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Pathogenic conversion of coagulase-negative staphylococci

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Abstract

Humans and animals are colonized by members of the genus *Staphylococcus*, however only some of these species evolved to cause invasive disease. The genetic basis for conversion of commensal staphylococci into pathogens is not known. We hypothesized that *Staphylococcus aureus* genes for coagulation and agglutination in vertebrate blood (*coa*, *vwb* and *clfA*) may support pathogenic conversion. Expression of *coa* and *vwb* in *Staphylococcus epidermidis* or *Staphylococcus simulans* supported a coagulase-positive phenotype but not the ability to cause disease in a mouse model of bloodstream infection. However, the simultaneous expression of *coa*, *vwb* and *clfA* in coagulase-negative staphylococci enabled bacterial agglutination in plasma and enhanced survival of *S. simulans* in human whole blood. Agglutination of *S. simulans* in the bloodstream of infected mice upon expression of *coa*, *vwb* and *clfA* provided also a mean for dissemination and replication in distal organs. Thus, the acquisition of genes for bacterial agglutination with fibrin appear sufficient for the conversion of commensal staphylococci into invasive pathogens.

Keywords

coagulation; clumping; agglutination; staphylococcus; abscess; virulence

1. Introduction

Staphylococcus aureus colonizes the nasopharynx, skin and gastrointestinal tract of humans and is also an invasive pathogen, frequently causing skin and soft tissue infection, bacteremia or abscess lesions in any organ tissue [1–3]. The genus *Staphylococcus* is comprised of more than 40 species, which share 16S ribosomal RNA sequence, low genomic DNA G+C content, cell wall composition (pentaglycine crossbridges, lysostaphin sensitivity), cytochrome and menaquinone profiles as well as susceptibility to erythromycin, bacitracin and furazolidone [4]. Humans and their domesticated animals are colonized by different species of the genus *Staphylococcus*: *S. aureus*, *S. auricularis*, *S. capitis*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. saprophyticus*, *S. simulans* and *S. warneri* [5].

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Conflict of interest

HKK, DM and OS declare a conflict of interest as inventors of patent applications related to the development of *S. aureus* vaccines.

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Of these, however, only *S. aureus* evolved to consistently cause invasive disease in healthy immuno-competent individuals [5].

Clinical diagnosis of *S. aureus* infection and initiation of appropriate antibiotic therapy requires laboratory identification of bacteria from superficial lesions, drainage of deep-seated abscesses or blood cultures [6]. As clinical samples may be contaminated with commensal *Staphylococcus* species, two laboratory tests, coagulation and clumping, exploit key microbiological traits associated with *S. aureus* to identify the pathogen [7, 8].

The coagulation test examines the ability of microbes inoculated into plasma of producing clots [9]. *S. aureus* isolates generate positive test results, owing to the expression of *coa* and *vwb*, whose products are secreted into the extracellular medium [10]. Coagulase (Coa) and von-Willebrand factor binding protein (vWbp) each associate with prothrombin (PT), also designated clotting factor II (FII), of the host coagulation cascade and generate enzymatically active complexes: Coa-PT and vWbp-PT [11]. Unlike thrombin, *i.e.* proteolytically activated FIIa, Coa-PT and vWbp-PT complexes cleave fibrinopeptides A and B off fibrinogen without cutting other thrombin substrates (FV, FVIII, FXI, FXIII, protein C, antithrombin and plasmin)[12, 13]. In addition, the vWbp-PT complex interacts and activates human FXIII in a non-catalytic manner [13].

The clumping test examines the agglutinating attributes of bacteria immersed in plasma. *S. aureus* isolates test positive in this assay owing to the secretion of coagulases (Coa and vWbp)[14] and to the assembly of clumping factor A (ClfA) in the bacterial envelope [15]. The joint action of coagulases in generating fibrin cables and of ClfA in promoting *S. aureus* association with fibrin protects bacteria from phagocytosis [14, 16]. Unlike *S. aureus*, coagulase-negative staphylococcal isolates, for example *S. epidermidis* or *S. simulans*, score negative in both coagulase and clumping tests [4].

Several *Staphylococcus* species produce coagulases, however these microbes (*S. delphini*, *S. intermedius* and *S. pseudintermedius*) adapted to causing invasive disease in other hosts: mink, fox, pigeon, cats or dogs [17, 18]. Genome sequence analysis of pathogenic and non-pathogenic *Staphylococcus* species suggested that horizontal gene transfer may be responsible for the evolution of pathogenic staphylococci [19]. However, it is not clear what genes may be sufficient for the conversion of commensal staphylococci into an invasive pathogen. This question is addressed here and we show that transfer of the *S. aureus* genes for coagulation and agglutination (*coa*, *vwb* and *clfA*) is sufficient to convert the coagulase-negative species *S. simulans* into a pathogen that coagulates vertebrate blood, agglutinates in human and mouse plasma and disseminates from the vasculature of infected mice to replicate in distal organs.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Wild-type isolate *S. aureus* Newman and its isogenic *coa/vwb/clfA* variant were described previously [14, 20]. *S. epidermidis* ATCC 12228 was obtained from American Type Culture Collection (ATCC.org). *S. simulans* MK148 (ATCC 27848) was a gift from Prof. Friedrich

Gotz. All staphylococcal strains were grown in Brain Heart Infusion (BHI) broth at 30°C. Strains harboring plasmids pOS1 and its derivatives were grown in BHI supplemented with 5 µg chloramphenicol/ml. *Escherichia coli* strain DC10B was cultivated in Luria broth with 100 µg ampicillin/ml at 30°C.

2.2. Cloning procedures and plasmids

The shuttle vector pOS1 (also referred to as vector) was used for all cloning procedures [21]. Plasmid *pcoa-vwb* expressing the *coa* and *vwb* genes under their respective promoters was described previously [10]. The *clfA* gene with its native promoter was cloned by amplification with the polymerase chain reaction (PCR) using genomic DNA from *S. aureus* Newman as template and the primer pair 5'-

CGGGGATCCAAGCTTTTCAAGCTAGGATTACATTAGGTA-3' and 5'-

GCGGAATTCGAATCATATGATTAATTTAATATCA-3'. The ends of the PCR product were cut with BamHI and EcoRI restriction enzymes and ligated into pOS1 cut with the same enzymes, thereby generating *pclfA*. The *clfA* PCR product was also cut and ligated into the BamHI and SmaI restriction sites of *pcoa-vwb* to generate *pcoa-vwb-clfA*. All cloning steps were performed in *E. coli* DC10B [22]. Plasmid clones were verified by DNA sequencing prior to electroporation into *S. epidermidis* ATCC 12228 and *S. simulans* MK148.

2.3. Culture fractionation and immunoblot analysis

Overnight cultures were diluted 1:100 into BHI and grown for 6 hours. Samples were normalized to the same absorbance at 600 nm of 4 units (A_{600} 4) and one milliliter of culture was centrifuged at 8,000 $\times g$ for 5 min. Supernatant containing coagulases was transferred to another tube. Bacterial cells containing cell wall bound ClfA were suspended in 1 ml 50 mM Tris-HCl (pH 7.5), 150 mM NaCl and incubated with 10 µg lysostaphin/ml at 37°C for 30 min. Ice-cold trichloroacetic acid (150 µl TCA) was added to each sample, mixed and incubated for 30 min on ice; TCA precipitated proteins were sedimented by centrifugation (10,000 $\times g$ for 15 min), washed with cold acetone, air-dried and solubilized in 100 µl SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.01 % bromophenol blue). Protein samples were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for 30 min with 10 ml TBS-T (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20) containing 5% milk and 50 µl human IgG (Sigma) prior to the addition of affinity-purified rabbit antibodies against Coa (α -Coa), vWbp (α -vWbp) or ClfA (α -ClfA) for 1 hour at room temperature. PVDF membranes were washed three times for 10 min with TBS-T and incubated with secondary anti-rabbit HRP-linked IgG for 1 hour at room temperature, washed, and developed using enhanced chemiluminescence substrates.

2.4. Coagulation and clumping assays

Overnight cultures were diluted 1:100 into 10 ml BHI, grown for 6 hours and normalized to A_{600} 4. For the coagulation assay, 10 µl bacterial suspensions were mixed with 90 µl anticoagulated mouse plasma (sodium citrate) or rabbit plasma (EDTA) and incubated at room temperature for 24 hours prior to tilting the tubes. Rabbit plasma anti-coagulated with EDTA was obtained from BBL™ (Coagulase Plasma). Mouse plasma was obtained by

drawing blood into 10 mM sodium citrate via cardiac puncture. Mouse blood was incubated at room temperature for 10 min, centrifuged at $2,000 \times g$ for 10 min and plasma was retrieved. For the clumping assay, bacteria from 10 ml staphylococcal culture were centrifuged, diluted in 1 ml PBS to A_{600} 4 and incubated with 10 μ l of 3 mg human fibrinogen (Sigma)/ml PBS. Clumping of staphylococci was observed by briefly inverting the tubes.

2.5. Agglutination assay

The agglutination assay was performed as described previously [14]. Briefly, 10 ml bacterial culture was centrifuged, washed and suspended in PBS and normalized to A_{600} 4. Staphylococci were incubated with SYTO 9 (1:500) (Invitrogen) for 15 min. The cells were washed twice with PBS and suspended in 1 ml PBS. Bacterial suspensions were mixed 1:1 (vol/vol) with human or mouse citrate-plasma or rabbit EDTA-plasma on glass slides and incubated for 30 min. Samples were viewed and images were captured on an IX81 live cell total internal reflection fluorescence microscope using a 20 $\times$ objective (Olympus). At least 10 images were captured for each sample. Areas of agglutination in each image were measured and quantified using ImageJ software. Statistical significance was determined by ordinary one-way analysis of variance (ANOVA) using Prism (GraphPad Software, Inc.).

2.6. Whole blood killing assay

Overnight cultures of *S. simulans* MK148 strains were diluted 1:100 into BHI and grown for 3 hours. Bacterial replication *ex vivo* was measured as described previously [16]. Briefly 0.475 ml of human blood anticoagulated with 5 μ g desirudin/ml was incubated with 25 μ l bacterial suspension (1.25×10^7 CFU). Following incubation at 37°C for 0 or 60 min, 0.5 ml of PBS containing 0.5% saponin, 100 U streptokinase K, 50 μ g trypsin, 1 μ g DNase, 5 μ g RNase was added to each sample for 10 min at 37 °C, prior to plating on solid medium for enumeration of CFU. Statistical analysis was performed with the two-way ANOVA. Human volunteers were enrolled under a protocol that was reviewed and approved by the University of Chicago's Institutional Review Board.

2.7. Murine infection model

Overnight cultures of *S. simulans* MK148 strains were diluted 1:100 into BHI and grown for 3 hours. Bacteria were centrifuged, washed and suspended in PBS and normalized to A_{600} 4. Colony forming units (CFU) of the inoculum were determined by serial dilutions of samples and plating on BHI agar plates. For infection, BALB/c mice (6 weeks old, female; Charles River Laboratories) were anesthetized via intraperitoneal injection with 65 mg/kg ketamine and 6 mg/kg xylazine. Mice in groups of 8–10 were infected by injection of a 100 μ l bacterial suspension (5×10^7 CFU) into the periorbital venous plexus. Mice received chloramphenicol in the drinking water (1 mg/ml) for the duration of the experiment to prevent plasmid loss in the infecting bacteria. On day 5 after infection, mice were euthanized by CO₂ inhalation and cervical dislocation. Bacterial loads in kidneys were determined by plating serial dilutions on BHI agar containing chloramphenicol using tissues homogenized in PBS with 0.1% Triton X-100; CFU were enumerated after incubation of plates at 30°C [23]. Kidneys were also fixed in 10% formalin for 24 h at room temperature, embedded in paraffin, thin-sectioned, and stained with hematoxylin and eosin. Stained tissues were

observed by light microscopy to enumerate abscess lesions. All statistical analyses were performed using the one-way ANOVA on ranks (Kruskal-Wallis test) with Dunn's post-hoc test. *P* values of < 0.05 were deemed significant. All mouse experiments were performed and conducted in accordance with the institutional guidelines following experimental protocol review and approval by the Institutional Biosafety Committee (IBC) and the Institutional Animal Care and Use Committee (IACUC) at the University of Chicago.

3. Results

3.1. Expression of agglutination factors in coagulase-negative staphylococci

E. coli/*Staphylococcus* shuttle vector pOS1 was used to generate expression plasmids for *clfA* (*pclfA*), *coa-vwb* (*pcoa-vwb*) as well as *coa-vwb-clfA* (*pcoa-vwb-clfA*) (Fig. 1A). Plasmids were electroporated into *S. simulans* MK148 (ATCC27848) and *S. epidermidis* ATCC12228, both coagulase-negative, non-pathogenic isolates from the human skin [24, 25]. Transformants of both bacterial species stably maintained their plasmids in the presence of chloramphenicol selection. To quantify the production of agglutination factors, staphylococci were grown to early stationary phase, cultures were normalized to the same absorbance at 600 nm, and centrifuged to separate the culture medium from the bacterial sediment. Staphylococci were suspended in buffer and peptidoglycan was digested with lysostaphin to generate a bacterial lysate. Proteins in culture medium and lysate samples were precipitated with trichloroacetic acid and analyzed by immunoblotting with rabbit antibodies specific for Coa, vWbp or ClfA (Fig. 1B). As expected, Coa and vWbp were detected in the culture medium of wild-type *S. aureus* strain Newman, whereas ClfA was detected in the bacterial lysate (Fig. 1B). As control, Coa, vWbp and ClfA were not detected in the culture medium or lysate of the isogenic *coa/vwb/clfA* mutant strain (Fig. 1B). Similarly, *S. simulans* and *S. epidermidis* carrying pOS1 only (vector) did not produce Coa, vWbp or ClfA (Fig. 1B). ClfA was, however, detected in the lysates of strains harboring *pclfA* but not in strains carrying *pcoa-vwb* (Fig. 1B). Reciprocal results were obtained when probing culture media with antibodies specific for Coa and vWbp (Fig. 1B). Finally, *S. simulans* or *S. epidermidis* carrying *pcoa-vwb-clfA* produced all three agglutination factors, Coa, vWbp and ClfA (Fig. 1B). Of note, the abundance of agglutination factors varied between *S. aureus*, *S. simulans* and *S. epidermidis*. In Fig. 1B, one tenth of *S. aureus* sample size was loaded on SDS-PAGE for a comparative analysis of Coa and vWbp production in coagulase-negative staphylococci. For the assessment of ClfA production, the *S. aureus* sample size loaded on a gel was concentrated 5-times as compared to *S. simulans* and *S. epidermidis* samples. Thus, the strains produced different levels of agglutination factors. Reduced production of some of these factors may at least in part be attributable to the *saeS* L18P allele of *S. aureus* Newman, which results in constitutive activation of the SaeS sensory kinase and high level expression of *coa* and *vwb* [26, 27].

3.2. Conversion of coagulase-negative staphylococci

Inoculation of *S. aureus* into rabbit plasma promotes clotting [7, 8], a diagnostic feature that is critically dependent of the expression of *coa* and *vwb* [10]. To determine whether plasmid-borne expression of *coa* and *vwb* converted the assay phenotype of coagulase-negative staphylococci, bacteria were inoculated into mouse or rabbit chelated-plasma and

incubated at 25°C. As controls, *S. aureus* Newman inoculation promoted coagulation of human and rabbit plasma in less than 2 hours, and in less than 12 hours in mouse plasma; coagulation was not observed when plasma was inoculated with the *coa/vwb/clfA* mutant (Fig. 2). Unlike *S. aureus*, neither *S. simulans* (vector) nor *S. epidermidis* (vector) was capable of clotting citrate-plasma. However, inoculation of *S. simulans* or *S. epidermidis* carrying *pcoa-vwb* or *pcoa-vwb-clfA* into plasma caused clotting in a manner similar to that of *S. aureus* (Fig. 2). Coagulase-negative staphylococci (*pclfA*) expressing *clfA* failed to clot vertebrate plasma (Fig. 2), in agreement with the concept that ClfA alone does not function as a coagulase [14]. Thus, expression of *coa* and *vwb* converts *S. simulans* and *S. epidermidis* into coagulase-positive staphylococci.

3.3. Conversion of clumping-negative staphylococci

Suspension of *S. aureus* in fibrinogen solutions promotes clumping of the bacteria, which spontaneously sediment to the tube bottom [28](Fig. 3). Earlier work had demonstrated *S. aureus* clumping to be dependent on the expression of *clfA* [15]. In agreement with earlier results, the *S. aureus coa/vwb/clfA* mutant did not display a clumping phenotype (Fig. 3). Further, suspension of *S. simulans* or *S. epidermidis* in fibrinogen solution did not result in bacterial sedimentation to the bottom of reaction tubes (Fig. 3). In contrast, coagulase-negative staphylococci harboring *pclfA* or *pcoa-vwb-clfA* sedimented following bacterial suspension in fibrinogen solution, similar to wild-type *S. aureus* (Fig. 3). *S. simulans* and *S. epidermidis* expressing coagulases without *clfA* (*pcoa-vwb*) also did not display a clumping phenotype (Fig. 3).

3.4. Conversion of agglutination-negative staphylococci

Another diagnostic tool, the slide agglutination test, monitors agglutination of bacteria immersed in calcium-chelated plasma (reviewed by [7]). This rapid test is used for positive identification of *S. aureus* and differential diagnosis with non-pathogenic staphylococci such as *S. simulans* and *S. epidermidis*. Bacterial agglutination requires two host factors, fibrinogen and prothrombin [29], as well as staphylococcal coagulases and clumping factor A [14]. As controls, Syto-9 stained wild-type, but not *coa/vwb/clfA* mutant *S. aureus*, generated aggregates of fluorescent bacteria in human plasma. *S. epidermidis* and *S. simulans* did not agglutinate in human plasma. However, coagulase-negative staphylococci expressing *coa*, *vwb* and *clfA* (*pcoa-vwb-clfA*) agglutinated in human plasma, whereas strains expressing only *coa-vwb* (*pcoavwb*) or *clfA* (*pclfA*) did not (Fig. 4A). Similar results were obtained when staphylococcal agglutination was measured in mouse or in rabbit plasma (Fig. 4B).

3.5. Expression of *coa-vwb-clfA* promotes virulence

Virulence associated with expression of coagulation and agglutination factors was measured using a whole blood killing assay and a sub-lethal mouse model of infection. For these experiments, only the *S. simulans* variants were used. All plasmid-bearing variants of *S. simulans* replicated similarly. However, the introduction of plasmid *pcoa-vwb-clfA* significantly reduced replication of *S. epidermidis* as compared to plasmids *pcoa-vwb* or *pclfA* (data not shown).

Earlier work demonstrated that *S. aureus* evades opsonophagocytosis and replicates in human blood via mechanisms involving *coa-vwb* induced fibrin formation and *clfA*-mediated assembly of fibrin shields [16]. This work established that *S. aureus* exploits the host factors prothrombin and fibrinogen to induce the formation of fibrin agglutinates and shield bacteria from phagocytes [16]. Enumeration of *S. aureus* in blood requires release of bacteria from fibrin cables, which is achieved by treating samples with streptokinase, an activator of plasminogen [30]. When incubated in fresh human blood, approximately 99% of the inoculum of *S. simulans* carrying vector alone (1.25×10^7 CFU) was killed within 60 min (Fig. 5). Expression of *coa-vwb-clfA*, but not of *coa-vwb* or *clfA* alone, significantly improved the survival of *S. simulans* in whole blood (Fig. 5).

Next, *S. simulans* variants were inoculated intravenously in mice. Earlier work has shown that inoculation of 1×10^7 CFU *S. aureus* Newman into immune-competent mice leads to disseminated abscess formation in many different organ tissues within 4–5 days [23]. Abscess lesions caused by *S. aureus* are highly structured [31]. The pathogenesis of these lesions requires expression of coagulases, i.e. *coa* and *vwb*, but not of *clfA* [10]. On the other hand, expression of all three genes, *coa-vwb-clfA*, contributes to the pathogenesis of staphylococcal bacteremia [14]. Intravenous inoculation of 5×10^7 CFU *S. simulans* strains into BALB/c mice did not cause lethal disease. Five days following infection, animals were euthanized, necropsied and kidneys removed for the enumeration of bacterial load in tissue homogenates (left kidney of necropsied animals) or subjected to histopathological analysis (right kidney). The data showed that mice infected with *S. simulans* (vector) or *S. simulans* (*pcoa-vwb*) harbored very modest bacterial loads (Fig. 6A). Most kidneys were sterile (Fig. 6A) and H&E staining of kidney sections revealed healthy tissues (Fig. 6CD) without lesions (Fig. 6B). Infection of BALB/c mice with *S. simulans* (*pclfA*) resulted in a modest enhancement of virulence whereby bacterial counts increased in some animals (Fig. 6A) and occasional lesions could be observed in the kidney sections (Fig. 6BE). ClfA-induced lesions presented as immune cell infiltrates without detectable staphylococcal community at the center of these lesions (Fig. 6E). Inoculation of BALB/c mice with *S. simulans* (*pcoa-vwb-clfA*) resulted in a dramatic increase in bacterial load in renal tissue (Fig. 6A). Histopathology analysis revealed numerous lesions in kidneys with large infiltrates of immune cells, predominantly granulocytes (Fig. 6BF). These lesions appeared heterogeneous in shape and staining but were found throughout the organ as if small hemorrhages enabled access of bacteria from the vasculature and into organ tissue. Taken together, these data reveal that expression of the three agglutination factors *coa*, *vwb* and *clfA* in coagulase-negative staphylococci promotes bacterial escape from the bloodstream and replication in infected host tissues.

4. Discussion

Members of the genus *Staphylococcus* evolved to colonize the epidermis, mucous membranes and gastrointestinal tracts of vertebrates [4]. Coagulase-negative staphylococci colonize a wide range of host species [32]. In contrast, pathogenic *Staphylococcus* species acquiring genetic traits for invasive disease target a narrow range of hosts [17, 19, 33]. Laboratory studies with *S. aureus*, the most prominent human pathogen of this genus, characterized coagulase and agglutination activities as hallmarks for the diagnosis of clinical

S. aureus isolates [7, 8]. Subsequent identification of the genetic determinants for coagulation and agglutination enabled experiments that assessed the contributions of these factors towards infectious disease pathogenesis in animal models by infecting animals with defined genetic mutants [10, 14]. This research revealed that genes encoding coagulases (*coa* and *vwb*) as well as clumping factor A (*clfA*) contribute to the establishment of *S. aureus* bacteremia [10, 34] and that mutants lacking all three determinants are essentially avirulent [14]. These experiments represent a classic genetic approach and ask whether a gene is required for disease. Here, we ask whether these genes are sufficient to cause disease by expressing *coa*, *vwb* and *clfA* in a heterologous and related bacterial host. We find that the simultaneous expression of genes for staphylococcal coagulation and agglutination is sufficient to convert coagulase-negative *S. simulans* into an invasive pathogen.

S. aureus agglutination with fibrin cables requires two host factors, prothrombin and fibrinogen (reviewed by [7]). More recent publications use the clumping test to describe the same reaction as agglutination [9]. However, Hawiger and colleagues showed that *S. aureus* binds to purified fibrinogen, specifically the C-terminal 17 residues of the γ -chain, which also functions as a pocket in the D domain of fibrin enabling polymerization of monomers into fibrin cables [35, 36]. This fibrinogen binding assay was subsequently used to identify *clfA* [15]. Several other *S. aureus* surface proteins also bind to fibrinogen, however ClfA is a key factor for staphylococcal agglutination with coagulase-derived fibrin cables [14]. Expression of *clfA* in the non-pathogenic commensal *Lactococcus lactis* leads to functional assembly of clumping factor A with fibrinogen binding activity in the bacterial envelope [37]. Injection of 1×10^9 CFU *L. lactis* expressing *clfA* into the tail vein of BALB/c mice kills the experimental animals within 24 hours, whereas animals challenged with *L. lactis* expressing *clfA*_{Y338A}, a variant that cannot bind fibrinogen [38], are not [39]. The pathophysiology of the lethal outcome in this model is not known, however we presume that the association of large numbers of Lactococci expressing *clfA* with fibrinogen generates thrombi blocking blood perfusion to vital organs. In the rat endocarditis model, *L. lactis* expressing *clfA* associate with and colonize the pre-damaged heart valve tissues for 48 hours but are subsequently eradicated by the host immune system [40]. As is shown here, expression of *clfA* in *S. simulans* is not sufficient to increase the virulence attributes of the heterologous host towards the establishment of abscess lesions. Similar results were obtained with the heterologous expression of *coa* and *vwb*. Although coagulase-negative staphylococci expressing *coa* and *vwb* produce abundant coagulase activity, this is not sufficient for the establishment of infectious lesions in mice. Co-expression of *clfA* and *coa-vwb*, which reconstitutes agglutination with fibrin in *S. simulans*, enhanced the virulence potential of this heterologous host. *S. aureus* abscess lesions present distinct morphological features at day 5 post infection [23]. Histology of *S. aureus* infected organs reveals that bacteria replicate at the center of abscess lesions and are protected by a fibrin deposit from concentric layers of immune cells which in turn are engulfed within eosinophilic deposits comprised of fibrin [10]. Infection with *S. simulans* (*pcoa-vwb-clfA*) resulted in the formation of small lesions with immune infiltrates not quite as mature as *S. aureus* abscesses yet numerous small lesions could be seen throughout the organ reminiscent of small hemorrhages or petechiae as is observed when capillaries leak blood into the skin. In *S. aureus* bloodstream infection, the agglutination factors Coa, vWbp and ClfA promote

endothelial adherence by inducing microthrombi with staphylococci embedded with fibrin shields [41]. This process has been proposed as one of the mechanisms responsible for the dissemination of *S. aureus* and subsequent invasion into organ tissues [7]. Data presented here support a model whereby Coa, vWbp, and ClfA are sufficient to promote *S. simulans* exit from the bloodstream by mechanically occluding blood flow in the microvasculature through the formation of bacterial agglutinates.

Stutzmann Meier and colleagues examined the virulence contributions of *coa* and *clfA* expressed in *Streptococcus gordonii*, a commensal of the human oropharynx [42]. Expression of *clfA* in *S. gordonii* enabled these bacteria (10^5 CFU inoculum) to adhere to rat endocardial tissue 12 hours post challenge [42]. However, adherence was not affected by co-expression of *coa* and *clfA* in *S. gordonii* [42]. It is not clear why co-expression of *coa* and *clfA* in streptococci does not generate increased virulence attributes in the rat endocarditis model. Nonetheless, we discuss several possibilities. First, *S. aureus* expresses two coagulases, *coa* and *vwb*, and cooperative effects between activators of hemostasis and *clfA* may require co-expression of all three genes, as is described here. In particular, adhesion of *S. aureus* to the wall of blood vessels under flow has been shown to be specifically mediated by vWbp [41]. Second, SD-repeat sequences of *S. aureus* ClfA are glycosylated and mutants lacking this post-translational modification display defects in agglutination with fibrin and in the murine abscess model [43]. Surface protein genes encoding SD repeats and fibrinogen binding activity are known to be expressed in coagulase-negative staphylococci [44]. The translational product of ClfA is likely glycosylated in *S. epidermidis* and *S. simulans*, as the mobility of ClfA on SDS-PAGE is similar to that of ClfA from *S. aureus* [43](Fig. 1). In agreement with this hypothesis, sequenced genomes of *S. epidermidis* and *S. simulans* isolates harbor homologues of *S. aureus* glycosyl-transferase genes (*sdgA* and *sdgB*) targeting SD repeats [43]. Third, Stutzmann Meier and colleagues measured adherence after a single 12 hour time interval, however it is not clear whether recombinant *S. gordonii* replicate on rat endocardial tissue to cause disease and whether such processes are impacted by the co-expression of *clfA* and *coa* [42].

In conclusion, expression of *S. aureus coa*, *vwb* and *clfA* is sufficient to confer onto coagulase-negative staphylococci the ability of agglutination with fibrin. Expression of *coa-vwb-clfA* increased the virulence of *S. simulans*, enabling the pathogenesis of invasive infections. We therefore propose that pathogenic conversion of commensal *Staphylococcus* species involves the acquisition of genes for agglutination with fibrin. In addition to *S. aureus*, similar conversions occurred in *S. delphini*, *S. intermedius*, *S. pseudintermedius* and *S. lugdunensis* [45, 46]. These microbes share the attributes of coagulating vertebrate plasma and of binding to fibrinogen/fibrin on the bacterial surface. The product of these reactions, a fibrin shield covering the bacterial surface, provides protection against host phagocytes [16, 34].

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Fig. 1. Production of Coa, vWbp and ClfA by *S. epidermidis* and *S. simulans*. (A) Diagram illustrating plasmid inserts carrying the *coa*, *vwb* or *clfA* genes from *S. aureus* Newman. (B) Culture supernatants were examined by immunoblotting with antibodies specific for Coa (α-Coa) and vWbp (α-vWbp) while whole culture lysates were used to examine ClfA production using antibodies specific for ClfA (α-ClfA). Cells cultures were normalized A_{600} 4 and *S. aureus* samples were diluted 10 times for Coa and vWbp or concentrated 5 times for ClfA analyses as compared to *S. epidermidis* and *S. simulans* extracts. Numbers to the left of blots indicate the position of molecular markers in kDa.

Strain	Mouse plasma		Rabbit plasma		Human plasma	
<i>S. aureus</i>		< 12 h		< 2 h		< 2 h
<i>S. aureus</i> Δ <i>coa/vwb/clfA</i>		—		—		—
<i>S. epi</i> (<i>pcoa-vwb</i>)		< 24 h		< 2 h		< 2 h
<i>S. epi</i> (<i>pclfA</i>)		—		—		—
<i>S. epi</i> (<i>pcoa-vwb-clfA</i>)		< 24 h		< 2 h		< 2 h
<i>S. epi</i> (vector)		—		—		—
<i>S. sim</i> (<i>pcoa-vwb</i>)		< 24 h		< 2 h		< 2 h
<i>S. sim</i> (<i>pclfA</i>)		—		—		—
<i>S. sim</i> (<i>pcoa-vwb-clfA</i>)		< 24 h		< 2 h		< 2 h
<i>S. sim</i> (vector)		—		—		—

Fig. 2.

Expression of *coa* and *vwb* promotes clotting by *S. epidermidis* and *S. simulans*. Anti-coagulated mouse, rabbit or human plasma was incubated with bacteria for up to 24 hours at 25°C. Tubes were tilted to assess coagulation. Data are representative of three independent experiments.

Fig. 3.

Expression of *clfA* promotes clumping by *S. epidermidis* and *S. simulans*. Sedimentation of bacteria was monitored immediately after mixing cultures with soluble fibrinogen and inverting the tubes. Data are representative of three independent experiments.

Fig. 4.

Staphylococcal agglutination in citrate-treated plasma. (A) Agglutination in human plasma of Syto-9 stained bacteria. Scale bar, 50 μ m. (B) Box and whisker plot representation of agglutination areas with anti-coagulated human, mouse and rabbit plasma. Statistical significance was determined using ANOVA (****, $P < 0.0001$).

Fig. 5.

Staphylococcal survival in human blood. Mid-log phase bacteria (1.25×10^7 CFU) were inoculated into freshly drawn human blood anti-coagulated with desirudin. At 0 min and 60 min, saponin- and streptokinase-containing buffer was added to lyse host cells and release agglutinated bacteria. Bacteria were counted by plating serial dilutions on solid medium and reported as CFU/ml. Data are from three independent experiments; error bars indicate standard deviation. Statistical significance was performed by two-way ANOVA analysis, (****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$).

Fig. 6.

Pathogenic conversion of *S. simulans* with *S. aureus* *coa*, *vwb* and *clfA*. Mice were injected in the peri-orbital venous plexus with 5×10^7 of *S. simulans* carrying either vector, *pcoa-vwb*, *pclfA* or *pcoa-vwb-clfA*, and euthanized 5 days following challenge. (A) Following necropsy, renal tissues were assessed for bacterial load. (B) Lesions observed in panels C through F were enumerated using 20–25 slides per infected group. The data in panels A and B represent the cumulative result of three independent experiments and were analyzed using the one-way ANOVA on ranks (Kruskal-Wallis test) with Dunn's post-hoc test (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant). (C-F) Microscopy images of hematoxylin-eosin-stained, thin-sectioned renal tissue. Whole kidney sections are shown and areas boxed in black are shown as a 20 $\times$ enlarged images. Images and insets in panels C and D did not reveal pathological features in renal tissues. In panels E and F, arrows point to replicating bacteria and immune cell infiltrates.