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***Staphylococcus aureus* β -Toxin Mutants Are Defective in Biofilm Ligase and Sphingomyelinase Activity, and Causation of Infective Endocarditis/Sepsis**

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Abstract

β -toxin is an important virulence factor of *Staphylococcus aureus*, contributing to colonization and development of disease.^{1–3} This cytotoxin has two distinct mechanisms of action: sphingomyelinase activity and DNA biofilm ligase activity. However, the distinct mechanism which is important for its role in infective endocarditis is unknown. We characterized the active site of β -toxin DNA biofilm ligase activity by examining deficiencies in site-directed mutants through *in vitro* DNA precipitation and biofilm formation assays. Possible conformational changes in mutant structure compared to wild type toxin were assessed via trypsin digestion analysis, retention of sphingomyelinase activity, and predicted structures based on the native toxin structure. We addressed the contribution of each mechanism of action in producing infective endocarditis *in vivo* in a rabbit model of infective endocarditis and sepsis. The H289N β -toxin *S. aureus* mutant, lacking sphingomyelinase activity, was decreased in lethality and infective endocarditis vegetation formation compared to wild type protein. β -toxin mutants disrupted in biofilm ligase activity did not decrease *S. aureus* lethality, but were deficient in infective endocarditis vegetation formation compared to wild type protein. Our study begins to characterize the DNA biofilm ligase active site of β -toxin and suggests β -toxin functions importantly in infective endocarditis through both of its mechanisms of action.

Keywords

Staphylococcus aureus; cytotoxin; biofilm; sphingomyelinase

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Author Contributions

All authors contributed to data collection. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

SUPPORTING INFORMATION

Figure Supplement 1 shows all strains used in the biofilm formation assay grew to similar absorbance 600 nm wavelength in wells for biofilm assays and representative wells of biofilm growth for each strain, stained with crystal violet. Additionally, there was no apparent defect or difference in growth between the strains as seen by a growth curve. Figure Supplement 2 shows COL strains carrying β -toxin or a β -toxin mutant on a plasmid expressed high levels of the protein, quantified by measuring zones of hemolysis compared to a standard curve made by recombinant β -toxin. It also shows representative images of heart vegetations recovered from rabbits infected at the lower inoculum. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Infective Endocarditis (IE) is a life threatening infection of native/prosthetic valves and the lining of the heart. IE is characterized by the formation of vegetations, “cauliflower-like” structures composed of bacteria and host factors.^{4–9} Each year there are as many as 100,000 cases of IE in the United States¹⁰. IE results in local and distal complications⁸. Often, pieces of septic vegetations detach leading to systemic embolization and metastatic infections resulting in strokes, infarcts, and/or abscesses that can cause persistent bacteremia, organ failure, and death in up to 66% of patients.^{9, 11, 12} *Staphylococcus aureus* is the most commonly identified pathogen in patients with healthcare-associated IE, accounting for 40% of cases, and is the leading cause of community-associated IE in the developed world.¹²

Thirty to forty percent of the population is asymptotically colonized with *S. aureus*. These carriers are more likely to develop *S. aureus* infections. *S. aureus* produces a variety of cell surface and secreted virulence factors which are important to its ability to cause disease such as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), cytotoxins, and superantigens (SAGs). Roughly 40% of *S. aureus* isolates from IE patients belong to the USA 200 clonal group, all of which produce the cytotoxin β -toxin.^{12, 13} Additionally, USA100 and USA400 clonal groups of *S. aureus*, are also capable of causing IE in part dependent on production of β -toxin^{1, 12, 13} through *in vivo* of excision of β -toxin-gene-inactivating bacteriophages.¹ Having *S. aureus* bacteremia is a critical factor for developing *S. aureus* IE. In the United States and Europe, *S. aureus* is the second most commonly isolated pathogen from bloodstream infections.¹³ Thirteen percent of septicemia isolates produce β -toxin, and β -toxin has been demonstrated to aid in *S. aureus* bloodstream survival.^{14, 15} However, the percentage of positive strains for β -toxin is likely to be higher given the loss of β -toxin-inactivating bacteriophages *in vivo*.¹

Huseby et al. have shown β -toxin is critical for causing IE in a rabbit model that strongly resembles human disease.² The *S. aureus* strain COL (*hly*⁺) forms large vegetations of about 220 mg, in comparison to a COL *hly*[−] strain forming vegetations of only about 2.5 mg.² Similarly, in the MW2 strain, a β -toxin⁺ variant produces larger vegetations than MW2 wild type in the same rabbit model.¹ Furthermore, the frequency of β -toxin producing variants increases within vegetations during infection with wild type MW2, thus favoring the production of β -toxin through bacteriophage excision.¹ There is also evidence β -toxin contributes to *S. aureus* colonization. Hiramatsu et al. demonstrated that when the β -toxin gene is intact, the strain NCTC 8325-4 persists longer in the nasal cavity than when the β -toxin gene is disrupted.³

β -toxin has a molecular mass of 35 kDa, a basic pI (>10.0), is a member of the DNase I superfamily, and is a neutral sphingomyelinase (SMase).^{1, 14} Unlike other cytotoxins, β -toxin does not form pores in plasma cell membranes, but instead hydrolyses the plasma membrane lipid, sphingomyelin, into ceramide and phosphorylcholine.¹⁵ The SMase activity of β -toxin is characterized by its ability to lyse sheep erythrocytes. Orthologs of *S. aureus* β -toxin can be found in *S. schleiferi*, *S. epidermidis*, and *Leptospira interrogans*; there is high sequence similarity of the *S. aureus* β -toxin gene and its structure to SMases in *Listeria ivanovii*, and *Bacillus cereus*.¹⁴

β -toxin also has DNA biofilm ligase activity. The active site for the DNA biofilm ligase activity is uncharacterized but is distinct from the SMase active site. The DNA biofilm ligase property of β -toxin is defined by its ability to cross-link to itself in the presence of DNA.² β -toxin covalently oligomerizes and precipitates in the presence of exogenous DNA, such as that found in biofilms. An *hlyB*⁻ strain of *S. aureus* shows reduced adherence in static assays and reduced biofilm growth in flow cell assays compared to an isogenic *hlyB*⁺ strain.²

While a role for β -toxin has been demonstrated in IE, the mechanism of action that it uses to cause its effects has not been elucidated. Considering the active site of the DNA biofilm ligase activity of β -toxin is unknown, we first began to characterize this active site through the construction of site-directed mutants. We determined mutants T149A, H162A, and D163A were deficient in a DNA precipitation assay. A biofilm formation assay was conducted to test further for disruption of the active site, in which mutants T149A and H162A were unable to form biofilms comparable to wild type β -toxin. Mutants T149A, H162A, and D163A were assessed for observable conformational changes by testing for retention of SMase activity, predicted structures, and comparable trypsin resistance as wild-type toxin. All mutants were able to lyse sheep erythrocytes and appeared to maintain native toxin overall conformation. Mutants H162A and D163A had similar stabilities as wild-type protein to heat and trypsin treatment.

We subsequently determined the contribution of each mechanism of action, SMase versus DNA biofilm ligase activity, of β -toxin in causing IE and sepsis in a rabbit model. The contribution of each activity to lethality and vegetation formation was determined. The SMase activity of β -toxin increased lethality and vegetation size. The DNA biofilm ligase activity of β -toxin did not affect lethality but increased vegetation size.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

COL, an early MRSA strain from the 1960's and RN450, a non-mutagenized version of *S. aureus* RN4220, both naturally encoding for β -toxin, were utilized from lyophilized stocks of low passage maintained in the laboratory. *S. aureus* strains were grown in Todd Hewitt (TH) broth (Becton Dickinson, Sparks, MD), and *E. coli* was grown in lysogeny broth (LB) Miller formulation, both at 37 °C with aeration (225 RPM). Plasmids were maintained using erythromycin (20 µg/ml) or carbenicillin (100 µg/ml).

Construction of RN450 *hlyB* nuc- and COL *hlyB*-

COL *hlyB*- was previously isolated from the original COL stock through disruption of the *hlyB* gene by insertion of a β C bacteriophage.² RN450 *hlyB* nuc- was generated by making in-frame deletions of *hlyB*. Briefly, PCR products were amplified with primers sets *hlyB*-upstream.F (5'-atctagaattcgagacgcttcatta-3')/*hlyB*-upstream.R (5'-GCTAGCACGCGTGTGTTTTTTCACCATC-3'), and *hlyB*-downstream.F (5'-cgatcgtgcgcagcctatagtaaata-3')/*hlyB*-downstream.R (5'-ATCCTACCCGGGGTCTGGTGAAAAC-3'). Amplifications were spliced together by overlapping PCR with *hlyB*-upstream.F/*hlyB*-downstream.R and inserted into pJB38. The

resulting plasmid was electroporated into RN4220, from which a lysogen of bacteriophage ϕ 11 containing the plasmid was made, the lysogen was used to introduce the plasmid into RN450 by transduction. The deletion was introduced via allelic exchange and verified by PCR, and loss of sheep blood hemolysis. The nuclease gene, *nuc*, was disrupted in this strain by using the Targetron Gene Knockout System (Sigma, TA0100) *nuc* specific insertion plasmid, and transduced into RN450 *hly* as previously described and confirmed by PCR.¹⁶

Expression of β -toxin in RN450 *hly**nuc*- and COL *hly*-

hly was amplified from RN4220 using primer set kpnI.btoxin.2F (5' - GCATCTTTATACTCAAAAACATTT-3'), and ecorI.btoxin.R (5' -AAAGAATTC GTTTGAGTGTAGAGTAAAGACTTG-3'). The PCR product was ligated into plasmid pCE104, and transformed into *E. coli* DH5 α . The resulting plasmid was electroporated into *S. aureus* RN4220, from which a lysogen of bacteriophage ϕ 11 containing the plasmid was made. This lysogen was used to move the plasmid into RN450 *hly* *nuc*- and COL *hly*-. Expression was characterized by sheep blood hemolysis. Mutations T149A, T151A, C158A, H162A, D163A, and H289N were made using QuikChange (Stratagene) site-directed mutagenesis and moved into RN450 *hly* *nuc*- and COL *hly*- as described for wild type β -toxin.

DNA Precipitation Assay

DNA precipitation assays were performed by Huseby et al..² Briefly, wild-type and β -toxin mutants were combined with 12 μ g of Qiagen maxi prep purified pCE104 plasmid DNA in molecular grade H₂O and 1.2 μ g of ethidium bromide, incubated at 37 °C for 30 minutes, incubated on ice for 10 minutes, and centrifuged for 10 minutes at 10,000 $\times$ *g*. DNA precipitation was visualized by photography.

Biofilm Formation Assay

Cultures were grown overnight in tryptic soy broth (TSB) (MP Biomedicals, LLC) at 37 °C with aeration. These cultures were diluted to 1.5 $\times$ 10⁸ colony-forming units (CFUs)/ml. These were further diluted 1:100 in TSB supplemented with 2% (wt/vol) glucose and 2% (wt/vol) NaCl. Approximately 200 μ l of these final dilutions was transferred to a 96-well plate (Costar, Corning Inc., NY, USA), previously coated with 20% human plasma diluted in bicarbonate/carbonate buffer. The cultures were incubated at 37 °C under static conditions overnight. Then, 150 μ l of culture was removed from each well and replaced with 150 μ l fresh TSB supplemented with 2% glucose and 2% NaCl, and then incubated under the same conditions overnight again. The absorbance was measured on a plate reader at 562 nm wavelength to measure total growth. The cultures were removed from the wells, and each was washed three times with 200 μ l PBS. The biofilm was fixed by adding 100 μ l 100% ethanol to each well. The plates were then air dried. Each biofilm was stained with 100 μ l of 0.1% crystal violet in deionized water at room temperature for 10 minutes. The crystal violet was removed, and each well was washed three times with 200 μ l PBS. To elute, 100 μ l of 80% ethanol/20% acetone was added per well and incubated at room temperature for 20 minutes. Each well was thoroughly mixed and diluted 1:10 in 80% ethanol/20% acetone. The absorbance of the dilution was measured on a plate reader at 562nm wavelength.

Protein Production

β -toxin used for DNA precipitation assays was collected from RN450 *hly nuc*-containing plasmid pCE104 and expressing *hly*. Overnight cultures were treated with 4 volumes of 100% EtOH to precipitate β -toxin. The precipitate was dried and resuspended in deionized water to 1/50th the original culture volume. The diameter of hemolysis was measured from each of the concentrates at various amounts on microscope slides coated with 1% agarose (Sigma Aldrich, St. Louis, MO) containing sheep erythrocytes, using NIH image J.^{1, 17} The β -toxin in the concentrates was then quantified by comparing lysis diameters to a standard curve made from lysis diameters of purified recombinant wild type β -toxin at known concentrations.

β -toxin was cloned into pTrcHis TOPO vector (Invitrogen Life Technologies, Grand Island, NY) using primer set bt toxin.3F (5'-GAATCTAAGAAAGATGATACTGATTTG-3')/bt toxin.2R (5'-CTATTACTATAGGCTTTGATTGGG-3') and expressed in *E. coli* DH5 α . *E. coli* expressing the vector was grown in 1 L terrific broth (TB) with 100 μ g/ml carbenicillin at 37 °C, shaken at 225 rpm to an OD₆₀₀ of 0.5. Protein production was then induced with 1 ml of 1 M isopropyl- β -D-thiogalactopyranoside (IPTG), followed by incubation at 30 °C for 18 hours. The cells were lysed to release protein via passage through a microfluidizer high shear fluid processor and batch purified using under native conditions with using cobalt-linked agarose resin (Sigma, St. Louis, MO). The eluted protein was dialyzed in 12–14kD molecular weight cutoff dialysis membrane against 1 L of PBS for 24 hours. Biofilm ligase mutants H162A and D163A were purified by the methods used for wild type β -toxin. With staphylococcal enterotoxin B as a standard, protein concentrations were determined by the Bradford assay (BioRad).

The structure of β -toxin was previously determined. Cartoon presentations of the structure of β -toxin was constructed using The PyMOL Molecular Graphics System, Version 1.8.0 Schrödinger, LLC. The location of mutated amino acid residues were mapped onto the structure.

Trypsin Treatment of β -toxin

Recombinant purified β -toxin (100 μ g/ml) was incubated at 37 °C with trypsin (50 μ g/ml in 10 mmol/L Tris-HCl, pH 8.0) to a final volume of 500 μ l for 0, 0.5, 1–6, 8, 12, and 24 hours. After incubation for the desired time, 50 μ l of the 500 μ l was removed and inactivated by incubation at 95 °C for 5 minutes followed by immediate storage at –20 °C. Each 50 μ l each time point was mixed with 50 μ l of SDS-PAGE sample buffer, and boiled for 5 minutes prior to assessment by SDS-PAGE.

Rabbit Model of Infective Endocarditis and Sepsis

All animal experiments were performed according to guidelines and protocols approved by the University of Iowa Institutional Animal Care and Use Committee (Protocol 1106140 which was replaced by current protocol 4071100). The experiment was performed as previously described.¹⁸ Briefly, New Zealand white rabbits, 2–3 kg of both sexes, were anesthetized with ketamine (25mg/kg) and xylazine (25mg/kg) (Phoenix Pharmaceuticals, Burlingame, CA). A catheter was inserted into the left carotid artery of each animal until the

aortic valve was reached and left in place for 2 hours to induce damage, removed, and incision site closed. Subsequently, bacteria were injected through the marginal ear veins. The experiments were allowed to proceed up to 4 days. Rabbits were treated with erythromycin to maintain the plasmid in the strains. Heart vegetations were dissected, weighed, homogenized, and plated to enumerate bacterial CFUs/total vegetations within each heart. Statistical significance in survival experiments was determined using the Log-rank, Mantel-Cox test (GraphPad Prism Software). Significance across means was carried out using the Mann-Whitney test (GraphPad Prism Software).

RESULTS

Site-Directed Mutagenesis Disrupts β -Toxin Oligomerization in a DNA Precipitation Assay

To begin to characterize the DNA biofilm ligase active site, mutagenesis was concentrated in the Asn143-Arg164 amino acid region of β -toxin as suggested in a prior study by Huseby et al.² (see Fig 1a). Site directed mutants T149A, T151A, C158A, H162A, and D163A were constructed and expressed in *S. aureus* RN450 *hlyB* nuc-. β -toxin was deleted, and nuclease was disrupted in the RN450 laboratory strain to allow for testing of mutants for deficiencies in the DNA precipitation assay without the interference of native β -toxin or DNA degradation due to nuclease. Mutations did not affect the SMase active site, as seen by retention of sheep erythrocyte lysis (Fig 1b). Protein concentrates were obtained from RN450 *hlyB* nuc- strains expressing wild type, T149A, T151A, C158A, H162A, or D163A β -toxin and β -toxin quantified. Proteins were tested in the DNA precipitation assay with β -toxin protein concentrations ranging from 35 μ g to 200 μ g. Wild type β -toxin formed a precipitate beginning at 60 μ g of protein. Mutant T151A was similarly able to form a precipitate beginning at 50 μ g, and mutant C158A beginning at 60 μ g of β -toxin. Mutants T149A, H162A, and D163A were not able to form precipitates at up to 200 μ g of β -toxin (Fig 1c).

Mutants Unable to Oligomerize in the DNA Precipitation Assay are Deficient in Biofilm Formation

Previous data demonstrate that a decrease in biofilm formation was seen in *S. aureus* strain COL following *hlyB* disruption, and therefore we investigated the ability of mutants, defective in the DNA precipitation assay, to form biofilms *in vitro* in this strain.² β -toxin wild type and mutants T149A, H162A, and D163A were expressed in COL *hlyB*- to test for deficiencies in biofilm formation without interference from endogenous β -toxin. Optical density of cultures in the 96-well plates was measured to rule out differences in growth between the strains that could account for deficiencies in biofilm formation; no growth differences were observed (Fig S1a). COL *hlyB*- was significantly reduced in its ability to form biofilms compared to COL or the complemented strain, COL *hlyB* + *hlyB*. Wild type β -toxin complemented COL *hlyB*- made biofilms comparable to COL expressing native β -toxin. COL *hlyB*- complemented with β -toxin T149A or H162A formed significantly less biofilms than COL *hlyB*- + *hlyB* at levels similar to COL *hlyB*-. COL complementation with β -toxin D163A was still able to form biofilms similar to COL *hlyB* + *hlyB* (Fig 2, S1b).

Protein Analysis Detect Minimal or No Major Conformational Changes in Mutants

The mutants were tested to determine if deficiencies in oligomerization and biofilm formation were the result of major conformational changes in the proteins caused by the mutagenesis. The β -toxin mutants T149A, T151A, C158A, H162A, and D163A retain their SMase activity as seen by sheep blood hemolysis, and are thus maintain correct conformation for this activity when expressed in *S. aureus* RN450 *hly* nuc- (Fig 1b). Furthermore, recombinant His₆-tagged wild type, H162A and D163A β -toxin proteins were purified and tested for their stability to heat and trypsin digestion. All purified proteins are readily detectable as a band at ~46 kDa corresponding to His₆-tagged β -toxin after treatment at 37 °C for up to 24 hours with a minimal decrease in quantity, as indicated by loss of band intensity, at 24 hours (Fig. 3a). Following trypsin treatment, both mutants were susceptible to proteolysis at rates and displayed patterns similar to wild type β -toxin. SDS-PAGE showed a shift of the prominent band to a smaller size and several smaller bands immediately following treatment, further fading with increased duration of treatment. Bands are visible at 12 hours but no longer detectable at 24 hours (Fig 3a). We located the mutagenized residues onto the native β -toxin structure as previously determined by Huseby et al. (Fig 3b).¹⁴ All residues were determined to be surface amino acids. Our prior experience from mutagenesis and structural analyses of large numbers of staphylococcal superantigens indicate that mutagenized surface amino acids, important in activities, do not alter the overall structure of the proteins, but instead alter only the amino acid side chains.

Sphingomyelinase Activity and Not DNA Biofilm Ligase Activity of β -toxin contributes to Lethality in a Rabbit Model of IE and Sepsis

S. aureus COL *hly*- expressing wild type β -toxin, a DNA biofilm ligase mutant, or a previously characterized SMase mutant, were tested in a rabbit model of IE and sepsis.^{2, 14} β -toxin production in tested strains was confirmed and quantified in sheep red blood cell hemolysis assays (Fig S2a). All strains made β -toxin at levels within 2-fold that of endogenous β -toxin produced by COL. Previous studies determined the lethal dose 50% endpoint (LD₅₀) of COL to be 2×10^9 CFUs. To account for the increased β -toxin production in the plasmid carrying strains, a lower dose was injected into the rabbits intravenously at 5×10^8 - 1×10^9 CFUs and experiment allowed to progress for up to 4 days to assess lethality. Rabbits with COL *hly*- plus *hly* had a statistically significant increase in lethality compared to those with the strain lacking β -toxin in the vector, COL *hly*-. Rabbits injected with the DNA biofilm ligase mutants had the same level of lethality as those rabbits injected with the wild type β -toxin carrying strain. However, rabbits infected with COL *hly*- complemented with the β -toxin SMase mutant H289N were significantly decreased in lethality compared to rabbits infected with COL *hly*- plus *hly*, with the same level of lethality as COL *hly*- (Fig 4a).

Sphingomyelinase and Biofilm DNA Ligase Activities of β -toxin Contribute to Vegetation Formation but Not Bacterial Burden within the Vegetations in a Rabbit Model of IE and Sepsis

To determine the contribution of the SMase and the DNA biofilm ligase activity to vegetation formation, the COL *hly*- strains complemented with wild type β -toxin, DNA

biofilm ligase mutants, or the SMase mutant were tested in the rabbit model of IE and sepsis. The rabbits were injected with 5×10^7 CFU, a dose that results in similar rabbit survival across strains and allows vegetation size to be compared within similar time frames (Fig 4b). Total vegetation size per rabbit was significantly larger in those infected with COL *hlyB*- plus *hlyB*, compared to those infected with the strain lacking β -toxin in the vector, COL *hlyB*- (averaging 19.2 mg versus 3.6 mg, respectively). COL *hlyB*- strains expressing DNA biofilm ligase mutants, T149A, H162A, or D163A were deficient in the ability to form vegetations compared to the wild type averaging 6.7 mg, 7.7 mg, and 3.1 mg respectively. Interestingly, COL *hlyB*- expressing the SMase mutant H289N was also deficient in vegetation formation averaging 2.0 mg, which is similar in size to COL *hlyB*- (Fig 4c, S2b). Despite the significant decrease in vegetation size seen in the DNA biofilm ligase and the SMase mutants, and the β -toxin deficient strain, there was no difference in CFUs recovered from dissected vegetations (Fig 4d), suggesting that the primary role of β -toxin in *S. aureus* IE is to promote accumulation of host factors and vegetation growth on infected valves.

DISCUSSION

S. aureus IE is a debilitating and rapidly progressing infection. Early diagnosis and treatment are difficult, and as such it results in death in up to 66% of cases.¹¹ IE can cause destruction of the heart valves, complete obstruction of blood flow leading to congestive heart failure, as well as causing infection in the lungs, kidneys, spleen, and brain.⁸ Previous research has shown a role for β -toxin in IE in a rabbit model. However, the specific mechanism of action the cytotoxin uses for its effects in IE is unknown.

In this study we begin to characterize amino acid requirements for the DNA biofilm ligase activity of β -toxin. We further examine the role each mechanism of action of β -toxin has in causing IE and lethal sepsis. Our data show residues T149, H162, and D163 are essential in the active site of the DNA biofilm ligase activity. Using the dual IE and sepsis rabbit model, we demonstrate SMase activity contributes to the lethality β -toxin causes. Additionally, our data clearly show both the SMase activity and DNA biofilm ligase activity of β -toxin promote formation of the vegetations pathognomonic of the disease. Vegetation formation is necessary for IE progression, this suggests β -toxin plays a critical role in the disease via both of its mechanisms of action.

Mutants T149A, H162A, and D163A were unable to oligomerize in the presence of DNA in a DNA precipitation assay, indicating these mutants are disrupted in the overall DNA biofilm ligase active site necessary for this function. Evidence showing that mutants T149A and H162A are deficient in a biofilm formation assay compared to wild type β -toxin confirms that the DNA biofilm ligase active site is disrupted in these mutants. The similar proteolytic rates and profiles of β -toxin mutants H162A and D163A compared to wild type in a trypsin digest analysis suggests that the single amino acid substitutions did not significantly change the protein conformation and stability. Furthermore, the predicted protein structures of mutants are likely to remain unaltered compared to wild type toxin. An intact SMase active site as tested in the sheep blood erythrocyte lysis assay also supports this conclusion. Future studies to characterize the biofilm active site more thoroughly are being directed to co-crystallization and structure determination of β -toxin in the presence of DNA.

To determine how the SMase and DNA biofilm ligase activities contribute to the role of β -toxin in IE, disruption mutants of each were tested in the rabbit model of IE and sepsis. We used a high bacterial inoculum to determine if β -toxin affects lethality and to examine which mechanism it uses to do so. We found all but one of the rabbits infected with wild type or biofilm ligase mutants succumbed on the first day following infection, suggesting β -toxin enhances lethality to which the DNA biofilm ligase activity does not contribute. In contrast, all but one rabbit infected with the SMase mutant or lacking β -toxin survived to the end of the experiment suggesting the SMase activity may be responsible for the increase in lethality associated with β -toxin production. How the SMase activity of β -toxin increases lethality is yet to be characterized. Rabbits infected with wild type or DNA biofilm ligase mutants, which have only the SMase activity intact, die too quickly to form vegetations at this inoculum, and thus vegetation related complications are not plausible reasons for their deaths. Sphingomyelin is found at varying degrees, depending on cell type, in cell membranes throughout the host. The SMase activity of β -toxin could therefore affect many cell types during infection, leading to lethality.

Since previous studies have shown β -toxin increases vegetation size, we used a lower inoculum to examine which mechanism promotes vegetation growth. We observed that both the DNA biofilm ligase mutants and the SMase mutant formed significantly smaller vegetations, on average 5.8 mg and 2 mg respectively, compared to wild type which formed vegetations averaging 19.2 mg. Both mutants had vegetations similar in size to the β -toxin deficient strain, suggesting that while both activities increase vegetation size in IE, their contributions are not additive.

The SMase activity of β -toxin may increase vegetation size, but how it does so is not clear. It is possible β -toxin helps initiate IE by causing inflammation and cytotoxicity directly at the site by: 1) destabilizing plasma membranes, and 2) generating by-products from sphingosine digestion. Previous work has shown β -toxin is capable of upregulating pro-inflammatory cytokines exaggerating the host inflammatory response which would lead to a subsequent influx of host immune cells and host factors to repair and control the damage.¹⁹ The resulting inflammation would serve as a better platform to which *S. aureus* could bind, leading to macrocluster formation and increasing vegetation size. While β -toxin is not a pore forming toxin, it has been shown to affect host cell plasma membranes including lysing blood erythrocytes and epithelial cells, which could lead to inflammation.²⁰ As a SMase, β -toxin digests sphingomyelin into phosphorylcholine and ceramide, a chemotactic signal. Studies have shown ceramide inhibits angiogenesis, induces apoptosis of various cardiac cell types, and might favor thrombosis.^{21, 22} Additionally, activation of a neutral SMase in aortic endothelial cells is associated with heart failure and endothelial dysfunction.²² Given the data showing ceramide has detrimental effects on the heart, it is possible the decrease in vegetation size observed with the SMase mutant may be linked to the ceramide this activity produces as a result of sphingomyelin digestion. Other by-products of sphingomyelin digestion are also involved in signaling and may also contribute to vegetation formation.

Consistent with its ability to bind to DNA and possibly other host factors aiding in biofilm formation, β -toxin also increases vegetation size via its DNA biofilm ligase activity. However, SMase mutant-infected rabbits show decreased vegetation size despite having the

DNA biofilm ligase activity still intact. This may be because the initial effects the SMase activity provides directly at the site are no longer present and might be necessary to initiate vegetation growth. Thus, without SMase activity there may not be sufficient vegetation formation to begin with for the DNA biofilm ligase activity to build upon. Following initial vegetation formation, the DNA biofilm ligase activity may quickly build up the vegetation before it breaks off to stabilize colonization by attaching to the host factors the SMase activity induced inflammation attracts.

It is important to note that while the DNA biofilm ligase activity increases vegetation size, it does not affect bacterial replication within the vegetation as can be seen by the similar CFUs recovered from mutants and wild type. This is consistent with the findings of Salgado-Pabón et al. and O'Callaghan et al.^{1, 23}, who despite finding increased pathogenesis in tissues with β -toxin, did not see an increase in CFUs recovered. The DNA biofilm ligase activity might serve to build up the vegetations so *S. aureus* is protected from stresses such as host immune defenses and to decrease its susceptibility to antibiotics. Biofilm dispersal and sensitivity to antimicrobials have been shown to increase with degradation of extracellular DNA.²⁴ It is possible *S. aureus* is using the DNA biofilm ligase activity to bind extracellular DNA to shield it from enzymatic degradation. β -toxin may also be binding to extracellular DNA to increase its proximity to other DNA binding proteins to further prevent DNA degradation.

Additional studies on endothelial cells and histopathology need to be done to determine if inflammation or cytotoxicity are present and responsible for the pathogenesis observed. Characterization of how β -toxin SMase activity induces inflammation and what host factors the DNA biofilm ligase active site binds to may provide targets for therapeutic treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CFUs	colony-forming units
IE	infective endocarditis
IPTG	isopropyl- β -D-thiogalactopyranoside
LD₅₀	lethal dose 50% endpoint
MSCRAAMs	microbial surface components recognizing adhesive matrix molecules
PBS	phosphate-buffered saline

SAg	superantigens
SMase	sphingomyelinase
TSB	tryptic soy broth
TB	terrific broth

References

1. Salgado-Pabon W, Herrera A, Vu BG, Stach CS, Merriman JA, Spaulding AR, Schlievert PM. Staphylococcus aureus beta-toxin Production is Common in Strains with the beta-toxin Gene Inactivated by Bacteriophage. *The Journal of infectious diseases*. 2014
2. Huseby MJ, Kruse AC, Digre J, Kohler PL, Vocke JA, Mann EE, Bayles KW, Bohach GA, Schlievert PM, Ohlendorf DH, Earhart CA. Beta toxin catalyzes formation of nucleoprotein matrix in staphylococcal biofilms. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107:14407–14412. [PubMed: 20660751]
3. Katayama Y, Baba T, Sekine M, Fukuda M, Hiramatsu K. Beta-hemolysin promotes skin colonization by Staphylococcus aureus. *Journal of bacteriology*. 2013; 195:1194–1203. [PubMed: 23292775]
4. Spaulding AR, Satterwhite EA, Lin YC, Chuang-Smith ON, Frank KL, Merriman JA, Schaefer MM, Yarwood JM, Peterson ML, Schlievert PM. Comparison of Staphylococcus aureus strains for ability to cause infective endocarditis and lethal sepsis in rabbits. *Frontiers in cellular and infection microbiology*. 2012; 2:18. [PubMed: 22919610]
5. Ferro, JM., Fonseca, AC. Chapter 7 - Infective endocarditis. In: José, B., José, MF., editors. *Handbook of Clinical Neurology*. Elsevier; 2014. p. 75-91.
6. Garcia-Cabrera E, Fernandez-Hidalgo N, Almirante B, Ivanova-Georgieva R, Noureddine M, Plata A, Lomas JM, Galvez-Acebal J, Hidalgo-Tenorio C, Ruiz-Morales J, Martinez-Marcos FJ, Reguera JM, de la Torre-Lima J, de Alarcon Gonzalez A. Neurological complications of infective endocarditis: risk factors, outcome, and impact of cardiac surgery: a multicenter observational study. *Circulation*. 2013; 127:2272–2284. [PubMed: 23648777]
7. Hoen B, Duval X. Infective Endocarditis. *New England Journal of Medicine*. 2013; 368:1425–1433. [PubMed: 23574121]
8. Thiene G, Basso C. Pathology and pathogenesis of infective endocarditis in native heart valves. *Cardiovascular Pathology*. 2006; 15:256–263. [PubMed: 16979032]
9. Spaulding AR, Salgado-Pabon W, Kohler PL, Horswill AR, Leung DY, Schlievert PM. Staphylococcal and streptococcal superantigen exotoxins. *Clin Microbiol Rev*. 2013; 26:422–447. [PubMed: 23824366]
10. Independent investigation by Immuvon Inc.
11. Baddour LM, Wilson WR, Bayer AS, Fowler VG Jr, Tleyjeh IM, Rybak MJ, Barsic B, Lockhart PB, Gewitz MH, Levison ME, Bolger AF, Steckelberg JM, Baltimore RS, Fink AM, O’Gara P, Taubert KA. Infective Endocarditis in Adults: Diagnosis, Antimicrobial Therapy, and Management of Complications: A Scientific Statement for Healthcare Professionals From the American Heart Association. *Circulation*. 2015; 132:1435–1486. [PubMed: 26373316]
12. Fowler VG Jr, Miro JM, Hoen B, Cabell CH, Abrutyn E, Rubinstein E, Corey GR, Spelman D, Bradley SF, Barsic B, Pappas PA, Anstrom KJ, Wray D, Fortes CQ, Anguera I, Athan E, Jones P, van der Meer JT, Elliott TS, Levine DP, Bayer AS. Staphylococcus aureus endocarditis: a consequence of medical progress. *JAMA: the journal of the American Medical Association*. 2005; 293:3012–3021. [PubMed: 15972563]
13. Nienaber JJ, Sharma Kuinkel BK, Clarke-Pearson M, Lamlertthong S, Park L, Rude TH, Barriere S, Woods CW, Chu VH, Marin M, Bukovski S, Garcia P, Corey GR, Korman T, Doco-Lecompte T, Murdoch DR, Reller LB, Fowler VG Jr. International Collaboration on Endocarditis-Microbiology, I. Methicillin-susceptible Staphylococcus aureus endocarditis isolates are associated with clonal complex 30 genotype and a distinct repertoire of enterotoxins and adhesins. *The Journal of infectious diseases*. 2011; 204:704–713. [PubMed: 21844296]

14. Huseby M, Shi K, Brown CK, Digre J, Mengistu F, Seo KS, Bohach GA, Schlievert PM, Ohlendorf DH, Earhart CA. Structure and biological activities of beta toxin from *Staphylococcus aureus*. *J Bacteriol*. 2007; 189:8719–8726. [PubMed: 17873030]
15. Vandenesch F, Lina G, Henry T. *Staphylococcus aureus* hemolysins, bi-component leukocidins, and cytolytic peptides: a redundant arsenal of membrane-damaging virulence factors? *Frontiers in cellular and infection microbiology*. 2012; 2:12. [PubMed: 22919604]
16. Kiedrowski MR, Kavanaugh JS, Malone CL, Mootz JM, Voyich JM, Smeltzer MS, Bayles KW, Horswill AR. Nuclease Modulates Biofilm Formation in Community-Associated Methicillin-Resistant *Staphylococcus aureus*. *PloS one*. 2011; 6:e26714. [PubMed: 22096493]
17. Schlievert PM, Case LC, Nemeth KA, Davis CC, Sun Y, Qin W, Wang F, Brosnahan AJ, Mleziva JA, Peterson ML, Jones BE. Alpha and beta chains of hemoglobin inhibit production of *Staphylococcus aureus* exotoxins. *Biochemistry*. 2007; 46:14349–14358. [PubMed: 18020451]
18. McCormick JK, Yarwood JM, Schlievert PM. Toxic shock syndrome and bacterial superantigens: an update. *Annual review of microbiology*. 2001; 55:77–104.
19. Walev I, Weller U, Strauch S, Foster T, Bhakdi S. Selective killing of human monocytes and cytokine release provoked by sphingomyelinase (beta-toxin) of *Staphylococcus aureus*. *Infection and immunity*. 1996; 64:2974–2979. [PubMed: 8757823]
20. Cifrian E, Guidry AJ, Bramley AJ, Norcross NL, Bastida-Corcuera FD, Marquardt WW. Effect of staphylococcal beta toxin on the cytotoxicity, proliferation and adherence of *Staphylococcus aureus* to bovine mammary epithelial cells. *Veterinary microbiology*. 1996; 48:187–198. [PubMed: 9054116]
21. Li H, Junk P, Huwiler A, Burkhardt C, Wallerath T, Pfeilschifter J, Förstermann U. Dual Effect of Ceramide on Human Endothelial Cells: Induction of Oxidative Stress and Transcriptional Upregulation of Endothelial Nitric Oxide Synthase. *Circulation*. 2002; 106:2250–2256. [PubMed: 12390956]
22. Levade T, Auge N, Veldman RJ, Cuvillier O, Negre-Salvayre A, Salvayre R. Sphingolipid mediators in cardiovascular cell biology and pathology. *Circulation research*. 2001; 89:957–968. [PubMed: 11717151]
23. O'Callaghan RJ, Callegan MC, Moreau JM, Green LC, Foster TJ, Hartford OM, Engel LS, Hill JM. Specific roles of alpha-toxin and beta-toxin during *Staphylococcus aureus* corneal infection. *Infection and immunity*. 1997; 65:1571–1578. [PubMed: 9125532]
24. Okshevsky M, Regina VR, Meyer RL. Extracellular DNAs as a target for biofilm control. *Current opinion in biotechnology*. 2015; 33:73–80.

Figure 1. Mutagenesis does not affect SMase activity but inhibits the ability of β -toxin to oligomerize. (a) Amino acid sequence of β -toxin. Highlighted areas are regions predicted to be biologically important by Huseby et al.^{2, 14} Black asterisks denote residues mutated, those in white indicate changes that disrupted the DNA biofilm ligase activity. Gray asterisk indicates residue mutated to disrupt SMase activity. (b) All DNA biofilm ligase mutants retain ability to lyse sheep blood erythrocytes similar to wild type as seen by zones of lysis surrounding growth on sheep blood agar plates. (c) DNA precipitation assays performed with β -toxin recovered from expression in *S. aureus* RN450 *hlyb nuc*-. Mutants T149a, H162A, and D163A were unable to form precipitates at up to 200 μ g of β -toxin. Wild type and mutant T151A formed a precipitate beginning at 50 μ g of protein, and C158A at 60 μ g.

Figure 2.

DNA precipitation assay-deficient mutants are poor at forming a biofilms in a static biofilm assay in the *S. aureus* COL strain. Mutants T149A and H162A made significantly less biofilms than wild type. Mutant D163A still made biofilms at similar levels to wild type.

Figure 3.

Mutagenesis does not alter β -toxin protein structure. (a) Trypsin digest analysis of mutants is comparable to wild type β -toxin proteolysis from 0-24 hours. Wild type and mutants H162 and D163 were stable at 37 °C for up to 24 hours, and had similar trypsin degradation rates and patterns. (b) Three-dimensional structure of β -toxin wild type compared to modeled mutants in DNA biofilm ligase and SMase activities. The T149, H162, and D163 DNA biofilm ligase inactivating residues are in magenta. The H289 SMase inactivating residue in blue. Residues H162 and D163 are in the same region toward the top of the protein, while T149 is near residue H289, obscured but partially on the surface. Amino acid substitutions at these residues do not appear to significantly change the predicted protein structure compared to wild type.

Figure 4.

β -toxin contributes to lethality and vegetation formation in a rabbit model of IE. (a) At an inoculum of $5 \times 10^8 - 1 \times 10^9$ CFUs, rabbits infected with COL *hlyB* + *hlyB* (■), COL *hlyB* + *hlyB* T149A (▲), COL *hlyB* + *hlyB* H162A (▼), or COL *hlyB* + *hlyB* D163A (◆), died rapidly. Mostly, rabbits infected with COL *hlyB* (○) and COL *hlyB* + *hlyB* H289N (◇) survived until the end of the experiment. (b) At an inoculum of 5×10^7 , there was no difference in survival between the strains. (c) COL *hlyB* + *hlyB* T149A, COL *hlyB* + *hlyB* H162A, COL *hlyB* + *hlyB* D163A, COL *hlyB* + *hlyB* H289N, or COL *hlyB*- infected rabbits produced little to no vegetations compared to COL *hlyB* + *hlyB* infected rabbits at the lower inoculum. Asterisks denote statistical significance compared to COL *hlyB* + *hlyB* (d) Vegetations from all rabbits infected at the lower inoculum had similar CFUs.