts. Amplification using 16S primers was performed as a calibration control. Numbers to the left of the gels indicate DNA markers and their corresponding lengths in base pairs. Positive and negative signs above gels indicate the inclusion or omission of RT during the experimental steps.

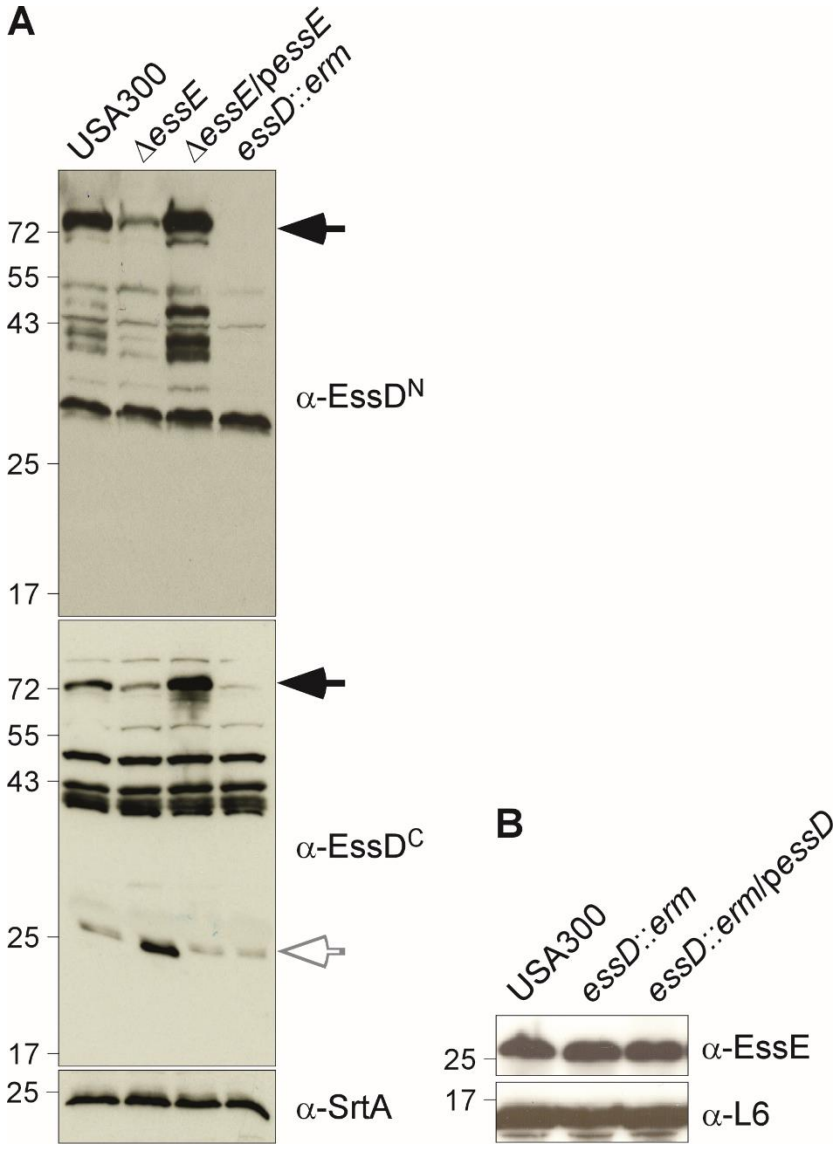

Figure 13. EssE is required to stabilize EssD. (A) *S. aureus* cultures of strain USA300 or its isogenic Δ*essE*, Δ*essE*/p*essE*, and *essD*::erm variants were grown to an A_{600} of 1.0, and lysostaphin was added to the whole culture. Proteins were TCA precipitated and separated by SDS-PAGE as described in the legend to Fig. 2A. Immunoblot analyses were performed using rabbit polyclonal antibodies specific for the N- and C-terminal domains of EssD (α-$EssD^N$ and α-$EssD^C$) and membrane protein SrtA. Arrows point to various polypeptides that are specifically recognized by EssD antibodies. (B) Extracts of the USA300 strain or its isogenic *essD*::*erm* variant with or without complementing plasmid (p*essD*) were prepared as described for panel A and examined by immunoblotting for the presence of EssE and ribosomal protein L6.

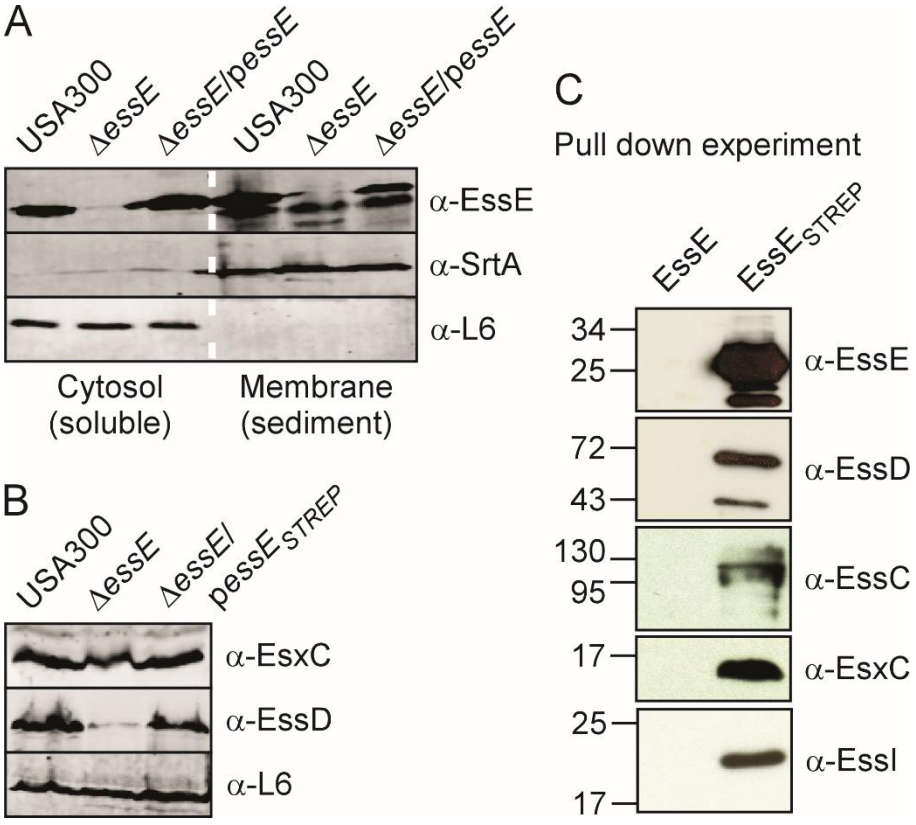

Figure 14. Identification of the EssE complex. (A) *S. aureus* cultures of strain USA300 or its isogenic Δ*essE* mutant with or without complementing plasmid (Δ*essE*/p*essE* strain) were grown to an A_{600} of 1.0. Cells were sedimented by centrifugation and lysed with lysostaphin, and lysates were subjected to ultracentrifugation (100,000 × *g* for 2 h). The supernatant and pellet fractions containing soluble proteins from the cytosol and membrane proteins from the sediment were separated, subjected to SDS-PAGE, and transferred to PVDF membranes for immunoblot analysis using antibodies against EssE as well as the membrane and cytosolic proteins SrtA and L6, respectively. (B) *S. aureus* cultures of strain USA300 or its isogenic Δ*essE* mutant with or without complementing plasmid producing EssE with a Strep-Tag (Δ*essE*/p*essE*$_{STREP}$ strain) were grown to an A_{600} of 1.0. Lysostaphin was added to the whole culture, and TCA was added to precipitate proteins and examine extracts as described in the legend to Fig. 2A. Immunoblot analyses were performed using anti-EssD^{N}, anti-EsxC, and anti-L6. (C) *S. aureus* cells producing EssE without (EssE) or with the Strep-Tag (EssE$_{STREP}$) were grown to an A_{600} of 2.0, sedimented, and lysed with lysostaphin. Cell lysates were suspended in DDM, and insoluble materials were removed by centrifugation at 100,000 × *g*. Detergent-soluble proteins were flowed by gravity over Strep-Tactin-Sepharose beads, washed extensively with PBS, and eluted with 2.5 mM desthiobiotin. Bound proteins in eluates were separated by SDS-PAGE and electrotransferred to PVDF membranes for identification by immunoblotting.

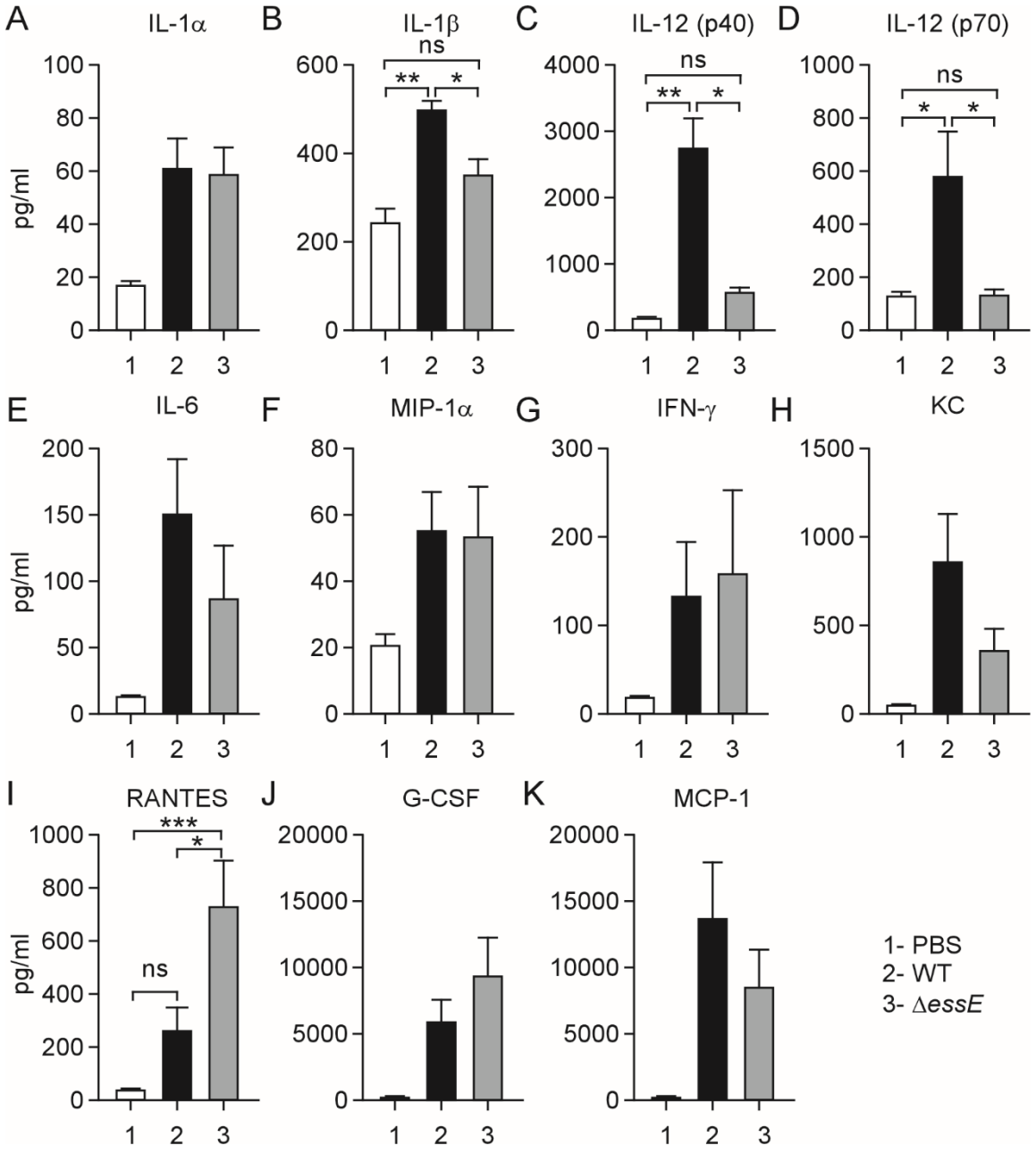

Figure 15. Cytokine profiling of mice infected with *S. aureus*. C57BL/6 mice (*n* = 5) were infected by intravenous injection with 5×10^7 CFU of *S. aureus* USA300 or its Δ*essE* variant or were mock infected (PBS). Blood was collected at 12 h postinfection, and serum cytokine levels were determined by enzyme-linked immunosorbent assay. Data were averaged, and standard errors of the means were calculated and analyzed for significant differences with one-way ANOVA using Bonferroni's multiple-comparison test. The statistical analysis is shown only for the groups that exhibited a significant difference between the wild-type (WT) and Δ*essE* strain data sets (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

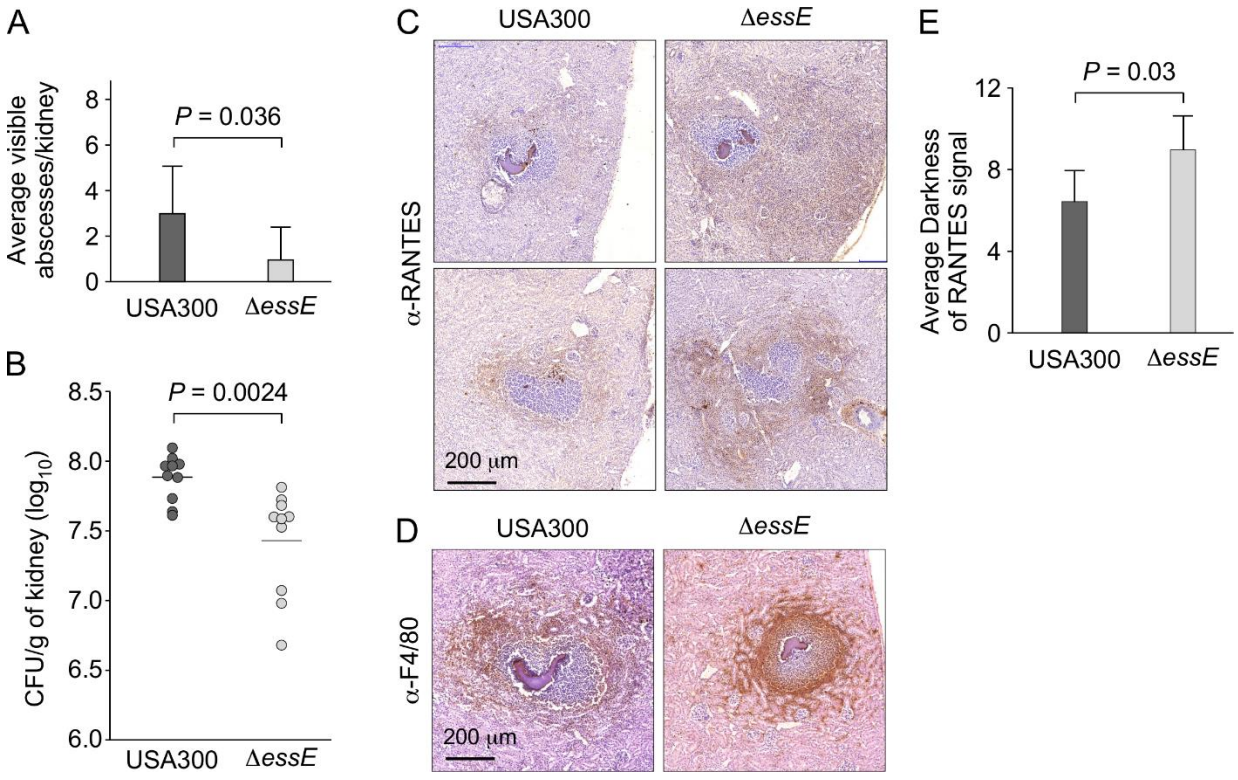

Figure 16. *essE* mutants exhibit altered abscesses. Staphylococcal replication in kidneys was measured 5 days postinfection after injection of 5×10^6 CFU of USA300 or its isogenic *essE* mutant in mice (groups of 8 to 10). (A and B) Kidneys were removed during necropsy to enumerate surface abscesses and subsequently homogenized in 1% Triton X-100 for plating and enumeration of CFU. Statistical analyses were performed using an unpaired two-tailed Student's *t* test with Welch's correction (A) and a two-tailed Mann-Whitney test (B). (C to E) Kidney sections were taken at 12-μm intervals, and abscesses were first stained with H&E to identify abscesses with staphylococcal communities (dark purple and denser stain in the center of slides) surrounded by immune cells. Next, the sections were incubated with anti-RANTES antibody or F4/80 antibody, a macrophage-specific marker. Following incubation with a secondary HRP-conjugated antibody and development, brown staining reflected the presence of macrophages outside the fibrin cuffs and surrounding USA300 lesions while the staining penetrated Δ*essE* lesions. Representative images are shown. Anti-RANTES signals were quantified using Fiji software, and values are reported as average darkness. Statistical analysis was performed using a two-tailed Mann-Whitney test.

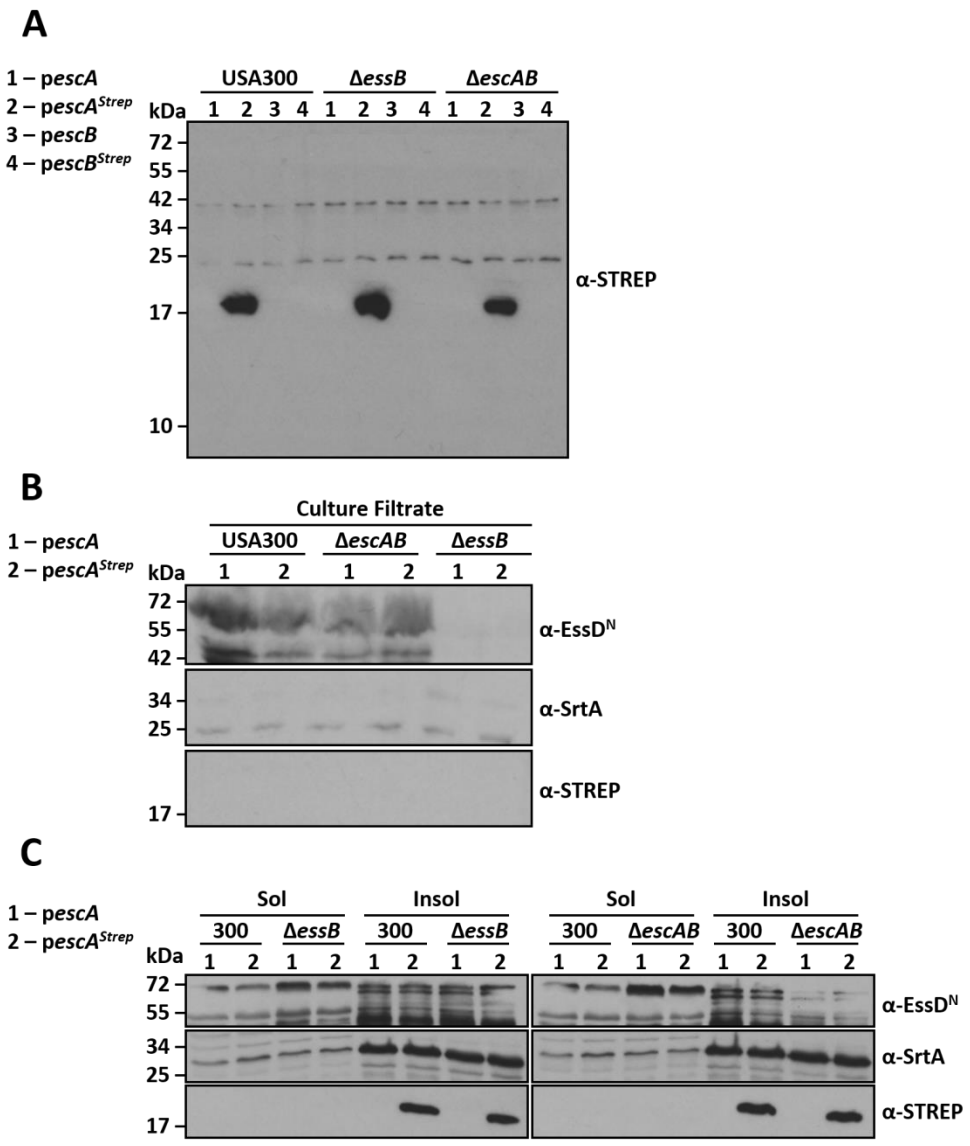

Figure 17. Subcellular localization of $EscA_{STREP}$. (A) *S. aureus* strain USA300, the isogenic Δ*essB* mutant, and the isogenic Δ*escAB* mutant were transformed with plasmids encoding EscA or EscB and STREP-tagged variants and examined for protein production. (B) Based on the presence of an immunoreactive band against $EscA_{STREP}$, subcellular localization was examined in the three strain backgrounds harboring either p*escA* or p$escA_{STREP}$. Cultures were grown at 37°C in TSB supplemented with serum. Cultures were either incubated with lysostaphin yielding whole culture lysates (A) or fractionated into culture filtrate (CF), soluble (Sol), and insoluble (Insol) subcellular contents (B, C). Proteins were precipitated by the addition of TCA, suspended in sample buffer prior to separation by SDS-PAGE, and transferred to PVDF membranes for immunoblot analyses with anti-STREP, anti-$EssD^N$, and anti-SrtA sera. Numbers to the left indicate the mobility of molecular mass markers.

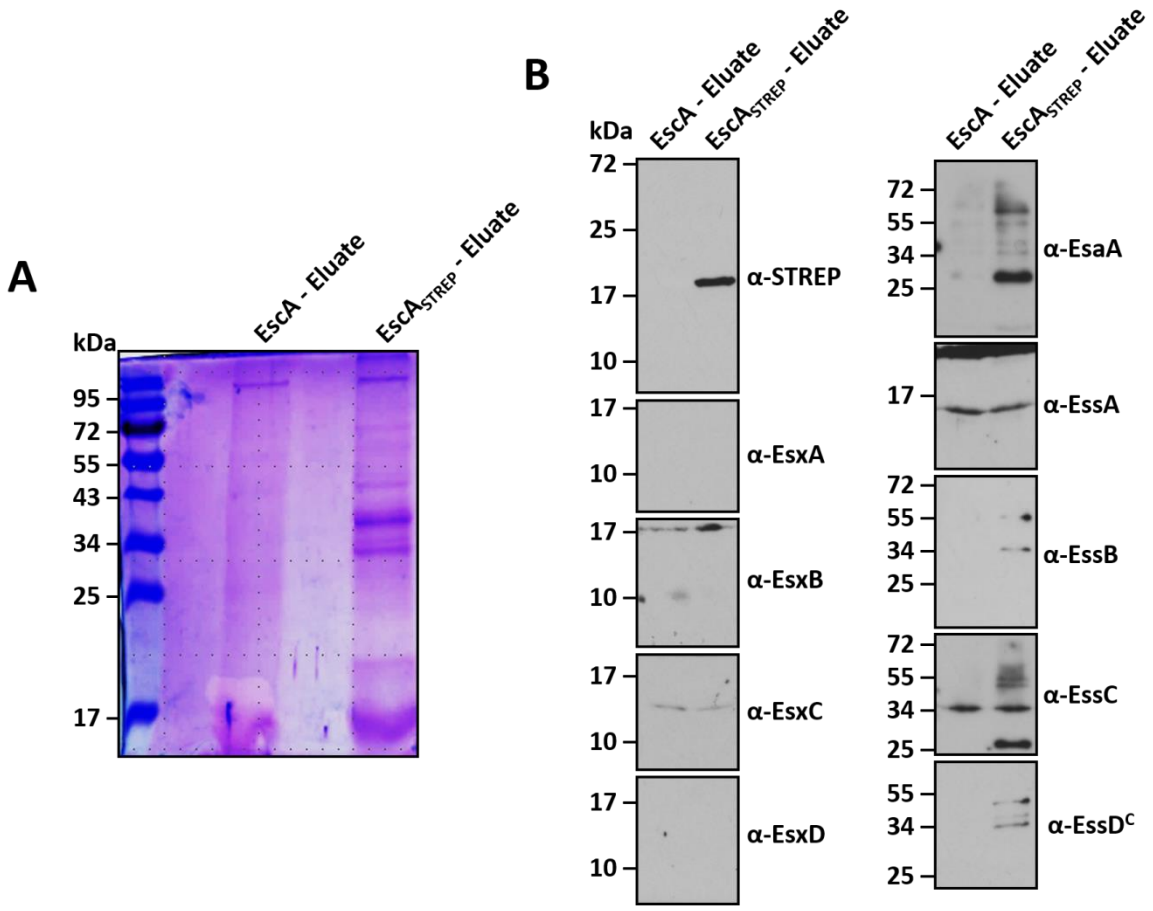

Figure 18. Identification of an ESS-EscA$_{STREP}$ complex. (A, B) *S. aureus* cultures of strain USA300 with EscA- and EscA$_{STREP}$-encoding plasmids were grown to an A_{600} of 2.0. Cells were sedimented by centrifugation and lysed with lysostaphin and two French Press passages, the whole cell lysates were subjected to 2% DDM solubilization. Detergent-solubilized whole cell lysates were flowed by gravity over Strep-Tactin-Sepharose beads, washed extensively with PBS, and eluted with 5 mM desthiobiotin. Eluates were separated by SDS-PAGE and Coomassie stained (A) or electrotransferred to PVDF membranes for immunoblotting with immune sera specific for STREP, EsxA, EsxB, EsxC, EsxD, EsaA, EssA, EssB, EssC, and EssD^{C} (B).

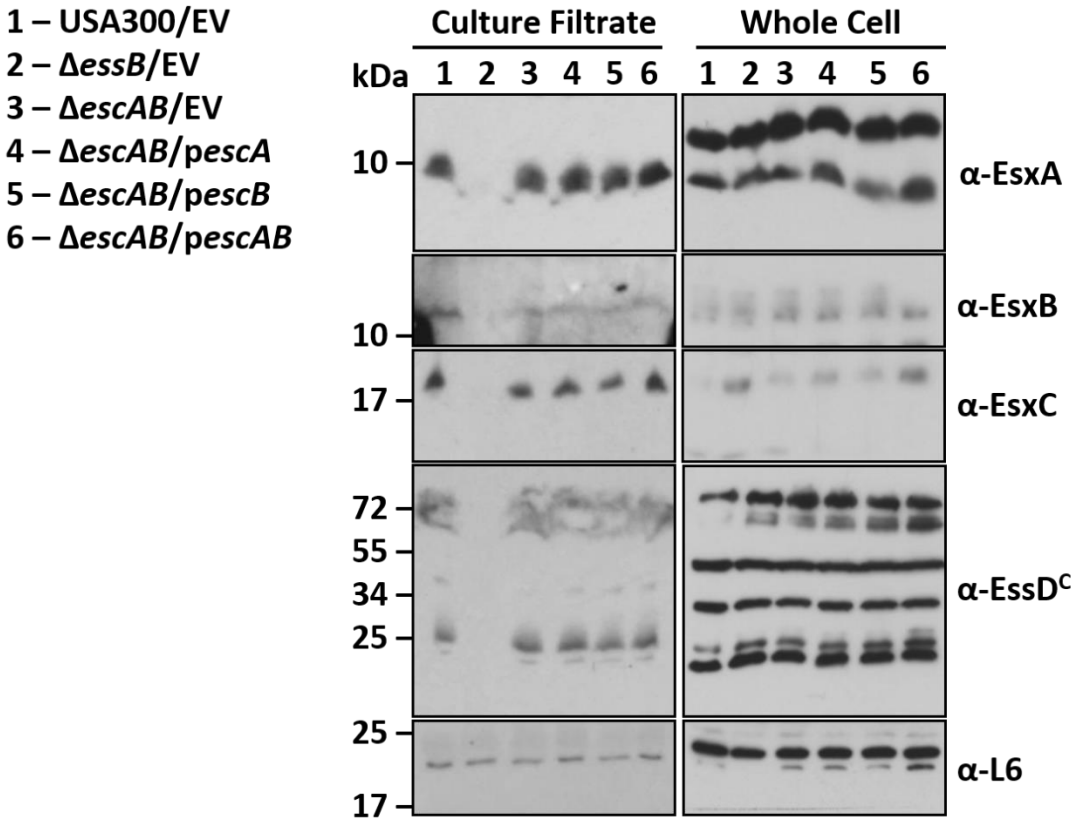

Figure 19. Secretion analysis of Δ*escAB* under ESS-inducing conditions. *S. aureus* strain USA300 harboring an empty vector (EV), the isogenic Δ*essB* mutant harboring an EV, and the isogenic Δ*escAB* mutant harboring an EV or p*escA*, p*escB*, or p*escAB* encoding EscA, EscB, or both EscA and EscB, respectively, grown at 37°C in TSB supplemented with serum at pH 5.5. Cultures were spun to separate the medium from intact cells. The spent culture medium was subjected to 0.22 µm filtration (Culture Filtrate), and the pelleted cells were subsequently lysed with lysostaphin to release all cellular content (Whole Cell). Proteins were TCA precipitated, suspended in sample buffer prior to separation by SDS-PAGE, and transferred to PVDF membranes for immunoblot analyses with anti-EsxA, anti-EsxB, anti-EsxC, anti-EssD^{C}, or anti-L6 polyclonal sera. Numbers to the left indicate the mobility of molecular mass markers.

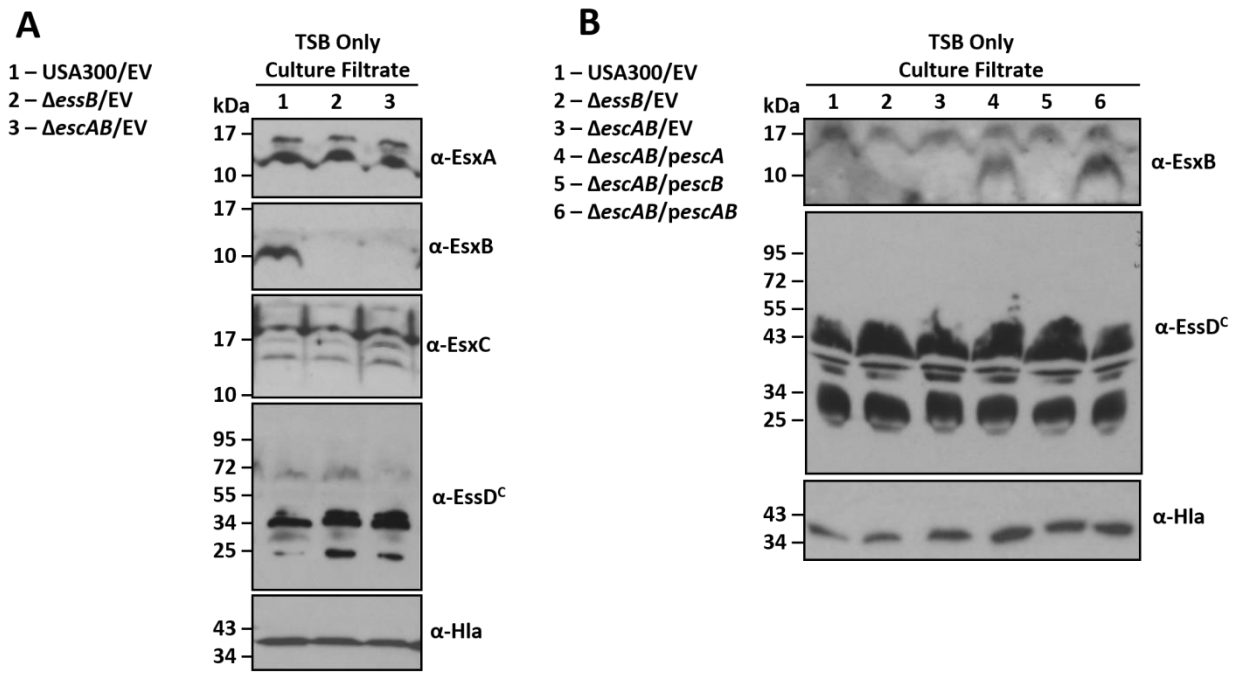

Figure 20. *escA* is required for EsxB accumulation in culture filtrates. (A) *S. aureus* strain USA300, the isogenic Δ*essB* mutant, and the isogenic Δ*escAB* mutant, all harboring an empty vector (EV), were grown at 37°C in TSB without supplementation. (B) *S. aureus* strain USA300 harboring an empty vector (EV), the isogenic Δ*essB* mutant harboring an EV, and the isogenic Δ*escAB* mutant harboring an EV or p*escA*, p*escB*, or p*escAB* encoding EscA, EscB, or both EscA and EscB, respectively, were grown at 37°C in TSB without supplementation. Cultures were spun to separate the medium from intact cells. The spent culture medium was passaged through a 0.22 µm filter (Culture Filtrate), and subjected to TCA precipitation, suspended in sample buffer prior to separation by SDS-PAGE, and transferred to PVDF membranes for immunoblot analyses. (A) Anti-EsxA, anti-EsxB, anti-EsxC, anti-EssD^C, or anti-Hla polyclonal sera was used. (B) Anti-EsxB, anti-EssD^C, or anti-Hla polyclonal sera was used. Numbers to the left indicate the mobility of molecular mass markers.