Beta toxin catalyzes formation of nucleoprotein matrix in staphylococcal biofilms

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Biofilms are surface-associated communities of microbes encompassed by an extracellular matrix. It is estimated that 80% of all bacterial infections involve biofilm formation, but the structure and regulation of biofilms are incompletely understood. Extracellular DNA (eDNA) is a major structural component in many biofilms of the pathogenic bacterium *Staphylococcus aureus*, but its role is enigmatic. Here, we demonstrate that beta toxin, a neutral sphingomyelinase and a virulence factor of *S. aureus*, forms covalent cross-links to itself in the presence of DNA (we refer to this as biofilm ligase activity, independent of sphingomyelinase activity) producing an insoluble nucleoprotein matrix in vitro. Furthermore, we show that beta toxin strongly stimulates biofilm formation in vivo as demonstrated by a role in causation of infectious endocarditis in a rabbit model. Together, these results suggest that beta toxin cross-linking in the presence of eDNA assists in forming the skeletal framework upon which staphylococcal biofilms are established.

Staphyloccus aureus | virulence | exotoxins

Biofilms are communities of microorganisms within a polymeric matrix that are attached to a surface. Bacterial biofilms are found on such diverse surfaces as rocks in the oceans, plaque on teeth, medical devices, and on damaged heart valves, in causing infectious endocarditis vegetations, and render infections markedly more resistant to effective antimicrobial treatment (1). Although biofilms are found in the majority of bacterial infections, their structure has not been completely characterized (2). The matrix of a biofilm typically contains exopolysaccharides (EPS), proteins, and extracellular DNA (eDNA) (3, 4) and protects embedded organisms from the effects of detergents, in vivo antibiotics, and the host–immune system. Here, we report that beta toxin in the presence of DNA forms covalent cross-links with itself, producing an insoluble matrix that promotes biofilm formation in vitro and in vivo.

Beta toxin of *Staphylococcus aureus* is a neutral sphingomyelinase (SMase). Its ability to lyse erythrocytes and kill proliferating human lymphocytes is linked to its SMase activity (5). Its structure (5) shows it belongs to the DNase I superfamily (CATH class 3.60) (6). The structures of beta toxin and DNase I have an rmsd of 3.3 Å over 220 Cαs (Fig. S1). This homology led us to hypothesize that beta toxin might bind and/or cleave DNA. Investigation of this hypothesis led to the unexpected finding that beta toxin plays a key role in the establishment of staphylococcal biofilms.

Results

When beta toxin is incubated with DNA, a decrease in the fluorescence of intercalated ethidium bromide was not observed as is seen with DNase I (Fig. S2), indicating no significant nuclease activity is associated with beta toxin. However, beta toxin binds both single-stranded and double-stranded oligonucleotides as evidenced by electrophoretic mobility shift assays (Fig. 1).

The most remarkable result from incubations of beta toxin with DNA was the immediate formation of a precipitate, commensurate with the amount of beta toxin added (Fig. S3). Strikingly, SDS/PAGE analysis of the precipitate revealed protein bands with molecular masses corresponding to dimers, trimers, and tetramers of beta toxin (Figs. 2*B* and 3*A*). Mass spectrometry confirmed that these bands were beta toxin oligomers (Fig. S4). In contrast, only the monomers of beta toxin were found in the supernatant and in control incubations (Fig. 2*A*). Thus, beta toxin oligomerization was DNA-dependent, and the oligomers were covalently linked (as mass spectrometry had indicated) and not susceptible to separation by denaturants. The high molecular mass bands remained even when the precipitate was treated with DNase I (Fig. 3*A*), showing that cross-linking was not by DNA molecules. Agarose gel electrophoresis of the precipitate showed that DNA was present but was unable to enter the matrix unless first treated with proteinase K (Figs. 2*D* and Fig. 3*C*). Thus, aggregation of DNA was by means of beta toxin molecules; all DNA could be removed from solution by adding sufficient beta toxin (Fig. 2*C*). We have termed this unique ability of beta toxin to cross-link in the presence of DNA biofilm ligase activity.

From homology between beta toxin and DNase I, H150 and H239 were identified as residues required for SMase activity and toxicity for human lymphocytes (5, 7). Mutating these residues individually to asparagine abolished both SMase activity and lymphotoxicity (5). However, neither of these mutations had any detectable effect upon DNA binding (Fig. 1) or precipitation (Fig. 4 *A* and *B*). These results suggest the active site residues for biofilm ligase activity are distinct from those for sphingomyelinase activity.

The nature of the cross-link must be considered. It is possible that binding DNA triggers a conformational change, refolding beta toxin into a molecule that forms an SDS- and urea-resistant interaction. Although this conformational change is conceivable, a far more likely explanation is the formation of a covalent bond, as implied by stability of oligomers in high concentrations of reducing agents, boiling in SDS, and mass spectrometric analysis (see below).

There are several possible residues that could be involved in cross-linking. Cysteine is unlikely because the cross-link is not subject to reductive cleavage, and both cysteines in beta toxin are involved in an intramolecular disulfide bond (5). Tyrosine is also unlikely, because the product has no color unlike most tyrosyl adducts. Although cross-links via a serine or threonine are possible, we believe an amino group (lysine), a carboxylate (aspar-

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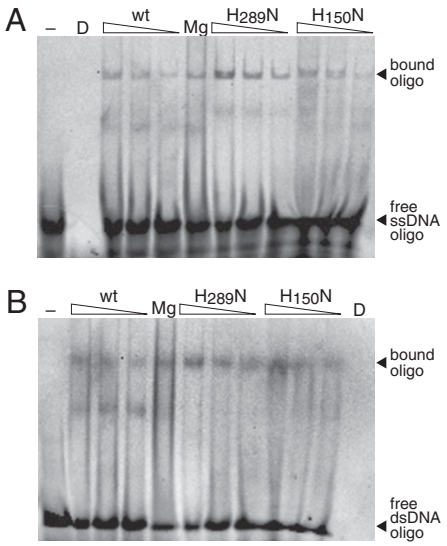

Fig. 1. Beta toxin binds single- and double-stranded DNA. (*A*) Ethidium bromide stained agarose gel analysis of beta toxin induced gel shifts of single-stranded DNA 42-mers. The reaction shown in the lane marked "–" contained no beta toxin. The reaction shown in lane "D" included 1 U DNase I. The reaction shown in the lane marked "Mg" lacked $MgCl_2$. The reactions shown in the other lanes contained 50, 25, or 10 μg of wild-type beta toxin or catalytically inactive beta toxin mutants H289N or H150N. (*B*) Similar gel shift experiments were performed with dsDNA oligonucleotides, with lane indications the same as in *A*.

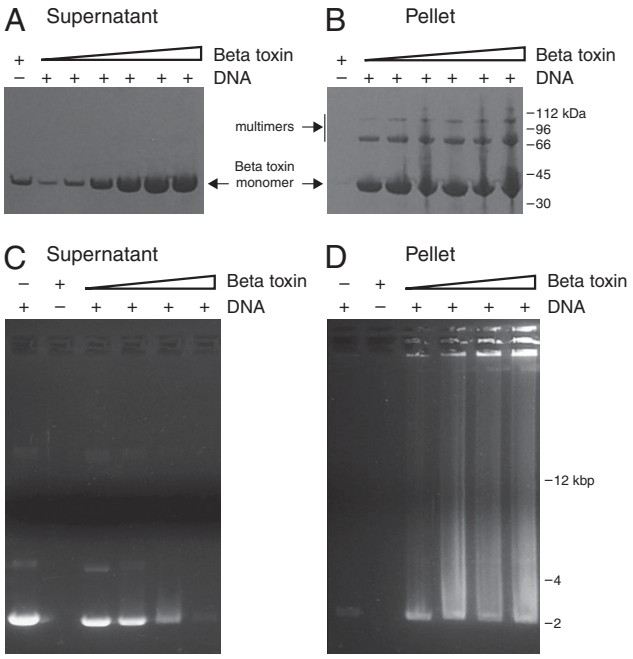

Fig. 2. DNA and beta toxin oligomerize and aggregate in the presence of each other. (*A*) Coomassie stained SDS/PAGE analysis of the supernatant (*Materials and Methods*) of the reaction of beta toxin with plasmid DNA. The reaction shown in the first lane contained 50 μg of beta toxin only. Those shown in lanes 2–7 contained 12, 50, 100, 200, 300, and 400 μg of beta toxin and 12 μg of pUC18 plasmid DNA. (*B*) SDS/PAGE analysis of the pellet of the reaction of beta toxin with plasmid DNA. Lane contents are identical to those in *A*. (*C*) Ethidium bromide stained agarose gel analysis of the same reactions in *A* and *B*. The reactions in lanes 3–6 contained 50, 100, 200 and 300 μg beta toxin and 12 μg pUC18 plasmid DNA. (*D*) Agarose gel analysis of the pellet of the reactions.

tate or glutamate), or a glutamine as in transglutaminases (8) are the most likely candidates.

Isopeptide bond formation, both spontaneous and enzymatically made are well documented in ubiquitination (9), transglutamination (10), and pili formation (11), and could also occur here. The SDS/PAGE band corresponding to the beta toxin dimer was cut out from the gel, the protein was extracted and subjected to trypsin digestion, and the resulting products were characterized by electrospray ionization mass spectrometry. Four of the predicted 24 peptides with masses >500 Da were not seen, suggesting that these parts of the protein engage in cross-linking (Fig. S4). Because the reaction depends on DNA binding, the biofilm ligase active site is likely to contact or include bound DNA. Given this constraint, tryptic peptides Asn143-Arg157 or Cys158-Arg164 may include the reactive residue(s).

Initial and subsequent mass spectrometric studies revealed no new detectable larger fragment(s) as one would expect if there were a covalent cross-link. These data can be reconciled if the target of the biofilm ligase activity is promiscuous. For example, suppose that the presence of DNA with its metal counterions activates the side chain carboxylate of a particular acidic residue in the ligase active site. If, like in ubiquination, this activated carboxylate can attack virtually any free amino group, i.e., any lysine side chain or amino terminus, 42 distinct products could be produced between two beta toxin monomers. If it were a lysine in the ligase active site attacking any carboxylate, there would also be 42 distinct products. Having so many products makes traditional mass spectrometric analysis problematic.

Given the role of eDNA in *S. aureus* biofilms (3, 4), we next investigated the impact of the *hlb* gene encoding beta toxin on biofilm development in vitro. The *hlb* gene in *S. aureus* COL (*hlb*$^+$) was disrupted by insertion of a βC bacteriophage (βC-Φ) to produce *S. aureus* COL *hlb*$^-$ (12). These organisms were compared with reference strain *S. aureus* UAMS-1 for the effect on adherence in a static assay (Fig. 5) and biofilm growth in a flow cell assay (Fig. S5).

The adherence assay indicated a 2-fold greater adherence for *S. aureus* COL (*hlb*$^+$) as compared with *S. aureus* UAMS-1 (Fig. 5) (13–15). It should be noted that *S. aureus* UAMS-1 does have a phage inserted into the *hlb* gene and, consequently, lacks the active beta toxin gene product. *S. aureus* COL *hlb*$^-$ showed half the adherence of *S. aureus* UAMS-1 and a quarter of that of *S. aureus* COL (*hlb*$^+$). Importantly, supplying beta toxin on plasmid pCN51 into *S. aureus* COL *hlb*$^-$ restored adherence nearly to levels similar to those of wild-type *S. aureus* COL (*hlb*$^+$).

Biofilm growth studies performed in a flow cell over 3 d showed that *S. aureus* COL (*hlb*$^+$) developed a more robust (faster growing, thicker) biofilm than *S. aureus* COL *hlb*$^-$ (Figs. S5 and S6). Indeed, when the biofilms were stained and thicknesses measured by confocal microscopy, *S. aureus* COL (*hlb*$^+$) biofilms were on average 60 μm thicker. A growth defect of *S. aureus* COL *hlb*$^-$ cannot be responsible for this difference, because growth curve analysis shows *S. aureus* COL *hlb*$^-$ grew faster than COL (*hlb*$^+$). The doubling time for COL *hlb*$^-$ was 21.9 ± 0.7 min; the doubling time for COL (*hlb*$^+$) was 35.6 ± 1.3 min. The discrepancy between the doubling times could be due to the heavy production of beta toxin; however, further experimentation will be necessary. In the flow cell studies (Fig. S5), *S. aureus* COL (*hlb*$^-$) grew as small groups of dispersed colonies, giving a thin star-like appearance to the flow cell, rather than the dense cell mass associated with the parent organism. Supplying *hlb* in *trans* on pCN51 to *S. aureus* COL *hlb*$^-$ partially restored the dense cell mass biofilm development that was observed with the parent organism.

As a final measure of role in biofilm formation, the same *S. aureus* strains, COL (*hlb*$^+$), COL (*hlb*$^-$), and COL (*hlb*$^-$) complemented with pCN51, were evaluated in vivo in a rabbit model of

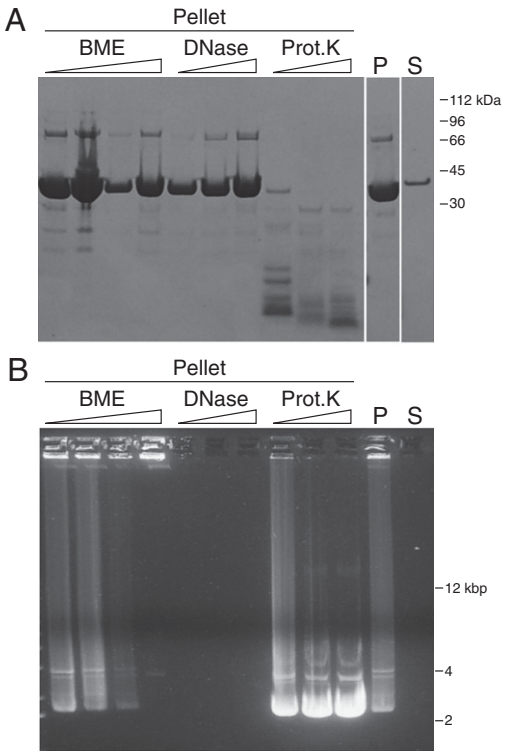

Fig. 3. Chemical characterization of beta toxin/DNA oligomers. (*A*) Coomassie stained SDS/PAGE analysis of the beta toxin-DNA reaction pellets treated with 0.05, 0.25. 0.5, or 2.5 M β-mercaptoethanol (BME) (lanes 1–4), 2, 10, or 20 U DNase I (lanes 5–7); 2, 20, or 100 μg proteinase K (lanes 8–10). Gel lanes P and S were rearranged for clarity. (*B*) SDS/PAGE analysis of untreated pellet (P) and supernatant (S) of the beta toxin-DNA reactions. (*C*) Ethidium bromide-stained agarose gel analysis of the beta toxin-DNA reaction pellet treated as above.

infectious endocarditis (16). The typical lesions formed in this model, referred to as vegetations, are considered biofilms composed of host cells and masses of growing bacteria. COL (*hlb*$^+$) formed highly significant vegetations in 4/4 animals (Table 1), averaging 220 ± 34 mg with $\log_{10}$ bacterial numbers of 8.28 ± 0.26. These vegetations nearly completely obstructed the rabbit aortas in the 4-d test period. In contrast, COL (*hlb*$^-$) formed minimal vegetations in four rabbits (Table 1), averaging 2.5 ± 4.3 mg vegetation weights and bacterial numbers of $\log_{10}$ 6.14 ± 0.22. Three of the 4 animals lacked vegetations, and bacterial counts are thus reflective of bloodstream infection. Both difference in vegetation weights and in $\log_{10}$ bacterial numbers between COL (*hlb*$^+$) and COL (*hlb*$^-$) were highly significant as determined by Student's *t* test of unpaired data (Table 1). COL (*hlb*$^-$) complemented with beta toxin on pCN51-restored vegetation formation (Table 1). The vegetation weights in the complemented strain compared with the knock-out strain were significantly different by Student's *t* test analysis. Because COL (*hlb*$^-$) complemented with pCN51 is isogenic with the knock-out strain COL (*hlb*$^-$) with respect to beta toxin, the difference in vegetation weights and $\log_{10}$ bacterial numbers is due to the difference in beta toxin production, rather than βC-Φ virulence genes (12).

Discussion

Lysozyme is another protein that has been shown to precipitate DNA (17). However, the precipitation of DNA by beta toxin is markedly different from that of lysozyme. The lysozyme-DNA precipitate can be dissolved in 300 mM NaCl; the beta-toxin-DNA pellet is stable at least up to 4 M NaCl. The high pI of lysozyme results in binding to DNA by simple electrostatics producing the susceptibility to high salt. Beta toxin also has a high pI, possibly greater than 10 (18), but is stable to high salt. More importantly, there is no cross-linking of lysozyme monomers in the lysozyme-DNA precipitate as seen in the beta toxin-DNA precipitate.

The connection between environmental signals and regulation of specific stages of biofilm formation is under investigation. Beta toxin, like toxic shock syndrome toxin-1, is regulated postexponentially by the *agr* locus (19). It is interesting that there have been several reports that *agr*$^-$ mutations tend to stimulate biofilm formation (20–24). This observation suggests that decreased beta toxin expression should produce thicker biofilms, rather than the opposite as we show. Some clues as to this seeming contradiction may be seen in the observations of Abdelnour et al. (25) and Xiong et al. (26) that persistent staphylococcal infections, which become *agr*$^-$ over time, nevertheless require expression of toxic shock syndrome toxin-1 (and presumably beta toxin) for the chronic infection. Clearly this matter requires further study.

Beta toxin is variably produced by *S. aureus* strains, depending on the presence or absence of βC-Φ whose insertion inactivates *hlb.* Of the 18 sequenced strains of *S. aureus*, *hlb* is intact in only 4. This inactivation mechanism is a unique mechanism to generate variation in virulence factor production in *S. aureus*, where most variable virulence factor traits, like *tst* and many enterotoxin genes, are controlled by the presence or absence of intact structural genes within pathogenicity islands or bacteriophages (12, 27). Nevertheless many clinical isolates of *S. aureus*, both methicillin-

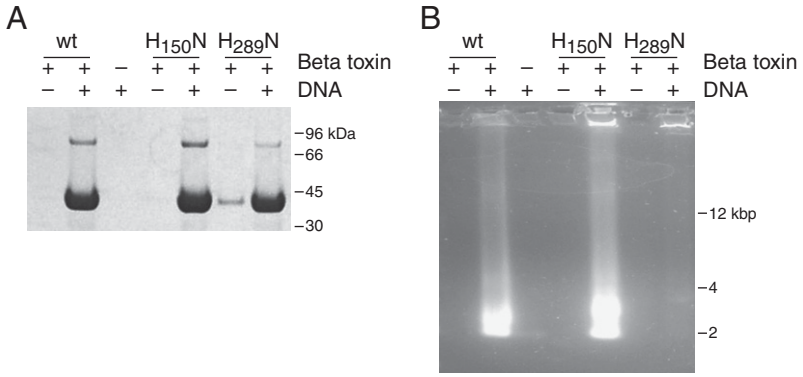

Fig. 4. DNA induces oligomerization of catalytically inactive beta toxin mutants. (*A*) Coomassie-stained SDS/PAGE analysis of native beta toxin, H150N, and H298N pellets reacted with DNA as described in the *Materials and Methods*. (*B*) Ethidium bromide-stained agarose gel analysis of reactions containing plasmid DNA and wild-type beta toxin, or catalytic mutants H150N, or H289N.

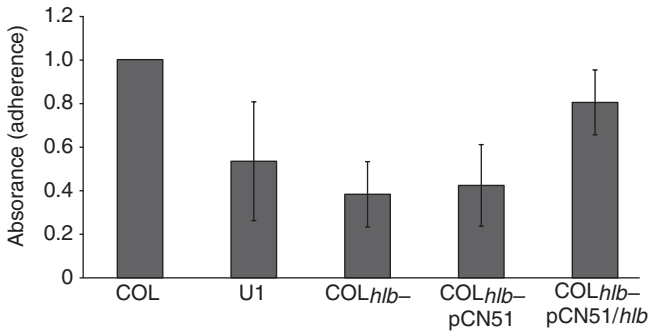

Fig. 5. Beta toxin-deficient (hlb$^-$) *S. aureus* strains do not adhere as well as beta toxin proficient strains. Adherence was measured as described in the *Materials and Methods* by using a 96-well plate coated with 20% human plasma. COL is an hlb$^+$ *S. aureus* strain; UAMS-1 (U1) is hlb$^-$. The COL$_{hlb}$$^-$ beta toxin-deficient strain was created as described in the text. Expression of beta toxin on the pCN51 plasmid in the COL *hlb*$^-$ background rescued the adherence phenotype (COL *hlb*$^-$ pCN51 *hlb*$^+$). Empty vector did not rescue the phenotype (COL *hlb*$^-$ pCN51). The observed difference between COL *hlb*$^+$ and COL *hlb*$^-$ is statistically significant at $P < 0.01$.

resistant (MRSA) and methicillin-sensitive (MSSA), are *hlb*$^+$, particularly those associated with animal infections (28) and certain types of human infections (26, 29). Importantly for humans, many MRSA and MSSA strains, belonging to pulsed-field gel electrophoresis type USA200 (typical menstrual toxic shock syndrome toxin-1 producing *S. aureus*), are *hlb*$^+$ (30). These strains commonly exist on human mucosal surfaces as biofilms (31, 32). Because *tst*$^+$ *S. aureus* is commonly found in infective endocarditis patients with persistent bacteremia (26), we hypothesize that these strains are also *hlb*$^+$, with beta toxin contributing to persistence of the endocarditis biofilms. These observations suggest that beta toxin, like these other staphylococcal toxins, may have definitive roles in selected types of infections, both in animals and humans. Our in vivo studies suggest that beta toxin may be highly important in infectious endocarditis when produced. Because no simple genetic switch has been indentified to cause planktonic bacteria to switch to biofilm formation (2), it is not surprising that beta toxin can fill multiple virulence roles, i.e., lysis of mammalian cells and formation of a biofilm matrix.

If the target of the beta toxin cross-link is a simple carboxylate, beta toxin could bind to other proteins or even to carbohydrates (Fig. 6). In addition, the family of putative DNase I homologs is very large. Similar activities might be present in other homologs, allowing them to participate in the formation of biofilms containing eDNA, both in other bacterial genera but also in eukaryotic sites e.g., in amyloid plaques (33).

Collectively, our data suggest that beta toxin contributes to biofilm formation in *S. aureus* strains that make the protein. However, *S. aureus* biofilms are complex and can be made by many strains that do not produce beta toxin, requiring many other factors dependent on strain. These factors include cell surface virulence factors, extracellular polysaccharides, other proteins, and extracellular DNA. These studies point to a molecular mechanism for forming the structural framework of many staphylococcal biofilms. This revelation opens the door to new avenues of treatment, as increased antibiotic resistance becomes more prevalent and as the critical role of biofilms in bacterial virulence becomes increasingly apparent.

Materials and Methods

Gel Shift Assays. Wild-type beta toxin and two active site mutants (H150N and H289N) were purified and produced as published (5). Pure protein (10, 25, or 50 μg) or 1 U DNase I (New England Bioscience) was combined with single-stranded (Biotin-ATTATTATTATTCCAATGGATTTATTTATTATTTATTTATTT-Fluorescein) or with annealed double-stranded (ATTATTATTATTCCGUGGATTTATTTATTTATT TATTTATTT-Fluorescein and AAATAAATAAATAAATAAATAAATCCGCGGAAT AATAATAAT) 0.02 nmol oligos. The entire reaction was mixed with sample buffer (Invitrogen) and electrophoresed on Novex 6.0% TBE gels (Invitrogen) for 1 h at 150 V after preelectrophoresis in 0.5× TBE buffer (Invitrogen) for 20 min. The gel shifts were visualized by using a Fujifilm FTA-5000 phosphoimager.

DNA Precipitation Assay. Beta toxin was combined with 12 μg of Qiagen maxi prep purified pUC18 plasmid DNA in 1× DNase I buffer (New England Biosciences) and 1.2 μg of ethidium bromide and then incubated at 37 °C for 30 min. The reaction was stopped by incubation on ice for 10 min, followed by centrifugation for 10 min in a microcentrifuge at 10,000 × *g*. The pellet was separated from the supernatant and washed twice with water, then resuspended in 10 μL of water. Half the pellet was electrophoresed on a NuPAGE Bis-Tris gel (Invitrogen NP0322) and then stained and destained according to the manufacturer's directions. The other half of the pellet was electrophoresed on a 0.8% agarose gel made with 1× TAE and separated at 100 V for 1.5 h. The supernatant was assayed identically to the pellet.

To treat the pellet, the reaction was scaled up 10-fold, and the protocol was followed as stated above. The pellet was split equally and treated with 0.05, 0.25, 0.5, or 2.5 M BME; 2, 10, or 20 U DNase I (NEB); and 2, 20, or 100 μg of proteinase K (Sigma). The treated pellets were electrophoresed on gels as stated above.

βC-Bacteriophage Isolation. Phages were isolated from the *S. aureus* strain Newman by diluting a saturated culture (1/500) into fresh Todd Hewitt broth and shaking for 1.5 h at 37 °C, followed by the addition of 1.0 μg of mitomycin C. The cells were shaken for 3 more hours, pelleted, and the supernatant was sterile filtered. *S. aureus* strain COL *hlb*$^+$ was diluted 1/500 from a stationary phase culture in Todd Hewitt broth and shaken at 37 °C for 3 h, and then placed on ice for 1.5 h. COL *hlb*$^+$ was plated (100 μL) onto Todd Hewitt agar plates and incubated upright for 30 min at room temperature. The Newman supernatant was then spotted onto the COL *hlb*$^+$ plate, the plate was incubated upright for 15 min at room temperature, and the plate then was inverted and incubated at 37 °C overnight. Colonies within a zone of lysis were picked and streaked onto blood agar plates. Lysogens with phage inserted into the beta toxin gene (COL *hlb*$^-$) no longer lysed red blood cells and were picked for further analysis; these lysogens presumably contained βC-Φ that inactivated *hlb* expression.

Adherence Assay. Adherence was measured as published (34). Briefly, cultures were grown overnight in tryptic soy broth (TSB) supplemented with 3% NaCl and 0.5% glucose. The cultures were diluted to OD_{600} equal to 0.01, 200 μL was placed in a 96-well plate (Costar 3596) in quadruplicate, and the plate previously coated in 20% human plasma was diluted in carbonate buffer at pH 9.6. The cultures were incubated statically at 37 °C overnight. The cultures were removed from the plate, and the plate was washed twice with 1× PBS, followed by 2-min room temperature incubation in 100 μL of 100% ethanol. To the ethanol, 100 μL of stain solution (0.41% crystal violet in 12.0% ethanol) was added and allowed to sit for 2 min at room temperature. The stain was removed, and the wells were washed with PBS three times. The absorbance of wells was determined on a plate reader at 595 nm.

In Vitro and in Vivo Biofilm Formation Assays. COL *hlb*$^+$ and COL *hlb*$^-$ (35) were grown overnight in TSB, diluted to 5×10^6 bacteria in TSB, then injected into flow cells for biofilm formation analysis as published (15). The reactions

Table 1. Beta toxin is essential for efficient vegetation growth in vivo

	COL (*hlb*$^+$)	COL *hlb*$^-$	COL *hlb*$^-$ + pCN51 (*hlb*$^+$)
Inoculate $\log_{10}$, cfu	8.98	9.00	9.08
Vegetation mass, mg	220 ± 35	2.5 ± 4.3	150 ± 30
$\log_{10}$, cfu per vegetation	8.28 ± 0.26	6.14 ± 0.22	7.90 ± 0.09

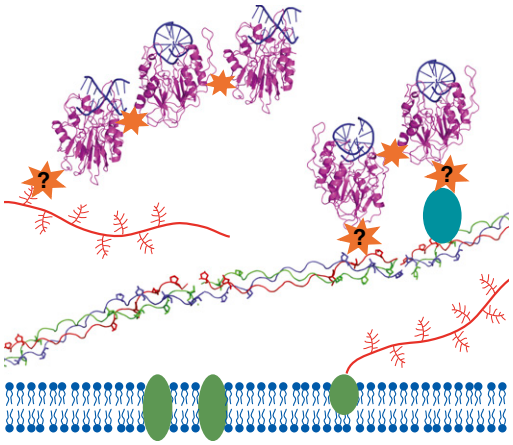

Fig. 6. Model of beta toxin forming a nucleoprotein network in the extracellular matrix. Beta toxin (magenta cartoon) binds eDNA (blue helix) and cross-links nonspecifically (orange star burst, question marks represent potential cross-links) to itself and other proteins. Other molecules that beta toxin may cross-link to are hyaluronic acid (red strands with branches), collagen (PDB entry 1CDG; red, green, and blue fibrils), or collagen binding proteins (blue oval). The plasma membrane (blue lipids) is punctuated with integral membrane proteins (light green ovals).

were monitored for 3 d, after which the biofilms were stained with Syto-9 and Toto-3 (Invitrogen) and the average thicknesses was calculated.

Rabbits (New Zealand White, either sex, 2–3 kg, 4 per group) were anesthetized with ketamine and xylazine according to an approved IACUC protocol (0908A71722) at the University of Minnesota, their left carotid arteries were exposed, and cannulae were inserted up against the aortic valves for 2 h (16). The University of Minnesota is AAALAC accredited. Subsequently, the cannulae were removed, the animals were closed, and *S. aureus*, COL *hlb*$^+$, COL *hlb*$^-$, or COL *hlb*$^-$ was complemented with beta toxin produced on pCN51, injected into the marginal ear veins. The animals were euthanized after 3 d, and vegetations on the aortic valves and within the aortas were removed, weighed, and homogenized for bacterial enumeration. When no vegetations were observed, a scalpel blade was used to scrape the surface of the aortic valves and adjacent aorta for bacterial enumeration. Differences in vegetation weights and $\log_{10}$ *S. aureus* numbers were determined by Student's *t* test analysis of unpaired data.

Protein Production. Beta toxin was cloned initially by the G.A.B. laboratory. The native promoter and signal sequence were cloned along with the ORF and inserted into the pCN51 plasmid (kindly provided by the K.W.B. laboratory). The primers used were 5′-GCATCTATTTTGTTGCACCTATATAAAAGGAGTC-3′ and 5′-GTTATTAGTTAGTGAATTCCTATTTACTATAGGCTTTG-3′. The native gene was PCR amplified and then digested with EcoRI and BamHI (New England Biolab). The digest was electrophoresed on a 1.5% agarose gel, the corresponding band cut out and gel purified, and the resultant DNA was ligated into the pCN51 plasmid digested with the same restriction enzymes. The ligation reactions were then used to transform *E. coli* XL-blue cells, and ampicillin resistant clones were picked for further DNA isolation.

Production of *S. aureus* COL *hlb*$^-$. pCN51 with the beta toxin insert along with the empty vector were transformed into competent *S. aureus* strain RN4220. The cells were made competent by diluting overnight cultures grown in TSB to an OD_{600} of 0.05 and shaking for 1 h at 250 RPM at 37 °C. The OD_{600} was assayed again, and the cells were used only if the OD_{600} had reached at least 0.4. The cells were spun down at 10,000 × *g*, the supernatant was discarded and resuspended in 15 mL of sterile room temperature double-distilled H_2O. This step was repeated, and the cells were resuspended in 2.5 mL of room temperature 10.0% sterile glycerol, and then spun down at 10,000 × *g*. The cells were resuspended in 1.25 mL of 10.0% sterile glycerol, transferred to Eppendorf tubes, and incubated without shaking at room temperature for 15 min, then spun down 10,000 × *g* for 2 min. The supernatant was discarded, and the cells were resuspended in 325 μL of 10.0% sterile glycerol, aliquoted 80 μL per tube, and immediately transformed.

Before transformation, 1.0 μg of DNA was drop dialyzed against double-distilled H_2O with a Millipore 0.025-μm membrane for 15 min, then added to 80 μL of fresh competent cells. The cells were electroporated at 100 ohms, 25 μf, 200 kV, and then 390 μL of B2 media (1.0% casein hydrolysate, 2.5% yeast extract, 0.1% K_2HPO_4 at pH 7.5, 0.5% glucose, and 2.5% NaCl) was added. The reaction was incubated at 37 °C without shaking for 1.5 h, then 100 μL of the transformation mixture was plated on tryptic soy agar containing 10 μg/mL erythromycin and incubated 37 °C overnight.

Transformants resistant to erythromycin were grown overnight in TSB at 37 °C, 250 RPM. The following day, the cultures were diluted 1:100 in TSB and incubated at 37 °C, 250 RPM for 1.5 h. βC-Φ (kindly provided by the K.W.B. laboratory) was added to the culture such that the multiplicity of infection (MOI) was 0.1. One milliliter of freshly made 10 mg/mL $CaCl_2$ was added. The cultures were incubated at room temperature without shaking for 30 min and then slowly rotated at 30 °C for 5 h or until lysis was complete. The cultures were then placed at 4 °C overnight. The phages were filtered through a sterile 0.45-μm filter and titered by diluting in duplicate from 10^{-6} to 10^{-9} in phage buffer (6.47 g of glycerol-2-phosphate, 60 mg of $MgSO_4$, 2.4 g of NaCl, 0.5 g of gelatin dissolved in 475 mL of double-distilled water H_2O, autoclaved, cooled, and followed by the addition of 22.5 mL of freshly made 10 mg/mL $CaCl_2$). *S. aureus* strain RN4220 was used for phage titering.

Production of Lysogens. COL *hlb*$^-$ was grown overnight in TSB at 37 °C with shaking at 250 RPM, then diluted 1:100 and incubated at 37 °C with shaking at 250 RPM for 1 h. The cells were pelleted at 9,000 × *g* in a microfuge for 5 min. The supernatant was discarded, the cells were washed with 5 mL of TSB and then pelleted at 9,000 × *g* for 5 min. The cells were resuspended in 1 mL of TSB, and 40 μL of fresh 10 mg/mL $CaCl_2$ and the appropriate amount of phage to give an MOI of 0.1 were added and incubated at room temperature for 10 min and then at 30 °C for 35 min without shaking. To this mixture, 5 mL of TSB was added, and the cells pelleted at 13,200 RPM. The cells were resuspended in 10 mL of TSB, transferred to a Falcon tube, and incubated at 37 °C, 250 RPM, for 1.5 h. The cells were pelleted at 13,200 RPM, the supernatant was discarded, the cells were resuspended in 1 mL TSB, and 0.1 mL of cells were plated on tryptic soy agar containing 10 μg/mL erythromycin; the plates were incubated at 37 °C overnight. Colonies that were resistant to erythromycin and lysogenized were chosen for further analysis.

Growth curve analysis. Growth curve analysis was done by diluting overnight cultures of COL *hlb*$^+$ and COL *hlb*$^-$ previously grown in TSB 1:100 in fresh TSB. The OD_{600} was assayed every 15 min for 3 h. The experiments were performed in triplicate and repeated twice.

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