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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) adhesin 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 · ml^{-1} resulted in reduced amounts of FnBP. Addition of the protease inhibitor α_2-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 *sar* (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.*

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MATERIALS AND METHODS

Cultures and growth conditions. *S. aureus* L530, L170, L786, L516, and L857 are clinical isolates from the University of Manitoba Health Sciences Clinical

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 fistula, osteomyelitis, and septic arthritis, respectively. *S. aureus* RN6390B (*agr*$^+$) and RN6112 (*agr* mutant) were generously provided by Richard Novick (Skirball Institute, New York, N.Y.) and have been described previously (37). Stock cultures were maintained in 20% glycerol at −70°C. As required, scrapings from frozen stock cultures were streaked on brain heart infusion (BHI) agar (BBL, Cockeysville, Md.) and cultured at 37°C. The culture medium for *S. aureus* RN6112 was supplemented with 10 μg of erythromycin · ml^{-1}.

Preparation of culture supernatant and purification of V8 protease. Two 2-liter Erlenmeyer flasks containing 600 ml of BHI broth prewarmed to 37°C 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 shaking platform (100 rpm) until stationary phase (OD_{600} of 9.0), and cells were removed by centrifugation at 5,000 × *g* for 30 min. The supernatant was concentrated 10-fold in an Amicon (Beverley, Mass.) stirred-cell ultrafiltration chamber equipped with a 10,000-molecular-mass cutoff membrane, filter sterilized, and stored at −20°C. For purification of V8 protease, the concentrated culture supernatant was dialyzed in 20 mM Tris-HCl (pH 7.4) (buffer A) and applied to a 56-ml column of Q-Sepharose Fast-Flow, with a Gradi-Frac chromatography system (Pharmacia, Piscataway, N.J.). The column was washed with buffer A and a 240-ml gradient of up to 0.5 M NaCl, with collection of 5-ml fractions. Fractions able to eliminate cell surface FnBP from exponential-phase cells of *S. aureus* L170 (see below) were pooled, dialyzed in buffer A, and subjected to a second anion-exchange step on a DEAE Mem-Sep 1010 cartridge (Millipore, Bedford, Mass.). Active fractions were pooled, concentrated, and applied at 0.5 ml · min^{-1} to a Sephacryl S-100 column (1.5 by 90 cm; Pharmacia) equilibrated in buffer A. The major protein peak eluted was dialyzed in 20 mM Tris-HCl–150 mM NaCl (pH 7.4), concentrated by ultrafiltration, and filter sterilized. The protein concentration was determined by an bicinchoninic acid protein assay (44). An aliquot was dialyzed in 20 mM ammonium bicarbonate and lyophilized for determination of the amino acid composition and N-terminal sequence analysis at the University of Calgary Protein Sequencing Core Facility. For composition analysis, protein was dissolved in 50% acetic acid, and a 4-μl aliquot containing 5 nmol of norleucine was hydrolyzed in 6 N HCl–0.1% β-mercaptoethanol under vacuum at 150°C for 60 min. The hydrolysate was vacuum dried and dissolved in sodium citrate buffer (pH 2.2), and amino acid analysis was performed with a Beckman model 6300 amino acid analyzer with ninhydrin detection. N-terminal sequence analysis was performed with an Applied Biosystems model 470A gas-phase protein sequencer on a trifluoroacetic acid-treated glass-fiber disk that had been precycled with Polybrene.

Preparation of cell surface proteins. Overnight cultures were subcultured (OD_{600} of 0.1) in 25 ml of prewarmed BHI broth in 125-ml Erlenmeyer flasks and grown to an OD_{600} of 0.8 at 37°C. Cultures were supplemented at the time of inoculation, where indicated, with concentrated culture supernatant, purified protease, or protease inhibitor. Cells were harvested by centrifugation (5,000 × *g* for 20 min), washed in ice-cold 50 mM Tris-HCl (pH 7.4), adjusted to 10^{10} cells · ml^{-1}, and converted into protoplasts by treatment with lysostaphin in an osmotically stabilized buffer (13). Protoplasts were centrifuged in an Eppendorf microcentrifuge at 12,000 rpm for 5 min, and the supernatant containing released cell surface proteins was stored at −20°C. To determine the influence of growth phase and endogenous proteolytic activity on the amount of cell surface FnBP, a 150-ml culture of *S. aureus* L170 was grown as described above in a 500-ml Erlenmeyer flask and supplemented with 0.125 U of an α_2-macroglobulin protease inhibitor (Boehringer Mannheim) · ml^{-1} where indicated. At hourly intervals, 10-ml samples were added to 250-ml flasks containing 10 ml of frozen 50 mM sodium azide. After the last addition, the samples were subjected to protoplast treatment as described above, except that the cell suspensions were first heat killed (88°C for 20 min).

Purification and labelling of Fn. Fn was purified from the cryoprecipitate fraction of human plasma, obtained from the Canadian Red Cross, according to standard methods (31). Purified Fn was then treated with thermolysin (Boehringer Mannheim), and the 29-kDa N-terminal fragment was isolated by affinity chromatography as described previously (29). The purified N-terminal Fn fragment (2 mg) was biotinylated with biotinamidocaproate *N*-hydroxysuccinimide ester, as outlined in the ImmunoProbe biotinylation kit (Sigma, St. Louis, Mo.). To quantify binding of Fn to *S. aureus* cells, human plasma Fn (Gibco/BRL, Gaithersburg, Md.) was labelled with ^{125}I to a specific activity of 27 MBq · nmol^{-1} by the chloramine T procedure (20). Binding of ^{125}I by suspensions of 5×10^8 heat-killed cells was quantified as described previously (29).

Western blotting for detection of FnBP and analysis of cell surface proteins. Aliquots of protoplast fluid containing released cell surface proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 7.5% acrylamide resolving gels, by the procedure of Laemmli (25). The gels were then stained with Coomassie blue or transferred to an Immobilon-P membrane (Millipore) with the Bio-Rad Trans-Blot apparatus and transfer buffer described by Towbin et al. (48). The membranes were then incubated for 1 h in phosphate-buffered saline (PBS) containing 3% (wt/vol) bovine serum albumin (BSA), washed in PBS–0.05% (vol/vol) Tween 20, and incubated overnight at 4°C on an orbital shaking platform in 50 ml of PBS–0.1% BSA–0.05% Tween 20 containing 0.5 μg of the biotinylated N-terminal Fn fragment · ml^{-1}. Membranes were then washed with PBS-Tween 20, incubated for 1 h in 5,000-fold-diluted alkaline phosphatase-conjugated streptavidin (Boehringer Mannheim), and diluted in PBS-BSA-Tween 20. After being washed extensively with PBS-Tween 20 and once in PBS, the membranes were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate alkaline phosphatase substrates (Bio-Rad) according to the supplier's recommendations. For analysis of cell surface proteins, proteins released by protoplast treatment of biotinylated cells (see below) were subjected to the same procedure, except that the incubation 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 × 600 dots per inch. The images were saved as TIFF files and imported into National Institutes of Health (NIH) Image (version 1.60) for computer-aided pixel densitometry analysis of the bands corresponding to FnBP.

Adhesion assay. Fetal rabbit lung fibroblasts 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 exposed as described previously (14) by treating a confluent cell monolayer with 0.5% (wt/vol) Triton X-100 (30 min at 37°C) followed by 25 mM ammonium hydroxide (10 min at 37°C). The wells were then rinsed with PBS, and the plates were stored at −20°C until needed. Wells of Corning 96-well microtiter plates were also coated with 1 μg of the 29-kDa N-terminal Fn fragment · ml^{-1} diluted in carbonate-bicarbonate buffer. Just prior to use for adhesion assays, plates coated with ECM or the N-terminal Fn fragment were incubated for 1 h with 200 μl of PBS containing 3% BSA. For adhesion assays, exponential-phase cells of *S. aureus* L170 were heat killed, adjusted to a cell density of 10^{10} ml^{-1} in PBS, and biotinylated by incubation for 2 h at room temperature with 50 μg of biotinamidocaproate *N*-hydroxysuccinimide ester · ml^{-1}. The biotinylated cells were washed extensively with PBS, suspended in PBS at a cell density of 10^{10} ml^{-1}, and stored in aliquots at −70°C. To quantify adhesion, 100-μl aliquots of biotinylated bacteria diluted in PBS–0.1% BSA–0.05% Tween 20 were added to the wells of microtiter plates coated with ECM or the N-terminal Fn fragment. After incubation for 1 h at room temperature on an orbital mixer, the wells were washed extensively with PBS-Tween 20 and then incubated for 1 h with 100 μl of streptavidin-alkaline phosphatase conjugate diluted 5,000-fold in PBS-BSA-Tween 20. The wells were again washed extensively with PBS-Tween 20 and then developed with 100 μl of a Sigma 104 phosphatase substrate in 0.1 M diethanolamine buffer (pH 9.8). The absorbance at 405 nm was quantified after 1 h with a TitreTek plate reader equipped with a 405-nm-wavelength filter.

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, strain L530 exhibited the poorest 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 a culture 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 (10^{10} 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 loading in each lane (Fig. 1A). The cell surface protein profiles of the exponential-phase cells cultured with exogenous stationary-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. When identical gels were transferred to Immobilon-P and probed with the biotinylated N-terminal Fn fragment (Fig. 1B), there was intense staining of proteins in the 200-kDa range from control cultures and lesser reactivity with a broad range of smaller products, which are presumably degradation products of FnBP. Remarkably, cell surface proteins from cultures supplemented with concentrated supernatant from strain L530 were devoid of FnBPs.

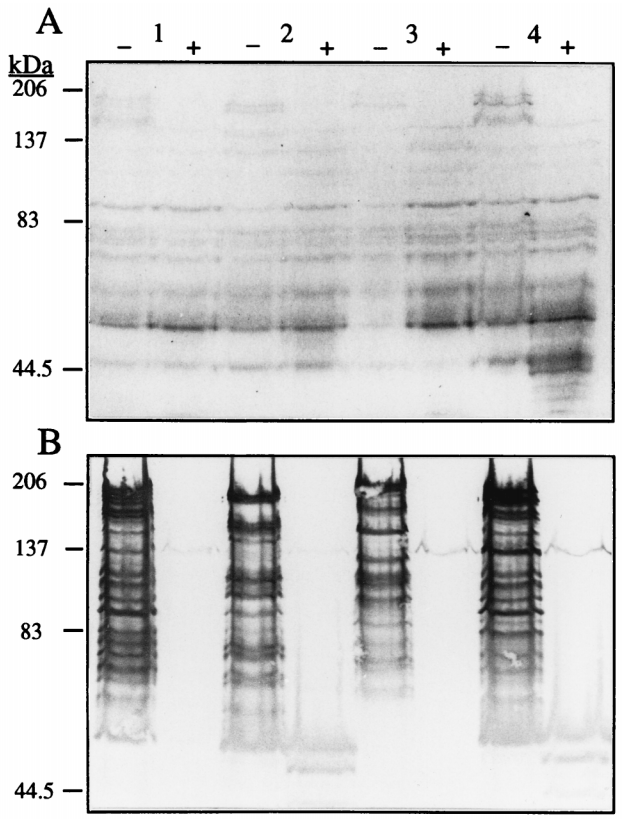

FIG. 1. Influence of exogenous stationary-phase culture supernatant from *S. aureus* L530 on cell surface FnBP expressed by exponential-phase cells of *S. aureus* L170 (lanes 1), L516 (lanes 2), L786 (lanes 3), and L857 (lanes 4). Cultures were supplemented at the time of inoculation with a 1/10 volume of 10× concentrated supernatant of *S. aureus* L530 (lanes labelled +), or sterile BHI broth (lanes labelled −). Cell surface proteins were released by protoplast treatment of exponential-phase cells that had been adjusted to a cell density of 10^{10} ml^{-1}. Subsequently, 30-μl aliquots representing cell surface proteins from 3×10^8 cells were subjected to SDS-PAGE with a 7.5% acrylamide resolving gel and stained for protein (A) or transferred to Immobilon-P and probed with the biotinylated N-terminal fragment of Fn (B). Molecular size markers are indicated on the left.

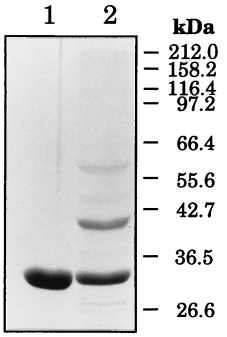

FIG. 2. Purification of a 31.4-kDa protein from culture supernatant of *S. aureus* L530, which is active in promoting the loss of cell surface FnBP from exponential-phase cells. Proteins were resolved by SDS-PAGE with a 12% acrylamide resolving gel and stained with Coomassie blue. Lanes: 1, 20 μg of purified protein; 2, 135 μg of concentrated culture supernatant protein from *S. aureus* L530. The locations and sizes of New England BioLabs broad-range protein marker standards are indicated on the right.

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 *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 *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 *S. aureus* V8 and with a related 31.3-kDa protease produced by *S. aureus* ATCC 12600, starting at residue 69 of the full precursor sequences 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 *S. aureus* L170 at the time of inoculation, 375 ng of exogenous V8 protease · ml^{-1} resulted in exponential-phase cells that were devoid of FnBP, and concentrations as low as 23 ng · 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 *S. aureus* L170 and L857 were grown to mid-exponential phase with or without 1 μg of exogenous V8 protease · 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-

TABLE 1. Comparison of amino acid composition of serine protease purified from *S. aureus* L530 with known compositions of mature *S. aureus* serine proteases based on published nucleotide sequences of *S. aureus* V8 (10) and ATCC 12600 (51)

Amino acid[a]	% Composition in *S. aureus* strain		
	L530	V8	ATCC 12600
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

[a] Asx, combined aspartate and asparagine; Glx, combined glutamate and glutamine.

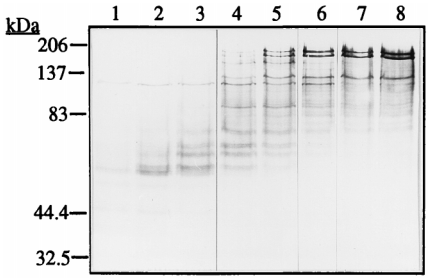

FIG. 3. Detection of cell surface FnBP after growth of *S. aureus* L170 to mid-exponential phase with the indicated concentrations of V8 protease. Lanes: 1, 6 μg · ml^{-1}; 2, 1.5 μg · ml^{-1}; 3, 0.375 μg · ml^{-1}; 4, 93.8 ng · ml^{-1}; 5, 23.4 ng · ml^{-1}; 6, 5.9 ng · ml^{-1}; 7, 1.5 ng · ml^{-1}; 8, control with no exogenous V8 protease. Proteins released by protoplast treatment of the exponential-phase cells (30 μl, representing protein from 3×10^8 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.

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 α_2-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 α_2-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 α_2-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 at hourly intervals throughout the growth phase, and the amount of ^{125}I-Fn bound by 5×10^8 cells was plotted as a function of the cell density when the cells 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 α_2-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 α_2-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

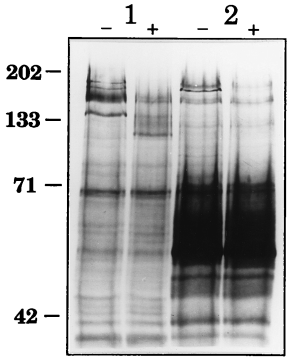

FIG. 4. Influence of exogenous V8 protease on the profile of cell-surface proteins expressed by exponential-phase cells of *S. aureus* L170 (lanes 1) or L857 (lanes 2) grown with (+) or without (−) 1 μg of exogenous V8 protease ml^{-1}. Suspensions (10^{10} ml^{-1}) of heat-killed exponential-phase cells were surface labelled with biotin and then subjected to protoplast treatment. Aliquots of the released cell surface proteins (15 μl, representing protein from 1.5×10^8 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.

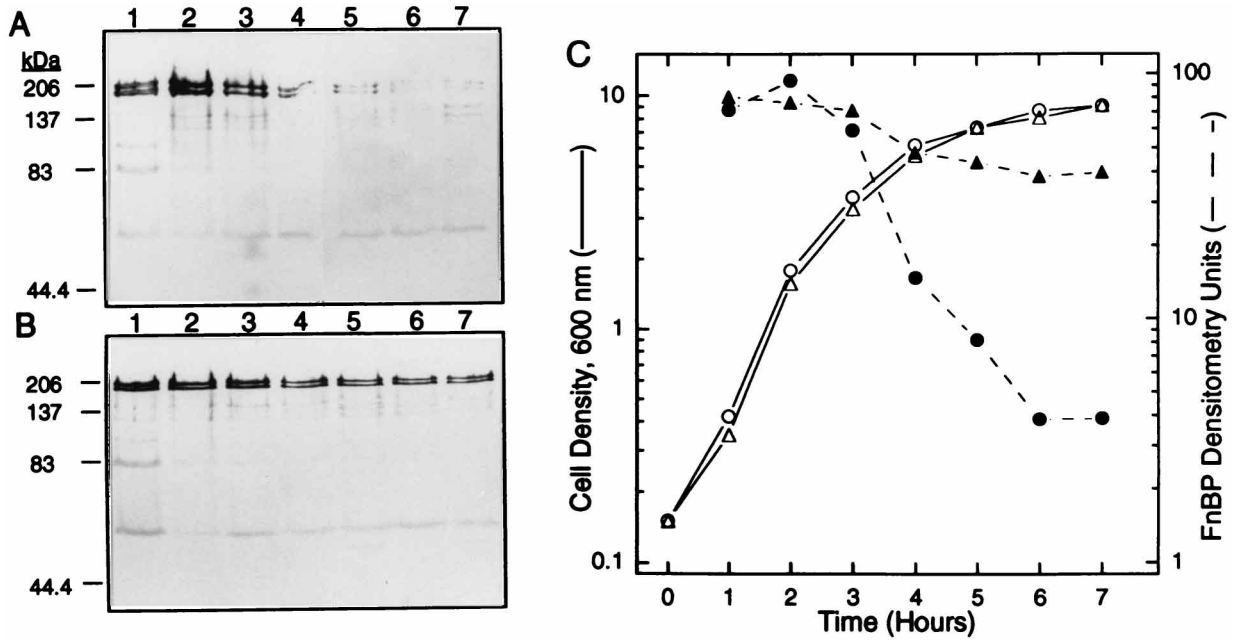

FIG. 5. Cultures of *S. aureus* L170 were supplemented at the time of inoculation with either 0.125 U of α_2-macroglobulin ml^{-1} (A) or an equal volume of sterile H_2O (B). At hourly intervals, corresponding to the lane numbers (A and B), cells were harvested and adjusted to a cell density of 10^{10} ml^{-1}. Aliquots (30 μl) of cell surface proteins released during subsequent protoplast treatment, representing protein from 3×10^8 cells, were subjected to SDS-PAGE, transferred to Immobilon P, and probed with the biotinylated N-terminal fragment of Fn. (C) cell density (OD_{600}) of the same cultures grown with (△) or without (○) exogenous α_2-macroglobulin and densitometric analysis of the intensity of bands corresponding to FnBP shown in panels A (●) and B (▲). Each datum point represents a single determination.

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

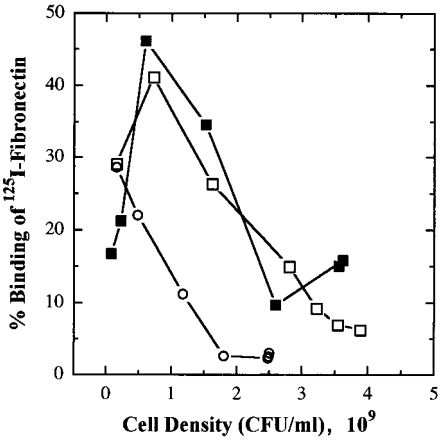

FIG. 6. Influence of growth phase on binding of ^{125}I-Fn by isogenic strains *S. aureus* RN6390B (*agr*$^+$) (○) and RN6112 (*agr* mutant) with 10 μg of erythromycin ml^{-1} (■) or without erythromycin (□). Cultures were grown in BHI broth at 37°C, and samples were taken at hourly intervals for determination of cell density and binding of ^{125}I-Fn. The percent binding of Fn by a suspension of 5×10^8 cells is plotted as a function of the cell density when the cells were harvested for binding assays. Each datum point represents the average of duplicate determinations.

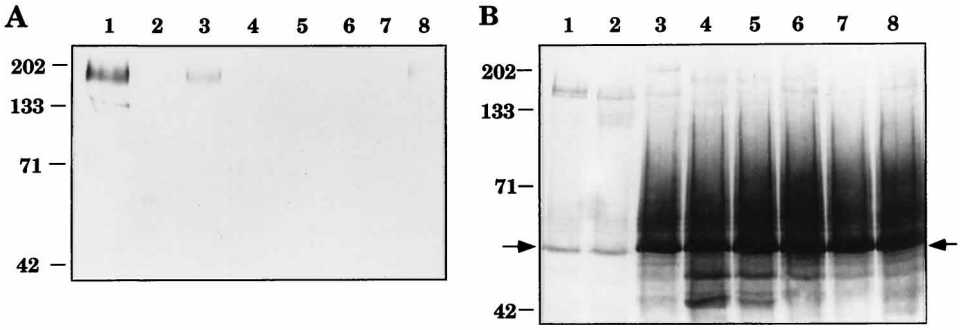

FIG. 7. Cell-surface FnBP (A) and protein A (B) expressed by exponential-phase cells of *S. aureus* L170 cultured without (lanes 1) or with 1 μg of exogenous V8 protease ml^{-1} (lanes 2) or *S. aureus* RN6112 (*agr* mutant) cultured without (lane 3) or with protease concentrations of 4, 1, 0.3, 0.1, and 0.025 μg ml^{-1} (lanes 4 to 8, respectively). After the release of cell surface proteins by protoplast treatment, 30-μl aliquots (representing cell surface protein from 3×10^8 cells) were subjected to SDS-PAGE with 7.5% acrylamide gels, transferred to Immobilon-P, and probed with the biotinylated N-terminal fragment of Fn (A) or 1 μg of preimmune immunoglobulin G ml^{-1} (B). (B) The arrows indicate the position of protein A. Molecular size markers are indicated on the left.

density, it is unlikely that the *agr* locus would be fully induced, and α_2-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. However, 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, fibrinogen, 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. subtilus*, 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 cysteine 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

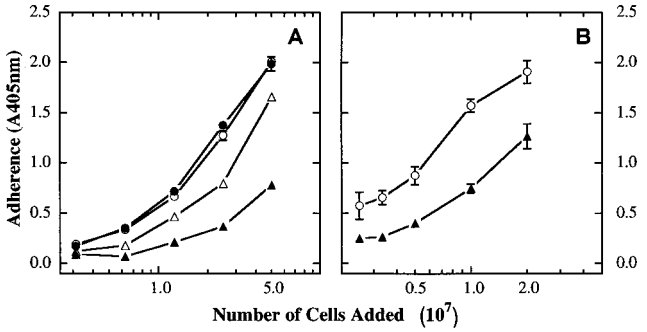

FIG. 8. Influence of exogenous α_2-macroglobulin or V8 protease on adhesion of *S. aureus* L170 to purified N-terminal fragment of Fn (A) or ECM synthesized by fetal rabbit lung fibroblasts (B). Each datum point represents the average of quadruplicate determinations; errors bars are standard deviations. Cultures of *S. aureus* L170 were grown to mid-exponential phase (○) or stationary phase (△) without supplement or to stationary phase with either 0.125 U of α_2-macroglobulin · ml^{-1} (●) or 2 μg of V8 protease · ml^{-1} (▲). Suspensions of heat-killed cells (10^{10} ml^{-1}) were then biotinylated, and the indicated numbers of bacteria were added to wells of microtiter plates coated with the N-terminal Fn fragment or fibroblast ECM. Adherent bacteria were detected with alkaline phosphatase-conjugated streptavidin, as described in Materials and Methods.

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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REFERENCES

1. **Abdelnour, A., S. Arvidson, T. Bremell, C. Rydén, and A. Tarkowski.** 1993. The accessory gene regulator (*agr*) controls *Staphylococcus aureus* virulence in a murine arthritis model. Infect. Immun. **61:**3879–3885.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Arvidson, S. O.** 1983. Extracellular enzymes from *Staphylococcus aureus*, p. 795–808. *In* C. S. F. Easmon and C. Adlam (ed.), Staphylococci and staphylococcal infections. Academic Press, London, United Kingdom.
4. **Balaban, N., and R. P. Novick.** 1995. Autocrine regulation of toxin synthesis by *Staphylococcus aureus*. Proc. Natl. Acad. Sci. USA **92:**1619–1623.
5. **Barret, A. J., and P. M. Starkey.** 1973. The interaction of alpha-2-macroglobulin with proteinases. Biochem. J. **133:**709–724.
6. **Berge, A., and L. Björk.** 1995. Streptococcal cysteine protease releases biologically active fragments of streptococcal surface proteins. J. Biol. Chem. **270:**9862–9867.
7. **Bjkorklind, A., and S. Arvidson.** 1980. Mutants of *Staphylococcus aureus* affected in the regulation of exoprotein synthesis. FEMS Microbiol. Lett. **7:**203–206.
8. **Borth, W.** 1992. α_2-Macroglobulin, a multifunctional binding protein with targeting characteristics. FASEB J. **6:**3345–3353.
9. **Boyle, M. D. P.** 1995. Variation of multifunctional surface binding proteins—a virulence strategy for Group A streptococci. J. Theor. Biol. **173:** 415–426.
10. **Carmona, C., and G. L. Gray.** 1987. Nucleotide sequence of the serine protease gene of *Staphylococcus aureus* strain V8. Nucleic Acids Res. **15:** 6757.
11. **Cheung, A. L., and S. J. Projan.** 1994. Cloning and sequencing of *sarA* of *Staphylococcus aureus*, a gene required for the expression of *agr*. J. Bacteriol. **176:**4168–4172.
12. **Cheung, A. L., K. J. Eberhardt, E. Chung, M. R. Yeaman, P. M. Sullam, M. Ramos, and A. S. Bayer.** 1994. Diminished virulence of sar^-/agr^- mutant of *Staphylococcus aureus* in the rabbit model of endocarditis. J. Clin. Invest. **94:**1815–1822.
13. **Cheung, A. L., and V. A. Fischetti.** 1988. Variation in the expression of cell wall proteins of *Staphylococcus aureus* grown on solid and liquid media. Infect. Immun. **56:**1061–1065.
14. **Creech-Tart, R., and I. van de Rijn.** 1991. Analysis of adherence of *Streptococcus defectivus* and endocarditis associated streptococci to extracellular matrix. Infect. Immun. **59:**857–862.
15. **Drapeau, G. R., Y. Boily, and J. Houmard.** 1972. Purification and properties of an extracellular protease of *Staphylococcus aureus*. J. Biol. Chem. **247:** 6720–6726.
16. **Finkelstein, R. A., M. Boesman-Finkelstein, Y. Chang, and C. C. Häse.** 1992. *Vibrio cholerae* hemagglutinin/protease, colonial variation, virulence, and detachment. Infect. Immun. **60:**472–478.
17. **Hart, M. E., M. S. Smeltzer, and J. J. Iandolo.** 1993. The extracellular protein regulator (*xpr*) affects exoprotein and *agr* mRNA levels in *Staphylococcus aureus*. J. Bacteriol. **175:**7875–7879.
18. **Hildén, P., K. Savolainen, J. Tyynelä, M. Vuento, and P. Kuusela.** 1996. Purification and characterisation of a plasmin-sensitive surface protein of *Staphylococcus aureus*. Eur. J. Biochem. **236:**904–910.
19. **Houmard, J., and G. R. Drapeau.** 1972. Staphylococcal protease: a proteolytic enzyme specific for glutamyl bonds. Proc. Natl. Acad. Sci. USA **69:**3506–3509.
20. **Hunter, W. M.** 1978. Radioimmunoassay, p. 14.1–14.40. *In* D. M. Weir (ed.), Handbook of experimental immunology. Blackwell Scientific Publications, Oxford, United Kingdom.
21. **Ji, G., R. C. Beavis, and R. P. Novick.** 1995. Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proc. Natl. Acad. Sci. USA **92:**12055–12059.
22. **Jolliffe, L. K., R. J. Doyle, and U. N. Streips.** 1980. Extracellular proteases modify cell wall turnover in *Bacillus subtilus*. J. Bacteriol. **141:**1199–1208.
23. **Jönsson, K., C. Signäs, H. P. Müller, and M. Lindberg.** 1991. Two different genes encode fibronectin binding proteins in *Staphylococcus aureus*. The complete nucleotide sequence and characterization of the second gene. Eur. J. Biochem. **202:**1041–1048.
24. **Kapur, V., S. Topouzis, M. W. Majesky, L. L. Li, M. R. Hamrick, R. J. Hamill, J. M. Patti, and J. M. Musser.** 1993. A conserved *Streptococcus pyogenes* extracellular cysteine protease cleaves human fibronectin and degrades vitronectin. Microb. Pathog. **15:**327–346.
25. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
26. **Lebeau, C., F. Vandenesch, T. Greenland, R. P. Novick, and J. Etienne.** 1994. Coagulase expression in *Staphylococcus aureus* is positively and negatively modulated by an *agr*-dependent mechanism. J. Bacteriol. **176:**5534–5536.
27. **McDevitt, D., P. Francois, P. Vaudaux, and T. J. Foster.** 1994. Molecular characterization of clumping factor (fibrinogen receptor) of *Staphylococcus aureus*. Mol. Microbiol. **11:**237–248.
28. **McGavin, M. H., D. Krajewska-Pietrasik, C. Rydén, and M. Höök.** 1993. Identification of a *Staphylococcus aureus* extracellular matrix-binding protein with broad specificity. Infect. Immun. **61:**2479–2485.
29. **McGavin, M. J., G. Raucci, S. Gurusiddappa, and M. Höök.** 1991. Fibronectin-binding determinants of the *Staphylococcus aureus* fibronectin receptor. J. Biol. Chem. **266:**8343–8347.
30. **Mekalanos, J. J.** 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. **174:**1–7.
31. **Miekka, S. I., K. C. Ingham, and D. Menache.** 1982. Rapid methods for isolation of human plasma fibronectin. Thromb. Res. **27:**1–14.
32. **Nakayama, K., F. Yoshimura, T. Kadowaki, and K. Yamamoto.** 1996. Involvement of arginine-specific cysteine proteinase (Agr-gingipain) in fimbriation of *Porphyromonas gingivalis*. J. Bacteriol. **178:**2818–2824.
33. **Passador, L., and B. H. Iglewski.** 1995. Quorum sensing and virulence gene regulation in *Pseudomonas aeruginosa*, p. 65–78. *In* J. A. Roth, C. A. Bolin, K. A. Brogden, F. C. Minion, and M. J. Wannemuehler (ed.), Virulence mechanisms of microbial pathogens. ASM Press, Washington, D.C.
34. **Patel, A. H., J. Kornblum, B. Kreiswirth, R. Novick, and T. J. Foster.** 1992. Regulation of the protein A-encoding gene in *Staphylococcus aureus*. Gene **114:**25–34.
35. **Patti, J. M., H. Jönsson, B. Guss, L. M. Switalski, K. Wiberg, M. Lindberg, and M. Höök.** 1992. Molecular characterization and expression of a gene encoding a *Staphylococcus aureus* collagen adhesin. J. Biol. Chem. **267:**4766–4772.
36. **Patti, J. M., B. L. Allen, M. J. McGavin, and M. Höök.** 1994. MSCRAMM-mediated adherence of microorganisms to host tissues. Annu. Rev. Microbiol. **48:**585–617.
37. **Peng, H.-L., R. P. Novick, B. Kreiswirth, J. Kornblum, and P. Schlievert.** 1988. Cloning, characterization, and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. J. Bacteriol. **170:**4365–4372.
38. **Proctor, R. A., D. F. Mosher, and P. J. Olbrantz.** 1982. Fibronectin binding to *Staphylococcus aureus*. J. Biol. Chem. **257:**14788–14793.
39. **Schweder, T., K.-H. Lee, O. Lomovskaya, and A. Matin.** 1996. Regulation of *Escherichia coli* starvation sigma factor (σ^s) by ClpXP protease. J. Bacteriol. **178:**470–476.
40. **Scott, J. E., and R. M. Das.** 1993. Production of fibroblast-pneumocyte-like factor by rabbit lung fibroblasts: isolation and effects of it and related factors on fetal type II cells in vitro. Life Sci. **53:**765–774.
41. **Setlow, P.** 1988. Small acid-soluble spore proteins of *Bacillus species*: structure, synthesis, genetics, function, and their degradation. Annu. Rev. Microbiol. **42:**319–338.
42. **Sheagren, J. N.** 1988. Inflammation induced by *Staphylococcus aureus*, p. 829–840. *In* J. I. Gallin, I. M. Goldstein, and R. Snyderman (ed.), Inflammation: basic principles and clinical correlates. Raven Press, New York, N.Y.
43. **Signäs, C., G. Raucci, K. Jönsson, P.-E. Lindgren, G. M. Anantharamaiah, M. Höök, and M. Lindberg.** 1989. Nucleotide sequence of the gene for a fibronectin-binding protein from *Staphylococcus aureus* and its use in the synthesis of biologically active peptides. Proc. Natl. Acad. Sci. USA **86:**699–703.
44. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150:**76–85.
45. **Sodeinde, O. A., Y. V. B. K. Subrahmanyam, K. Stark, T. Quan, Y. Bao, and J. D. Goguen.** 1992. A surface protease and the invasive character of plague. Science **258:**1004–1007.
46. **Sodeinde, O. A., A. K. Sample, R. R. Brubaker, and J. D. Goguen.** 1988. Plasminogen activator/coagulase gene of *Yersisinia pestis* is responsible for degradation of plasmid-encoded outer membrane proteins. Infect. Immun. **56:**2749–2752.
47. **Sun, Q., G. M. Smith, C. Zahradka, and M. J. McGavin.** 1997. Identification of D motif epitopes in *Staphylococcus aureus* fibronectin binding protein for the production of antibody inhibitors of fibronectin-binding. Infect. Immun. **65:**537–543.
48. **Towbin, M., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.

49. **Veenstra, G. J. C., F. F. M. Cremers, H. van Dijk, and A. Fleer.** 1996. Ultrastructural organization and regulation of a biomaterial adhesin of *Staphylococcus epidermidis*. J. Bacteriol. **178:**537–541.
50. **Wolf, B. B., C. A. Gibson, V. Kapur, I. M. Hussaini, J. M. Musser, and S. L. Gonias.** 1994. Proteolytically active streptococcal pyrogenic exotoxin B cleaves monocytic cell urokinase receptor and releases an active fragment of the receptor from the cell surface. J. Biol. Chem. **269:**30682–30687.
51. **Yoshikawa, K., H. Tsuzuki, T. Fujiwara, E. Nakamura, H. Iwamoto, K. Matsumoto, M. Shin, N. Yoshida, and H. Teraoka.** 1992. Purification, characterization and gene cloning of a novel glutamic acid specific endopeptidase from *Staphylococcus aureus* ATCC 12600. Biochim. Biophys. Acta **1121:**221–228.

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