Modification of the *Staphylococcus aureus* Fibronectin Binding Phenotype by V8 Protease

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The amount of cell surface fibronectin (Fn)-binding protein (FnBP) adhesins expressed by *Staphylococcus aureus* is maximal during exponential growth but disappears rapidly as the culture progresses into stationary phase. To identify factors responsible for the loss of cell surface FnBP, a culture of *S. aureus* L170, which shows high levels of Fn binding, was supplemented at the time of inoculation with concentrated stationary-phase supernatant from *S. aureus* L530, a strain which binds Fn poorly. The resulting exponential-phase cells were devoid of FnBP. The factor responsible for this activity was purified from the culture supernatant and identified as V8 protease. When cultured with 375 ng of exogenous V8 protease ml\(^{-1}\), exponential-phase cells of *S. aureus* L170 were devoid of cell surface FnBP, and concentrations as low as 23 ng m\(^{-1}\) resulted in reduced amounts of FnBP. Addition of the protease inhibitor \(\alpha\)-macroglobulin to the culture medium prevented the growth-phase-dependent loss of cell surface FnBP, whereas growth with exogenous V8 protease resulted in reduced adherence to the solid-phase N-terminal fragment of Fn and to the extracellular matrix synthesized by fetal rabbit lung fibroblasts. Although FnBP was extremely sensitive to V8 protease, exogenous protease did not exert a significant influence on the amount of cell surface protein A. However, a limited number of other high-molecular-weight cell surface proteins were also sensitive to V8 protease. Therefore, both the adhesive phenotype and cell surface protein profile of *S. aureus* can be modified by V8 protease activity.

The microbial infectious process can be described as two major phases in which a pathogen must first adhere to and colonize host tissue and then overcome or elude host defense mechanisms and cross tissue barriers (9). However, a phenotype that is optimal for colonization may not be conducive to subsequent persistence and invasion. Consequently, the expression of virulence factors is coordinated to meet the changing requirements of a pathogen during the different phases of infection (30). As a paradigm of this concept, *Staphylococcus aureus* can efficiently colonize host tissues through its ability to express adhesins specific for extracellular matrix (ECM) proteins, such as collagen (35), fibrinogen (27), and fibronectin (Fn) (23, 43), and also an adhesin with broad ligand binding specificity for several different ECM proteins (28). Following colonization, *S. aureus* can then quickly invade deeper tissues, including bone and joints, and frequently reaches the bloodstream, where it excels in the ability to cause metastatic infection in virtually every tissue and organ system of the body (42). Therefore, *S. aureus* appears to be particularly adept at orchestrating the transition between the colonization and invasive phases of infection.

This transition is coordinated through three interactive global regulatory networks, agr (37), xpr (17), and sur (11), of which agr is the best characterized in terms of its global influence. Mutations in agr block the post-exponential-phase induction of several secreted proteins that promote the invasive phase of infection, including serine protease, lipase, fibrinolysin, alpha-hemolysin, beta-hemolysin, delta-hemolysin, enterotoxin B, and toxic shock syndrome toxin (7). In contrast, coagulase, protein A (immunoglobulin G binding), and Fn-binding protein (FnBP) are expressed maximally by exponentially growing cells (3, 7, 38), and strains that are defective in agr exhibit elevated expression of protein A (34) and FnBP (1, 12) and a constitutive intermediate level of coagulase (26). Therefore, agr appears to exert a negative influence on the expression of genes involved in the colonization phase of infection. Potentially, constitutive adherence could retard or prohibit microbial invasion of deeper tissue. In this situation, the transition to the invasive phase of infection could be accelerated by the down-regulation of adhesin expression and by the elimination of cell surface adhesins following successful colonization. Accordingly, we have noted a dramatic reduction in the amount of cell surface FnBP as *S. aureus* cultures progress toward the stationary phase of growth.

To identify the mechanism of this phenomenon, we considered recent findings that a secreted octapeptide pheromone is responsible for the induction of the agr locus (4, 21) and also that a secreted cysteine protease of *Streptococcus pyogenes* releases biologically active fragments of cell surface proteins (6). In either situation, we anticipated that a soluble factor secreted by *S. aureus* cells would elicit a reduction in the amount of cell surface FnBP as the culture progresses towards stationary phase. To test this hypothesis, we selected a clinical isolate of *S. aureus* that binds Fn poorly. When concentrated stationary-phase culture supernatant from this strain was added to a culture of *S. aureus* that binds large amounts of Fn, cells harvested in the exponential growth phase were devoid of FnBP. The factor responsible for this activity was purified and identified as V8 (serine) protease. Herein, we present evidence that V8 protease can moderate the adhesive phenotype of *S. aureus*.

**MATERIALS AND METHODS**


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Microbiology Laboratory. Strain L530, obtained from a blood culture of a patient with endocarditis, binds Fn poorly, while strain L170, obtained from a blood culture of a patient with a wound infection, exhibits high levels of Fn binding. Strains L786, L516, and L857 were obtained from blood cultures of patients with dialysis peritonitis, and septic arthritis, respectively. S. aureus cultures were inoculated with an overnight culture of S. aureus L530 to achieve an initial optical density at 600 nm (OD 600) of 0.1. The culture was grown at 37°C on a shaker at 4°C on an orbital shaking platform in 50 ml of PBS–0.1% BSA–0.05% NaCl. Aliquots of protoplast fluid containing released cell surface proteins were subjected to Immobilon-P and probed with the biotinylated N-terminal fragment of Fn to quantitate binding of Fn to cell surface proteins. Preloading of Immobilon-P with the biotinylated N-terminal fragment of Fn was omitted. Where indicated, blots were scanned on a Hewlett-Packard ScanJet model 3C at a resolution of 600 dots per inch. The images were saved as TIFF files and imported into Adobe Photoshop as black-and-white images. Computer-aided pixel density analysis of the bands corresponding to FnBP.

Preparation of culture supernatant and purification of V8 protease. Two 24-h cultures of S. aureus (National Institute of Allergy and Infectious Diseases) were inoculated with an overnight culture of S. aureus L530 to achieve an initial optical density at 600 nm (OD 600) of 0.1. The culture was grown at 37°C on an orbital shaking platform in 50 ml of PBS–0.1% BSA–0.05% NaCl. Aliquots of protoplast fluid containing released cell surface proteins were isolated as described previously (40) and cultured in 96-well microtiter plates (Nunc), with minimal essential medium containing 10% fetal bovine serum. Fibroblast ECM was added to each well (1:1, v/v) and grown to an OD 600 of 0.8 at 37°C. Cultures were supplemented at the time of inoculation to four different clinical isolates of S. aureus.

RESULTS

Cell surface FnBP is absent from cells cultured with exogenous supernatant from S. aureus L530. Of 26 commensal and clinical methicillin-susceptible S. aureus isolates assayed for binding of Fn with strain L530, the best binding, amounting to 8% of the added ligand, compared to a median value of approximately 33% (unpublished observations). To determine if a secreted factor could contribute to the growth-phase-dependent reduction in the amount of cell-surface FnBP, concentrated stationary-phase culture supernatant was prepared from cultures of S. aureus L530 and added at the time of inoculation to four different clinical isolates of S. aureus. Cells harvested from the exponential-phase cultures were adjusted to equivalent cell densities (1010 cells ml−1) and subjected to protoplast treatment to release cell surface proteins. Subsequent SDS-PAGE and Coomassie blue staining showed that several different proteins common to each preparation exhibited a similar intensity of staining, indicative of similar protein profiles. A signal for FnBP was observed in each preparation (Fig. 1A). The cell surface protein profiles of the exponential-phase cells cultured with exogenous station-ary-phase supernatant from S. aureus L530 were similar to those of control cultures. However, in each case, a notable exception was an approximately 200-kDa protein that was absent from cultures supplemented with concentrated supernatant from S. aureus L530.

Cell surface proteins from cultures supplemented with concentrated supernatant from strain L530 were devoid of FnBP.
V8 protease promotes the loss of cell surface FnBP. To identify the factor responsible for the loss of cell surface FnBP, the concentrated culture supernatant was subjected to chromatography on Q-Sepharose Fast-Flow, DEAE MemSep, and Sephacryl S-100. Throughout the purification, fractions enriched in a 31.4-kDa protein consistently promoted the loss of cell surface FnBP in cultures of \textit{S. aureus} L170 (data not shown), and a homogenous preparation of this protein was obtained after chromatography on Sephacryl S-100 (Fig. 2). From 1 liter of culture supernatant containing 254 mg of protein, a total of 6 mg of purified protein was recovered, giving a 2.4% yield of total culture protein. As the 31.4-kDa protein is the most abundant protein in the culture supernatant of \textit{S. aureus} L530 (Fig. 2), it is likely that endogenous protein in the BHI broth culture medium resulted in an erroneously high estimation of the amount of culture supernatant protein. N-terminal sequence analysis of the purified protein produced the sequence VILPNNDRHQITDTTNGHYA. A BLAST search (2) of the GenBank protein sequence database identified a perfect match with the 29.0-kDa V8 protease of \textit{S. aureus} V8 and with a related 31.3-kDa protease produced by \textit{S. aureus} ATCC 12600, starting at residue 69 of the full precursor sequence and residue 1 of the mature active peptides (10, 51). Amino acid composition analysis also indicated a close match with the known amino acid composition of both proteases (10, 51) (Table 1).

When added to cultures of \textit{S. aureus} L170 at the time of inoculation, 375 ng of exogenous V8 protease \(\text{ml}^{-1}\) resulted in exponential-phase cells that were devoid of FnBP, and concentrations as low as 23 ng \(\text{ml}^{-1}\) caused a noticeable reduction in the amount of FnBP relative to that in control cultures (Fig. 3). To examine the influence of V8 protease on other cell surface proteins, cultures of \textit{S. aureus} L170 and L857 were grown to mid-exponential phase with or without 1 \(\text{mg}\) of exogenous V8 protease \(\text{ml}^{-1}\) and then surface labelled with biotin prior to protoplast treatment. The released cell surface proteins were then subjected to SDS-PAGE and transferred to an Immobilon-P membrane, allowing biotinylated proteins to be detected with alkaline phosphatase-conjugated streptavidin. This process revealed that only a limited number of high-

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Amino acid & \textit{L530} & \textit{V8} & \textit{ATCC 12600} \\
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Asx & 23.07 & 24.25 & 27.68 \\
Thr & 7.22 & 7.09 & 6.57 \\
Ser & 4.11 & 3.73 & 3.46 \\
Glx & 9.45 & 8.95 & 7.96 \\
Pro & 8.10 & 9.33 & 11.07 \\
Gly & 9.58 & 8.21 & 7.61 \\
Ala & 7.11 & 6.34 & 5.88 \\
Val & 5.46 & 7.09 & 6.57 \\
Met & 1.16 & 1.12 & 1.04 \\
Ile & 4.82 & 5.60 & 5.19 \\
Leu & 3.32 & 2.99 & 2.77 \\
Tyr & 2.94 & 2.61 & 2.42 \\
Phe & 3.58 & 3.36 & 3.11 \\
His & 2.89 & 2.99 & 2.77 \\
Lys & 5.32 & 4.85 & 4.50 \\
Trp & 1.03 & 0.75 & 0.69 \\
Arg & 0.85 & 0.75 & 0.69 \\
\hline
\end{tabular}
\caption{Comparison of amino acid composition of serine protease purified from \textit{S. aureus} L530 with known compositions of mature \textit{S. aureus} serine proteases based on published nucleotide sequences of \textit{S. aureus} V8 (10) and ATCC 12600 (51)}
\end{table}


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\textsuperscript{a} Asx, combined aspartate and asparagine; Glx, combined glutamate and glutamine.

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molecular-mass cell surface proteins ranging in size from 133 to 202 kDa were sensitive to exogenous V8 protease, whereas the stability of smaller proteins was not affected (Fig. 4).

**Loss of cell surface FnBP is reversed during growth with exogenous protease inhibitor.** To determine if endogenous proteolytic activity contributes to the growth-phase-dependent reduction in the amount of cell surface FnBP, *S. aureus* L170 was cultured with or without exogenous α2-macroglobulin, a universal protease inhibitor (5). In control cultures, cell surface FnBP was maximal after 2 hours of growth and then disappeared rapidly as the culture progressed towards stationary phase (Fig. 5A and C). However, there was only a minor reduction in the amount of cell surface FnBP when the culture was supplemented with α2-macroglobulin (Fig. 5B and C). Densitometry analysis indicated a 24-fold difference between the maximum (OD_{600} of 1.7) and minimum (OD_{600} of 8.7) expression of FnBP in the control culture, compared to only 2-fold in the culture supplemented with α2-macroglobulin (Fig. 5C). Therefore, endogenous proteolytic activity contributes to the elimination of cell surface FnBP. Although V8 protease is a member of the serine protease family and is strongly inhibited by diisopropylfluorophosphate (15), the less toxic inhibitor phenylmethylsulfonyl fluoride had no effect on V8 protease activity (data not shown). Consequently, we did not grow cultures with exogenous phenylmethylsulfonyl fluoride, as others have done to demonstrate the role of serine protease in controlling cell wall turnover in *B. subtilus* (22). Collectively, these data suggest that endogenous proteolytic activity exhibits a significant influence on the growth-phase-dependent reduction in the amount of cell surface FnBP.

**Exogenous V8 protease promotes elimination of cell surface FnBP in an agr mutant strain of *S. aureus*.** Expression of FnBP and protein A is elevated in strains of *S. aureus* that are defective in agr (1, 12), and agr regulates expression of protein A at the transcriptional level (34). To assess the influence of agr on growth-phase-dependent variation in Fn binding, cells from cultures of *S. aureus* RN6390B (agr-) and *S. aureus* RN6112 (agr mutant) were harvested (Fig. 6). Inclusion of erythromycin (10 μg·ml⁻¹) in the culture medium is necessary to maintain the agr mutant phenotype in *S. aureus* RN6112, so this strain was also grown without antibiotic to differentiate the effects of being agr negative from a potential erythromycin effect. Relative to the time of growth at which Fn binding was optimal, stationary-phase cells of *S. aureus* RN6390 exhibited a 10-fold growth-phase-dependent reduction in Fn binding, compared to only 3-fold for *S. aureus* RN6112 cultured with erythromycin. Stationary-phase cells of *S. aureus* RN6112 cultured without erythromycin bound less Fn than did the cells cultured with the antibiotic, but the relationships between Fn binding and cell density were otherwise very similar for the two cultures. When serial dilutions of the stationary-phase cultures were plated on BHI agar with or without 10 μg of erythromycin·ml⁻¹ determination of the viable cell counts indicated that about one third of the cells cultured in the absence of antibiotic had reverted to erythromycin sensitivity (data not shown). When *S. aureus* RN6112 was cultured to mid-exponential phase with various concentrations of exogenous V8 protease, the cell surface proteins released by protoplast treatment were devoid of FnBP, which could be detected at only the lowest concentration of protease tested (Fig. 7A). The same concentrations of V8 protease did not cause a major reduction in the amount of cell surface protein A expressed by RN6112 or by *S. aureus* L170 at a single concentration of 1 μg·ml⁻¹ (Fig. 7B). Therefore, in the absence of direct agr-mediated regulation of gene expression, exogenous V8 protease can promote the loss of cell surface FnBP but exerts a negligible influence on protein A.

**Cells cultured with exogenous V8 protease exhibit reduced adhesion.** To determine the influence of protease activity on the adhesive phenotype of *S. aureus* L170, cells were labelled with biotin after growth to both mid-exponential and stationary phases or to stationary phase with either 2 μg of V8 protease·ml⁻¹ or 0.125 U of α2-macroglobulin·ml⁻¹. Based on the number of cells required to achieve an absorbance value at 405 nm of 1.0, cells grown to stationary phase exhibited a twofold reduction in adherence to the N-terminal Fn fragment relative to exponential-phase cells (Fig. 8A). However, when cells were grown to stationary phase with exogenous α2-macroglobulin, the degree of adherence was identical to that of exponential-phase cells. Finally, cells grown to stationary phase with exogenous protease exhibited an estimated four- to fivefold reduction in adherence relative to that of exponential-phase cells. Furthermore, cells grown to stationary phase with exogenous protease exhibited two- to threefold-less adherence to ECM.

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**FIG. 3.** Detection of cell surface FnBP after growth of *S. aureus* L170 to mid-exponential phase with the indicated concentrations of V8 protease. Lanes: 1, 6 μg·ml⁻¹; 2, 1.5 μg·ml⁻¹; 3, 0.375 μg·ml⁻¹; 4, 93.8 ng·ml⁻¹; 5, 23.4 ng·ml⁻¹; 6, 5.9 ng·ml⁻¹; 7, 1.5 ng·ml⁻¹; 8, control with no exogenous V8 protease. Proteins released by protoplast treatment of the exponential-phase cells (30 μl representing protein from 3 × 10⁸ cells) were subjected to SDS-PAGE, transferred to Immobilon P, and probed with the biotinylated N-terminal fragment of Fn. Molecular size markers are indicated on the left.

**FIG. 4.** Influence of exogenous V8 protease on the profile of cell-surface proteins expressed by exponential-phase cells of *S. aureus* L170 (lanes 1) or L857 (lanes 2) grown with (+) or without (−) 1 μg of exogenous V8 protease·ml⁻¹. Suspensions (10⁸ ml⁻¹) of heat-killed exponential-phase cells were surface labelled with biotin and then subjected to protoplast treatment. Aliquots of the released cell surface proteins (15 μl, representing protein from 1.5 × 10⁶ cells) were then subjected to SDS-PAGE and transferred to Immobilon-P membranes as described in the legend to Fig. 1. Biotinylated cell surface proteins were detected with alkaline phosphatase-conjugated streptavidin. Molecular size markers are indicated on the left.
synthesized by fetal rabbit lung fibroblasts, relative to that of cells harvested during mid-exponential growth (Fig. 8B).

**DISCUSSION**

*S. aureus* is noted for its ability to efficiently colonize host tissues followed by rapid invasion of deeper tissue, bacteremia, and metastatic infection (42). Our data establish that the Fn-binding phenotype of *S. aureus* can be moderated by a secreted protease that is either identical or very similar to the previously characterized V8 (serine) protease (10, 51). In terms of the significance of this finding, a number of studies suggest that adherent bacteria remain localized at the site of infection, and proteolytic activity often plays a key role in promoting the spread of infection. This is exemplified by a *Yersinia pestis* plasminogen activator, which converts tissue plasminogen into the active protease plasmin and also degrades outer membrane proteins encoded by the *Yersinia* virulence plasmid (46). A mutant strain that is defective in the plasminogen activator is unable to initiate bacteremia and remains localized at the site of infection (45). In other examples, synthesis and secretion of the protease elastin are induced at a high cell density through a quorum-sensing mechanism in *Pseudomonas aeruginosa* (33), and *Vibrio cholerae* expresses a hemagglutinin/protease that degrades epithelial cell receptors to which the bacteria adhere (16). In these situations, microbial proteases promote the spread and transmission of infection by degrading host cell receptors and/or microbial adhesins, which would otherwise retain adherent bacteria at the site of infection.

A similar mechanism may also promote the spread and dissemination of infections initiated by *Staphylococcus* and *Streptococcus* species. *Streptococcus pyogenes* cysteine protease releases biologically active fragments of cell surface proteins, including M protein and C5a peptidase (6), degrades human Fn and vitronectin (24), and releases active urokinase receptor from monocytes (50). A trypsin-like proteolytic activity of *Staphylococcus epidermidis* degrades an adhesin that promotes microbial adherence to biomaterials (49), and our present data support a role for V8 protease activity in controlling the Fn binding phenotype of *S. aureus*. The function of V8 protease in this respect would probably be limited to colonized tissue, as the expression of V8 protease requires *agr* (7), which is itself maximally induced at a high cell density by a quorum-sensing mechanism (4, 21). Therefore, during bacteremia at a low cell density of 10^6 ml^-1, the V8 protease activity would be limited to colonized tissue, but at high cell densities, the V8 protease activity would be induced and promoted by the quorum-sensing mechanism, allowing for the spread and dissemination of infection.
density, it is unlikely that the agr locus would be fully induced, and α₂-macroglobulin, which is abundant in plasma (5, 8), would also contribute to the stability of cell surface FnBP, as we have shown in vitro. However, within a microcolony on host tissue, the cell density would presumably be sufficient to activate agr and promote the synthesis and secretion of V8 protease, analogous to the cell-density-dependent induction of elastin synthesis by P. aeruginosa (33).

In identifying a quorum-sensing mechanism in S. aureus, it was first observed that concentrated supernatant from a stationary-phase culture could induce the agr response in exponentially growing cells (4). Partial purification of the factor responsible for this activity identified a culture supernatant fraction enriched in a 38-kDa protein. However, it was later established that the activator of agr was an octapeptide pheromone that copurified with the 38-kDa protein (21). The relationship, if any, between this 38-kDa protein and the 31.4-kDa protein that we have identified as V8 protease is unknown. Possibly, purified V8 protease may contain some of the agr autoinducer as a contaminant. Exogenous V8 protease could eliminate cell surface FnBP from the agr mutant strain S. aureus RN6112. Furthermore, although expression of protein A is regulated at the transcriptional level by agr (34), exogenous V8 protease did not cause a dramatic reduction of cell surface protein A in S. aureus L170 or RN6112. Therefore, the effects attributed to V8 protease appear to be due to proteolytic activity alone and not to the induction of a global response affecting gene expression.

The sensitivity of FnBP to V8 protease can be explained by the substrate specificity of the enzyme and the amino acid composition of FnBP. V8 protease cleaves on the carboxyl side of glutamic acid (19), and when the enzyme was first characterized, it was suggested that this restricted specificity would liberate relatively large peptides which would be of limited value in satisfying the nutritional requirements of the organism (15). However, glutamic acid is the most abundant amino acid of FnBP, comprising 12.1% of the amino acid content of the mature 982-amino-acid protein (43). Furthermore, the binding domain of FnBP consists of three tandem repeats of a 37- or 38-amino-acid motif in which glutamic acid residues are essential for Fn binding, and treatment of synthetic peptides representing the individual motifs with V8 protease generated fragments that were unable to bind Fn (29). In view of these considerations and our present findings, V8 protease may play a significant role in moderating the adhesive phenotype of S. aureus during the course of an infection. In this respect, V8 protease activity also influenced a limited number of other cell surface proteins in the 130- to 200-kDa size range (Fig. 4). Other investigators have noted that a 230-kDa cell surface protein expressed by certain methicillin-resistant strains of S. aureus is sensitive to the protease plasmin (18). However, in this situation, treatment of the cells with plasmin promoted increased adherence to solid-phase Fn, fibronectin, and immunoglobulin G. Consequently, different proteolytic activities of both the microbe and the host could play complex roles in controlling the adhesive phenotype of S. aureus during the course of an infection.

In B. subtilis, proteolytic activity controls spore germination and cell wall turnover (22, 41), while the ClpXP protease of Escherichia coli degrades a sigma factor required for expression of genes in response to starvation stress (39), and a cytotoxic protease of Porphyromonas gingivalis may function as a processing protease required for maturation of fimbrial adhesins (32). Therefore, microbial proteases can act as regulators of complex physiological processes, including microbial adherence. Of significance in terms of controlling S. aureus infection, FnBP represents a target for therapeutic agents.
aimed at preventing the initiation of infection by blocking microbial adherence (36, 47), while V8 protease could constitute a target for therapeutic agents aimed at controlling the spread of infection, for which *S. aureus* is well-known. Work is in progress to inactivate the structural gene for V8 protease so that its role in controlling the adhesive phenotype and cell surface protein profile of *S. aureus* can be comprehensively evaluated.

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