Università degli Studi del Piemonte Orientale

"Amedeo Avogadro"

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Dottorato di Ricerca in Biotecnologie Farmaceutiche ed Alimentari

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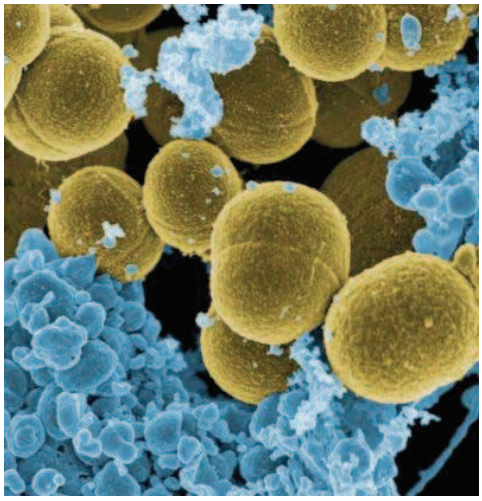

Giuliana Balsamo

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Functional characterization of the ESAT-6 secretion system of *Staphlococcus aureus*

Candidate

Giuliana Balsamo

Supervisors

Meera Unnikrishnan, PhD
Novartis Vaccines and Diagnostics, Siena

Prof. Menico Rizzi
University of Piemonte Orientale, Novara

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A Stefano e Leonardo

Contents

Introduction

1. *Staphylococcus aureus*

In an elegant series of clinical observations and laboratory studies published in 1880 and 1882, Ogston described staphylococcal disease and its role in sepsis and abscess formation. *Staphylococcus aureus* (*S. aureus*) is a Gram positive coccus and a member of the Micrococcaceae family. The name "*aureus*" is derived from characteristic gold pigmentation. On microscopical examination, the organisms appear as Gram-positive cocci in clusters [1] . The staphylococcal genome consists of a circular chromosome (of approximately 2800 bp) with prophages, plasmids, and transposons. Genes governing virulence and resistance to antibiotics are found on the chromosome, as well as in the extrachromosomal elements [2]. These genes, on the extrachromosomal elements, are usually transferred between staphylococcal strains, species, or other Gram-positive bacteria [3].

2. Antibiotic resistance in Staphylococcus aureus

Meticillin-resistant *S. aureus* (MRSA) was discovered in 1960, within a year of the introduction of semi-synthetic anti-staphylococcal penicillins. Over the following 40 years MRSA was a problem confined largely to hospitalized patients and to occasional outpatients who had readily identifiable predisposing risk factors, such as recent hospitalization, presence of an invasive device, history of surgery, haemodialysis or residence in a nursing home [4, 5].

Invasive MRSA infections result in more deaths annually (~18,500) than any other single infectious agent in the United States, exceeding the number of deaths associated with HIV/AIDS, viral hepatitis and influenza combined [6]. MRSA infections are endemic in hospitals worldwide; in addition, community-associated MRSA (CA-MRSA) can cause infections in otherwise healthy individuals [7] and is responsible for a significant percentage of *S. aureus* skin and soft tissue infections in the United States (>50%), Asian countries (~17%) and Europe (ranging from <1% to 32%, depending on the country) [8, 9]. Unlike HA-MRSA, CA-MRSA is remarkably fit and able to spread within communities; it is virulent

and often susceptible to multiple narrow-spectrum antimicrobial agents. For example USA300 strain has emerged as the most prevalent CA-MRSA isolate in the United States and is commonly associated with skin and soft tissue infections [7].

Resistance to methicillin, and in consequence to β-lactam antibiotics, is mediated by the *mecA* gene, which codes for the penicillin-binding protein 2A. The *mecA* gene is located on a genetic island called the staphylococcal cassette chromosome mec (SCCmec), differences in which are used to categorise MRSA. HA-MRSA strains carry SCCmec types I–III, whereas CA-MRSA strains carry SCCmec IV and the more recently isolated SCCmec [10]. Vancomycin resistance was first reported for *Enterococcus faecium*, and transfer of vancomycin resistance from enterococci to *S. aureus* has been shown to occur[11]. In 1996 a vancomycin reduced sensitivity strain of *S. aureus* was isolated. The first highly-vancomycin-resistant strain was isolated in 2002. This strain was shown to carry a plasmid harboring, among other resistance genes, the *vanA* gene. The proteins encoded by these genes are responsible for replacing the C-terminal D-alanyl–D-alanine (D-Ala–D-Ala) of the disaccharide pentapeptide cell wall precursor with a depsi-peptide, D-alanyl–D-lactate (D-Ala–D-Lac), thereby lowering the cell wall affinity for vancomycin [12].

3. Virulence factors of *Staphylococcus aureus*

Pathogenicity of *S. aureus* is caused by the expression of an arsenal of virulence factors which can lead to superficial skin lesions, such as styes, furunculosis, and paronychia, or to more serious infections, such as pneumonia, mastitis, urinary tract infections, osteomyelitis, endocarditis, and even sepsis. In rare cases *S. aureus* causes meningitis [13] (Fig. 1).

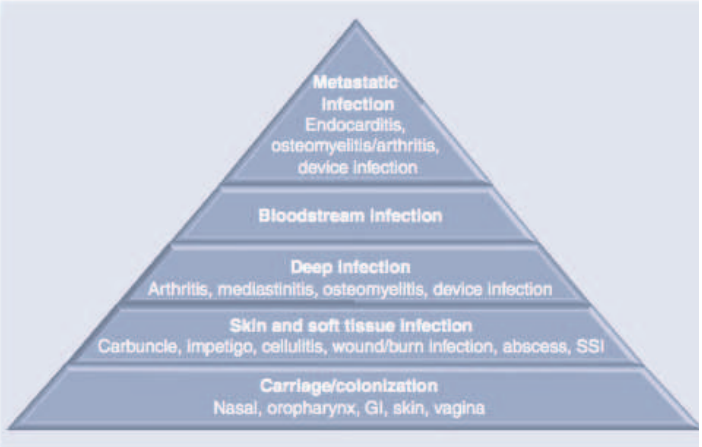


Fig. 1 The broad spectrum of *S. aureus* disease. Starting from benign colonization, throught increasingly more viulent and medically burdensome presentations. The degree of severity of these disease decreases from top to bottom of the triangle whereas the size of the affected population increases from top to bottom.

Virulence factors that *S. aureus* employs to cause these diseases are displayed at the surface of the staphylococcal cell or secreted into host milieu [14]. Specifically, these virulence factors include: surface proteins that promote adhesion to and colonization of host tissues, invasins that are exported to an extracytoplasmic location and promote bacterial spread in tissues (leukocidin, kinases, and hyaluronidase), surface factors that inhibit phagocytic engulfment (capsule and protein A), biochemical properties that enhance staphylococcal survival in phagocytes (carotenoid and catalase production), immunological disguises (protein A, coagulase, and clotting factor), membrane damaging toxins that disrupt eukaryotic cell membranes (hemolysins and leukotoxin), super-antigens that contribute to the symptoms of septic shock (SEA-G, toxic shock syndrome toxin [TSST], and ET) and determinants for inherent and acquired resistance to antimicrobial agents [15]. Staphylococci produce various enzymes, such as proteases, lipases, and hyaluronidases that destroy tissues. These bacterial products may facilitate the spread of infection to adjoining tissues, although their role in the pathogenesis of disease is not well defined.

Some of the key virulence associated factors are discussed in more details below.

Capsule: More than 90% of *S. aureus* strains can produce *in vitro* capsular polysaccharide (CP), which can be divided into 11 serologically distinct capsular types, CP1 – CP11 [16]. The majority of isolates from different sources, are CP5- or CP8-positive. Expression of CP has been shown to enhance the bacterial ability to evade opsonophagocytosis, whereas CP-specific antibodies mediate type-specific opsonophagocytosis and killing by polymorphonuclear cells. *S. aureus* capsules also promote abscess formation, animal studies suggest that the capsule promotes bacterial colonization and persistence on mucosal surfaces. Loss of capsule expression, however, may lead to *S. aureus* persistence in a chronically infected host. Experimental studies conducted with a mouse model of staphylococcal mastitis demonstrated that lack of capsular polysaccharide expression results in an increased capacity for persistence in the host. This capacity was attributed to more effective interaction between unmasked staphylococcal surface ligands and cell receptors, leading to internalization into epithelial cells [17]. CPs have been shown to be protective antigens in animal models, leading to their investigation as potential vaccine targets [18, 19].

Protein A: Staphylococcal protein A (SpA) is a key virulence factor that enables *S. aureus* to evade innate and adaptive immune responses [20]. SpA is secreted as a precursor with an N-terminal signal peptide and a C-terminal LPXTG sorting signal [21]. SpA binds to the Fcγ portion of human and animal immunoglobulins, a defense mechanism that provides *S. aureus* with protection from opsonophagocytic killing. Previous works suggested that the mutated form of the protein SpAKKAA may be useful as a vaccine to prevent *S. aureus* disease in humans. SpAKKAA MAbs promoted opsonophagocytic killing of MRSA in mouse and human blood, provided protection from abscess formation, and stimulated pathogen-specific immune responses in the mouse model [22].

Toxins: Staphylococci produce numerous toxins that are grouped on the basis of their action's mechanisms. Lysis of red blood cells is primarily mediated by the

hemolysins known as alpha (α), beta (β) and delta (δ) toxins. The α toxin encoded by the *hla* gene is important for *S. aureus* pneumonia, sepsis, septic arthritis, brain abscess and corneal infections[23, 24]. This pore forming toxin (33-kDa) is secreted by majority of *S. aureus* clinical isolates and is active against a wide range of mammalian cells. In addition to its pore forming ability, α toxin induces the release of cytokines and chemokines such as IL-6, IL-1β, IL-1α, IL-8, TNF-a, KC and MIP-2 [25-27]. Immunization with inactive α toxin was recently shown to protect mice against lethal *S. aureus* pneumonia [28]. Certain strains of *S. aureus* also secrete beta (β) toxin, a 35-kDa sphingomyelinase encoded by the *hlb* gene. In contrast to α toxin, β toxin is highly hemolytic for sheeps but not for rabbits erythrocytes. Delta (δ) hemolysin or toxin is a 26 amino acid peptide encoded by the *hld* gene [29]. This toxin is produced by 97% of *S. aureus* isolates and lyses erythrocytes, a variety of mammalian cells and sub-cellular structures such as membrane bound organelles, spheroplasts and protoplasts. In contrast to the α and β toxins, δ toxin does not possess a cleavable signal sequence, and its secretion mechanism is not completely understood.

Phenol-soluble modulins (PSMs): PSMs are a family of amphipathic, a-helical peptides that comprise four shorter and two longer PSM-like peptides, whose genes are arranged in two gene clusters. Although the PSM genes are present in all sequenced *S. aureus* strains, much higher *in vitro* PSM production was detected in the CA-MRSA compared to HA-MRSA, raising the possibility that PSMs contribute to the enhanced virulence of CA-MRSA [30]. Within the past 5 years it has been demonstrated that PSMs have a variety of biological functions that are crucial to staphylococcal pathogenesis. Consequently, deletion mutants of the *psma* operon in *S. aureus* are severely attenuated in animal infection models, indicating a central role of PSM peptides in staphylococcal virulence [31]. Some studies revealed that all *S. aureus* PSMs are involved in different biological functions.

They efficiently lyse red and white blood cells, control biofilm development [32, 33] and trigger receptor-mediated inflammatory responses [34].

4. Secretion systems in *Staphylococcus aureus*

Staphylococcal proteins are exported thougth different secretion system that have been characterized experimentally or that can be deduced from sequenced genomes. Since these pathways are likely used for the export of virulence factors to the cell surface and the milieu of the host, can be regarded as a subcellular road map to staphylococcal pathogenesis. The secretion of proteins across biological membranes is in most cases mediated by translocation machinery recognising a specific sequence motif in the protein to be secreted [35].

The most commonly used pathway for bacterial proteins transport is the general secretory "Sec" pathway. Specifically, this pathway is responsible for the secretion of the majority of the proteins found in *Bacillus subtilis*, and other Gram-positive bacteria, including *S. aureus*. Proteins that are exported via the Sec pathway contain signal peptides with recognition sites for so called type I or type II SPases. Furthermore, the Sec-dependent export of proteins can be divided into the following three stages: I) targeting to the membrane translocation machinery by export-specific or general chaperones, II) translocation across the membrane by the Sec machinery, and III) posttranslocational folding and modification [15].

Another well characterized secretion system is the twin-arginine translocation "Tat" pathway that exists in many bacteria, archaea, and chloroplasts. This pathway was named after the consensus double (twin) Arg residues that are present in the signal peptides[36] . In contrast to the Sec machinery, where only unfolded proteins are translocated across the membrane, the Tat machinery is capable of translocating folded proteins. In Gram-negative bacteria, an active Tat pathway seems to require three core components, named TatA, TatB, and TatC [37]. In all Gram-positive bacteria except streptomycetes and *Mycobacterium smegmatis*, the Tat pathway involves only TatA and TatC [38].

Type VII secretion system. Recent evidence shows that *S. aureus* has a novel and specialized secretion systems for the transport of extracellular proteins across their hydrophobic and highly impermeable cell wall [39]. The identification of this specialized secretion system began with the isolation of the tuberculosis vaccine strain *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) at the Pasteur Institute (Lille, France) in 1921 [40]. This novel secretion secretion mechanism Type-VII secretion is responsible for the secretion of two highly immunogenic proteins: ESAT-6 (early secretory antigenic target) of 6 kDa and CFP-10 (culture filtrate protein) of 10 kDa protein. They are missing from the closely related attenuated live vaccine (BCG) due to the deletion of region of difference 1 (RD1) in which lie the two genes [41]. Strikingly, mycobacterial genomes encode five of these transport systems. Two of these systems ESX-1 and ESX-5, are involved in virulence: they both affect the cell-to-cell migration of pathogenic mycobacteria. ESX-3, a paralogous system present in all mycobacterial species, is required for growth *in vitro*. Was shown that mycobacteria lacking ESX-3 are defective in acquiring iron [42].

In *Mycobacterium tuberculosis* (*M. tuberculosis*), ESAT-6 (EsxA) and CFP-10 (EsxB) belong to the WXG100 family of 23 small secreted proteins that share a size of approximately 100 amino acids, a helical structure, and a characteristic hairpin bend formed by the conserved Trp-Xaa-Gly (W-X-G) motif. The genes encoding these proteins, are called *esx* genes in *M. tuberculosis* (*esxA-W*) and are arranged in tandem pairs at 11 genomic loci. In five of these genomic loci (ESX-1–ESX-5), the esx genes are flanked by genes coding for components of secretion machineries involved in the export of the corresponding Esx proteins [43].

ESX-1 components have been implicated in the ability of the bacterium to trigger macrophage production of IFN-β [44], activate the inflammasome, modulate macrophage cytokine production and signaling, and escape from the phagolysosome [45, 46]. The ESX-1 substrate proteins are also important targets of

the adaptive immune response and are recognized by both $CD4^+$ and $CD8^+$ T cells in a majority of infected individuals [47].

In addition to ESAT-6 and CFP-10, four other substrates of the ESX-1 locus have been reported in *M. tuberculosis*. This system includes a multi-transmembrane protein, Rv3877 (Snm4), and two putative SpoIIIE/FtsK adenosine triphosphatase (ATPase) family members, Rv3870 (Snm 1) and Rv3871 (Snm2). ESX-1 secretes also a transcriptional regulator, EspR (Rv3849) and two proteins of unknown function, EspA (Rv3616c) and EspB (Rv3881). EsxA, EsxB, EspA and EspB require each other for secretion[48].

The two proteins CFP-10 and ESAT-6 form a 1:1 complex. A striking feature of the complex is the long flexible arm formed by the C-terminus of CFP-10, which was found to be essential for binding to the surface of cells. The unstructured C-terminus of the CFP-10 substrate was recognized by Rv3871, a cytosolic component of the ESX-1 system that itself interacts with the membrane protein Rv3870. Point mutations in the signal that abolished binding of CFP-10 to Rv3871 prevented secretion of the CFP-10/ESAT-6 virulence factor complex.[49].

Pallen *et al*. discovered ESAT-6 homologues in the sequenced genomes of other Gram-positive bacteria including *Bacillus subtilis, Bacillus anthracis, Clostridium acetobutylicum, Listeria monocytogenes*, and *S. aureus*[43]. The genes for the ESAT-6-like proteins are clustered with at least one gene encoding an FSD-type membrane protein, suggesting that FSD ATPases may represent a universal portal for excretion of Esx-like proteins (Fig. 2)[50].

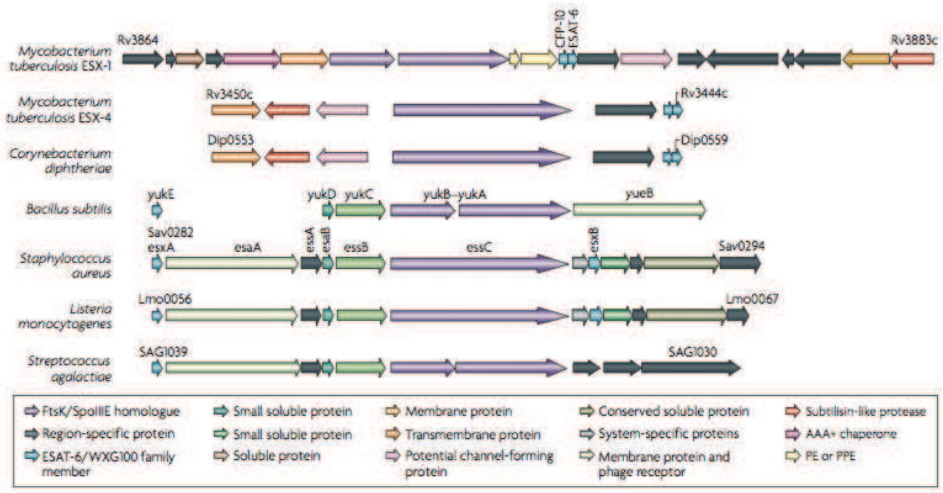


Fig. 2 Comparison of different gene clusters that encode Type-VII secretion system

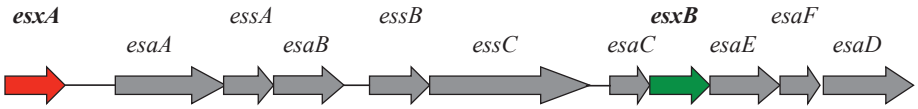
S. aureus ESAT-6 secretion system. Protein sequence alignment of *S. aureus* EsxA and EsxB with *M. tuberculosis* Esx proteins show that *M. tuberculosis* and *S. aureus* EsxA display 20.8% identity and 25% similarity, whereas *S. aureus* EsxB and *M. tuberculosis* EsxA are 17.8% identical and 35% similar. The peptide sequences of *S. aureus* EsxA and EsxB encompass the WXG motif, a signature sequence of ESAT-6-like proteins [51] (Fig. 3A). It appears that both EsxA and EsxB carry the C-terminus translocation signal, which means that each protein is transported and acts independently as a virulence factor with no requirement for heterodimerization. It may be significant that the C-terminus of EsxA adopts a helical conformation, a structural feature that may be important in the molecular recognition of EsxA by the transport machinery.

Unlike *M. tuberculosis, S. aureus* contains only a single Type-VII secretion sytem (Ess) gene cluster and EsxA and EsxB are encoded within a cluster comprised of eight predicted ORFs (*esxA, esaA, essA, esaB, essB, essC, esxY,* and *esxB*) as shown in Fig. 3B.

Missiakas *et al*., attributed a biological role to Esx proteins during staphylococcal infection and abscess formation. Mutants that failed to secrete EsxA and EsxB displayed defects in the pathogenesis of *S. aureus* murine abscesses, suggesting that this specialized secretion system may be a general strategy of human bacterial pathogenesis. Also they have shown that mutations in *essA*, *essB*, or *essC*, that are predicted encode for transmembrane proteins, abolished synthesis and therefore secretion of EsxA and EsxB without affecting transcription of *esxA* and *esxB*. Without secretion, EsxA and EsxB may be rapidly degraded in the bacterial cytoplasm or posttranscriptional feedback inhibition may reduce *esxA* and *esxB* expression [39]. Contrary to ESAT-6 and CFP-10 genes that are co-trascribed, altought *esxA* and *esxB* are located in the same locus, they are separated by 6 other genes and they are not co-trascribed. The *esxA* gene is under complex control. EsxA has been reported to be regulated by sigma factor B and σB-controlled SpoVG, and is a monocistronic transcript that is driven by a σA promoter. It was demonstrated that the transcription of *esxA* is controlled by a regulatory cascade involving downstream σB-dependent regulatory elements, including the staphylococcal accessory regulator SarA, the ArlRS two-component system and SpoVG. The regulation of EsxB is currently unknown [52].

A

B



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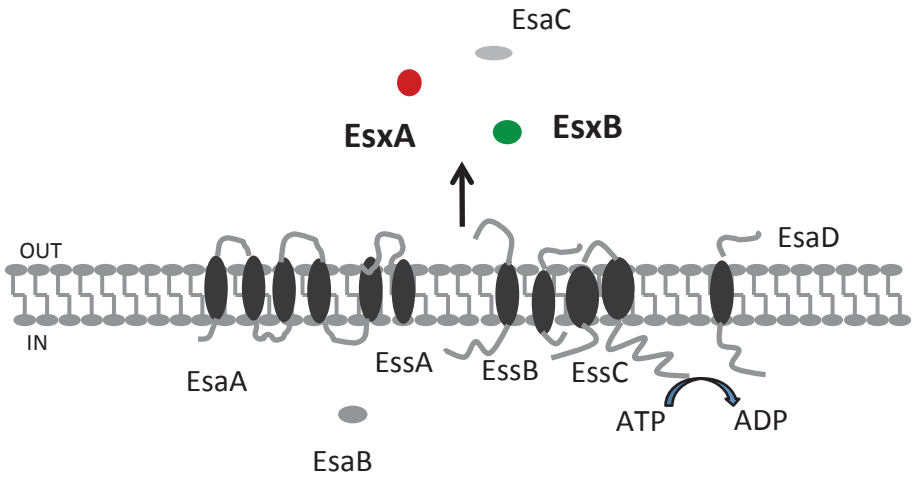
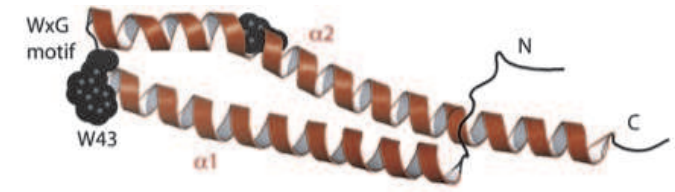


Fig. 3 A Sequence alignment of *S. aureus* EsxA and EsxB with ESAT-6 and CFP-10 of *M. tuberculosis*. *S. aureus* EsxA shares 12% sequence identity with ESAT-6, 14% with CFP-10, and 13% with *S. aureus* EsxB. EsxB shares 8% sequence identity with ESAT-6 and 13% with CFP-10. **B** *esx* locus in *S. aureus* **C** Membrane topology of Ess secretion system in *S. aureus*

The crystal structures of EsxA was determined. The asymmetric unit of each crystal form is a dimer. The EsxA subunit forms an elongated cylindrical structure created from side-by-side α-helices linked with a hairpin bend formed by the WXG motif (Fig 4). Structural and sequence comparisons, exploiting biological data on

related proteins found in *M. tuberculosis*, suggest that this family of proteins may contribute to pathogenesis by transporting protein cargo through the secretion system exploiting a C-terminal signal that facilitate interactions with host receptor proteins[51].

A



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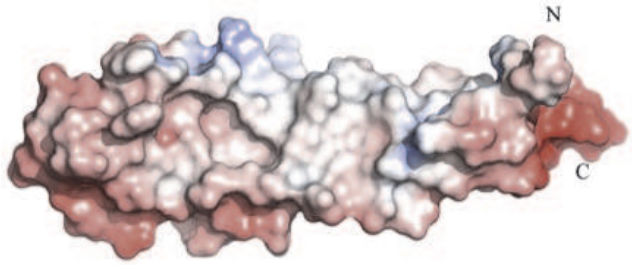

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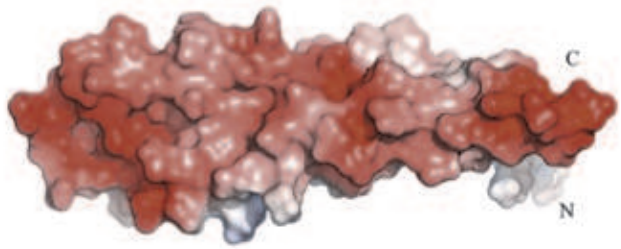


Fig 4. Structure of EsxA
A A subunit of EsxA protein. The position of the conserved WXG motif is marked and Trp43 is depicted as black Van der Waals spheres. also the N- and C-terminal region are marked. **B** The electrostatic surface of EsxA protein (blu, positive; red, negative; white, neutral) show the hydrophobic side of the protein. **C** Similar representation rotated 180°C show the hydrophilic side of the protein.

Secretion of EsxA and EsxB into culture medium during growth *in vitro* was demonstrated previously, with mutation of EsxB affecting secretion of EsxA and *vice versa* [39]. Very recent work further reports that deletions in either gene also

affects secretion of additional Ess substrates, EsxC and EsxD. EsxA and EsxB were reported to interact with different Ess substrates such as EsxC and EsxD respectively [53].

5. Staphylococcal Biofilms

Staphylococci are recognized as the most frequent causes of biofilm-associated infections [54]. This exceptional status among biofilm-associated pathogens is due to the fact that staphylococci are frequent commensal bacteria on the human skin and mucous surfaces. *S. epidermidis* and *S. aureus* are the most frequent causes of nosocomial infections and infections on indwelling medical devices, which characteristically involve biofilms[55]. Often, *S. aureus* biofilm-associated infections are difficult to treat with antibiotics and devices need to be replaced [56]. In addition, they represent a reservoir for dissemination of infection to other sites in the human body. In contrast to many other medical biofilms, such as multi-species dental plaque formation, biofilm-associated infections with staphylococci are usually not mixed with other species . Research performed in many biofilm-forming organisms has revealed that the development of a biofilm is a multi step process involving an initial attachment and a subsequent maturation phase and a final dispersal phase that involves the detachment of the bacterial cells, believed to be crucial for the dissemination of the bacteria [54] (Fig 5).

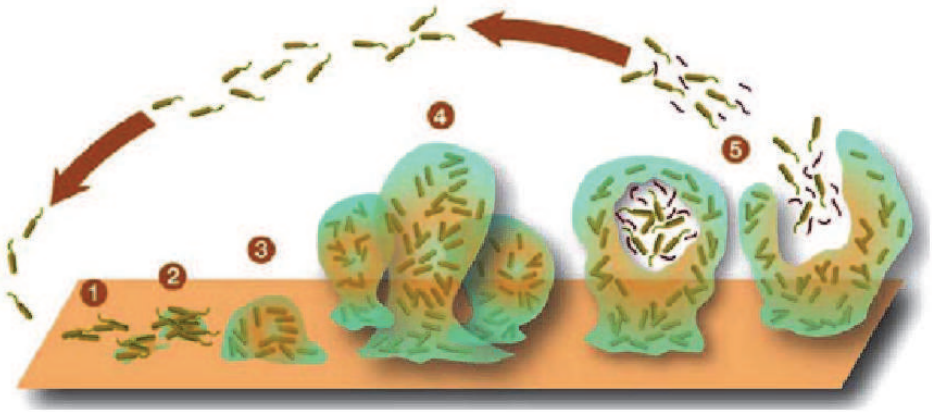


Fig. 5 Schematic representation of development of a biofilm as a five-stage preocess. Stage1: Initial attachment of cells to a surface. Stage2: Production of

EPS resulting in more firmly adhered "irreversible"attachement. Stage3: Early development of biofilm architecture. Stage4: Maturation of biofilm architecture. Stage5: Dispersion of single cells from the biofilm.

In the human body, the attachment to human matrix proteins represents the first step of biofilm formation. *S. aureus* express dozens of so-called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) that have the capacity to bind to human matrix proteins such as fibrinogen or fibronectin, and often combine binding capacity for several different matrix proteins. The subsequently phase is the maturation of biofilm formation is characterized by 1) intercellular aggregation that can be accomplished by a variety of molecules such as adhesive proteins or – usually polysaccharide-based - exopolymers, and 2) biofilm structuring forces that lead to the typical 3-dimensional appearance of mature biofilms with a fluid-filled channels. Biofilm detachment is crucial for the dissemination of bacteria to other colonization sites. It may occur by the detachment of single cells or larger cell clusters. Several factors may contribute to detachment: 1) mechanical forces, 2) cessation of the production of biofilm building material and 3) detachment factors sensu strictu [57]. It has long been recognized that biofilms have dramatically increased resistance to antibiotics. Two main mechanisms contribute to biofilm resistance: 1) prevention of the antibacterial substance from reaching its target and 2) the specific physiology of a biofilm, which limits the efficacy of antibiotics, mainly of those that target active cell processes and which may also include specific subpopulations of resistant cells. Recent studies of staphylococcal biofilm development have demonstrated that there are some key structural and regulatory factors that determine the form and physiology of staphylococcal biofilms.

6. Intracellular life of *Staphylococcus aureus*

In addition to its armor of virulence factors, the capacity of *S. aureus* to successfully evade host defenses has been recently attributed to its ability to invade immune and non-immune cells. *S. aureus* is mainly an extracellular pathogen, but

an accumulating number of studies have shown that it can invade and replicate in many types of host cells *in vitro* [58].

This property potentially contributes to bacterial persistence and several benefits for the pathogen. It has been proposed that intracellular *S. aureus* evades exposure to antibiotics and host immunity [59]. Clinical studies have reported the presence of intracellular staphylococci from nasal epithelial cells, indicating that this could serve as a reservoir for recurrent infections[60]. The intracellular enviroment leads to the formation of small-colony variants (SCVs) characterized by slow growth and a range of morphological and metabolic changes including altered antibiotic resistance profiles [61, 62].

The intracellular process of *S. aureus* is mediated by the interaction between fibronectin binding protein and host-cell α5β1integrin [63]. Other bacterial surface proteins like clumping-factor A or host cell Src kinase also appear important in the mediation of *S. aureus* uptake and intracellular persistence [64]. After internalization, the behavior of the bacterium varies according to cell-line or bacterial strain. In order to subvert host cell functions to their benefit, bacterial pathogens have developed various strategies. In particular, intracellular bacteria have adapted mechanisms to modulate the apoptotic pathway. The resulting induction or inhibition of apoptosis is often crucial for a successful infection of the host. Several bacteria elicit an inflammatory process which, in the place of infection, leads to the disruption of tissue barriers and thus may secure efficient microbial spread in the host [65].

Some studies have reported an active intracellular bacterial replication within vacuoles. Others have described both a rapid bacterial escape from vacuole with a consequently induction of cellular apoptosis and persistence for several days before induction of escape processes[66].

In the case of *S. aureus* the affected apoptotic pathways appear to depend on the strain and host cell type. Several studies reported that after infection the Cell Death Program occurs trought caspases activation [67-69]. During *S. aureus* infection the

production of α-toxin appears correlated with the induction of apoptosis[70]. In contrast, inhibition of apoptosis may be essential for intracellular pathogens to establish chronic infection. Pathogen-triggered anti-apoptosis of infected host cells facilitates a slow microbial replication process and enables persistence in the infected host. *S. aureus* was recently reported to induce anti-apoptotic factors in epithelial cells. It seems, like for many other bacteria, *S. aureus* may also be able to block apoptosis [71].

7. New therapeutic and vaccine strategies for *Staphylococcus aureus* treatment

Mortality, morbidity, and cost from invasive *S. aureus* infections remain disturbingly high despite the introduction of several new antibiotics to treat methicillin-resistant *S. aureus* infections [72]. *S. aureus* infections are now the most common cause of hospitalization. To address these problems, investigators at universities, biotechnology companies, and large pharmaceutical companies have tried to develop an effective vaccine. Despite much effort, no clinical trials have succeeded to date. What has made *S. aureus* vaccine so difficult? First, the protective immunity against *S. aureus* is not completely understood and animal models, especially murine models, have not predicted success in humans as they did for the other successful vaccines. Second, the situation for *S. aureus* is much more complex, as this bacterium has multiple virulence factors that neutralize the host immune responses than these other bacterial pathogens. Third, *S. aureus*–infected patients present with a very broad range of diseases, which means that vaccine development must focus on preventing a wide spectrum of disease presentations. The need for an effective *S. aureus* vaccine is increasing in view of broad antibiotic resistance and apparent increasing virulence of the community strains[73, 74].

Careful consideration of the patient populations in which candidate vaccines are initially evaluated for their efficacy will also play a key role for vaccine development. So far the vaccines tested in clinical trials targeted a single *S. aureus*

component. Studies using antigen combinations have shown greater efficacy than single antigen vaccines in animal models. Therefore, multivalent vaccines will likely work better in humans as well so that it is able to cover different pathologies that *S. aureus* causes. On this basis, Bagnoli *et al.* have proposed a model in which vaccine efficacy is gained through three major immune responses: 1) antibodies to directly inhibit bacterial viability and/or toxicity 2) antibodies mediated opsonophagocytosis 3) cell-mediated immunity to stimulate recruitment of phagocytes at the site of the infection. A combination of staphylococcal antigens with different properties and functions is able to elicit a potent antibody production, and a proper cellular response. In that way is possible to cover different pathologies caused by *S. aureus* infections [75].

Outline of thesis

Staphylococcus aureus is a versatile pathogenic bacterium capable of rapidly developing or acquiring multiple antibiotic resistances, and is now recognized as a global major health-care associated problem. *S. aureus* is responsible for a wide spectrum of human diseases, ranging from skin infections to severe diseases, such as rhinosinusitis, otitis, arthritis, osteomyelitis, endocarditis or fatal sepsis.

These infections are difficult to eradicate and often relapse even after prolonged and adapted antibiotic therapy, suggesting that *S. aureus* has developed specific strategies for persistence such as the ability to live within biofilms or inside cells, and thereby evading antibiotics and immune responses. Due to the increasing antibiotic resistance, a new strategy for prevention the disease has become an important medical need.

During my PhD I have focused my studies on the functional characterization of two secreted proteins, EsxA and EsxB secreted by the specialyzed ESAT-6 like secretion system. These proteins are important for virulence in murine infection during kidney abscess formation and they are considered as potential vaccine candidates.

My research encompasses many areas including: 1) the genetic manipulation of *S. aureus* to understand the function of these proteins, 2) gene expression studies to explore environmental conditions under which the Esx proteins are induced, 3) the mechanisms invesigation of Ess-mediated secretion of EsxA and EsxB and finally 4) the functional characterization of Esx proteins during intracellular staphylococcal infection in *in vitro* cellular models.

Data from these studies will better characterize promising vaccine candidates and support development of an effective vaccine against *S. aureus* infection.

Generation and functional characterization of *esxA* and *esxB* deletion mutants of *Staphylococcus auresus*

Giuliana Balsamo, Fabio Bagnoli, Davide Serruto and Meera Unnikrishnan

Unpublished Results

Novartis Vaccines and Diagnostics, Siena, Italy

Introduction

Staphylococcus aureus (*S. aureus*) a Gram-positive coccus, is a major human pathogen causes a wide spectrum of nosocomial and community-acquired infections that are associated with high morbidity and mortality [1]. It causes infections ranging from superficial skin lesions to serious conditions like pneumonia and endocarditis. Systemic and chronic infections, in particular, place a major burden on healthcare systems worldwide [2, 3].

Treatment is made more difficult by the increasing number of MRSA, VRSA strains that are resistant to multiple antibiotics. One of the factors that contribute to staphylococcal resistance includes biofilm formation. Formation of biofilms or complex bacterial communities occurs in a multistep process and has been demonstrated to be important in staphylococcal virulence. Numerous reports in the past two decades have shown that especially biofilm-forming staphylococci cause a severe infections [4]. Biofilm formation by pathogenic staphylococci on implanted medical devices leads to “chronic polymer-associated infections”. Bacterial cells in a biofilm have been reported to alter metabolic activity [5, 6].

S. aureus pathogenicity is multifactorial and depends on the expression of a large class of gene products comprising cell-wall associated and extracellular proteins. These secreted virulence factors including enzymes and toxins are commonly exported by the Sec secretion system, a well-described secretion pathway [7]. An Ess secretion system similar to the Type-VII secretion system of *M. tuberculosis* was reported in *S. aureus* [8]. The ESAT-6 secretion system (Ess) consists of 12 proteins, including EsxA and EsxB that are similar to the ESAT-6 and CFP-10 of *M. tuberculosis*. ESAT-6 (EsxA) and CFP-10 (EsxB) are well-characterized virulence factors of *M. tuberculosis* [9].

The Esx substrates are typically small proteins (100 amino acids) with a conserved WXG motif. These WXG100 proteins are a class of effector molecules found in other Gram-positive bacteria. Bioinformatic analyses have shown that one WXG gene is frequently positioned near, or directly adjacent to a second related WXG

gene [10]. The identification of WXG proteins encoded by the pathogens *M. tuberculosis* and *S. aureus* has created significant interest in the proteins biological activity.

EsxA and EsxB factors, secreted by the system, are well conserved across clinical *S. aureus* strains and are currently being considered as potential vaccine candidates. Although well conserved between strains, expression and secretion of Ess-encoded substrates are poorly studied, both *in vitro* growth and during human staphylococcal infection. While the mycobacterial Esx proteins are important in several host-associated functions such as cell survival and granuloma formation[11, 12], the biological functions of the *S. aureus* Esx proteins are however not known and the mechanism of action during the pathogenesis is still unclear. Missiakas *et al.* have showed that EsxA, EsxB and other Ess proteins are important during kidney abscess formation in a murine infection and that some of the Ess proteins are important for bacterial persistence in a staphylococcal abscess [8, 13].

In *M. tuberculosis* the Esx substrates ESAT-6 and CFP-10 were shown to interact each other and form a 1:1 complex [14]. A striking feature of the complex is the long flexible arm formed by the C-terminus of CFP-10, which was found to be essential for the secretion [15] and for binding to the surface of cells strongly suggest a key signalling role for the complex, in which the binding to cell surface receptors leads to modulation of host cell behaviour to the advantage of the pathogen [14].

The structure of the *S. aureus* secretion apparatus or the mechanisms of Esx protein secretion are still unclear. The interactions between the various Ess components have not yet been mapped completely.

Sundaramoorthy *et al.* reported that EsxA and EsxB purified from *E. coli* do not form a complex. They obtained crystals of EsxA and there are no current evidence that the two proteins could interact each other [16]. Recent study shown that EsxB associates with a novel substrate, EsxD, and EsxA dimerizes with itself or EsxC (EsaC). In particular EsxA and EsxC form both homo- and heterodimers whereas

EsxB and EsxD appear to function as a heterodimer [17]. EsxD carries the C-terminal motif YxxxD/E that has been proposed to target Type VII substrates for secretion in mycobacteria [18]. Was shown that the deletion, in this motif prevent secretion of EsxA and EsxC but not EsxB or EsxD [19]. The genetic inactivation of *esxA, esxB, esxC* or *esxD* leads to loss of secretion of all four substrates. Moreover the deletion of *esxD* abrogated the production of EsxB altogether and affected the secretion, but not the production, of EsxA and EsxC.

Identifying all the bacterial interactors of the Esx proteins would help to understand how the secretion apparatus function. Futhermore, identification host proteins that may interact with these proteins could indicate a role in host-pathogen interactions. In this study genetic and biochemical approaches were used to characterize the Esx proteins of *S. aureus* in order to better understand their mechanisms of action during staphylococcal infection.

Material and methods

1. Bacterial RNA extraction and qRT-PCR

Over night (O/N) cultures were diluted (1/100) in 5ml of TSB and grown until specific OD_{600}. Cells were sedimented by centrifugation, suspended in TSM (100 mM Tris·HCl, pH 7.0/500 mM sucrose/10 mM MgCl2) and digested with lysostaphin (5µg/ml) for 5 min. The resulting protoplasts were suspended with RLT buffer and RNA was isolated using the Qiagen RNeasy mini kits (Qiagen) using manufacturer's protocol. For intracellular bacterial RNA extraction, after infection of cells total RNA was extracted with TRIzol (1ml/well) (Life Technologies) and purified using Zymo Quick RNA purification Kit (The Epigenetics Company). Followed by on column DNase I treatment (Qiagen) and then DNase I treatment in solution (Roche). Reverse transcription was performed using Superscript II (Invitrogen). Real-time PCR was done using TaqPlatinum Syber Green polymerase SuperMix UDG (Invitrogen). A relative quantification was performed using 16S rRNA as internal control. Primers used for qRT-PCR are listed in the table 1.

Primers	**Sequence**
qRT*esxA* FW	AGGTGAAATTGCAGCGAACT
qRT*esxA* RW	CTTGTTCTTGAACGGCATCA
qRT*esxB* FW	AGCGGCAAAAACAGCTAAAG
qRT*esxB* RW	TATTGGCGAACTGTCCTTCC
qRT16S FW	TACGGCTTACCAAGGCAAC
qRT16S RW	CGGAAGATTCCCTACTGCTG

Table 1: Sequence of qRT-PCR primers specific for *esxA, esxB* and 16S.

2. Construction of bacterial mutants

For deletion of *esxA* and *esxB* a 2-kbp DNA fragments flanking the *esx* genes were amplified by PCR and cloned into the *Escherichia coli*/*S. aureus* shuttle/suicide vector pKOR1 [20] with abutted XhoI restriction sites and *att* site using primers listed in the table 2. Constructs containing flanking genomic regions of *esxA* and *esxB* were cloned first in *E. coli* then into RN4220 (restriction negative, methylation positive strain). Plasmid DNA extracted was used to transform *S. aureus* USA300 MRSA. Integration of plasmid into the chromosome was obtained at non permissive conditions for pKOR1 replication (incubation twice at 43°C in TSB) and the selection for homologous recombination and pKOR1 integration into the bacterial chromosome was induced by growing bacteria at permissive temperature (incubation twice 2 at 30°C in TSB). Anhydrotetracycline-mediated induction of pKOR1- encoded secY antisense RNA which inhibits growth was used for selecting for chromosomal excision and loss of plasmid at 30°C.

Primers	**Sequence**
esxA flanking region 1 FW	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAAAATTGATCCTCGTGTTG
esxA flanking region 1 RW	GAGAGAC TCGAGAACTAGAAACCTCCTGAAT
esxA flanking region 2 FW	GAGAGACTCGAGGC ATTCTGAAATTGGCAAAG
esxA flanking region 2 RW	GGGGACCACTTTGTACAA GAAAGCTGGGTGTATC TTGCATACTTGATTG
esxB flanking region 1 FW	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAGCAATGATTTCATCAGAA
esxB flanking region 1RW	GAGAGACTCGAGCATATCTTCACCTCAATATTAAT
esxB flanking region 2 FW	GAGAGACTCGAGTGAAAGATGTTAAGCGAAT
esxB flanking region 2RW	GAGAG AATGCTCGAGAACCCATGATGAAAGATGTTAAGCGAAT

Table 2. Sequence of primers for knoching out *esxA* and *esxB* gene.

For complementation of mutant strains, the full length *esxA* and *esxB* genes were amplified using specific primers for the *esx* genes and cloned into plasmid pOS1CK, which was generated by cloning the P1 constitutive promoter of the *sarA*

gene into the pOS1 plasmid. For complementing with both genes, *esxB* was cloned downstream of *esxA* in the pOS1CK*esxA* construct. For site direct mutagenesis of *esxA* the leucine, serine double mutations were introduced into the *esxA* gene cloned into a pET vector by PIPE [21]. To create the mutations in glycine95 we used primers carrying the mutated amino acid sequence. *esxA* truncated gene was amplified using primers listed in the table 3. All mutant forms were cloned into the episomal plasmid pOS1CK.

Primers	**Sequence**
esxALSGFW:	GCGCTGCAGTT GAGAGGAGAGAAAATGGCAATGATTAAGATG
esxALSGRW:	GCGCCCGGTTATTGCAAAC CGAAATTAT
esxAtruncFW	GCGCTGCAGCCGCTCGAGATGGCAATGATTAAG
esxAtruncRW	TCCCCCGGGTTGTTGGTCTTGTTC

Table 3: Sequence of primers for *esxA* mutation

3. Tandem Affinity Purification (TAP)-Tagging and Pull Downs

esxA/B gene was cloned in-frame with the two tags: FLAG protein and the Streptavidine Binding Peptide (SBP) at N- and C-terminus into episomal plasmid pAM401. *ΔesxA* or *ΔesxB*, were transformed with the plasmid. As negative control the same strains were transformed with empty plasmid. After growing the bacteria until late log phase, the total extracts were prepared using buffer containing lysostaphin 5ug/ml, 10mM HEPES, 150 mM NaCl pH 7.5, 0.4% NP40 and complete protease inhibitor. The affinity pull down was performed as described in Schluepen *et al.* article [22]. After binding of the EsxA-SBP-FLAG to the streptavidine beads the sample were washed using stringent solution and after the elution step performed with biotin, the purified complex was subjected to SDS-PAGE. The sample was trypsin-digested and analyzed by LC-MS/MS in PPI group

in Novartis. Anti-FLAG immunoblotting was used to verify the presence of the flagged EsxA protein.

4. Preparation and analysis of bacterial fractions

Bacterial lysates and supernatants were prepared as modification of described previously [23]. Bacterial strains were grown O/N from glycerol stocks in TSB at 37°C with shaking. Cultures were diluted 1:100 in fresh broth and shaken at 37°C until they reached an OD_{600} 1. For whole culture lysates, 5ml of cultures were centrifuged, washed once with PBS and suspended 1ml di Tris-HCl pH 6.8 with protease inhibitors and incubated in the presence of lysostaphin (Sigma) at a final concentration of 100μg/ml for 1h at 37°C at 750 rpm, followed by freezing/thawing three times in a dry ice/thermomixer at 37°C. The bacterial lysates were centrifuged at 14,000 rpm for 10 min, the supernatants were filtered and the proteins in the lysates were precipitated with TCA at final concentration of 10%.

5. Biofilm formation *in vitro*

To reproduce biofilm *in vitro*, bacteria grown in a rich medium BHI supplemented with 1% glucose and 3% NaCl at different time points: 6, 12, 24, 48 and 72h at 37°C and in 5% of CO_2 rich environment without shaking in a 24 well plate. Biofilm formation was made visible by staining *S. aureus* cells with 0.2% of crystal violet for 15 minutes. After three washes biofilm was quantified by measuring OD at540nm.

Results

1. Gene expression analysis for *esxA* and *esxB*

1.2 Quantitative Real Time PCR in different *in vitro* conditions

In order to better identify the molecular mechanisms of action of *esxA* and *esxB* it was necessary first define the conditions in which *esxA* and *esxB* are expressed. Previous studies involving the analysis of expression of virulence factors were mainly performed *in vitro* during growth in rich medium [24]. However, when *S. aureus* infects a host, the bacterial growth conditions are quite different from those in a medium, which may be related to the different expression of virulence factors in the host [24]. It has been previously reported that stress conditions, such as growth in presence of serum, CO_2, anaerobic conditions and biofilm are responsible for the induction of different virulence factors [24].

In this study we investigated the expression of *esxA* and *esxB* during different phases of growth, in presence of serum, during biofilm formation and within eukaryotic cells after *in vitro* infection using quantitative real-time PCR.

We compared the expression of *esxA* and *esxB* in two relevant clinical isolate Newman a meticillin sensitive strain (MRSS) and USA300 a meticillin resistent strain (MRSA) during growth in rich medium Tryptic Soy Broth (TSB). The result obtained shown an increase of transcript levels for both genes in USA300 in comparison to Newman strain (Fig. 1A).

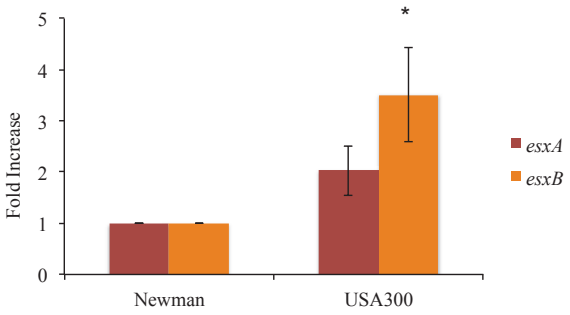
Then we investigated the expression kinetics of the *esxA* and *esxB* genes. We extracted the bacteria RNA at early, late and stationary phase. The data, normalized to expression during early phase showed that *esxA* is expressed more during the late phase of growth (Fig. 1B).

Since is known from literature that many virulence factors, are significantly increased during growth in serum compared to that in bacterial medium, we investigated the same hypotesis for *esxA* and *esxB* expression. We quantified the level of transcript of *esxA* and *esxB* during growth in DMEM medium

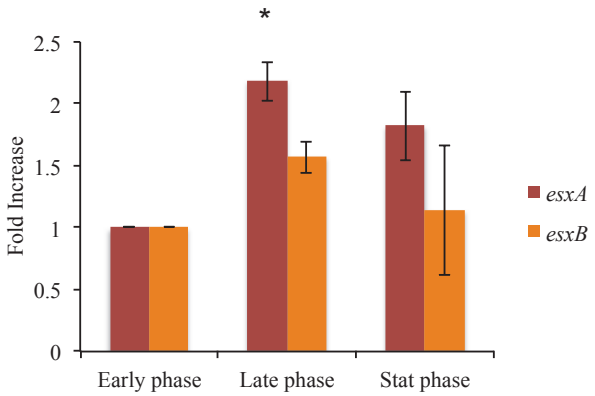
supplemented with different concentration of Fetal Bovine Serum (FBS) 2%, 10% and 20%. The bacterial RNA was extracted at late log phase and the data were normalized to transcript levels measured during growth in DMEM medium. The result showed that the transcript levels of *esxA* and *esxB* decrease in medium supplemented by 2-20% of serum (Fig. 1C). The biofilm-forming ability is an attribute of several pathogens. In particular *S. aureus* is one of the most frequent cause of biofilm-related infections [4]. A comparative *S. aureus* global gene expression transcriptome analysis has shown several genes differentially expressed under biofilm formation in comparison to planktonically growth[6]. In order to test if our genes could be expressed in biofilm, we set up an *in vitro* biofilm assay where the bacteria grow in a rich media Brain Heart Infusion broth (BHI) supplemented with 1% of glucose and 3% of NaCl. We detected an increase in expression of *esxB* gene after 24h of biofilm growth in static condition compared to planktonic growth after 24h of incubation at 37°C (Fig. 1D).

A transcriptome analysis by Garzoni *et al.* shows an extensive alteration of bacterial gene expression following internalization of epithelial cells [25]. These findings suggest that *S. aureus* extensively reprograms its transcriptome to adjust the intracellular environment. As the mycobacterial Esx proteins are known to function intracellularly [12], we wanted to investigate if *esxA* and *esxB* are differentially expressed when *S. aureus* is within eukaryotic cells. In order to study this, we set up an *in vitro* infection assay where A549 human lung epithelial cells were infected with USA300 strain (as described in Methods of the fourth chapter of the thesis). RNA was extracted from intracellular bacteria 6h after infection (p.i.). Quantitative real time PCR analysis for *esxA* and *esxB* showed that both genes, in particular *esxB,* are up-regulated upon internalization when normalized to expression in the infection medium, DMEM with 10% FBS (Fig. 1E).

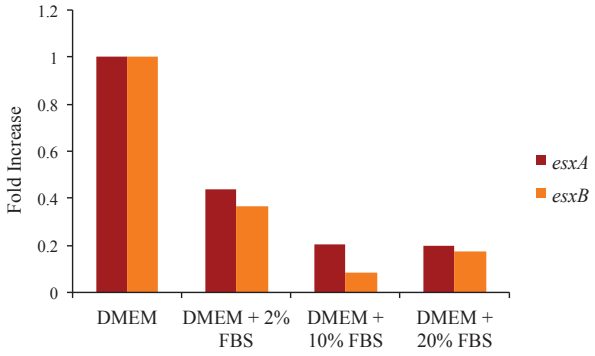
A


5
4
3
2
1
0
Fold Increase
*
esxA
esxB
Newman
USA300

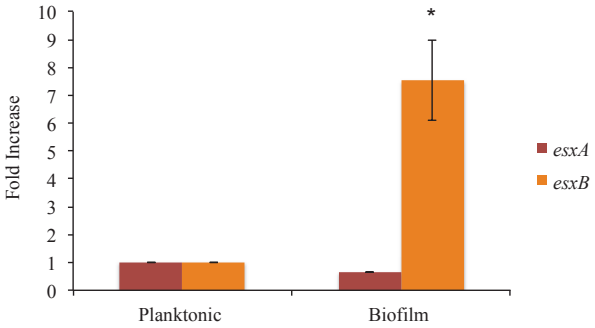
B


2.5
2
1.5
1
0.5
0
Fold Increase
*
esxA
esxB
Early phase
Late phase
Stat phase

C


1.2
1
0.8
0.6
0.4
0.2
0
Fold Increase
DMEM
DMEM + 2% FBS
DMEM + 10% FBS
DMEM + 20% FBS
esxA
esxB

D


10
9
8
7
6
5
4
3
2
1
0
Fold Increase
*
Planktonic
Biofilm
esxA
esxB

E

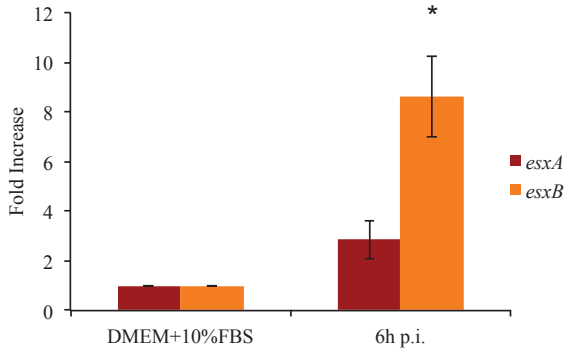

12
10
8
6
4
2
0
Fold Increase
*
DMEM+10%FBS
6h p.i.
esxA
esxB

Fig. 1 Gene expression analysis for *esxA* and *esxB* genes by quantitative real-time PCR (qRT-PCR).
A Expression analysis of *esxA* and *esxB* genes in different strains Newman and USA300 and **B** in different growth of phases during growth in TSB medium: early, late and stationary. **C** *esxA* and esxB transcripts level from DMEM plus fetal bovine serum. **D** Expression during biofilm formation and **E** upon internalization of epithelial cells 6h post infection. A relative quantification was performed using 16S rRNA as internal control. * indicates significant differences as compared with reference control condition ($P < 0.05$). The data presented are the mean of 3 independent experiments +/- s.d. in all the experiment. Data shown in **C** are representative of 2 independent experiments.

2. Generation and characterization of *esx* mutants

2.1 Generation of deletion mutants

In order to characterize the functions of EsxA and EsxB we made unmarked, single deletion mutants for *esxA* and *esxB* genes in two relevant clinical isolates of *S. aureus*, Newman (MSSA) and USA300 (MRSA) strains. To create deletion of these genes in the genome of *S. aureus* we used pKOR1, an *Escherichia coli/S. aureus* shuttle/ suicide vector that permits rapid cloning via lambda recombination and *ccdB* selection (Fig 2A). A method for rapid selection of allelic replacement mutations in the chromosome of *S. aureus* was described by Bae *et al.* [20]. Staphylococci were transformed and grown at 43°C, a non-permissive condition for pKOR1 replication that allows the selection for homologous recombination and plasmid integration into the bacterial chromosome. Anhydrotetracycline-mediated induction of pKOR1-encoded *secY* antisense transcript via the Pxyl/tetO promoter, a condition that is not compatible with staphylococcal growth, selects for chromosomal excision and loss of plasmid. Using this strategy, allelic replacements in *S. aureus* were generated at frequencies that obviated the need for antibiotic marker selection (Fig. 2B).

In order to characterize the function of both proteins, double mutant for *esxA* and *esxB* was generated in both strains. Δ*esxA* genetic background was used to make deletion in *esxB* gene. Δ*esxA* competent cells were transformed using the same strategy describe before.

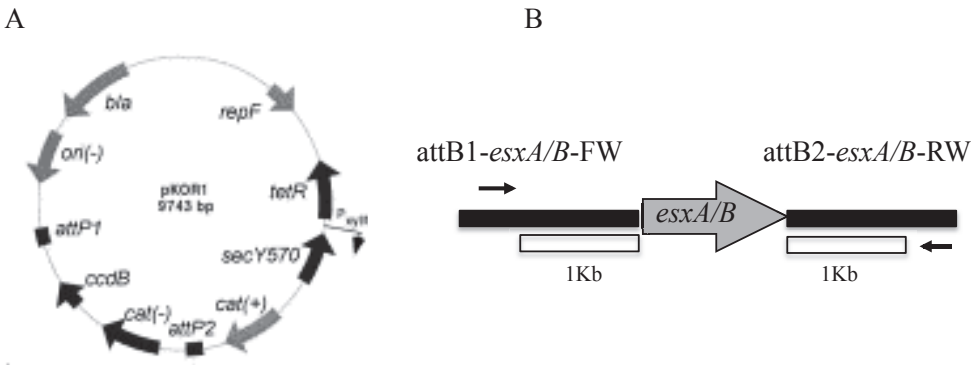


Fig. 2 pKOR1 allelic replacement generates staphylococcal chromosomal deletions
A pKOR1 vector map. **B** Chromosomal map of *esxA/B* gene and the location of PCR primers to make *esx* deletion constructs. The location of primers (attB1-*esxAB*-FW and attB2- *esxAB*-RW) are shown as short black arrows.

The mutants were confirmed by PCR using two different sets of primers: one annealing to the flanking region of *esxA* or *esxB* gene and the other specific for each gene. As shown in Fig 3A we verified the different size of fragment around the *esx* gene for mutant (2Kb) compared to WT (2.5Kb) and the lack of *esxA* and *esxB* (Fig. 3A). The lack of the protein was verified by western blot analysis (Fig. 3B). The deletion was further confirmed by qRT-PCR and sequencing.

A B

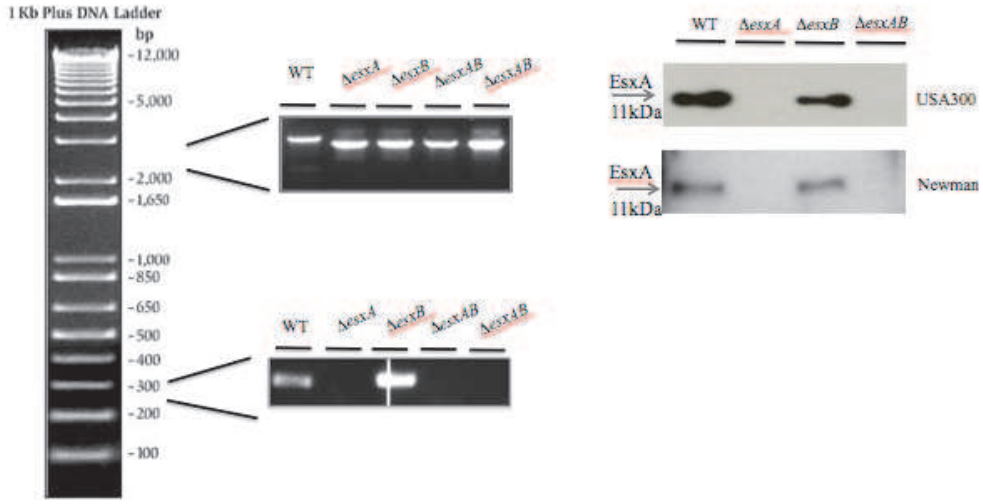


Fig. 3 Genetical and biochemical analysis of *esxA*, *esxB* and *esxAB* deletion mutants

A PCR was performed on chromosomal DNA from WT and Δ*esxA,* Δ*esxB or* Δ*esxAB* mutants using primers specific for flanking region of *esx* gene or binding to the *esx* genes. PCR products were analyzed on 0.8% agarose gel. The expected size for PCR products from genomic DNA obtained from the WT or mutant strains is 2.5 or 2 Kb, respectively. **B** Western blot analysis anti-EsxA on total extract of WT and *esx* mutants generated in two different strains USA300 and Newman.

3. Characterization of *esx* mutant strains during biofilm formation

3.1 Role of Esx proteins in staphylococcal biofilm

It is reported in a previous transcriptome analysis on *S. aureus* biofilms that several virulence factors are differentially expressed during biofilm formation [4] and our gene expression analysis showed an up-regulation of *esxB* gene in biofilm. Hence we wondered if these two small proteins could have a role during biofilm development. We investigated the behavior of the *esx* deletion mutants in an *in vitro* biofilm assay. We found that, although the mutants have no growth defects in BHI medium (Fig. 4A), a reproducible 1.5-2 fold increase in biofilm formation for the *esxB* deletion mutant as compared to WT strain at later time point (Fig. 4B, C). No differences were detected at early time points 6 and 12h after incubation.

Our hypothesis is that EsxB protein could play a role at later steps of biofilm development, i.e during the dispersal phase of staphylococcal biofilms. It could be directly involved in biofilm detachment by affecting cell-cell interactions or it could act to destroy the biofilm matrix. It is also possible that EsxB plays indirect role acting as a transcriptional regulator (up-regulates proteases, adhesins etc.), or by controlling post-translationally the secretion of proteins that may be involved in biofilm formation.

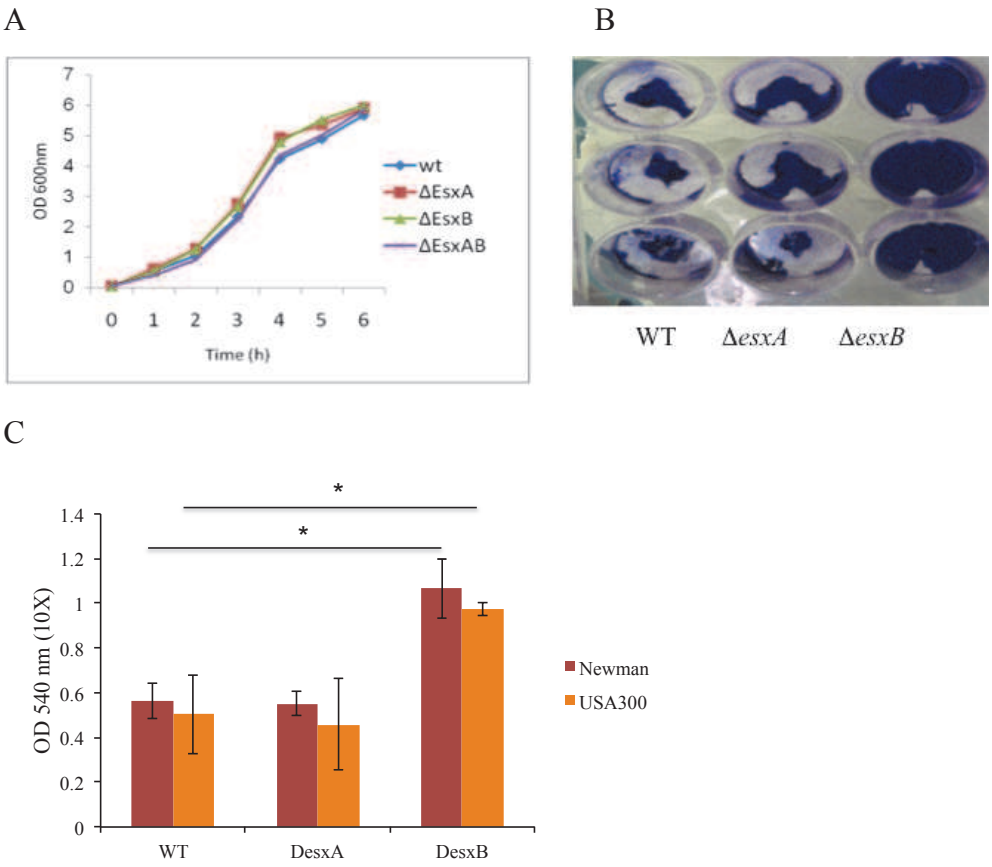


Fig. 4 *esx* mutants do not display any *in vitro* growth defects in media tested; increase of biofilm formation for *esxB* and *esxAB* at later time points

A Growth curves of WT and the various *esx* mutants in Brain Heart Infusion + 1%glucose, 3%NaCl at 37°C with shaking **B** Image showing biofilm formed by WT Newman and USA300 *S. aureus* strains and the various *esx* mutants after 24h in BHI containing NaCl and glucose at 37°C in 5% CO2 and stained with 0.2% crystal violet **C** A reproducible 2-fold increase in biofilm accumulation was observed for the Δ*esxB* mutants as compared with the WT at later time points. * indicates significant differences as compared with WT ($P < 0.05$). The data are representative of 3 independent experiments.

3.2 Transcriptome analysis of *esx* mutants

The mycobacterial ESX-1 substrate EspR is known to regulate the expression on other *esx* genes [26]. It is possible that the staphylococcal Esx proteins may have regulatory roles, which may mediate the effects observed above in biofilm formation. In order to investigate if EsxA and EsxB have a role in regulating the expression of other *S. aureus* factors, we performed a comparative gene expression analysis between WT Newman strain and various *esx* deletion mutants using *S. aureus* oligonucleotide microarrays.

Microarrays were first validated using various genomic DNA and RNA controls. Gene expression analysis showed no significant differences in the expression profiles of WT and *esx* mutant during growth in BHI.

4. Characterization of the Esx proteins secretion mechanisms

4.1 Interactions between *Staphylococcus aureus* Esx components

Interactions between staphylococcal Esx components and mechanisms by which they mediate secretion of substrates are not clear, very recent work further reports that deletions in either gene also affects secretion of additional Ess substrates [19]. The homologues mycobacterium Esx proteins ESAT-6 and CFP-10 was shown to be secreted as an heterodimer and function only when interacted each other and with other ESX-1 components. In order to investigate the proteins that interact with EsxA and EsxB, we used *in vitro* pull down approach. We employed a Tandem Affinity Purification (TAP) method in combination with mass spectrometry to identify possible interactors of EsxA and EsxB. This method is based on the sequential utilization of two different affinity tags to purify protein assemblies [22]. *esxA* and *esxB* genes were cloned in frame with a dual tag: a streptavidin binding protein plus a FLAG-peptide epitope at either the N- or the C-terminus of the proteins, and expressed in Δ*esxA* and Δ*esxB* (Fig 5A). Eluates from the respective affinity purifications were analysed by western blot showing only the C termed FLAG tag EsxA protein (Fig 5B, C). EsxB- FLAG was not detected probably due to the probable tag cleavage. However when we analysed the eluates by Mass Spectrometry analysis we did not identify any *S. aureus* proteins that directly bound to EsxA. This might be because the conditions under which the pull downs were performed were not suitable to allow stable interactions. Also, it is possible that the presence of a FLAG tag in these small proteins could interfere with the interactors binding to the proteins.

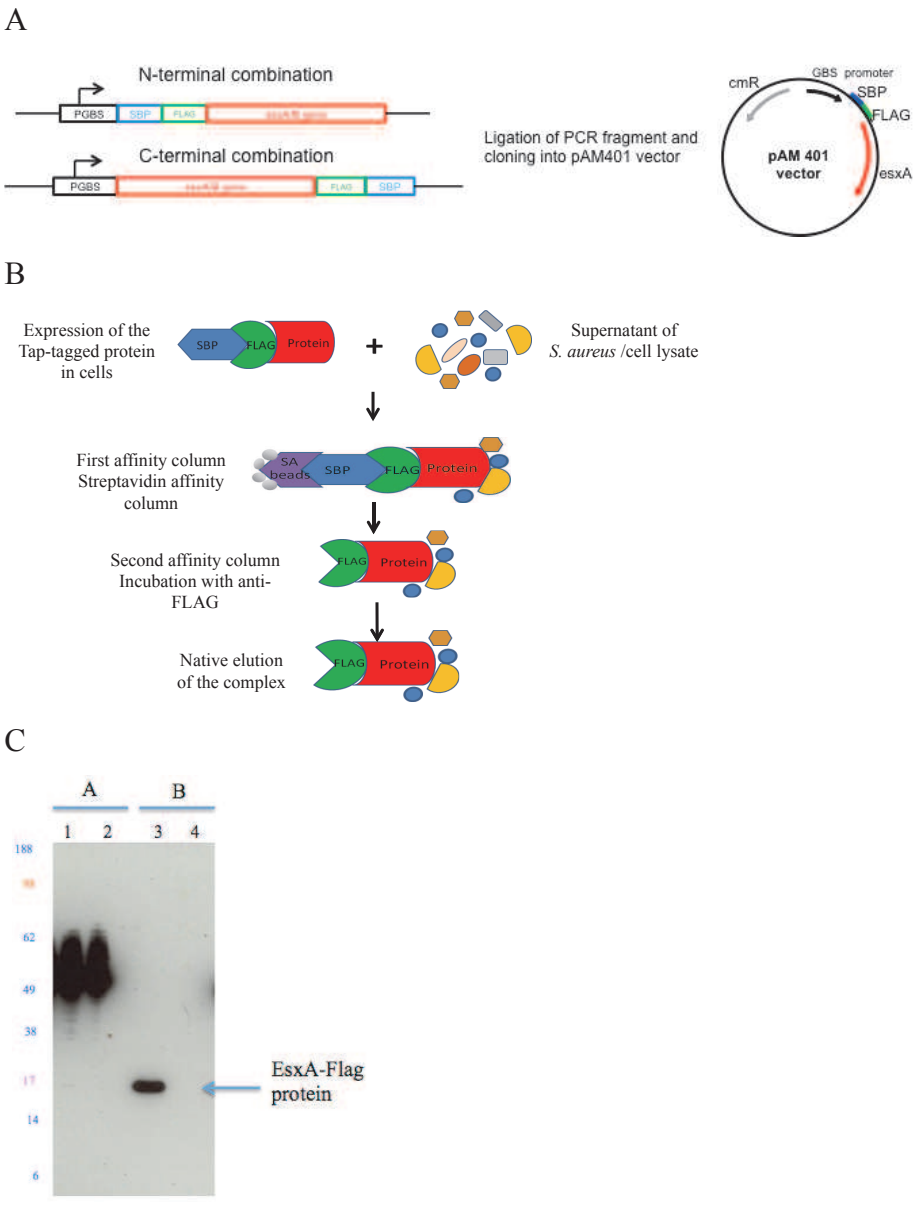


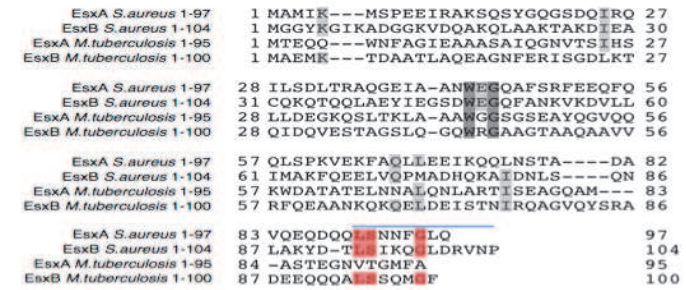
Fig. 5 TAP-tag Pull down

A Schematic representation of TAP-tagged *esx* gene constructs. **B** Schematic representations of TAP tag pull down of interacting proteins. **C** Western blot analysis to detect FLAG-tagged protein. A: unbound fraction B: eluate fraction after pulling down total lysate with anti-FLAG antibody Lines 1 and 3: Δ*esxA* expressing EsxA-FLAG protein. Lines 2 and 4: negative control, Δ*esxA*-empty plasmid.

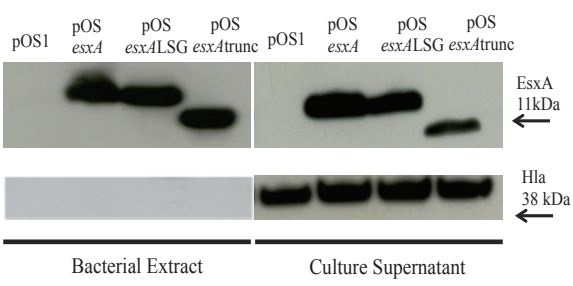
4.2 Secretion of EsxA

The homologues mycobacterial EsxA and EsxB proteins are secreted thought the Esx secretion system with non–classical signal sequence. As the signal sequences controlling *S. aureus* Esx protein secretion are not known, we wanted to determine the residues that are required for secretion of the protein *in vitro*. Based on the homology between *M. tuberculosis* and *S. aureus* Esx proteins, we identified amino acids (a.a.) conserved between the proteins at the C-terminus: leucine90, serine91 and glycine95 (Fig. 6A). EsxA bearing mutations in 3 a.a. or with a C-terminal 8 a.a. deletion were expressed episomally in the Δ*esxA* mutant to create strains Δe*sxA*pOS1CK*esxA*-LSG (Δ*esxA*-*esxA*LSG) and Δ*esxA*pOS1CK*esx*Atrunc (Δ*esxA*-*esx*AT) respectively. Immunoblotting analysis showed that the triple mutation in EsxA reduced the secretion of the protein into the culture supernatant while truncation of the C-terminal tail of EsxA severely impaired secretion of the protein compared with secretion of native EsxA (Fig. 6B, C).

A



B



C

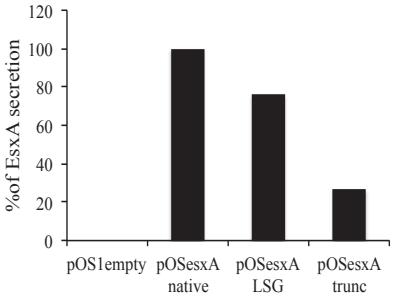


Fig. 6 C-terminal residues are important for secretion of EsxA

A Alignment of amino acid sequence of *M. tuberculosis* and *S. aureus* EsxA and EsxB proteins. In red are shown the high conserved residues that were replaced with alanine and the blue line indicates the 8 a.a deleted in the truncated EsxA protein. **B** Immunoblotting analysis of total extracts (TE) and supernatants (SN). Proteins in each fraction were precipitated with TCA, separated on SDS-PAGE, and detected by immunoblotting with anti-EsxA and anti-Hemolysin (loading control). Loading was normalized by OD_{600} of bacterial culture. **C** The graph shows the densitometry analysis performed using Image J software.

Discussion

Little is known about the biological functions of EsxA and EsxB during *Staphylococcus aureus* pathogenesis. Missiakas's group demonstrated that the transposon mutants defective in EsxA and EsxB secretion show decreased virulence in an abscess model of staphylococcal infection indicating a key role of these two proteins in a kidney abscess formation [8]. The high conservation among the *S. aureus* clinical strains suggests their importance as virulence factors. Although the mechanisms of action of the two orthololegous proteins in *Mycobacterium tuberculosis* ESAT-6 (EsxA) and CFP-10 (EsxB) are very well characterized, the biological functions of the *S. aureus* counterparts during infection and the mechanisms of the secretion are not well defined.

In this study we have analyzed different aspects of EsxA and EsxB proteins including expression, secretion and role during *in vitro* biofilm formation.

A gene expression analysis, performed in different *in vitro* condition, showed that *esxB* is induced under selective conditions such as biofilm, and repressed in presence of serum. Both genes are more expressed in MRSA USA300 compared to MSSA Newman strain indicating differencies in regulation of the two proteins in diverse clinical strains. Expression analysis during *in vitro* infection of epithelial cells show and up-regulation 6h post infection suggesting that *esxA* and *esxB* could play a role in *S. aureus* intracellular infection.

The development of a biofilm is a multistep process involving an initial attachment to human matrix proteins, a subsequent maturation phase characterized by intercellular aggregation and a final dispersal phase that involves the detachment of single cell or cell clusters by various mechanisms [4]. We found that *esxB* is significantly up-regulated in bacteria within biofilms. Consequently, employing isogenic mutants we observed that Δ*esxB* accumulated more biofilm *in vitro* at later

time point. In order to understand if EsxB had a direct role in biofilm formation, we analysed the differences in gene expression profile between WT and mutant strains. We observed that none of other staphylococcal genes were significantly differential expressed in the *esxB* mutant strain, indicating a potential direct role of this protein in biofilm. We still do not understand the precise role of EsxB in biofilms. However our hypothesis is that EsxB may be important in dissemination of biofilms so could play a role in the dispersal of staphylococcal biofilms being directly involved in biofilm detachment by affecting cell-cell interactions. It could act to destroy the biofilm matrix or it is also possible that EsxB has post-translationally control on the secretion of proteins that may be involved in biofilm development.

Interaction between the various components of the apparatus of secretion was also analyzed. While the mycobacterial EsxA and EsxB function as a heterodimer [15], the staphylococcal EsxA was crystallized as a homodimer, and there is no current evidence suggesting that the two proteins interact [16]. Unlike ESAT-6 and CFP-10, EsxA and EsxB do not interact each other. In a very recent work using a biochemical approach to detect protein-protein interaction, EsxA and EsxB of *S. aureus* were reported to interact with different Ess substrates such as EsxC and EsxD respectivel[19]. We employed a Tandem Affinity Purification method (TAP) to identify any other interactors of *S. aureus*. It combines affinity purification and mass spectrometry analysis to identify the interactors. However our experiments performed on the total lysate of *S. aureus* did not reveal any interactions between Esx proteins. Hence as discussed earlier, the conditions used and the presence of tags on the proteins may have interfered with our analysis.

Staphylococcal EsxA and EsxB are small secreted proteins that lack a canonical topogenic sequence. The secretion of EsxA and EsxB into culture medium during growth *in vitro* was previously demonstrated [8]. C-terminal signals were reported previously to mediate secretion of CFP-10 and other Esx locus encoded substrates

secreted by mycobacterial ESX-1 system [15]. We show that the C-terminus of EsxA, that contains residues conserved in the Esx substrates, is required for secretion *in vitro*. While single or multiple changes in amino acids did not appear to affect secretion, a truncation of the C-terminus resulted in a significant decrease in secretion, as reported recently for secretion of another Ess substrate-EsxD [19]. This indicates that the C-terminus may control secretion by mediating interactions with other proteins.

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The staphylococcal Esx proteins modulate apoptosis and release of intracellular *Staphylococcus aureus* from epithelial cells

Charalampia G. Korea[1], Giuliana Balsamo[1], Alfredo Pezzicoli, Simona Tavarini, Fabio Bagnoli, Davide Serruto and Meera Unnikrishnan*

Novartis Vaccines and Diagnostics, Siena, Italy

1. These authors contributed equally to this work

Running title: Esx proteins block host cell apoptosis

*meera.unnikrishnan@novartis.com

Microbial Molecular Biology,

Novartis Vaccines Research Centre

Via Fiorentina, 1,

53100 Siena, Italy

Tel. +390577539391

Abstract

The opportunistic pathogen *Staphylococcus aureus* is one of the major causes of healthcare-associated infections. *S. aureus* is primarily an extracellular pathogen, but recently it has been reported to invade and replicate in several host cell types. The ability of *S. aureus* to persist within cells has been implicated in resistance to antimicrobials and recurrent infections. However, few staphylococcal proteins that mediate intracellular survival have been identified. Here, we examine if EsxA and EsxB, substrates of the ESAT 6-like secretion system (Ess), are important during intracellular *S. aureus* infection. The Esx proteins are required for staphylococcal virulence but their functions during infection are unclear. While isogenic *S. aureus esxA* or *esxB* mutants are not defective for epithelial cell invasion *in vitro*, a significant increase in early/late apoptosis was observed in *esxA* mutant-infected cells as compared to wildtype. Impeding secretion of EsxA, by deleting C-terminal residues of the protein, also resulted in a significant increase of epithelial cell apoptosis. Furthermore, cells transfected with *esxA* showed an increased protection from apoptotic cell death. A double mutant lacking both EsxA and EsxB also induced increased apoptosis, but remarkably was unable to escape from cells as efficiently as the single mutants or wildtype. Thus, using *in vitro* models of intracellular staphylococcal infection, we demonstrate that EsxA interferes with host cell apoptotic pathways and, together with EsxB, mediates the release of *S. aureus* from the host cell.

Introduction

Staphylococcus aureus (*S. aureus*) is a Gram-positive coccus that causes infections ranging from superficial skin lesions to serious conditions like pneumonia and endocarditis. *S. aureus* is also a major cause of hospital-acquired infections of surgical wounds and of indwelling medical devices. Staphylococcal infections, in particular systemic and chronic infections, place a major burden on healthcare systems worldwide (1, 2). Antibiotic resistance still remains a challenge in the management of staphylococcal infections, especially with the recent rise in methicillin and vancomycin resistant strains (MRSA and VRSA) (3, 4). During infection, *S. aureus* expresses a wide array of secreted and cell surface-associated virulence factors to evade immune responses by a variety of mechanisms such as promoting adhesion to host cells, binding proteins in blood or resisting immune cell attack (5-7).

In addition to its armor of virulence factors, the capacity of *S. aureus* to successfully evade host defenses has been recently attributed to its ability to invade immune and non-immune cells. *S. aureus* is mainly an extracellular pathogen, but an accumulating number of studies have shown that it can invade and replicate in many types of non-phagocytic host cells *in vitro* (8). Clinical studies have reported the presence of intracellular staphylococci from nasal epithelial cells, indicating that this could serve as a reservoir for recurrent infections (9, 10). Although the intracellular presence of *S. aureus* during *in vivo* staphylococcal infection remains unclear, a transient, intracellular lifestyle potentially provides protection against exposure to antibiotics and host immune responses, as well as a favorable environment for the formation of resistant variants (11, 12).

S. aureus possesses the Sec and Tat secretion systems, which presumably transport the majority of the known virulence factors (13, 14). A specialized ESAT-6 secretion system (Ess), similar to Esx-1 secretion system described in *Mycobacterium tuberculosis* (*Mtb*) was also identified in *S. aureus* (15). Ess consists of 12 proteins, including EsxA and EsxB that are similar to the ESAT-6

and CFP-10 of *Mtb*. ESAT-6 (EsxA) and CFP-10 (EsxB) are well-characterized virulence factors of *Mtb* implicated in survival in macrophages, host cell lysis and dissemination (16-18). For the staphylococcal Esx proteins, mutants that failed to secrete EsxA and EsxB displayed defects in *S. aureus* abscess formation in mice, suggesting that these proteins are important during staphylococcal disease (15). Other Ess proteins such as EsaD were reported to be important for staphylococcal virulence, while EsaC was required for persistent staphylococcal infection in mice (19, 20). Importantly, to date no clear biological function has been attributed to the staphylococcal Esx proteins.

The precise structure of the Ess secretion apparatus is currently not known. Structural analysis of EsxA suggests that this protein may act as a chaperone or as an adaptor protein to facilitate interactions with host receptor proteins (21). Co-dependent secretion of Ess substrates has been reported, similar to that observed for mycobacterial substrates (15). C-terminal residues are important for interaction of the mycobacterial EsxB with other proteins of the apparatus and for secretion (22, 23)**.** Recently, a C-terminal motif (YxxxD/E) of the Ess substrate EsxD, was shown to be required for secretion of EsxA and EsaC (24).

In this study we examine a potential intracellular role for the staphylococcal EsxA and EsxB, employing an *in vitro* cellular model of *S. aureus* infection. We demonstrate that EsxA interferes in *S. aureus*-induced apoptosis in human epithelial cells *in vitro*. This inhibitory effect is associated with the secretion of EsxA, which is mediated by C-terminal residues of the protein. Our data also suggest that EsxA and EsxB together affect the release of intracellular *S. aureus* from host cells.

Materials and Methods.

Bacterial strains and growth conditions. *S. aureus* strain USA300 (lac) was used for all the experiments. Complemented *S. aureus* strains were grown in presence of chloramphenicol 10μg/ml. For infection experiments bacteria were grown in Tryptic Soy Broth (TSB) overnight (O/N) at 37°C, diluted 1/100 in fresh TSB and cultured until exponential phase of growth (A_{600}= 0.6-0.7) and then re-diluted 1/100 in DMEM supplemented with 10% fetal bovine serum (FBS).

Construction of bacterial mutants. For deletion of *esxA* and *esxB* a 2kb DNA fragments flanking the *esx* genes were amplified by PCR and cloned into the *Escherichia coli*/*S. aureus* shuttle/suicide vector pKOR1 using previously described methods (25). The *esxA* and *esxB* double mutant was obtained by deleting *esxA* in the Δ*esxB* mutant. All mutants were confirmed by PCR using external primers of flanking regions and sequencing. For complementation of mutant strains, the full length *esxA* and *esxB* genes were amplified and cloned into plasmid pOS1CK, which was generated by cloning the P1 constitutive promoter of the *sarA* gene into the pOS1 plasmid. For complementing with both genes, *esxB* was cloned downstream of *esxA* in the pOS1CK*esxA* construct. *esxA* with site directed mutations were cloned into the episomal plasmid pOS1CK. For generation of fluorescent bacteria, pOS1CK-GFP was cloned into WT and Δ*esxAB* strains.

Eukaryotic cell culture. Human lung epithelial cell line A549 (**CCL-185™)** was obtained from the ATCC and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Biological Industries) and incubated at 37°C, 5% CO_2. For cell passages, 100μg/ml primocin (Invivogen) was supplemented in the medium, whereas no antibiotics were used 24h prior and during infection assays.

S. aureus infection of eukaryotic cells. At 24h before infection $2x10^5$ cells/ml A549 cells were seeded in a 24-well plate (Nunc, Wiesbaden, Germany) so as to have 80% cell confluency. *S. aureus* cultures, as described above were diluted in DMEM-10 and added on A549 at an MOI of 7:1. After 2h of incubation,

extracellular bacteria were killed by adding 20µg/ml lysostaphin (Sigma Aldrich) for 30 min at 37°C. To remove lysostaphin and dead bacteria, cells were washed with growth medium once and, in the case of further incubation, fresh growth medium supplemented with 5µg/ml lysostaphin was added in each well. Infected host cells were trypsinized and lysed with cold water in a final volume of 1ml. Lysates were diluted with PBS and plated in serial dilutions on TSA plates to calculate the CFU/ml.

Confocal Microscopy. For fluorescence microscopy, cells were grown in chamber slides with polylysine coating and infected with *S. aureus* as described above. After lysostaphin treatment, cells were rinsed once with warm culture medium and fixed for 15 min with 4% paraformaldehyde at room temperature (RT). For observing intracellular infections with Vancomycin-Bodipy FL staining (Molecular Probes), cells were incubated with 2µg/mlvancomycin-1% BSA in PBS at 4°C in the dark for 15 min, washed once. Phalloidin (Molecular Probes) at a dilution 1:40 was used for staining F-actin. For analysis, 60-150 cells were counted/field and at least 4 fields were counted for each experiment. For microcolony visualization, samples were incubated with rabbit anti-staphylococcus at dilution of 1:1000 (ProSci), Alexa Fluor 488-labeled secondary antibodies (1:1000), together with biotinylated wheat germ agglutinin (1:500) and streptavidin-Alexa Fluor568 (1:1000) for 15 min at RT. The images were acquired with the confocal microscopes Leica LSM700 and LSM710 and analyzed by Leica Confocal Software (Leica Microsystems, Heidelberg, Germany).

Preparation and analysis of bacterial fractions. Bacterial lysates and supernatants were prepared as modification of described previously (26). ***Flow cytometry analysis.*** For analysis of apoptosis, cells were grown on 24-well plates and infected with *S.aureus* as described above. At various time points post infection, medium was aspired and $1x10^7$ cells were detached using cell dissociation Buffer (Invitrogen). Cells were washed once at 3000g, pelleted, resuspended in 50µl Aqua Live-Dead 1:500 (Invitrogen) and then incubated for 20 min in the dark at 4°C.

Cells were pelleted at 3000g, washed once with PBS and resuspended in 200µL Annexin-V (eBioscience) and incubated for 20 min in the dark at 4°C. The cells were washed, fixed with PFA 4% and resuspended in 150µl PBS. The double staining was analyzed by a FACS Canto II flow cytometer using the FlowJo software on a subpopulation of entire single cells selected from the initial population, based on the morphology. Each experiment was repeated at least three times.

Expression of bacterial esxA in A549 cells. We constructed p*esxA*-EYFP by cloning the full length *esxA* gene upstream of and in frame with the EYFP of plasmid pEYFP-N1 (Clontech). For transfection of the A549 cells, $8x10^5$ cells/well were seeded on a 6-well plate O/N and transfected using Lipofectamine 2000 (Invitrogen) using manufacturer's protocols. After 6h, the medium was changed and 24h post transfection cells labeled with Aqua Live-Dead and Annexin-V. Cells were analyzed by flow cytometry selecting for cells that were positive for EYFP.

Statistical analysis. A non-parametric, one way ANOVA Tukey's or Dunnett's multiple comparisons test at 95% confidence interval was applied to data in multiple groups, and a two tailed Mann Whitney U test was used for 2 groups. A $P<0.05$ was considered statistically significant.

Results

EsxA modulates apoptosis in S. aureus infected cells. To study a potential role of EsxA and EsxB, two proteins encoded by the Ess locus of *S. aureus* (15), in the invasion and survival of human epithelial cells, isogenic single mutants Δ*esxA* and Δ*esxB,* were generated in USA300. The absence of expression of *esxA* or *esxB* in the mutants was confirmed by qRT-PCR (Fig. S1A) and the analysis of the growth phenotypes of Δ*esxA* and Δ*esxB* in TSB medium showed no growth defects (Fig S1B). A549 epithelial cells were used for infection assays, given their high infection efficiency with wildtype (WT) USA300 (50-60% cells infected) at MOI's from 1:1 to 10:1. A549 invasion assays performed comparing the *esx* mutants to the WT did not reveal any major differences in internalization efficacy (Fig. 1). Intracellular trafficking studies of the *esx* mutants by confocal microscopy showed presence of bacteria both in vacuoles and in the cytoplasm at 4h *p.i*., and mostly in the cytoplasm at 6h *p.i*. (data not shown). However, there were no significant differences in trafficking of the mutants as compared to WT. Interestingly, analysis of infected cells showed that very often the presence of intracellular bacteria in the cytoplasm was followed by actin polymerization, leading to rounding of infected cells. As compared with WT, higher numbers of cells infected with Δ*esxA* showed increased accumulation of polymerized actin (Fig. S2A), a phenotype linked with the earlier steps of apoptosis (27), as compared with WT-infected cells. This observation suggested that EsxA may play a role in host cell apoptosis during staphylococcal infection.

To further investigate the potential role of EsxA in apoptosis, we performed flow cytometry analysis on cells infected with WT and the *esx* mutants at different time points after infection. Cells were stained with AquaLive-Dead that stains lysed cells (a marker of cell death) and Annexin-V that specifically binds to the phosphatidylserine (PS) exposed on the plasma membrane of apoptotic cells. Staining with Annexin-V showed an increased number of early and late apoptotic cells at 6h *p.i.* for Δ*esxA*-infected but not the Δ*esxB*-infected A549 cells (Fig. 2A,

B). As shown in the scatter plots, the cells that are infected by Δ*esxA* mutant consist of increased subpopulations of early (bottom right quarter of the scatter plot) and/or late apoptotic (top right quarter of the scatter plot) cells compared to WT-infected cells. The increase in apoptosis observed for the mutant was reversed upon episomal expression of *esxA* in the Δ*esxA* mutant, *Δesx*ApOS1CK*esxA* (*ΔesxA-esxA*) (Fig. 2A, B). As reported previously, the WT-infected cells also showed an increase in apoptosis compared with uninfected cells (28, 29). These differences in the number of apoptotic cells were found to decrease at later time points (8h and 16h), possibly because during infection cells containing intracellular bacteria die and detach from the monolayer (data not shown).

These data suggest that the EsxA protein may modulate *S. aureus*-induced apoptosis during epithelial cell infection *in vitro*.

C-terminal residues are important for secretion of EsxA: impacting EsxA secretion affects EsxA-mediated modulation of apoptosis. As EsxA is a secreted protein, we wanted to test if preventing secretion of EsxA could also result in increased apoptosis. As the signal sequences controlling EsxA secretion are not known, we first identified the residues that are required for secretion of the protein *in vitro*. Based on the homology between *Mtb* and *S. aureus* Esx proteins, we identified the amino acids that are conserved at the C-terminus of the staphylococcal proteins, a region thought to be important for secretion of Esx proteins in mycobacteria (22). Leucine90, serine91 and glycine95 were conserved in both the staphylococcal Esx proteins and the mycobacterial EsxB (Fig. 3A).

In an attempt to define targeting sequences responsible for mediating secretion by the Ess system, we studied effects of site-directed mutations and deletions at the C-terminus of the EsxA protein. EsxA bearing mutations in three amino acids (L90A, S91A, and G95A) or with an 8 amino acid (a.a.) deletion was expressed episomally in the Δ*esxA* mutant to create strains Δ*esxA*pOS1CK*esxA*-LSG (Δ*esxA-esxA*LSG) and *Δesx*ApOS1CK*esxA*trunc (Δ*esxA-esxA*T) respectively. Immunoblotting

analysis showed that the triple mutation in EsxA mildly reduced (by ~25%) secretion of the protein into the culture supernatant (Δ*esxA*-*esxA*LSG) as compared with secretion of native EsxA (Δ*esxA*-*esxA*). Truncation of the C-terminal tail of EsxA comprising the last 8 a.a. severely impaired secretion of the protein (by ~75%) (Fig. 3B).

To understand if hindering secretion of EsxA affects cellular apoptosis, we investigated if the strains Δ*esxA*-*esxA*LSG and Δ*esxA*-*esxA*T were able to reverse the increase in apoptosis induced by the Δ*esxA* mutant, as seen above with the Δ*esxA* mutant complemented with native EsxA (Δ*esxA*-*esxA*) (Fig. 2A, B). Flow cytometry analysis showed that the Δ*esxA* mutant expressing the truncated EsxA (Δ*esxA*-*esxA*T) was able to induce more apoptotic cells when compared to the mutant expressing native EsxA protein (Fig. 3C) and had similar profile to the control *i.e* Δ*esxA* mutant transformed with empty plasmid (Δ*esxA*-pOS1CK). On the other hand the Δ*esxA*-*esxA*LSG strain did not show a similar increase in apoptosis; probably due to the minimal effects of the LSG mutation on EsxA secretion (Fig. 3B).

Thus, these data suggest that impacting secretion of EsxA can affect the EsxA-mediated modulation of host cell apoptosis in epithelial cells *in vitro*.

EsxA delays apoptosis when expressed in the host cell. In order to further confirm that EsxA interferes with host apoptotic pathways, A549 cells were transfected with the plasmid pEYFP expressing full length *esxA* or truncated (8 a.a.) as N-terminal fusions with EYFP (p*esxA*-EYFP). Using flow cell cytometry, we measured by Annexin-V and AquaLive-Dead staining the apoptotic subpopulations in the transfected cells (gated by expression of EYFP). The expression of full length *esxA* in the cytoplasm resulted in a 2.5-fold decrease of the number of apoptotic cells compared to the control cells transfected with the empty vector pEYFP (Fig. 4A). These data are in agreement with the results described above for the *esxA* mutant. Expression of *esxA* lacking the C terminal 8 a.a. on the other hand

did not induce a decrease in apoptosis, indicating that the C-terminal residues needed for secretion are not important in mediating apoptosis (Fig. S3).

Staurosporine, a potent inducer of apoptosis, is known to induce caspase-dependent and caspase-independent apoptotic pathways (30). We tested if EsxA suppresses staurosporine-induced apoptosis. 24h after transfection with control plasmid pEYFP or p*esxA*-EYFP, cells were treated with 2.5μM staurosporine for 30min. Staurosporine-induced apoptosis, as measured by Annexin-V staining was significantly reduced in cells transfected with p*esxA*-EYFP, compared with the control pEYFP-transfected cells (Fig. 4B).

The data in all support a role for the staphylococcal EsxA in interfering with cell apoptotic pathways.

Esx proteins affect escape of bacteria from host cells. The mycobacterial counterparts EsxA and EsxB are known to form a heterodimer and also function together to cause host cell lysis (31, 32). Recent data have shown that EsxA and EsxB can interact with different Ess substrates, however do not interact directly each other (21, 24). To understand the effects of EsxA and EsxB taken together, we constructed a double mutant of *esxA* and *esxB*. As observed for the single mutants, Δ*esxAB* was similar to WT for invasion into epithelial cells (data not shown). As observed for Δ*esxA* in Fig 2, Δ*esxAB* induced more apoptosis in epithelial cells (Fig. S4). Interestingly, colony counts from infected A549 cells showed a significant increase for intracellular Δ*esxAB* at later time point after cell invasion (16h *p.i.*) as compared to WT (Fig. 5A). Examination of cells by confocal microscopy revealed presence of more intracellular bacteria in epithelial cells infected with Δ*esxAB* as compared to the WT (Fig. 5B). The differences between WT and the double mutant were also confirmed using GFP-expressing *S. aureus* strains; cells containing more fluorescent bacteria were observed for the Δ*esxAB*-infected as compared to WT-infected cells (Fig. 5C).

To understand if the Δ*esxAB* strain was accumulating within cells as a result of its inability to exit cells, we used a continuous infection assay, where infected A549 monolayers were first treated with lysostaphin to remove extracellular bacteria, but thereafter incubated in medium without lysostaphin for different time periods. Bacteria were seen to exit cells and progressively form extracellular bacterial aggregates on the cell layer. After 9h post infection we observed the formation of several such *S. aureus* 'microcolonies' by immunofluorescence staining of cells infected with WT, Δ*esxA* and Δ*esxB,* but very few for cells infected with the double mutant Δ*esxAB* (Fig. 6). Equal infection efficiency in this continuous infection assay was confirmed for all strains by quantitating intracellular counts after 2h (data not shown). Again, the *esxAB* mutant was able to eventually exit cells at later times as seen for the WT.

These results may suggest that in our *in vitro* infection assay, EsxA and EsxB together modulate cellular escape of *S. aureus*.

Discussion

The human pathogen *S. aureus* has been shown to invade and survive in a range of host cells *in vitro*. (8, 12, 33). Despite several studies describing intracellular staphylococcal infection, there is very little understanding of the bacterial factors or the mechanisms involved in intracellular survival of *S. aureus*. Our data show that EsxA, a protein secreted by the specialized staphylococcal Ess secretion system, modulates host cell survival by interfering with apoptotic pathways. To our knowledge this is the first description of staphylococcal factor that has an anti-apoptotic function in epithelial cells. Our data also support intracellular functions for the staphylococcal Esx proteins in cellular models of infection.

The interplay between bacterial virulence factors and host cell proteins is of utmost importance for a successful bacterial infection and staphylococcal infection is no exception to the rule. In order to persist in the hostile environment of the host cell, invading pathogens have developed various mechanisms to survive intracellularly, e.g. by inhibiting the lysosomal killing or by fine-tuning cell mechanisms such as apoptosis or autophagy (34, 35). It is known that apoptosis, a well-known mechanism of cell death, is induced by intracellular *S. aureus* (29, 36, 37). The staphylococcal toxin, alpha hemolysin (Hla) was reported to induce apoptosis upon intracellular staphylococcal infection (37). The apoptotic pathways affected by *S. aureus* appear to depend on the strain and host cell type used and several studies argue for or against the employment of molecules such as caspases (28, 38, 39) or calcium, (40) in *S. aureus*-induced apoptosis in epithelial cells. On the other hand, as seen for many other bacteria, *S. aureus* may also be able to block apoptosis. Although an anti-apoptotic effect of *S. aureus* has not been demonstrated as yet, *S. aureus* was recently reported to induce anti-apoptotic factors in epithelial cells (41). During staphylococcal infections, EsxA alone and/or with other Ess effectors, may transiently block cell apoptosis induced by extracellular staphylococci or by other staphylococcal proteins, to allow for intracellular replication of the bacteria. Indeed, it has been shown in other pathogens, such as *Helicobacter pylori* and

enteropathogenic *E.coli* (42, 43) that bacterial proteins can have both pro- and anti-apoptotic activities and that bacterial anti-apoptotic factors are active in the host cell cytoplasm. *S. aureus*, like other bacterial intracellular pathogens, is capable of manipulating the host cell to its advantage.

EsxA and EsxB, substrates of a novel ESAT-6 like secretion (Ess) system, are located in the same locus, however are separated by 6 other genes (15). EsxA has been reported to be regulated by sigma factor B and σB-controlled SpoVG, and is expressed as a single transcript, while we do not know how EsxB is regulated (44). Secretion of EsxA and EsxB into culture medium during growth *in vitro* was demonstrated previously, with mutation of EsxB affecting secretion of EsxA and *vice versa* (15). Very recent work further reports that deletions in either gene also affects secretion of additional Ess substrates, EsxC and EsxD (24). EsxA and EsxB were reported to interact with different Ess substrates such as EsxC and EsxD respectively (24). Hence it is not surprising that EsxA and EsxB mutants behave differently in terms of modulating apoptosis. EsxA and EsxB may affect stability and secretion of specific subsets of Ess substrates inside cells.

C-terminal residues were reported previously to mediate secretion of EsxB (CFP-10) and other Esx locus encoded substrates secreted by mycobacterial Esx-1 and Esx-1 paralogs (22, 23)**.** We show that the C-terminal tail of EsxA which contains residues conserved in mycobacterial and the staphylococcal Ess substrates, EsxA (and EsxB), is important for secretion of EsxA *in vitro*. The effect of these residues on the secretion of other Ess substrates was not examined in this study, however we believe that this could be the case as reported recently (24). Also, secretion of EsxA is not completely abolished, hence there are likely to be other residues or interactions governing secretion. Defective secretion of EsxA mimics the increase in apoptosis seen for the Δ*esxA* mutant. This suggests that secretion of EsxA is important for the EsxA-mediated apoptosis. However, we are yet to demonstrate directly the intracellular secretion of this protein.

As discussed above, disrupting secretion of EsxA may affect EsxA-dependent Ess

substrates. However, our transfection studies indicate that EsxA may directly mediate an anti-apoptotic effect in the host cytoplasm. EsxA lacking the C-terminal 8 a.a. was not important for cellular effects, indicating that the any interactions with host or bacterial factors may involve alternate regions of the protein. EsxA may function by either blocking one of the caspase-mediated pathways or by imitating or inducing host anti-apoptotic factors, such as Bcl2. Preliminary analyses of *hla* expression or secretion in the *esxA* mutant showed no changes (also as shown in Fig 3B), indicating no direct interactions between Hla and EsxA. Further investigations of the *S. aureus*-induced cell death pathways intercepted by EsxA are currently ongoing and will reveal molecular mechanisms involved in subversion of host cell death.

In other pathogens, an important step in bacterial dissemination, after intracellular replication, is the bacterial exit from the host cells. Previous studies have demonstrated that staphylococcal factors such as PSMs and leukocidins may affect bacterial escape from neutrophils (45, 46). The roles of these factors in non-immune cells are at present not clear. While both Δ*esxA* and Δ*esxAB* mutants show increased apoptosis, in continuous *in vitro* invasion assays the exit of bacteria from epithelial cells is affected only when both proteins are absent. The mycobacterial EsxA and EsxB function as a heterodimer (31), but recently the mycobacterial EsxA alone was demonstrated to mediate lipid membrane lysis (47). The staphylococcal EsxA was crystallized as a homodimer, and there is no current evidence suggesting that the two proteins directly interact (21). We propose two possible explanations for our results with the double mutant: 1. EsxA and EsxB directly mediate cell lysis, in association with host proteins in a multiprotein complex, 2. EsxA and EsxB modulate secretion of specific subsets of other Ess effectors which mediate cell lysis; deletion of both proteins results in total loss of all required effectors.

The orthologous Esx proteins in mycobacteria have been implicated in several aspects of pathogenesis including survival of mycobacteria in macrophages,

granuloma formation, induction of apoptosis and autophagy, phagosomal rupture and host cell lysis (16, 48). Although other Gram-positive bacteria such as *Listeria monocytogenes* contain orthologs, their functions are unclear (49, 50). The EsxA proteins from *S. aureus* and *Mtb* demonstrate only 20.8% identity (15), although they show structural similarities (21). It is very intriguing that the Esx proteins seem to have an intracellular role in *Mtb* and *S. aureus*, two very diverse pathogens. The mechanisms, by which they affect intracellular survival, however, appear different. Our data suggest that staphylococcal Esx proteins are not involved in avoiding phagosomal lysis, as indicated by lack of differences in the cytoplasmic vs vacuolar bacteria in infected epithelial cells, but may be involved in lysing the host cell. The staphylococcal proteins do not appear to affect the survival of the bacteria within the cell *per se,* as bacterial numbers by colony counts (Fig. 1) and by microscopy (data not shown) appear to be unaltered. Such differences with *Mtb* may reflect differences between the pathogens, but could also be attributed to the cell type under study *i.e.* macrophages vs epithelial cells. Both pathogens however may use the host cell as a niche to replicate and disseminate, with a key role for Esx proteins. Indeed, for *S. aureus*, the relevance of intracellular bacteria to infection and the role of the Esx proteins in modulating this remains to be demonstrated.

The facultative intracellular pathogen, *S. aureus* may use the host cell to escape unfavorable extracellular environments. This intracellular phase may play a key role in determining persistence of the bacteria within an infected tissue. Esx proteins have been implicated in persistence within abscesses in infected mice (19). Based on our data from an *in vitro* model of infection, we propose that the Esx proteins by delaying host cell death and controlling bacterial exit from host cells, may facilitate persistence and spread of the pathogen in the infected host. Future studies on the how the intracellular effects of these proteins contribute to virulence will clarify their role during infection.

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Figures

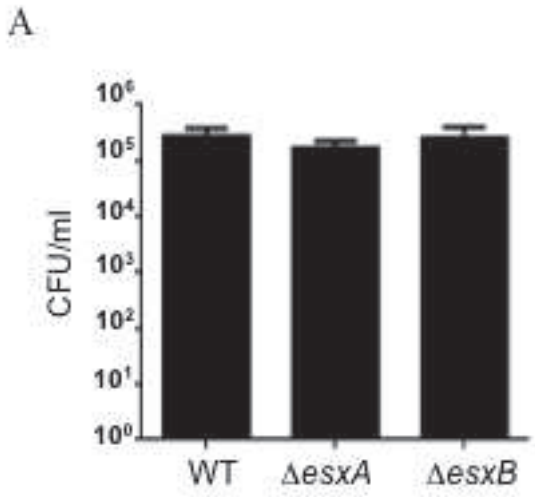
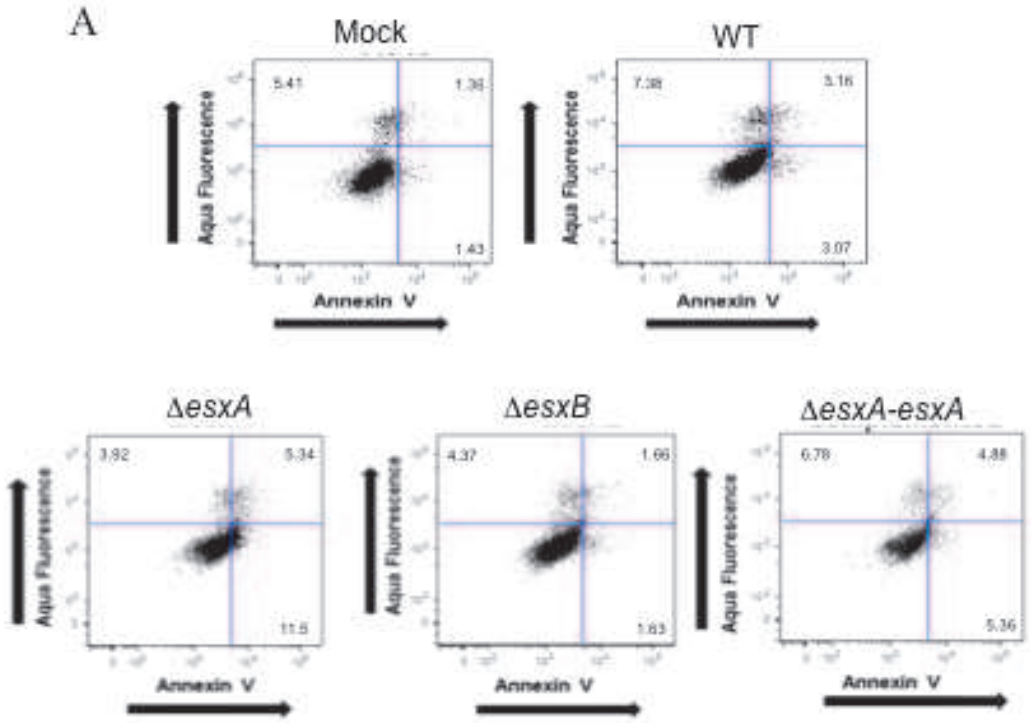


Fig. 1 WT and mutant strains do not show any differences in cell invasion
Invasion of WT and *esx* mutants into A549 epithelial cells 2h *p.i*. A549 cells were infected as described in Methods. 2h *p.i*. Extracellular bacteria were removed with lysostaphin treatment, cells were lysed and intracellular bacteria were quantitated by colony counts.


A
Mock
WT
ΔesxA
ΔesxB
ΔesxA-esxA
Aqua Fluorescence
Annexin V
5.41
1.36
1.43
7.38
3.16
3.07
3.92
5.34
11.5
4.37
1.66
1.63
0.78
4.88
5.36

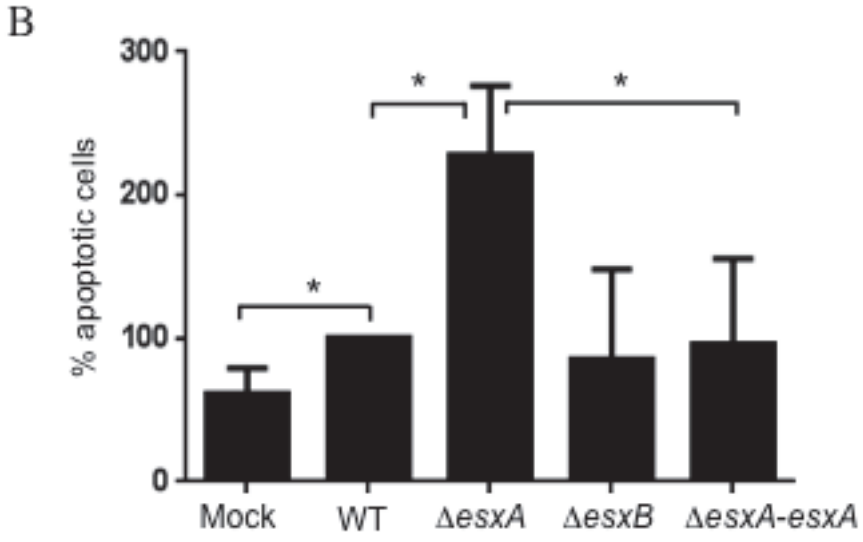

B
% apoptotic cells
300
200
100
0
*
*
*
Mock
WT
ΔesxA
ΔesxB
ΔesxA-esxA

Fig. 2 EsxA modulates host cell apoptosis
A Representative flow cytometry scatterplots depicting the early apoptotic (bottom right quadrant), late apoptotic (top right quadrant) and dead cells (top left quadrant) 6h *p.i*. The numbers in the plots represent the percentage of gated A549 cell populations in each quadrant. **B** Flow cytometry analysis of *S. aureus*-infected A549 cells. Quantitation of apoptotic cells using flow cytometry for A549 cells infected by WT, Δ*esxA,* Δ*esxB* and Δ*esxA-esxA* at 6h *p.i*. Cells were stained with AquaLive-Dead 1:500, for the detection of lysed cells, and with Annexin V-FITC for the detection of apoptotic cells. Graphs represent percentage of early and late apoptotic cells in the parent population for the mutant-infected cells in relation to the WT, which was set at 100%. * indicates significant differences using a one way ANOVA with a Tukey's multiple comparisons test ($P< 0.05$). The data presented are the mean of 3 independent experiments +/- s.d.

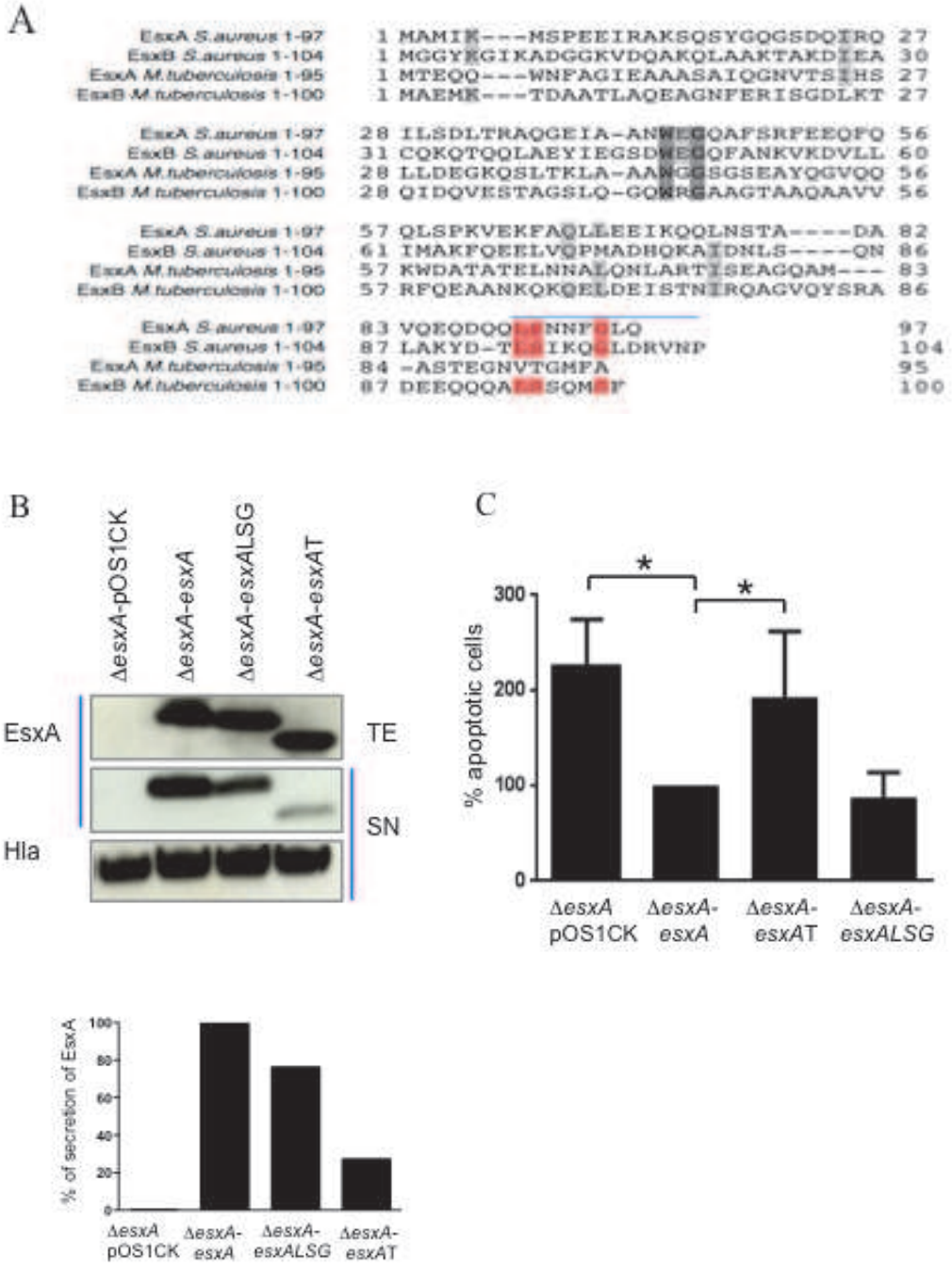

A
EsxA S.aureus 1-97 1 MAMIK---MSPEEIRAKSQSYGQGSDQIRQ 27
EsxB S.aureus 1-104 1 MGGYKGIKADGGKVDQAKQLAAKTAKDIEA 30
EsxA M.tuberculosis 1-95 1 MTEQQ---WNFAGIEAAASAIQGNVTSIHS 27
EsxB M.tuberculosis 1-100 1 MAEMK---TDAATLAQEAGNFERISGDLKT 27
EsxA S.aureus 1-97 28 ILSDLTRAQGEIA-ANWEGQAFSRFEEQFQ 56
EsxB S.aureus 1-104 31 CQKQTQQLAEYIEGSDWEGQFANKVKDVLL 60
EsxA M.tuberculosis 1-95 28 LLDEGKQSLTKLA-AAWGGSGSEAYQGVQQ 56
EsxB M.tuberculosis 1-100 28 QIDQVESTAGSLQ-GQWRGAAGTAAQAAVV 56
EsxA S.aureus 1-97 57 QLSPKVEKFAQLEEEIKQQLNSTA----DA 82
EsxB S.aureus 1-104 61 IMAKFQEELVQPMADHQKAIDNLS----QN 86
EsxA M.tuberculosis 1-95 57 KWDATATELNNALQNLARTISEAGQAM--- 83
EsxB M.tuberculosis 1-100 57 RFQEAANKQKQELDEISTNIRQAGVQYSRA 86
EsxA S.aureus 1-97 83 VQEQDQQ NNF LQ 97
EsxB S.aureus 1-104 87 LAKYD-T IKQ LDRVNP 104
EsxA M.tuberculosis 1-95 84 -ASTEGNVTGMFA 95
EsxB M.tuberculosis 1-100 87 DEEQQQA SQM F 100
B
ΔesxA-pOS1CK
ΔesxA-esxA
ΔesxA-esxALSG
ΔesxA-esxAT
EsxA
TE
SN
Hla
C
% apoptotic cells
300
200
100
0
*
*
ΔesxA pOS1CK
ΔesxA-esxA
ΔesxA-esxAT
ΔesxA-esxALSG
% of secretion of EsxA
100
80
60
40
20
0
ΔesxA pOS1CK
ΔesxA-esxA
ΔesxA-esxALSG
ΔesxA-esxAT

Fig. 3 C-terminal residues are important for secretion of EsxA: impeding EsxA secretion affects EsxA-mediated modulation of apoptosis
A Alignment of amino acid (a.a) sequence of *M. tuberculosis* and *S. aureus* EsxA and EsxB proteins. In red are shown the high conserved amino acid residues that were replaced with alanine and a blue line indicates the 8 a.a deleted in the truncated EsxA protein. **B** Immunoblotting analysis of total extracts (TE) and supernatants (SN). Proteins in each fraction were precipitated with TCA, separated on SDS-PAGE, and detected by immunoblotting with anti-EsxA and anti-Hemolysin (loading control). Loading was normalized by OD_{600} of bacterial culture. The graph shows the densitometry analysis performed using Image J software. **C** Flow cytometry analysis of apoptotic A549 cells infected with *esxA* mutant expressing native or mutant forms of EsxA. At 6h *p.i*. cells were dissociated and stained with AquaLive-Dead and Annexin V-FITC. The graph represents percentage of early and late apoptotic cells induced by the *esxA* mutant containing empty vector (Δ*esxA*-pOS1CK) or the different mutant forms (Δ*esxA*-*esxA*T, Δ*esxA*-*esxA*LSG) in relation to the *esxA* mutant expressing native *esxA (*Δ*esxA*-*esxA*) (set at 100%). Data are the mean of 3 independent experiments +/- s.d. * indicates significant differences using a one way ANOVA Tukey's multiple comparisons test ($P< 0.05$).

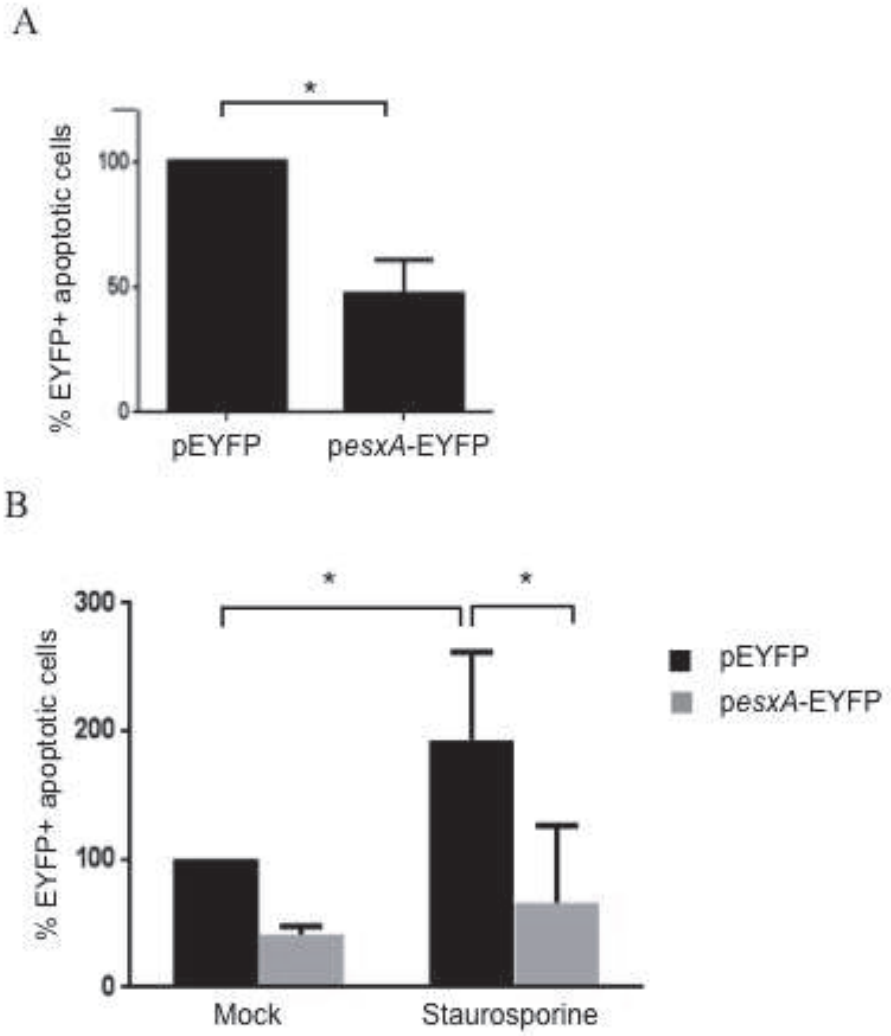


Fig. 4 *esxA* interferes with apoptosis when expressed in the host cell
A Flow cytometry analysis of apoptotic A549 cells transfected with *esxA*. 24h post transfection cells were dissociated and then stained with AquaLive-Dead and Annexin V-APC. The percentage of apoptotic cells (early and late apoptotic cells) was measured in the subpopulation bearing EYFP, *i.e.* transfected cells. Data are shown as a percentage of cells transfected with the control vector. Data are the mean of 3 independent experiments +/- s.d. * indicates significance by a two-tailed Mann Whitney test. **B** Flow cytometry analysis of apoptosis after treatment with staurosporine. 24h post transfection, A549 cells transfected with the plasmid p*esxA*-EYFP were treated with 2.5 mM staurosporine for 30 min, followed by AquaLive-Dead and Annexin V-APC staining. The percentage of apoptotic cells was measured in the subpopulation of transfected cells expressing EYFP. Data are

the mean of 3 independent experiments +/- s.d. * indicates significant differences using a one way ANOVA Dunnett's multiple comparisons test ($P< 0.05$).

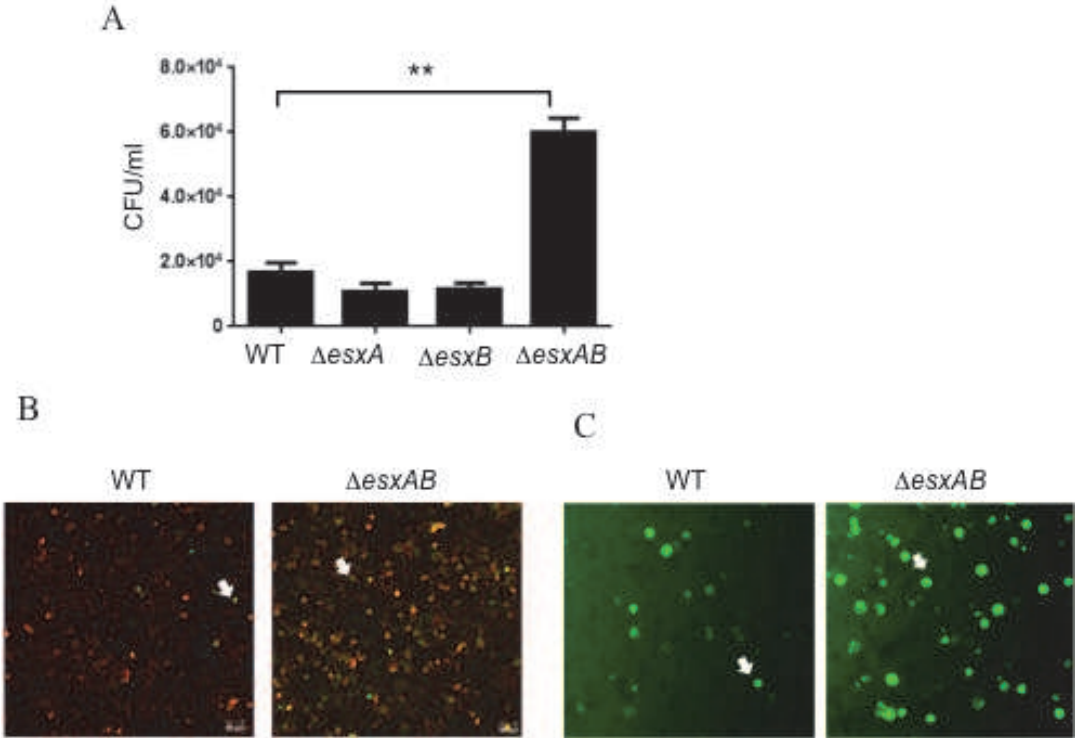


Fig. 5 Increased numbers of intracellular bacteria in epithelial cells infected with Δ*esxAB*

A Colony counts of intracellular WT, single and double mutants for the Esx proteins from A549 epithelial cells infected for 16h. A 5-fold increase can be seen between the WT and the double mutant. ** indicates significant differences by one way ANOVA with a Tukey's multiple comparison test ($P<0.001$). Data are representative of 3 independent experiments. **B** Confocal microscopy of A549 infected with WT or Δ*esxAB* after 16h infection. Intracellular bacteria were stained with vancomycin-BODIPY (green) and cells were stained with phalloidin (red). **C** Representative images (10X) of A549 cells with intracellular fluorescent (GFP-expressing) WT and Δ*esxAB*. The white arrows indicate the intracellular bacteria.

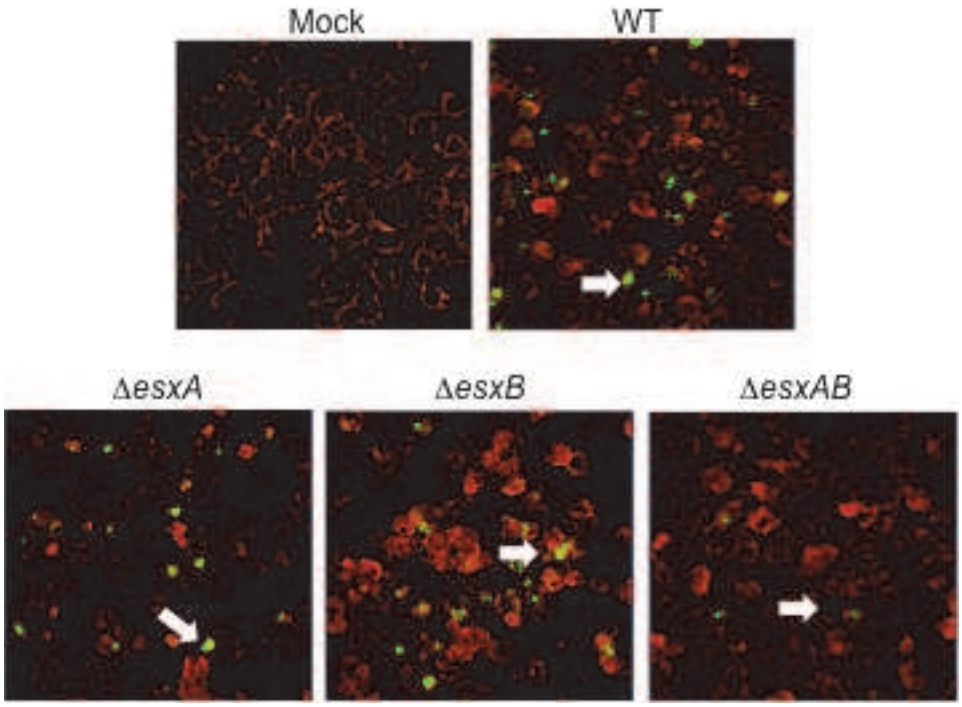


Fig. 6 The staphylococcal EsxA and EsxB proteins may mediate bacterial release from infected cells

Confocal microscopy analysis of A549 cells continuously infected by WT and mutant strains 9h 30min *p.i.*. Extracellular bacterial microcolonies and bacteria exiting infected dying cells can be seen on the cell layer (white arrows). Very few microcolonies were observed for the Δe*sxAB*. Bacteria were stained with anti-*S. aureus* (green) and cells were stained with wheat germ agglutinin (WGA) (red). All the images are representative of at least 3 independent infection experiments. The white arrows indicate bacterial 'microcolonies' outside epithelial cells.

Supplementary Figures

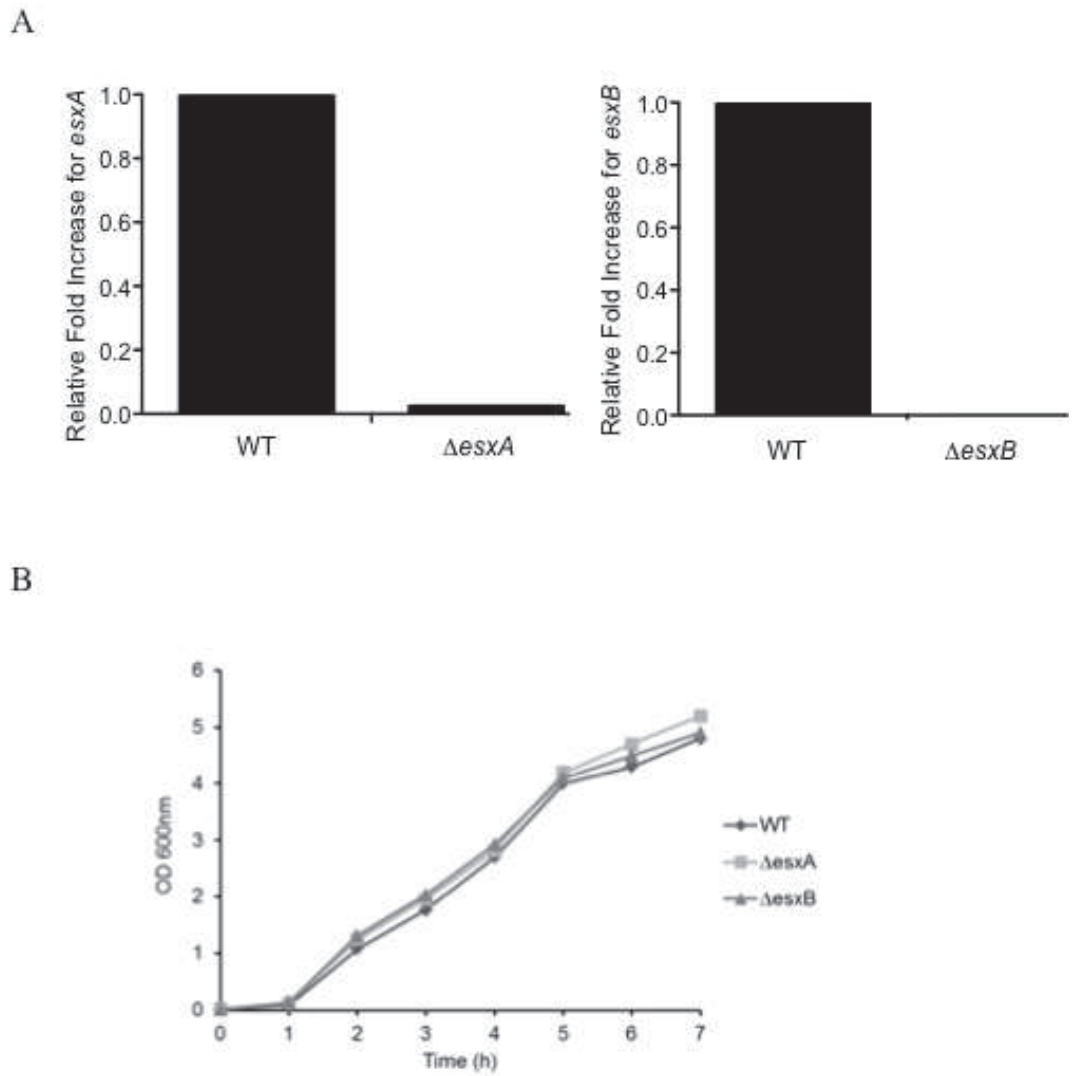


Fig. S1 Expression analysis of *esx* deletion mutants and growth curves.
A Expression analysis of *esxA* and *esxB* genes in USA300 WT strain and in different *esx* mutants by quantitative real-time PCR (qRT-PCR). Relative quantification was performed using 16S rRNA as internal control. **B** Growth curve in TSB medium does not reveal any growth defects.

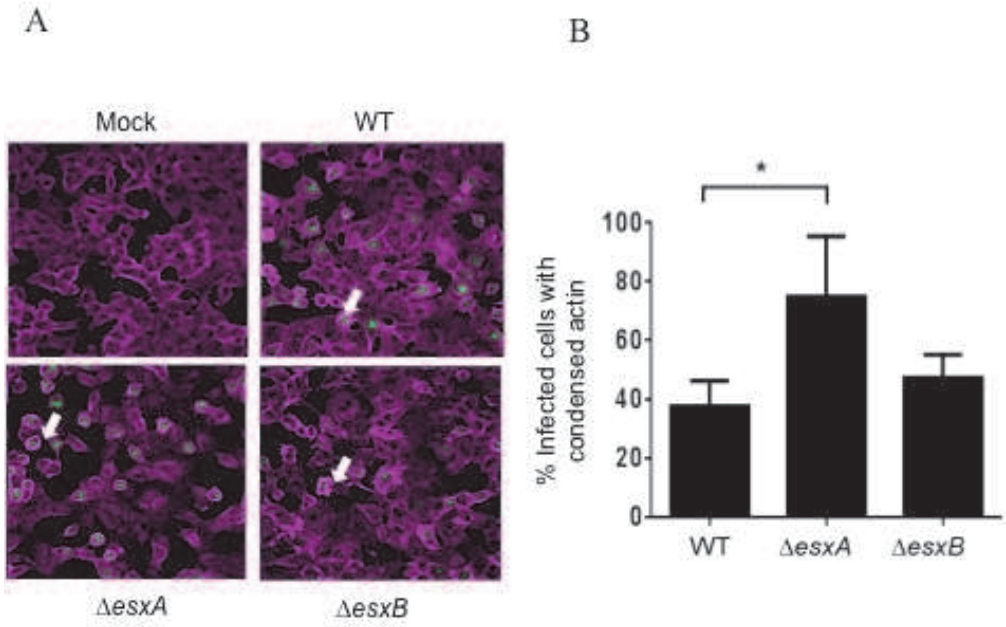


Fig. S2 *esxA* mutant is not defective for invasion, but induces more actin polymerization.

A Confocal microscopy of A549 cells infected by the WT and the *esx* mutant strains Δ*esxA* and Δ*esxB* 4h *p.i.* Vancomycin-Bodipy-FL stains *S. aureus* wall, while phalloidin stains F-actin. Polymerized actin (condensed form) can be observed as brightly stained cell membranes on rounded cells (white arrows). **B** The graph shows quantitation of rounded infected cells with cell membranes bearing polymerized actin for the WT and *esx* mutant-infected cells. Data are representative of 3 independent experiments. * indicates significant differences, one way ANOVA, Tukey's multiple comparisons test $P<0.05$.

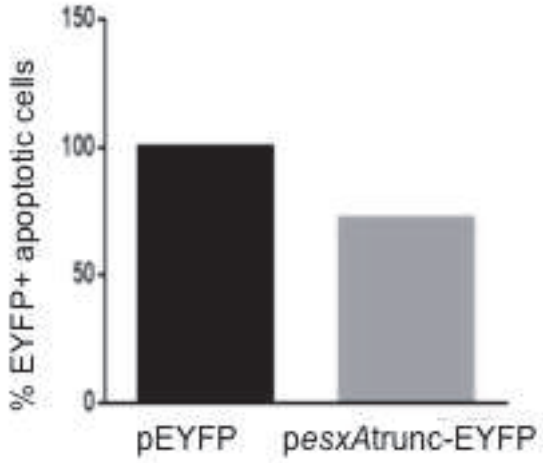


Fig. S3 Flow cytometry analysis of apoptotic A549 cells transfected with truncated *esxA*

24h post transfection cells were dissociated and then stained with Aqua Live-Dead and Annexin V-APC. The percentage of apoptotic cells (early and late apoptotic cells) was measured in the subpopulation bearing EYFP, *i.e.* transfected cells. Data are shown as a percentage of cells transfected with the control vector. Data are from one representative experiment

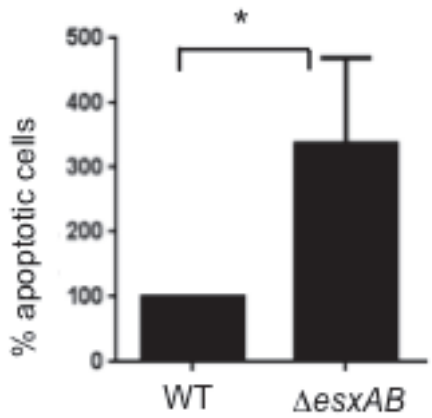


Fig. S4 Δe*sxAB* mutant induces more apoptosis in epithelial cells compared to WT.

Quantitation of apoptotic cells using flow cytometry for A549 cells infected by WT and Δe*sxAB* strain at 6h *p.i*. Cells were stained with Aqua Live-Dead (1:500), for the detection cell dead, and with Annexin V-FITC for the detection of apoptotic cells. Data are the mean of 3 independent experiments +/- s.d. * indicates significant differences using a one tailed Mann Whitney test ($P< 0.05$).

Conclusions

The human pathogen *Staphylococcus aureus* is responsible for the vast majority of healthcare-associated infections worldwide [5]. Over the past 30 years, *S. aureus* strains have acquired resistance to different antibiotics, most notably β-lactam compounds (MRSA, methicillin-resistant *S. aureus*) [6]. In the USA, MRSA strains are isolated in more than 60% of nosocomial infections and are also a frequent cause of community-acquired disease. The recent emergence of vancomycin-resistant MRSA strains has been viewed as testimony for the possible return to a pre-antibiotic era [8]. For these reasons the treatment of *S. aureus* infections is becoming problematic.

The manifestations of staphylococcal diseases vary greatly, ranging from minor skin infections to life threatening pneumonia, septicemia and endocarditis. Depending on the body site and time of infection, *S. aureus* virulence factors are known to be differentially expressed, thus posing a significant challenge to targeted vaccine development [13].

The diverse immune evasion strategies exhibited by *S. aureus* are well known. *S. aureus* can dwell in niches that are out of reach of normal humoral responses, such as in biofilms [54] and there is recent evidence that auxotrophic small-colony variants of *S. aureus* may persist intracellularly. This, perhaps transient, intracellular lifestyle may provide protection against exposure to antibiotics and host immune responses, as well as a favorable environment for the formation of resistant variants [61, 62]. A major future task is to find new and effective treatment for *S. aureus* infections. More specifically, there is a pressing need to identify new therapeutic strategy against *S. aureus*. To overcome the spectrum of diseases caused by this pathogen, a vaccine should ideally target virulence factors expressed in different stages and types of disease as well as those factors involved in immune evasion.

S. aureus pathogenesis is multifactorial and depends on the expression of different

secreted and surface exposed proteins of *S. aureus*. Therefore, a better understanding of which genes and proteins are differentially expressed during pathogenesis and infection is necessary to understand the increased persistence and resistance of *S. aureus*. Thus, secreted proteins are critical in mediating virulence. Recently, a Type VII secretion systems have been described for several Gram-positive bacteria including *S. aureus* (Ess secretion-system) [39, 43]. These secretion systems have been best studied in mycobacteria, where one such Type VII locus, the ESX-1 was shown to be important in virulence and granuloma formation [45].

EsxA and EsxB, virulence factors secreted by the Ess system are well conserved across clinical *S. aureus* strains and are currently being considered as potential vaccine candidates. The structurally similar *M. tuberculosis* Esx proteins have previously been successfully used to engineer new vaccines for TB since in the current BCG vaccine the ESX-1 locus is deleted.

Although *esxA* and *esxB* genes are located in the same locus and are in the same gene clusters, it appears that they are regulated in different way and in contrast to the *M. tuberculosis* ESAT-6 and CFP-10 genes, they are not co-transcribed. The staphylococcal EsxA has been reported to be regulated by sigma factor B and σB-controlled SpoVG, and is expressed as a single transcript, while we do not know how EsxB is regulated [52]. While the mycobacterial Esx proteins are important in several host-associated functions such as cell survival and granuloma formation little is known about staphylococcal Esx proteins during pathogenesis. The Esx proteins are believed to be crucial for bacterial persistence in a staphylococcal abscess[39].

During my study I have analyzed different aspects of these proteins including expression, secretion and role during *in vitro* infection. We characterized the expression of *esxA* and *esxB* in representative methicillin sensitive (MSSA)-Newman and resistant *S. aureus* (MRSA)-USA300 isolates under *in vitro* conditions. Although the genes are completely conserved, Esx proteins were

expressed and secreted to different extents in these two strains. Differences in the expression showed that both genes are more expressed by MRSA USA300 strain and this suggests that the regulation of expression or secretion of these proteins may differ in different strains of *S. aureus*. The gene expression analysis, performed during different *in vitro* conditions, indicate that these proteins are induced under selective conditions. A quantitative real time results showed that *esxA* and *esxB* are switched on during late growth phase in a rich medium and are little down-regulated in particular stress conditions as in presence of serum. Furthermore, these genes are modulated in biofilms and during intracellular infection that are likely to occur during infection. An induction of *esxB* expression was detected both during biofilm formation and after infection, while a mild increase of *esxA* transcript levels was detected intracellularly.

In order to better characterize the biological function of these two virulence factors, we made single unmarked deletion mutants in two relevant clinical strains, Newman and USA300. Employing isogenic mutants we observed that Δ*esxB* accumulated more biofilm *in vitro*. Using expression microarray approaches to investigate changes in the transcriptome we observed that none of other staphylococcal genes were significantly differential expressed in the *esxB* mutant comparing to WT strain, indicating a potential direct role of this proteins in biofilms. Altought we do not understand the precise role of EsxB in biofilms, we speculate that EsxB may be important in dissemination of biofilms.

Clinical studies have reported the presence of intracellular staphylococci from nasal epithelial cells, indicating that this could serve as a reservoir for recurrent infections [60]. Despite several studies describing intracellular staphylococcal infection *in vitro,* there is very little understanding of the bacterial factors or the mechanisms involved in intracellular survival of *S. aureus*. In this study we investigate the biological role of EsxA protein during intracellular infection of epithelial cells and we show that EsxA modulates apoptosis, a well-known mechanism of cell death. An isogenic *esxA* mutant induced more apoptosis

compared with WT in *S. aureus* infected epithelial cells and expressing EsxA within the cells resulted in delayed host cell apoptosis. While the staphylococcal toxin, alpha hemolysin (Hla) was reported to induce apoptosis upon intracellular staphylococcal infection[70]. As seen for many other bacteria, *S. aureus* may be also able to block apoptosis during *in vitro* infection of epithelial cells, thus allowing efficient replication of bacteria.

Another aspect that was studied was the mechanisms underlying secretion via the Ess apparatus. While the homologus mycobacterial EsxA and EsxB function as a heterodimer [49] the staphylococcal EsxA was crystallized as a homodimer, and there is no current evidence suggesting that the two proteins interact each other [51]. On the contrary to what is already known for mycobacterial Esx proteins, the interactions between the various Ess components have not yet been mapped completely. Recent work further reports that deletions in either gene also affects secretion of additional Ess substrates, EsxC and EsxD. EsxA and EsxB were reported to interact with different Ess substrates such as EsxC and EsxD respectively[53]. In order to detect which are the other proteins involved in these interactions, we employed a tandem affinity purification method (TAP). TAP-based purification in combination with mass spectrometry was used to detect specific interactions in various systems. Use of tagged proteins expressed by *S. aureus* will allow detection of any native bacterial proteins. The pull down experiments did not revealed any interaction between Esx proteins during the *in vitro* growth. This may be due to the possibility that the tags at the C- or the N-terminus of the protein interfere in interactions. However we cannot exclude that the multiprotein complex acts during alternate more optimal conditions. It is possible that the proteins function together in a multiprotein complex with other host and/or staphylococcal proteins during infection.

Co-dependent secretion of Ess substrates has been reported, similar to that observed for mycobacterial substrates with mutation of EsxB affecting secretion of EsxA and *vice versa* [39]. The signal sequence that mediates the Esx secretion in *S.*

aureus is not known. In an elegant series of studies in *M. tuberculosis* was reported that C-terminal residues are important for interaction of the mycobacterial EsxB with other proteins of the apparatus and for secretion [49]. Recently, a C-terminal motif (YxxxD/E) of the Ess substrate EsxD, was shown to be required for secretion of EsxA and EsaC [53]. Based on the homology between the *S. aureus* and *M. tuberculosis* Esx proteins we identified the high conserved residues that could have a functional role in secretion. The truncation of C-terminal tail of EsxA protein, which contains residues conserved in at least the Ess substrates EsxA and EsxB, was shown to be required for secretion *in vitro* and during *in vitro* intracellular infection.

The studies from my PhD wished to extend the scientific knowledge about the functional role of two key staphylococcal virulence factors: EsxA and EsxB, in order to define their relevance in *S. aureus* pathogenicity as well as their importance as vaccine candidates.

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List of publications

The staphylococcal Esx proteins modulate apoptosis and release of intracellular *Staphylococcus aureus* from epithelial cells

Charalampia G. Korea*, Giuliana Balsamo*, Alfredo Pezzicoli, Simona Tavarini, Fabio Bagnoli, Davide Serruto and Meera Unnikrishnan

*co-first author

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