Secreted Proteases Control Autolysin-mediated Biofilm Growth of Staphylococcus aureus*

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Background: Esp, a secreted protease of Staphylococcus epidermidis, blocks biofilm formation of Staphylococcus aureus and its ability to colonize human nares.

Results: Esp cleaves autolysin, thereby preventing the release of staphylococcal DNA as biofilm matrix.

Conclusion: Secreted proteases control S. aureus biofilm development and host colonization.

Significance: Methods that promote autolysin degradation may also prevent S. aureus colonization of humans.

Staphylococcus epidermidis, a commensal of humans, secretes Esp protease to prevent Staphylococcus aureus biofilm formation and colonization. Blocking S. aureus colonization may reduce the incidence of invasive infectious diseases; however, the mechanism whereby Esp disrupts biofilms is unknown. We show here that Esp cleaves autolysin (Atl)-derived murein hydrolases and prevents staphylococcal release of DNA, which serves as extracellular matrix in biofilms. The three-dimensional structure of Esp was revealed by x-ray crystallography and shown to be highly similar to that of S. aureus V8 (SspA). Both atl and sspa are necessary for biofilm formation, and purified SspA cleaves Atl-derived murein hydrolases. Thus, S. aureus biofilms are formed via the controlled secretion and proteolysis of autolysin, and this developmental program appears to be perturbed by the Esp protease of S. epidermidis.

Staphylococcus aureus is both a commensal and an invasive pathogen that causes skin and soft tissue infections, sepsis, and endocarditis (1). The primary niche for S. aureus colonization are the human nares (2). Approximately 20% of the human population is colonized persistently, whereas 30% represent intermittent carriers, and 50% are noncarriers (3). Nosocomial S. aureus bacteremia is three times more frequent in carriers than in noncarriers (3, 4). Colonization of S. aureus with highly virulent, multidrug-resistant strains (methicillin-resistant S. aureus, MRSA) is associated with invasive disease and treatment failure (5). S. aureus is currently the most frequent cause of infectious disease morbidity and mortality in the United States (6). Thus, strategies are needed to prevent S. aureus nasal colonization without selecting for antibiotic resistance and with the ultimate goal of reducing the incidence of staphylococcal infections.

Colonization of human nares is thought to involve the establishment of S. aureus biofilms (7). Work from many laboratories suggests that S. aureus biofilm growth occurs as a developmental program whereby bacteria initially adhere to host epithelial surfaces and subsequently release some of their DNA as extracellular matrix to replicate as biofilm communities (8, 9). Biofilm growth is also associated with the shedding of staphylococci, where released bacteria promote invasive disease or disseminate within host tissues (9). Several secreted products have been reported to function as adhesins for S. aureus biofilm formation, including fibronectin binding proteins (FnBAP and FnBP) (10, 11), the extracellular adhesion protein (Eap) (12, 13), and the extracellular matrix protein (Emp) (13). S. aureus biofilms use bacterial DNA as an extracellular matrix (14, 15), which is released via Atl, a multifunctional murein hydrolase (16, 17). In addition to atl, the release of DNA by S. aureus grown in biofilms is also dependent on the cidABC and lrgAB operons, which appear to function as holins/antiholins by either initiating or preventing staphylococcal entry into a programmed cell death pathway (17). The expression of the cid and lrg operons is controlled in response to environmental signals via the LysR type regulator CidR and the two-component regulator LytRS, respectively (8, 18).

The 1256-residue autolysin precursor is secreted via its N-terminal signal peptide. Following signal peptide removal, pro-Atl is cleaved at two sites, residues 302 and 874, thereby generating the mature amidase (N-acetylmuramoyl-L-alanine amidase (AM, residues 303–874) and N-acetylglycosaminidase domains (GL, residues 875–1276)) (19). Each of the two enzymes is endowed with repeat domains (R1-R2-R3) that are tethered both to the C-terminal end of AM (R1-R2, residues

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‡ The abbreviations used are: Atl, Staphylococcus aureus autolysin; AM, Atl derived N-acetylmuramoyl-L-alanine amidase; Esp, S. epidermidis extracellular serine protease; GL, Atl derived glucosaminidase; PI, propidium iodide; R1, R2, and R3, repeat domains 1, 2, and 3, respectively, of S. aureus Atl; TSB, tryptic soy broth; PDB, Protein Data Bank.
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EXPERIMENTAL PROCEDURES

Bacterial Strains and Reagents—S. aureus Newman (27) and its variant with a *bursa aurealis* insertion in *atl* (28) have been previously described. The *atl* mutational lesion was transduced with bacteriophage φ85 into wild-type *S. aureus* Newman. Staphylococci were grown in tryptic soy broth (TSB) or on tryptic soy agar plates. Erythromycin (10 μg/ml) was used to select *S. aureus* isolates expressing *esp* (7). Decreased cell wall teichoic acids (24). Deletion mutations in the *atl* gene abolish *S. aureus* biofilm formation, and *atl* mutants form large clusters of cells with incompletely separated cell wall envelopes (25).

Studying nasal colonization in human volunteers, Iwase et al. (7) observed a negative correlation between the colonization of *Staphylococcus epidermidis* strains expressing *esp* and *S. aureus*. Co-culturing of *S. epidermidis* strains expressing *esp* inhibited *S. aureus* biofilm formation (7). Although Esp does not affect the viability of *S. aureus*, the purified protease prevents biofilm formation and promotes disassembly of pre-established biofilms (7). Esp was found to degrade 75 different proteins in *S. aureus* biofilms (26). Nevertheless, previous work left unresolved by what mechanism Esp may interfere with *S. aureus* biofilms (26).

### Esp Expression and Purification—Pro-Esp (Met1–Gln282) with an N-terminal His tag was cloned into pET28b, expressed in *E. coli* BL21 (DE3) cells and purified using nickel affinity chromatography (nickel-nitritolriatric acid Superflow agarose resin; Qiagen) (29). Mature Esp was purified by cleaving pro-Esp with thermolysin followed by gel filtration chromatography (Superdex 75 10/30 column; GE Healthcare) with 20 mM Tris-HCl (pH 7.2), 150 mM NaCl. Briefly, purified pro-Esp was incubated with thermolysin at 37 °C for 4 h, and cleavage was quenched by the addition of 5 mM EDTA. The purity and proteolytic activity of Esp were confirmed by SDS-PAGE and azocasein assay, respectively (29). Esp was concentrated to 22 mg/ml using an Amicon ultrafiltration system.

### Esp Crystallization and Structure Determination—Concentrated, mature Esp was crystallized using the hanging drop vapor diffusion method (29). A droplet consisting of 1 μl of protein (22 mg ml⁻¹ in 20 mM Tris-HCl, pH 7.2, 150 mM NaCl) and 1 μl reservoir solution (0.25 M potassium acetate, 22% PEG 3350) was equilibrated against 1 ml of reservoir solution at 22 °C. Native diffraction data were collected to 1.8 Å resolution on a R-Axis IV imaging plate detector mounted on an in-house RIGAKU® rotating anode x-ray generator operating at 100 mA and 50 kV and using 20% (v/v) ethylene glycol as a cryoprotectant. Diffraction data were processed with D*TREK (30). The native Esp crystals belonged to the monoclinic space group P21 with one molecule in the asymmetric unit. Data collection and processing statistics are reported in Table 1.

| Data collection | Resolution range (Å) | Space group | P21 |
|-----------------|----------------------|-------------|-----|
| Data processing | Unit cell parameters (a, b, c in Å; β in °) | 39.4, 60.4, 42.3; 98.6 |
|                | Unique reflections   | 17,810      |
|                | Multiplicity         | 5.2 (5.1)   |
|                | Mean I/s (I)         | 202.2 (5.9) |
|                | Completeness (%)     | 97.4 (95.1) |
|                | Rmerge (%)           | 3.9 (20.1)  |
|                | Overall R factor from Wilson (Å²) | 23.8 |

**Table 1**

Data collection, processing, and refinement statistics

Numbers in parentheses correspond to the values in the highest resolution shell.

### Esp Cleavage of GST-Atl Hybrids by Esp and V8—GST-AM

GST-GL*ΔR₃* and GST-GL were purified as described previously (20). GST-AM was purified via a modified protocol (37). *E. coli* BL21 (DE3) harboring pGST-AM was grown in 2 liters of Luria broth at 37 °C to an *A*₆₀₀ of 0.5, expression was induced with 1 mM of isopropyl β-D-thiogalactopyranoside, and culture was incubated for an additional 3 h at 30 °C. The cells were harvested by centrifugation, suspended in STE lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 100 μg/ml lysozyme), and incubated on ice for 15 min following the addition of 5 mM DTT and 1.5 mM PMSE. Sarkosyl was added to a final concentration of 2% (w/v). Bacteria were disrupted in a French pressure cell at 6,000 p.s.i. followed by centrifugation for 10 min at 13,000 × g. The supernatant was transferred to a new tube, Triton X-100 was added to a final concentration of 2%, and samples were incubated at room temperature for 30 min and loaded onto 1 ml of glutathione-Sepharose 4B column (GE Healthcare) pre-equilibrated with STE. The column was washed with 100 ml of STE buffer. GST-AM was eluted with 10 ml of 20 mM glutathione, 10% glycerol, 10 mM Tris-HCl (pH 8.0), 120 mM NaCl. Purified GST-Atl hybrids (5 μg of GST-AM, 534–874) or the N-terminal end of GL (R3, residues 875–1016) (20). Each repeat domain folds into two half-open barrel subunits that bind polyglycerol-phosphate lipoteichoic acid at accessible sites in the bacterial envelope (21). Surface access is limited to peptidoglycan in the vicinity of the cell division septum (22, 23), because these sites are not occluded by polyribitol-phosphate wall teichoic acids (24). Deletion mutations in the *atl* gene abolish *S. aureus* biofilm formation, and *atl* mutants form large clusters of cells with incompletely separated cell wall envelopes (25).
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GST-AM\(_{\Delta R1R2}\) / GST-GL, or GST-GL\(_{\Delta 3R} \) were incubated with 400 nM Esp or V8 for 20 min at 37 °C. Samples were subjected to SDS-PAGE, and proteins were stained with Coomassie Brilliant Blue.

Biofilm Assays—Static biofilm assays were performed using a previously described protocol (38). Single S. aureus colonies were used to inoculate 2 ml of TSB with 0.2% glucose and incubated overnight at 37 °C with shaking (250 rpm). Cultures were diluted to \(A_{600}\) 0.05, and 200-µl aliquots were added to Costar 3596 96-well polystyrene plates (Corning, Lowell, MA) pre-treated with 100 µl of 1 µg/ml human fibronectin (BD, Franklin Lakes, NJ) in PBS overnight at 4 °C. Plates were incubated at 37 °C with 5% CO2 for 24 h and washed with 200 µl of PBS twice. Washed samples were treated with 100 µl of ethanol for 2 min and then stained with 100 µl of 0.41% crystal violet in 12% ethanol for 2 min. Excess stain was removed by three washes with 200 µl PBS. The remaining crystal violet staining was solubilized with 100 µl of 95% ethanol for 10 min, and absorbance of 595-nm light was measured. The average absorbance values of media-only wells were subtracted from wells that had been inoculated with S. aureus.

For biofilm restoration experiments, 2.5 µg of purified GST-Atl hybrids were incubated with S. aureus Newman on fibronectin-coated microtiter plates at 37 °C with 5% CO2 for 24 h. For biofilm disassembly experiments, S. aureus and S. epidermidis biofilms were formed for 24 h. Purified Esp or V8 (2.5 µg) were added, and samples were incubated for 24 h at 37 °C. Biofilms were quantified as described above and analyzed with the Student’s t-test using GraphPad Prism version 5.0 for Windows (GraphPad Software, La Jolla, CA.)

Biofilm Substrates of Esp—S. aureus biofilms were formed during growth in iron-depleted CRPMI (RPMI 1640) medium. Culture medium was depleted of iron by batch incubation with 6% (w/v) Chelex 100 and then supplemented with 10% RPMI 1640 to provide trace amounts of divalent cations for growth (13). S. aureus overnight cultures in TSB were diluted to \(A_{600}\) 0.05, and 50 µl were added to FALCON 150-mm culture dishes coated with 1 µg/ml human fibronectin. Plates were incubated at 37 °C with 5% CO2 for 24 h and washed three times with 35 ml of PBS each. Biofilm was removed with a cell scraper, suspended in 1 ml of PBS, and incubated with 400 nM Esp or left untreated for 16 h at 37 °C with rotation. Biofilm samples were subsequently boiled in sample buffer, and proteins separated by 10–20% gradient SDS-PAGE and visualized with Coomassie Blue staining. Protein bands were excised and identified with liquid chromatography tandem mass spectrometry at the Taplin Biological Mass Spectrometry Facility (Harvard Medical School).

Extracellular DNA in Staphylococcal Biofilms—S. aureus biofilms were formed in Costar 12-well polystyrene plates with 12-mm glass coverslips that had been pretreated with 1 µg/ml human fibronectin. The wells were washed three times with PBS and stained with 1 µl SYTO 9/propidium iodide (PI) at room temperature for 20 min. The wells were washed with PBS, and the samples were fixed with 4% paraformaldehyde. Coverslips were mounted on glass slides and viewed via light microscopy. Microscopy and image acquisition were performed with the Olympus “live cell” DSU spinning disk inverted confocal microscopy (Integrated Microscopy Core Facility, The University of Chicago). Images were obtained using a 40× objective. Fluorescence intensities from 15 random fields were quantified using ImageJ software.

Peptidoglycan Cleavage Assay—S. aureus peptidoglycan was purified as described previously (39). Briefly, staphylococci were grown in 2 liters of TSB to \(A_{600}\) 0.6 and centrifuged, and bacteria were washed in water, suspended in 4% SDS, and boiled for 30 min. Detergent was removed by washing staphylococci extensively in water. Staphylococci were subjected to bead beating, glass beads were removed, and cell debris was sedimented by centrifugation. The extract was incubated with 100 µg/ml amylase for 2 h, followed first by the addition of 10 µg/ml DNase and 50 µg/ml RNase for 2 h and then by incubation with 100 µg/ml trypsin for 16 h at 37 °C. Peptidoglycan extracts were centrifuged, washed with water, suspended in 1% SDS, and boiled for 15 min to heat-inactivate all enzymes. Peptidoglycan was extensively washed with water to remove all traces of SDS, followed by washing with 8 M LiCl, 100 mM EDTA, and acetone. The cell walls were then washed with water and lyophilized. Hydrofluoric acid was added and incubated for 48 h at 4 °C to remove teichoic acid. Peptidoglycan was neutralized, and sedimented murein sacculi were treated with alkaline phosphatase for 16 h at 37 °C. Purified peptidoglycan was boiled for 5 min, washed with water, and stored at 4 °C. Peptidoglycan was incubated with 5 µg of purified GST-AM, GST-AM\(_{\Delta R1R2}\), GST-GL, or GST-GL\(_{\Delta 3R} \) in 0.1 m phosphate buffer (pH 7.0) for 16 h at 37 °C. Peptidoglycan cleavage was determined by measuring the \(A_{600} \) before and after incubation.

Staphylococcal Cell Cluster Analysis—Overnight cultures of S. aureus were diluted 1:100 in 100 µl of TSB and added to 96-well plates at 37 °C with shaking. Culture growth was monitored by reading \(A_{600} \) at 30-min intervals. Overnight cultures were diluted 1:100 in 1 ml of TSB and incubated at 37 °C for 2 h with shaking with or without 25 µg of GST-Atl hybrids. Cultures were subsequently centrifuged at 7,000 × g for 1 min and fixed with 4% paraformaldehyde prior to washing and suspension in 1 ml of PBS. Staphylococci were then analyzed at the University of Chicago flow cytometry facility using BD LSRII Blue flow cytometer to measure the cluster size of S. aureus cells.

Activity Measurements of Staphylococcal Proteases—Concentrated extracellular media, obtained as the supernatant following centrifugation of overnight cultures of S. aureus, were concentrated 15-fold using the Amicon ultrafiltration system. Concentrated culture media in 20-µl aliquots were incubated with 480 µl of reaction mixture containing 1% azocasein, 100 mM Tris-HCl (pH 8.0) at 37 °C overnight. Following incubation, 25 µl of 100% TCA was added to quench each reaction; following centrifugation at 15,000 × g for 10 min, soluble material was recovered with the supernatant, and absorbance at 440 nm was measured to determine protease activity.

RESULTS

Esp Cleaves Atl in Staphylococcal Biofilms—Following signal peptide cleavage, the pro-form of Esp (pro-Esp) is cleaved in the extracellular medium of S. epidermidis cultures to generate mature Esp protease, which mediates the disassembly of
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S. aureus biofilms (7). We expressed six-histidyl-tagged pro-Esp in E. coli and purified recombinant protein by affinity chromatography (Fig. 1A). Thermolysin cleavage and gel filtration chromatography were used to obtain purified Esp (Fig. 1B). The variant EspS235A harbors an alanyl substitution at the active site serine residue of Esp (Fig. 1A). When examined for protease activity with azocasein substrate (40), Esp cleaved significantly more substrate than pro-Esp, whereas EspS235A did not display protease activity (Fig. 1C). Wild-type S. aureus strain Newman was used to form staphylococcal biofilms using human fibronectin as a matrix, which were quantified by crystal violet staining (41). Treatment with Esp, but not pro-Esp or EspS235A, triggered disassembly of staphylococcal biofilms (Fig. 1D). Proteins in biofilms with or without Esp treatment were separated by 10-20% gradient SDS-PAGE, stained with Coomassie Blue, and identified via LC-MS/MS (Fig. 1). Table 2. Although the genes for some of these secreted proteins contribute to S. aureus biofilm formation, they are not essential for this developmental process. Of note, in S. aureus Newman biofilms, Atl is a highly abundant component and effectively degraded during Esp treatment (Fig. 1E). Considering the importance of Atl in biofilm development, we focused our experimental approach on the interactions between Esp and Atl.

**Esp Treatment of Biofilms Formed from atl Staphylococci—Mutations in the autolysin gene (atlE) of S. epidermidis cause a dramatic reduction in biofilm formation (42). AtlE was initially shown to function as a S. epidermidis adhesin (purified AtlE binds host vitronectin, fibronectin, and Hsc70 receptor (42)). More recent work highlighted the contribution of atl in S. aureus UAMS-1, BH1CC, and many other MSSA and MRSA isolates toward releasing DNA as an extracellular matrix for staphylococcal biofilm formation (16, 38). This discovery was accompanied by the insight that S. aureus, but presumably not S. epidermidis (43), forms biofilms in vitro and in vivo without the icaABCD locus (16), which provides for the synthesis of (81-6)-poly-N-acetylgalcosamine exo-polysaccharide (44). We wondered whether atl mutant S. aureus Newman can form biofilms and, if so, whether atl bacterial communities can be disassembled by treatment with Esp. Compared with wild-type staphylococci, the atl mutant formed only a rudimentary biofilm that, when subjected to treatment with Esp, did not show

![Table 2](image)
significant disassembly (Fig. 2A). As a control, Esp treatment caused disassembly of biofilms formed by \textit{S. aureus} Newman. When subjected to growth assays with rotating cultures, the \textit{atl} mutant replicated at a rate indistinguishable from that of wild-type staphylococci (Fig. 2B), although the \textit{atl} mutants formed large clusters of incompletely separated staphylococci (23) (see below). The growth of wild-type and \textit{atl} mutant staphylococci was not perturbed when cultures were treated with Esp, indicating that protease treatment kills neither wild-type nor mutant strains (Fig. 2B). As a measure for the direct dispersal of bacteria from biofilms, staphylococci were labeled with SYTO9 and then subjected to Esp treatment. Esp released approximately half of wild-type staphylococci from biofilms and almost all \textit{atl} bacteria from their rudimentary biofilm (Fig. 2C). These experiments identify Atl as a key target of Esp, whose degradation prevents biofilm formation and is associated with the disassembly of biofilms. Moreover, the contributions of other targets of Esp, with known auxiliary functions in biofilm formation or stability (Eap, Emp, SpA, FnbA, and FnbB), are revealed as protease treatment eliminates the rudimentary biofilm of \textit{atl} mutants.

\textit{Esp Cleavage of Atl}—To determine which of the functional domains of Atl are cleaved by Esp, we purified AM (N-acetylmuramyl-L-alanine amidase), AM\textsubscript{ΔR1R2} (lacking the C-terminal repeat domains R1 and R2 of AM), GL (N-acetylglucosamine-N-acetylmuramic acid glucosaminidase), and GL\textsubscript{ΔR3} (lacking the N-terminal R3 domain of GL) as hybrids fused to the C-terminal end of GST (Fig. 3. Esp treatment cut AM, AM\textsubscript{ΔR1R2}, and GL, but not GL\textsubscript{ΔR3} (Fig. 3B). Esp treatment generated several cleavage fragments from AM, AM\textsubscript{ΔR1R2} or GL, suggesting that the protease can cut at multiple sites within the amidase and the R1-R3 domains (Fig. 3B). Edman degradation of cleaved peptides identified glutamic acid residues (for example Glu\textsuperscript{862} in GL) as Esp cleavage sites (Fig. 3B).

\textit{Murein Hydrolase Activities of Esp-treated Atl}—To explore the effects of Esp treatment on Atl murein hydrolase activities, we purified murein sacculi from wild-type \textit{S. aureus} and extracted wall teichoic acids via hydrofluoric acid treatment (45). The murein hydrolase activities of AM and AM\textsubscript{ΔR1R2} as well as GL and GL\textsubscript{ΔR3} were determined by incubating GST hybrids fused to the C-terminal end of GST with murein sacculi while monitoring absorbance at 600 nm. Similar to lysostaphin (46), a glycyl-glycine endopeptidase that cleaves staphylococcal cell wall cross-bridges (47), GST-AM treatment of peptidoglycan caused a large decrease in absorbance (Fig. 4A). Esp treatment abolished all peptidoglycan hydrolase activity of AM (Fig. 4A). Removal of the R1-R2 repeat
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Esp Treatment and Staphylococcal Release of Extracellular DNA—We sought to test the hypothesis that Esp blocks the release of extracellular DNA during biofilm formation and signaling. Extracellular DNA release may be quantified by flow cytometry, which revealed that 6.82% of wild-type but 57% of trans-Atl mutants exist as large cell clusters (Fig. 5). The cluster reducing activity of GST-AM and GST-GL was abolished by treatment with Esp (Fig. 5).

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FIGURE 3. Esp treatment of GST-Atl hybrids. A, diagram illustrating the primary structure of GST hybrids with Atl domains including GST-AM, GST-AM(ΔR1R2), GST-GL, and GST-GL(ΔR3). B, purified GST hybrids (5 μg) were incubated with 400 nM Esp (+) or mock treated (−) for 20 min at 37 °C. Proteins were separated on SDS-PAGE followed by Coomassie Blue staining. Black arrowheads identify the migratory positions of GST-AM, GST-AM(ΔR1R2), GST-GL, and GST-GL(ΔR3). The white arrowhead identifies an Esp cleavage species of GST-GL, which had been cut after glutamyl 862 (E/VKTTQK), as identified by Edman degradation.

FIGURE 4. Peptidoglycan hydrolase and biofilm promoting activity of GST-Atl hybrids in the presence or absence of Esp treatment. A, S. aureus Newman murein sacculi were obtained with a bead beater instrument and extracted with detergent as well as hydrofluoric acid to remove membranes and wall teichoic acids, respectively. Cleavage of peptidoglycan by 5 μg of purified lysostaphin (lyso), GST-AM (AM), GST-AM(ΔR1R2) (AM(ΔR1R2)), GST-GL (GL), or GST-GL(ΔR3) (GL(ΔR3)) in the presence (+) or absence (−) of 400 nM Esp was monitored by measuring absorbance at 600 nm (A600). The data represent average ± sample standard deviation of three independent experimental determinations, and the standard error of the means is indicated by brackets. Statistical significance was assessed in pairwise comparison with the two-tailed Student’s t test. **, p < 0.001; ***, p < 0.0001; *, p < 0.01; **, p < 0.05. B, biofilm formation of the atl mutant on fibronectin-coated microtiter plates at 37 °C with 5% CO2 for 24 h was analyzed in the presence or absence (mock) of 5 μg of purified GST-AM (AM), GST-AM(ΔR1R2) (AM(ΔR1R2)), GST-GL (GL), GST-GL(ΔR3) (GL(ΔR3)), or 400 nM Esp (+) or −). Following incubation, the plates were washed and stained with crystal violet to measure biofilm formation as absorbance at 595 nm (A595). Biofilm data were averaged from three independent determinations. The standard error of the means is indicated as brackets. Statistical significance was assessed with the two-tailed Student’s t test. **, p < 0.001; ***, p < 0.0001; **, p < 0.05.

Domains of AM reduced the peptidoglycan hydrolase activity of AM(ΔR1R2); however, this activity was also abolished by treatment with Esp (Fig. 4A). Finally, GL displayed very little activity in the reducing the absorbance at 600 nm, which can be explained by the relatively short glycan chains and intricate cross-linking (>99%) of the staphylococcal cell wall (20). The murein hydrolase activity of GL was abolished by Esp treatment (Fig. 4A). GL, which had been cut after glutamyl 862 (E/VKTTQK), as identified by Edman degradation.

trans-Complementation of atl Mutant Biofilms—The addition of purified GST-AM or GST-GL restored (trans-complementation) the biofilm formation defect of atl mutant S. aureus Newman grown on fibronectin-coated microtiter plates (Fig. 4B). This activity was abolished following treatment of GST-AM with Esp (Fig. 4B). Esp treatment did not affect peptidoglycan hydrolase activity of GST-GL and did not affect GST-GL biofilm trans-complementation either. GST-AM(ΔR1R2) also did not display biofilm trans-complementation for atl mutants, and Esp treatment did not affect this phenotype (Fig. 4B). GST-GL(ΔR3) did not degrade murein sacculi and did not trans-complement the atl mutant biofilm defect (Fig. 4B). Esp treatment did not affect biofilm formation in the presence of GST-GL(ΔR3) (Fig. 4B).
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**FIGURE 5. Effect of Esp treatment on the staphylococcal cluster dispersing activity of GST-Atl hybrids.** A, overnight cultures of *S. aureus* Newman wild-type (wt) or *atl* variant were diluted to A600 0.05 in 1 ml of TSB and incubated at 37°C for 2 h in the presence or absence of 25 μg of purified GST-AM (AM), GST-AMΔR1ΔR2 (AMΔR1ΔR2), GST-GL (GL), GST-GLΔR1 (GLΔR1), or 400 nM Esp (+ or −). Staphylococci were fixed with 4% paraformaldehyde, washed, suspended with 1 ml of PBS, and analyzed by flow cytometry. B, percentage of bacteria in large cell clusters were quantified for wild-type and *atl* mutant staphylococci with or without Atl hybrids and Esp treatment.

added the protease to either wild-type or *atl* mutant *S. aureus* Newman. Biofilms were stained with either PI as a measure for extracellular DNA or with SYTO9 to quantify viable staphylococci (Fig. 6). Treatment with Esp reduced the amount of extracellular DNA and bacterial cells, as quantified by PI and SYTO9 staining (Fig. 6). Treatment with Esp did not improve biofilm formation of *S. aureus* Newman *atl* or *sspA* mutants (Fig. 7C). These results suggest that the expression and/or activity of secreted V8 protease must be carefully controlled during *S. aureus* biofilm formation, because treatment with exogenous, active V8 protease cannot complement the *sspA* mutant phenotype (Fig. 7C). Of note, neither V8 nor Esp protease treatment affected biofilm formation of *S. epidermidis* RP62a (Esp+), suggesting that the biofilm program of this microbe is not controlled by secreted serine proteases or their protease-sensitive substrates (Fig. 7C).

**Crystallographic Structure of Esp**—Purified Esp was crystallized, and its three-dimensional structure was determined using x-ray crystallography. Esp displays a β-barrel fold assembled from two discrete domains and a C-terminal α-helix, similar to eukaryotic serine proteases of the chymotrypsin family (Fig. 8, a and b) (53–55). Even though Esp exhibits a highly conserved, compact β-barrel fold, the five or more intradomain disulfide bonds that are responsible for the structural rigidity of eukaryotic serine proteases are absent (54). The five or more intradomain disulfide bonds that are responsible for the structural rigidity of eukaryotic serine proteases are absent (54). Each of the two Esp domains is comprised of six antiparallel β-strands, and the solvent-accessible catalytic and substrate binding sites are situated at the interface of the two domains. The N-terminal domain (chymotrypsin nomenclature) is comprised primarily of residues Gln77–Ile183, whereas the C-terminal domain encompasses Ser184–Ile264. Although the position of the C-terminal α-helix (Asp266–Ile276) is conserved with that of other serine proteases, the N-terminal segment (Val67–Gln76) contains a short β-strand that is associated with the substrate-binding S1 pocket and distinct from eukaryotic serine proteases (Fig. 8b).

In addition to the conserved position of putative catalytic triad residues (Ser235, Asp159, and His117), the substrate-binding region (S1 pocket) and the oxyanion hole, which together constitute the critical functional elements of activated serine proteases, are also conserved in Esp (Fig. 8a). A search for structural homologues of Esp identified *S. aureus* V8, a serine protease with a Z-score of 39.7 and 59% primary sequence identity (PDB code 1QY6) (33).

**Structural Comparison of Esp and V8**—The distances between Ne of the Esp active site His117 and Oγ of Ser235 and...
FIGURE 6. Esp treatment and the release of extracellular DNA in *S. aureus* biofilms. A, purified 400 nM Esp or DNase I were incubated with *S. aureus* Newman wild-type (wt) or its *atf* variant during biofilm assembly on fibronectin-coated microtiter plates at 37 °C with 5% CO₂ for 24 h. Following incubation, plates were washed and stained with PI to reveal extracellular DNA or SYTO 9 to reveal viable staphylococci and analyzed via DIC and fluorescence microscopy. DIC, differential interference contrast. B, fluorescence intensity staining of PI, SYTO 9, or PI/SYTO 9 staining in samples from A was quantified with ImageJ. The data were averaged from three independent determinations, and the standard error of the means is indicated as brackets. Statistical significance was assessed in pairwise comparison using the two-tailed Student’s t test. ***, p < 0.0001; **, p < 0.001; *, p < 0.05.
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**FIGURE 7.** *S. aureus* V8 protease and biofilm formation. A, protein sequence alignment of mature Esp and V8, B, GST-AM, GST-AM_{ATRIH2}-GST-GL, and GST-GL\_{F3} (5 µg) were incubated with 400 nM Esp (Esp), V8 protease (V8), or mock treatment (−) for 20 min at 37 °C. Proteins were separated on SDS-PAGE followed by Coomassie Blue staining. Arrowheads identify the migratory positions of GST-AM, GST-AM_{ATRIH2}, GST-GL, and GST-GL_{F3}. C, purified Esp, V8, or mock treatment were added during biofilm formation of *S. epidermidis* RP62a and *S. aureus* Newman wild-type and *atl* and *sspA* mutant strains on fibronectin-coated microtiter plates at 37 °C with 5% CO2 for 24 h. Following incubation, plates were washed and stained with crystal violet, and biofilm formation was measured as absorbance at 595 nm. D, *S. aureus* Newman wild-type (wt) or its *sspA* mutant without plasmid (−) or with *pspA* or vector control (pWW412) was incubated on fibronectin-coated microtiter plates at 37 °C with 5% CO2 for 24 h. Following incubation, the plates were washed and stained with crystal violet to measure biofilm formation as absorbance at 595 nm (A595). Biofilm data were averaged from three independent determinations. The standard error of the means is indicated as brackets. Statistical significance was assessed with the two-tailed Student’s t test. ***, p < 0.001; *, p < 0.05; NS, no significant difference. E, the activity of extracellular proteases secreted by *S. aureus* wild-type (wt), *atl* and *sspA* mutant cultures were quantified with the azocasein assay, and product cleavage was measured as absorbance at 440 nm. The protease activity data were averaged from three independent determinations. The standard error of the means is indicated as brackets. Statistical significance was assessed with the two-tailed Student’s t test. NS, no significant difference.
Research over the past two decades has identified bacterial genes and mechanisms supporting biofilm growth, which can be thought of as a developmental program with three or more discrete steps. Biofilm formation initially requires bacterial adhesion to solid surfaces, and this includes bacterial adhesion to surfaces at liquid-air interfaces. Bacterial replication into a biofilm is dependent on cell-cell adhesions and on the establishment of an extracellular matrix, which is often comprised of DNA released from a subpopulation of biofilm bacteria but may also involve the synthesis of extracellular polysaccharides.

Eventually, biofilms must release planktonic cells for dissemination in tissues of an infected host and/or development of new biofilm structures. These paradigms also appear to apply to the biofilms formed by Staphylococcus aureus, a pathogen that colonizes human nares. Iwase et al. reported that Staphylococcus epidermidis Esp, a secreted serine protease, can disperse S. aureus biofilms. Furthermore, colonization with Esp+ S. epidermidis strains was associated with protection from S. aureus colonization and administration of Esp+ S. epidermidis into the nares of human volunteers diminished S. aureus colonization. These findings provide strong support for the model of S. aureus biofilm formation in human nares; however, others have challenged this view and proposed that S. aureus may replicate as planktonic bacteria in the nasal cavity.

Here we investigated the molecular basis of S. epidermidis Esp interference with S. aureus biofilm formation. Our data suggest that Atl is the premier target of Esp-mediated biofilm interference. Esp treatment diminished Atl-dependent release of extracellular DNA by cleaving the AM and GL murein hydrolase activities. Esp treatment did not affect biofilm formation for atl and sspa mutants of S. aureus Newman. X-ray crystallography revealed the three-dimensional structure of Esp, which is highly similar to that of S. aureus V8 (SspA) (33). V8 protease also cleaved Atl AM and GL and blocked biofilm formation. These data suggest that S. aureus biofilms are formed under conditions of controlled secretion and proteolysis of...
autolysin, a determinant for the release of DNA biofilm matrix. This developmental program can be perturbed by the Esp protease of S. epidermidis and by the V8 protease.

Earlier work reported that spsA expression in S. aureus SH1000, a variant of laboratory strain 8325-4 (RN6390B) in which the rsblU mutational lesion has been repaired (65), is required for biofilm formation when this strain is grown in 2% tryptic soy broth supplemented with 0.2% glucose but not when the strain is grown in TSB alone (52). In S. aureus SH1000, mutations in spsA and in other genes for extracellular serine proteases (splABCDEF) trigger a relative increase in extracellular protease activity (52, 66), which is associated with a reduction in biofilm formation. This phenotype is abolished in a genetic background where the structural gene for aureolysin (aur) has been deleted (52); aureolysin is a metalloproteinase that, following secretion into the extracellular medium, activates the serine proteases of S. aureus via removal of their pro-peptides (67, 68). Presumably, a cascade of secretion reactions and the sequential activation of extracellular proteases (aureolysin > cysteine proteases > serine proteases) control the activity of secreted Atl and the assembly or disassembly of staphylococcal biofilms (62).

We also solved the three-dimensional structure of Esp. A search for structural homologues of Esp using the DALI server (69) identified seven structures with less than 2.0 Å root mean square deviation value. S. aureus V8 protease was the best fit with a Z-score of 39.7 and 59% primary sequence identity (PDB code 1QY6) (33). Staphylococcal epidermyotic toxin A (ETA) (PDB code 1AGI) was the second best with Z-score of 29.9 and 28% sequence identity (70). Staphylococcal secreted serine proteases SplB (PDB code 1VID) and SplA (PDB code 2W7U) displayed 32% and 28% identity, respectively (71). Glutamyl-endopeptidase (PDB code 1P3C, 26% identity) and exo-lytive toxin B (PDB code 1QTF, 29% identity), with root mean square deviation less than 2 Å were also identified (72).

Nemoto and co-workers (73, 74) characterized S. aureus glutamyl endopeptidase V8 and identified, in addition to catalytic Ser237 (Esp Ser235), the N-terminal Val69 (Esp Val67) residue as essential for substrate cleavage. S. aureus V8 protease with an N-terminal truncation to Ile70 (Esp Ile69) was inactive, and mutants with an altered N-terminal residue Val69 (even with conserved substitutions) were also inactive, which is indicative of a strict requirement of the N-terminal Val residue for enzyme activity (75). Crystal structures of Esp and V8 display identical disposition for their N termini, which associate with respective S1 pockets more intimately than other active serine proteases (Fig. 8c). The N-terminal segment of Esp and V8 crosses over loop 1 into the bottom of the S1 pocket, and the N-terminal Val69 (V8 Val67) amino group is suitably positioned to act as an acceptor of the negative charge of P1 residue side chain (33). The N-terminal Val67 in Esp is positioned with its α-amino group located adjacent to the conserved Thr230 (V8 Thr232) and Asn259 (V8 Asn261), pointed into S1 pocket, within hydrogen bonding distance. Similarly, the His250 (V8 His252) residue on loop 2, conserved among glutamyl endopeptidases (73), having hydrogen bonds with side chains of conserved Tyr226 (V8 Tyr228) and Thr230 (V8 Thr232), is also suitably positioned to interact with the substrate acidic P1 residue.

Nevertheless, there are some notable differences between V8 and Esp in and around their S1 pockets (Fig. 8d) that can be associated with differences in substrate specificity. Extensive mutational analysis of V8 and Esp by Nemoto et al. (73) localized the difference in their specificities to Tyr251 (V8 Trp253) and Asp255 (V8 Pro257) residues on Esp loop 2. Substitutions at these positions affected mainly the K cat with constant k cat values, suggesting that these residues affect only substrate binding affinities (73). The K cat of native Esp harboring Tyr251-Val254-Asp255 on loop 2 was larger than that of V8 with Trp253-Val256-Pro257, but with almost similar k cat values (76). Tyr251 of Esp is hydrogen-bonded with the side chain of Glu223, which is replaced by Ala223 in V8. In addition, the Lys257-Tyr258-Asn259-Ser260-Asn261 segment on loop 2 of Esp is replaced by Glu259-Tyr260-Asn261-Gly262-Ala263 in V8, all side chains pointing out of the S1 pocket, but into the known specificity determining secondary sites of serine proteases. Other notable residue differences between Esp and V8 in the vicinity of the S1 pocket include Tyr92 (V8 Gln94) and Tyr99 (V8 Thr101) on loop A. Thus, the S1 pockets of Esp and V8 preferentially bind negatively charged substrate side chains that are held in place by the amino group of the N-terminal Val67 (V8 Val69) residue and stabilized by conserved Thr230, His250, and Asn259 in Esp. However, the specificity differences between equally efficient Esp and V8 enzymes toward acidic P1 residue could be assigned to the differences observed in loop 2, specially to the Asp255 (V8 Pro257) present at the bottom of S1 pocket and pointing toward the catalytic site and other secondary residues on either side of the substrate P1 residue to the difference in loop 2 and loop D segments. These features of staphylococcal serine proteases may explain why Esp and V8 are able to cleave multiple domains of Atl and, when added exogenously during the early stages of biofilm formation, can interfere with the establishment of these structures. The V8 protease does contribute to biofilm formation of S. aureus Newman presumably by controlling the autolytic activity of Atl-derived AM and GL enzymes. Thus, secreted serine proteases can be viewed as biofilm regulatory factors that impact the production of biofilm matrix and

![Figure 9. Model illustrating S. aureus atl-dependent biofilm formation and the impact of serine proteases, i.e., S. epidermidis Esp or S. aureus V8 (SspA), on controlling Atl activity and biofilm disassembly. The model distinguishes five steps in the biofilm developmental process: attachment, eDNA release, maturation, detachment, and dissemination. Three surface proteins (Esp, FnbA, and FnbB) are thought to promote S. aureus attachment to fibronectin (attachment). The secretion of Atl promotes the release of eDNA as an extracellular matrix for biofilm formation (eDNA release). Activation of secreted SspA (V8 protease) inactivates Atl, thereby promoting staphylococcal replication in the newly formed matrix (biofilm maturation). The continued activation of SspA promotes the detachment of staphylococcal cells from the biofilm (detachment). Detached staphylococci disseminate and adhere elsewhere by binding to fibronectin and establishing another biofilm. S. aureus biofilm formation is perturbed by the S. epidermidis secreted protease Esp. We propose that exuberant expression of S. epidermidis Esp (unlike S. aureus SspA) perturbs biofilm formation of S. aureus.](image-url)
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the release of planktonic bacteria to initiate invasive disease (Fig. 9). If so, application of serine proteases (Esp or V8) as a treatment of nasal colonization with S. aureus may disperse planktonic staphylococci with invasive disease potential.

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