

## The N-terminal A Domain of Fibronectin-binding Proteins A and B Promotes Adhesion of *Staphylococcus aureus* to Elastin\*

Received for publication, February 26, 2004, and in revised form, July 2, 2004  
Published, JBC Papers in Press, July 2, 2004, DOI 10.1074/jbc.M402122200

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The ability of *Staphylococcus aureus* to adhere to components of the extracellular matrix is an important mechanism for colonization of host tissues during infection. We have previously shown that *S. aureus* binds elastin, a major component of the extracellular matrix. The integral membrane protein, elastin-binding protein (EbpS), binds soluble elastin peptides and tropoelastin via its surface-exposed N-terminal domain. In this study, we demonstrate that some strains of *S. aureus* adhere strongly to immobilized human elastin and that this interaction is independent of EbpS but instead is mediated by the fibronectin-binding proteins, FnBPA and FnBPB. Our results show that EbpS mutant cells adhere to elastin-coated plates, whereas the cells negative for FnBPA and FnBPB do not adhere to the plates. Furthermore, only wild-type cells from the exponential phase of growth adhered when FnBPs were expressed maximally. We show that adherence to elastin promoted by FnBPA was not affected by soluble fibronectin, suggesting that the elastin binding domain is distinct from the fibronectin binding regions. Recombinant FnBPA<sub>37–544</sub> (rFnBPA<sub>37–544</sub>) protein corresponding to the A region of FnBPA and anti-FnBPA<sub>37–544</sub> antibodies inhibited FnBPA-mediated bacterial adherence to immobilized elastin. Finally, recombinant A domain proteins, rFnBPA<sub>37–544</sub> and rFnBPB<sub>37–540</sub>, bound immobilized elastin dose-dependently and saturably. This interaction was inhibited by soluble elastin peptides, suggesting a specific receptor-ligand interaction.

The initial step in the infection process is colonization of host tissue. *Staphylococcus aureus* is a human pathogen that can express several proteins on its cell surface that promote bacterial adherence to proteins of the mammalian extracellular matrix (ECM).<sup>1</sup> Collectively, these proteins are called microbial surface components recognizing adhesive matrix molecules

(MSCRAMMs). The binding to the ECM allows bacteria to adhere to and colonize the host tissue. Surface proteins that interact with collagen (1), fibrinogen (2, 3), fibronectin (4), and von Willebrand factor (5) have been characterized. The importance of the MSCRAMM proteins to the virulence of *S. aureus* has been demonstrated by studying isogenic mutants in animal models of infection. The collagen-binding protein, Cna, was implicated in the pathogenesis of osteomyelitis and septic arthritis (1, 6, 7), and the fibrinogen-binding protein, ClfA, promoted experimental endocarditis (8) and septic arthritis (9).

Elastin, along with microfibrillar components, is a major component of the elastic fiber ECM. Elastin is a hydrophobic protein that provides resilience and elasticity to tissues such as lung, aorta, and skin. Tropoelastin, the soluble precursor of elastin, is produced by smooth muscle cells, endothelial cells, chondrocytes, and fibroblasts (10, 11). The protein consists of alternating hydrophobic and hydrophilic domains with the hydrophilic domains often consisting of lysine residues that are involved in cross-linking (12). A thermodynamically controlled process called coacervation is thought to orientate the tropoelastin monomers into an ordered fibrillar structure via interactions between the hydrophobic domains of neighboring molecules (13). Lysine residues in the hydrophilic cross-linking domains of secreted tropoelastin then are rapidly cross-linked both intermolecularly and intramolecularly by the enzyme lysyl oxidase to form mature elastin (14).

*S. aureus* infects elastin-rich tissues such as lung and heart valves, causing pneumonia and native-valve endocarditis, respectively (15, 16). The membrane-associated elastin-binding protein (EbpS) was shown to promote binding of soluble elastin peptides and tropoelastin to *S. aureus* cells (17, 18). It was suggested that EbpS might be the adhesin responsible for the bacterial colonization of elastin-rich tissue (17). If EbpS is to function as an elastin adhesin, it must be able to promote bacterial adherence to immobilized elastin. In this study, we show that the ability of *S. aureus* to adhere to immobilized elastin is not dependent on EbpS but rather on the fibronectin-binding proteins FnBPA and FnBPB (Fig. 1), cell wall-associated proteins that promote bacterial adherence to immobilized fibronectin *in vitro* (19), to plasma clots, and to *ex vivo* coverslips removed from subcutaneous tissue cages in guinea pigs (20, 21). They are encoded by two closely linked genes, *fnbA* and *fnbB*, which are tandemly arrayed but transcribed separately (19, 22). Transcription of the *fnb* genes is restricted to the early exponential phase of growth (19) under the control of Agr and SarA (23, 24). Fibronectin binding activity is lost as cultures enter stationary phase due to degradation of both FnBPA and FnBPB by V8 serine protease (25) and masking of the proteins by capsular polysaccharide (26). The A domains of FnBPA and FnBPB are 45% identical. They also share 25%

\* This work was supported by grants from Enterprise Ireland, the Health Research Board of Ireland, and Inhibitex Inc. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: ECM, extracellular matrix; rFnBP, recombinant FnBP; MSSA, methicillin-sensitive *S. aureus*; MSCRAMM, microbial surface components recognizing adhesive matrix molecules; ClfA, clumping factor A; ClfB, clumping factor B; FnBPA, fibronectin-binding protein A; FnBPB, fibronectin-binding protein B; EbpS, elastin-binding protein of *S. aureus*; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

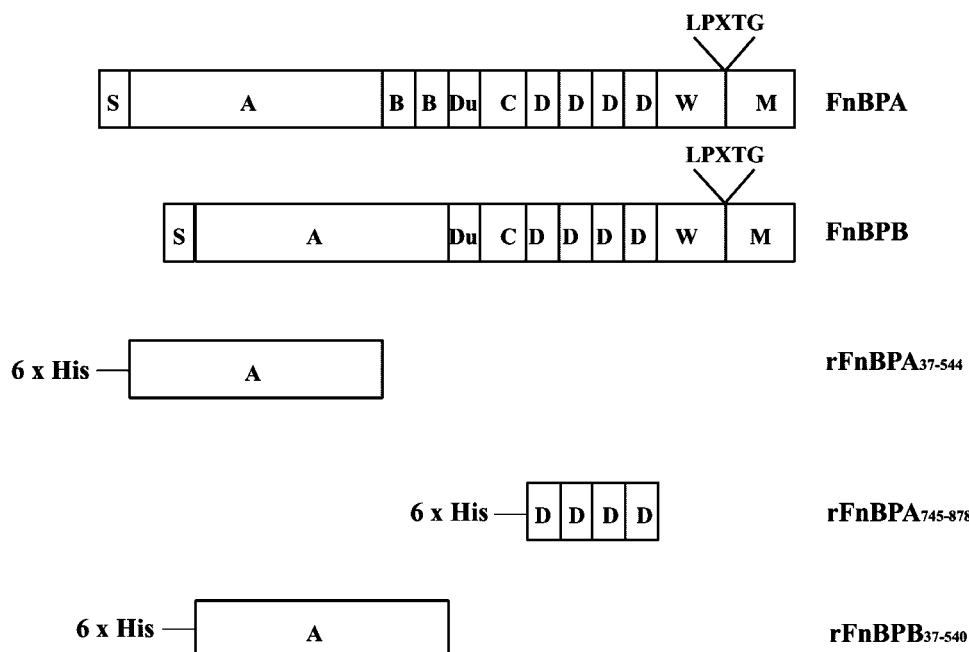


FIG. 1. Structural organization of the fibronectin-binding proteins of *S. aureus* strain 8325-4, FnBPA and FnBPB, and recombinant truncated derivatives. A secretory signal sequence (S), regions A, B, Du, C, and D, cell-spanning domains (W), and membrane-spanning domains (M) are indicated. The LPXTG motifs involved in anchoring the proteins to the cell wall peptidoglycan are also indicated. The recombinant proteins rFnBPA<sub>37-544</sub>, rFnBPB<sub>37-540</sub>, and rFnBPA<sub>745-878</sub> with N-terminal His<sub>6</sub> are shown.

identity to the A domains of ClfA and ClfB. Indeed, the FnBPA and FnBPB A domains bind to the C terminus of the  $\gamma$ -chain of fibrinogen at the same site as ClfA and the platelet integrin (20, 27). 10 fibronectin binding domains extend throughout domains B, C, and D of FnBPA (28, 29), although most binding studies have been performed with the D repeats, such as D1D2D3 (30), which share 95% identity between both FnBPA and FnBPB. In this study, we have analyzed the molecular basis of adherence of *S. aureus* to immobilized elastin. We show that this involves FnBPA and FnBPB, and we have localized the elastin binding site in each to the N-terminal A domain.

#### MATERIALS AND METHODS

**Bacterial Strains**—Cloning was routinely performed in *Escherichia coli* strain XL-1 blue *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI<sup>q</sup>ZΔM15 Tn10 (Tet<sup>r</sup>)*] (Stratagene). *E. coli* strains were transformed by the calcium chloride method (31). *E. coli* strain Topp 3 Rif [F' *proAB lacI<sup>q</sup>ZΔM15 Tn10 (Tet<sup>r</sup>) (Kan<sup>r</sup>)*] (Stratagene) was used for the expression of recombinant truncates of the fibronectin-binding proteins.

*S. aureus* strains P1 (32), Newman (33), Cowan (NCTC 8530), MSSA476 (34), methicillin-resistant *S. aureus* 252 (34), RN4220 (35), COL (36), and 8325-4 (37) were used in this study. Transduction in *S. aureus* using bacteriophage 85 was performed as described by Foster (38). The *ebpS::Em<sup>r</sup>* mutation that had previously been constructed by allele replacement by insertion of a cassette-encoding erythromycin resistance into the *ebpS* gene was transduced from strain DU5979 (18) into strain P1 to produce strain P1 *ebpS* (DU5997). Similarly, the *fnbA::Tc<sup>r</sup> fnbB::Em<sup>r</sup>* insertion mutations were co-transduced by virtue of their close linkage into *S. aureus* strain P1 from strain DU5883 (19) to produce strain P1 *fnbA fnbB* (DU5998). Plasmids pFNBA4 and pFNBPB4 (19) were transduced from strain DU5883 (19) into strain DU5998.

**Expression and purification of rFnBPA<sub>37-544</sub>, rFnBPA<sub>745-878</sub>, and rFnBPB<sub>37-540</sub>**—The region of the *fnbA* gene encoding residues 37–544 was PCR-amplified from plasmid pFNBA4 using the forward primer FAA-Fwd, 5'-AAAGGATCCGCATCAGAACAAGACAAC-3', incorporating a BamHI restriction site (*underlined*) and the reverse primer FAA-Rev, 5'-AGAGTCGACCTATTCACAGTAGTACTAAATTC-3', incorporating a SalI restriction site. The region of the *fnbA* gene encoding residues 745–878 was PCR-amplified from plasmid pFNBA4 using the forward primer FAD-Fwd, 5'-CGCGGATCCGGCCAAAATAGCGGTAAC-3', incorporating a BamHI restriction site (*underlined*) and the

reverse primer FAD-Rev, 5'-CCCAAGCTTCTATGGCAGATTGGAGGTG-3', incorporating a HindIII restriction site (*underlined*). The region of the *fnbB* gene encoding residues 37–540 was PCR-amplified from plasmid pFNBB4 using the forward primer FAB-Fwd, 5'-CGCGGATCCGCATCCGAACAACAATAC-3', incorporating a BamHI restriction site (*underlined*) and the reverse primer FAB-Rev, 5'-AGAGTCGACCTATTCAGTTTCAATGGTACCTTC-3', incorporating a SalI restriction site (*underlined*). The PCR conditions were 30 cycles of 94 °C for 1 min, 48–50 °C for 1 min, and 72 °C for 1–4 min using *Pfu* polymerase (Promega). The PCR products were cloned into the N-terminal six-histidine tag expression vector pQE-30 (Qiagen). Recombinant proteins were expressed and purified by Ni<sup>2+</sup> chelate chromatography followed by Q-Sepharose chromatography as described previously (34). SDS-PAGE analysis showed a single major band of >95% purity.

**Antibodies**—Specific pathogen-free rabbits were immunized with rFnBPA<sub>37-544</sub> and rFnBPB<sub>37-540</sub>. Immunoglobulin was purified as described by Owen (39). Chicken anti-human fibronectin antibodies were supplied by Gallus Immunotech (Fergus, Ontario, Canada) after immunization with the purified N-terminal 29-kDa fragment containing the type I modules to which FnBPs bind.

**Western Immunoblotting and Western Ligand Affinity Blotting**—Samples were boiled for 5 min in an equal volume of 2× sample buffer before electrophoresis. SDS-PAGE (40) was performed through a 4.5% stacking and 10% polyacrylamide-separating gel. Proteins were transferred to polyvinylidene difluoride membrane (Roche Applied Science) by Western blotting. Rabbit polyclonal anti-human fibrinogen-horse-radish peroxidase antibodies (Dako 1:4000) and goat polyclonal anti-human fibronectin antibodies (Dako 1:2000) were used in an attempt to detect contaminants in 30  $\mu$ g of elastin peptides fractionated by SDS-PAGE and Western blotted onto polyvinylidene difluoride membranes. Protein A conjugated to horseradish peroxidase (Sigma) was used to detect any bound antibodies using the LumiGLO chemiluminescence substrate (New England Biolabs).

**Bacterial Adhesion to Immobilized Elastin Peptides**—Microtiter plate wells (Povair) were coated with 50- $\mu$ l volumes of various concentrations (0.001–1  $\mu$ g) of  $\kappa$ -elastin from bovine ligament, human aortic elastin, or human lung elastin (Elastin Products Company) that had been dissolved in coating buffer (0.1 M sodium bicarbonate, pH 9.4). The elastin peptides were air-dried under UV light (366 nm) at room temperature for 18 h. Wells were blocked for 2 h at room temperature with 5% (w/v) bovine serum albumin in PBS. *S. aureus* cultures were grown at 37 °C to an A<sub>600 nm</sub> of 0.5 (early exponential phase) or 12 (stationary phase) in 20 ml of trypticase soy broth (Difco) in a 250-ml flask with shaking at 200 rpm. Cells were washed in PBS and resuspended to an

$A_{600\text{ nm}}$  of  $1 (1 \times 10^8)$  colony forming units/ml). Bacterial cell adherence was measured using a fluorescent nucleic acid stain SYTO-13 (Molecular Probes). SYTO-13 was supplied in 5 mM dimethyl sulfoxide. It was diluted 1:10 in 10 mM Tris-HCl (pH 7.5). Bacterial cells were incubated with SYTO-13 (2.5  $\mu\text{M}$ ) at room temperature for 15 min in the dark. Elastin-coated wells were washed three times with PBS. 100  $\mu\text{l}$  of stained cells then were added and incubated in the dark at room temperature for 90 min. Wells were washed three times with PBS, and adherent bacteria were measured using a LS-50B spectrometer (PerkinElmer Life Sciences) with excitation at 488 nm and emission at 509 nm.

**Effect of Soluble Fibronectin on the Adherence of *S. aureus* to Immobilized Fibronectin and Elastin**—A culture of *S. aureus* P1 *fnbA fnbB* (pFNBA4) was grown to early exponential phase, and cells were collected as described above. Cells were preincubated with 100  $\mu\text{g/ml}$  human fibronectin (Calbiochem) for 1 h at ambient temperature before being stained with SYTO-13 and tested for adherence to microtiter plates (Povair) coated with human aortic elastin (10  $\mu\text{g/ml}$ ) as described above or to control wells coated with human fibronectin (5  $\mu\text{g/ml}$ , Calbiochem).

**Inhibition of Bacterial Adherence to Immobilized Elastin with Antibodies**—*S. aureus* P1 *fnbA fnbB* (pFNBA4) cells were grown to early exponential phase, washed, and resuspended in PBS. Cells were then diluted with an equal volume of dilutions of anti-rFnBPA<sub>37-544</sub> or anti-rEbpS<sub>1-267</sub> (18) antibodies (100–0.8  $\mu\text{g/ml}$ ) and incubated at room temperature for 1 h. Cells then were stained with SYTO-13, 100  $\mu\text{l}$  was added to the microtiter wells coated with human aortic elastin, and fluorescence was measured as described above.

**Inhibition of Bacterial Adherence to Immobilized Elastin with Recombinant Proteins**—Human aortic elastin peptides (10  $\mu\text{g/ml}$ ) were coated onto the wells of a microtiter plate as described previously. Wells were washed three times with PBS, and various concentrations (0.2–60  $\mu\text{M}$ ) of rFnBPA<sub>37-544</sub>, rFnBPA<sub>745-878</sub>, rFnBPB<sub>37-540</sub>, or recombinant protein A (Sigma) were added in 50- $\mu\text{l}$  volumes and incubated at room temperature for 30 min. Cells of *S. aureus* P1 *fnbA fnbB* (pFNBA4<sup>+</sup>) grown to early exponential phase and stained with SYTO-13 were added, and bacterial adherence was measured as described above.

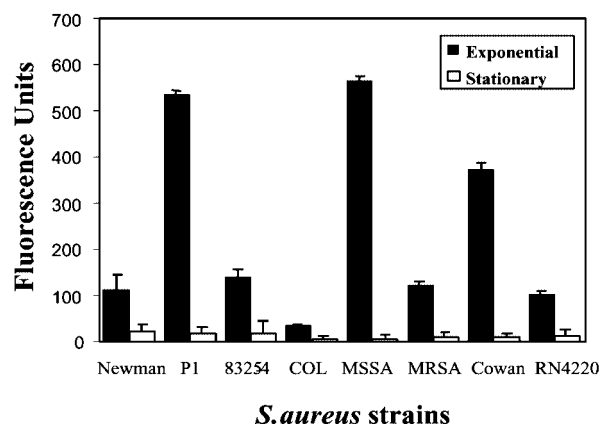
**Direct Binding of Recombinant Proteins to Immobilized Elastin and Fibrinogen**—Human aortic elastin (20  $\mu\text{g/ml}$ ) and human fibrinogen (10  $\mu\text{g/ml}$ ) were coated onto the wells of microtiter plates for 18 h under UV light and for 18 h at 4 °C, respectively. Plates were washed three times with PBS and blocked with 5% (w/v) bovine serum albumin in PBS for 2 h at 37 °C. Following three washes with PBS, different concentrations of purified rFnBPA<sub>37-544</sub> and rFnBPB<sub>37-540</sub> protein in PBS were added and incubated for 1 h at room temperature. The A domain of ClfA (rClfA<sub>40-559</sub>) was used as a control for fibrinogen binding. Wells were washed with PBS, and bound protein was detected by incubation with a 1:2000 dilution of polyclonal anti-rFnBPA<sub>37-544</sub>, anti-rFnBPB<sub>37-540</sub>, or anti-rClfA<sub>40-559</sub> antibodies for 1 h at room temperature. After three washes with PBS, goat anti-rabbit-horseradish peroxidase-conjugated antibodies (Dako, 1:2000) were added and bound horseradish peroxidase was detected as described above.

**Inhibition of Recombinant Protein Binding to Immobilized Elastin with Soluble Elastin Peptides**—The wells of a microtiter plate were coated with human-aortic elastin peptides (50  $\mu\text{g/ml}$ ) and blocked with bovine serum albumin as described previously. Recombinant rFnBPA<sub>37-544</sub> and rFnBPB<sub>37-540</sub> were preincubated for 1 h at room temperature in PBS containing soluble human aortic elastin peptides (0–250  $\mu\text{g/ml}$ ) before being added to elastin-coated wells and incubated for 1 h at room temperature. After washing in PBS, bound rFnBPs were detected as described above.

## RESULTS

**Adherence of *S. aureus* to Immobilized Elastin Peptides**—It was reported previously that soluble <sup>125</sup>I-labeled  $\alpha$ -elastin peptides from bovine ligament and <sup>125</sup>I-labeled human recombinant tropoelastin, the monomeric precursor of elastin, bound specifically to *S. aureus* cells (41) and that this was mediated by the elastin-binding protein, EbpS (17, 18). Binding experiments with an *ebpS* mutant of *S. aureus* strain, Newman, indicated that EbpS is the dominant factor for binding soluble elastin but also suggested that the bacteria express a second elastin binding moiety (18).

ELISA-based assays have been used to study the adherence of *S. aureus* cells to immobilized mammalian ECM proteins such as fibronectin, collagen, and fibrinogen (42). To assess the



### *S. aureus* strains

FIG. 2. Adherence of cells from cultures of *S. aureus* strains Newman, P1, 8325-4, COL, MSSA476, methicillin-resistant *S. aureus* 252, Cowan, and RN4220 grown to early exponential phase ( $A_{600\text{ nm}} = 0.5$ ) and stationary phase ( $A_{600\text{ nm}} = 12$ ) to immobilized  $\kappa$ -elastin from bovine ligament (10  $\mu\text{g/ml}$ ).

ability of *S. aureus* strains to adhere to immobilized elastin peptides,  $\kappa$ -elastin from bovine ligament was coated onto the wells of a microtiter plate. Cultures of laboratory strains, Newman, P1, 8325-4, Cowan, COL, and RN4220, and clinical strains, MSSA476 and methicillin-resistant *S. aureus* 252, were grown to early exponential phase and stationary phase, and the ability of cells to adhere to immobilized elastin peptides was measured. The cells of strains P1, Cowan, and MSSA476 from exponential phase cultures adhered strongly to immobilized elastin peptides, but cells from stationary phase cultures did not adhere (Fig. 2). Thus, adherence to immobilized elastin is a feature of cells from the exponential phase of growth. MSCRAMMs, such as FnBPs (19) and ClfB (3), are known to be expressed exclusively on cells from the exponential phase.

**Adherence to Immobilized Elastin Is Mediated by the Fibronectin-binding Proteins and Not by EbpS**—Strain P1 was used for further investigation of *S. aureus* adherence to elastin, because this strain is amenable to genetic manipulation and exhibits strong adherence to immobilized elastin peptides. To investigate whether EbpS was responsible for this interaction, *S. aureus* P1 wild type and an isogenic *ebpS* mutant were tested for adherence to immobilized  $\kappa$ -elastin from bovine ligament. Both strains adhered in a dose-dependent manner, but there was no significant difference in elastin binding activity of the two (Fig. 3A), indicating that EbpS is not an MSCRAMM for elastin.

It then was shown that a mutant of P1 *fnbA fnbB* defective in the expression of the fibronectin-binding proteins, FnBPA and FnBPB, did not adhere to immobilized  $\kappa$ -elastin (Fig. 3B). P1 adhered in a dose-dependent manner, whereas no binding was observed with strain P1 *fnbA fnbB*. This defect was partially complemented by pFNBA4 (expressing FnBPA) and fully complemented by pFNBB4 (expressing FnBPB). Partial complementation by pFNBA4 could be attributed to lower expression of the 8325-4 *fnbA* gene in the heterologous P1 host. These data strongly suggest that FnBPA and FnBPB are the surface proteins responsible for bacterial adherence to immobilized elastin peptides. A further analysis of FnBP-mediated adherence to immobilized elastins was performed with P1 *fnbA fnbB* (pFNBA4).

***S. aureus* Adheres to Human Aortic Elastin and Human Lung Elastin in an FnBP-dependent Manner**—*S. aureus* adheres to immobilized elastin peptides purified from bovine neck ligament in a FnBP-dependent manner (Fig. 3). If the interaction between the fibronectin-binding proteins and elastin is an important factor in human diseases of elastin-rich tissue, such

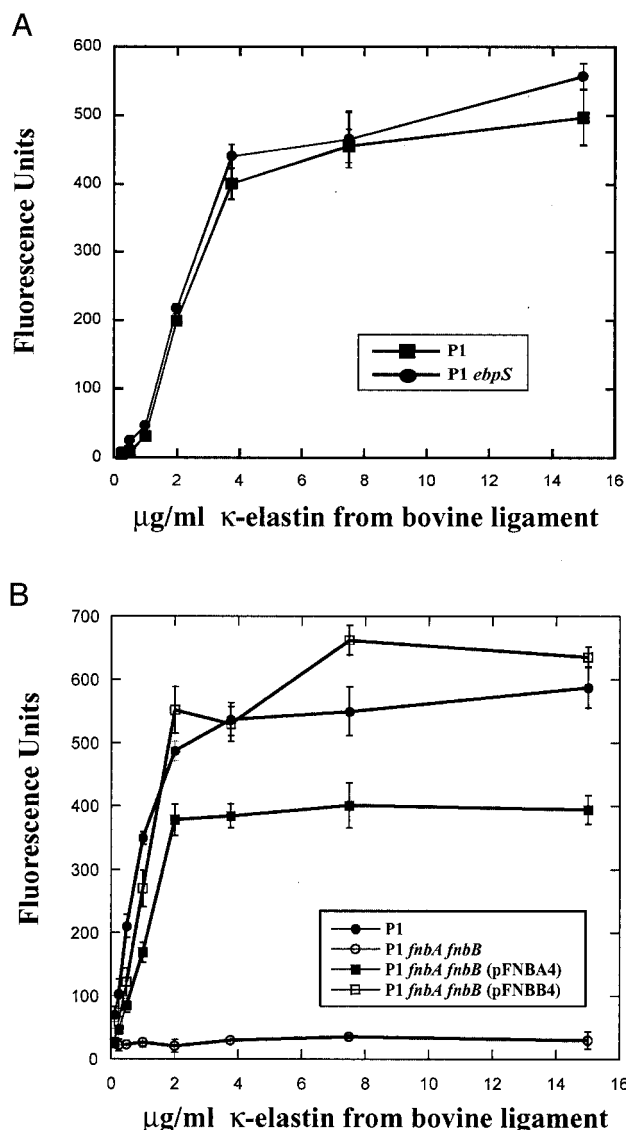


FIG. 3. *A*, adherence of cells from exponential phase cultures ( $A_{600\text{ nm}} = 0.5$ ) of *S. aureus* strains P1 and P1 *ebpS* to increasing concentrations of immobilized  $\kappa$ -elastin peptides from bovine ligament. *B*, adherence of cells from early exponential phase cultures ( $A_{600\text{ nm}} = 0.5$ ) of *S. aureus* strains P1, P1 *fnbA fnbB*, P1 *fnbA fnbB* (pFNBA4), and P1 *fnbA fnbB* (pFNBB4) to immobilized  $\kappa$ -elastin peptides from bovine ligament.

as endocarditis and pneumonia, bacteria should be able to adhere to immobilized human elastin. Human and bovine tropoelastin share a 70.3% identity at the primary amino acid sequence level and are structurally and anti-genetically similar (43).

Elastins from bovine ligament, human aorta, and human lung were coated onto the wells of microtiter plates. Cells of strain P1 from an exponential phase culture bound in a dose-dependent manner to all three forms of immobilized elastin (Fig. 4). Strain P1 *fnbA fnbB* did not adhere (data not shown), and the defect was complemented by plasmids pFNBA4 and pFNBB4 (data not shown). Thus, the adherence to both immobilized human aorta and lung elastin was FnBP-dependent. Strain MSSA adhered to immobilized elastins in a similar fashion to strain P1 (data not shown).

To determine whether human aortic elastin dried under UV light onto the wells of a microtiter plate behaves similarly as the native protein, the ability of anti-human aortic elastin antibodies to bind was measured by ELISA. The antibodies bound in a dose-dependent manner (data not shown), indicat-

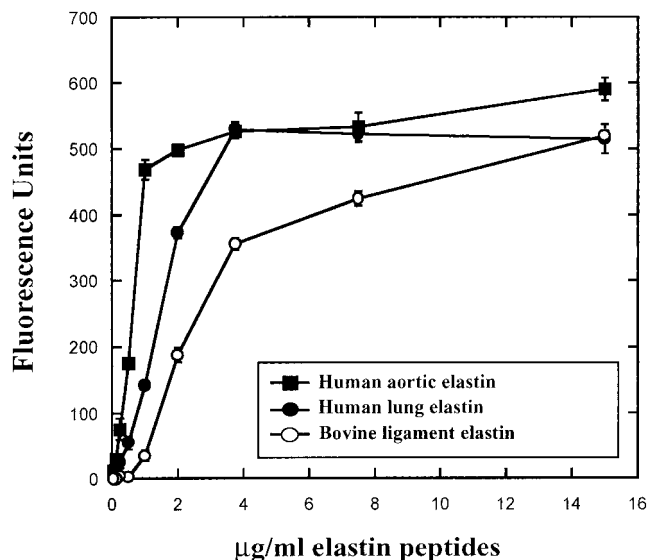


FIG. 4. Adherence of cells from an early exponential phase culture of *S. aureus* strain P1 to immobilized elastin from human lung, human aorta, and bovine ligament.

ing that many epitopes from native elastin are present in the immobilized peptides.

Because the FnBPs can adhere to both fibronectin (44) and fibrinogen (20), it was important to determine whether the commercially available elastin peptides used in adherence assays were contaminated with either of these host proteins. No immunoreactive proteins were detected in the preparations of human lung elastin or human aortic elastin by Western immunoblotting with polyclonal chicken, goat, or rabbit anti-human fibrinogen or polyclonal anti-human fibronectin antibodies (data not shown). In the case of chicken antibodies, the limit of detection of immobilized fibronectin by ELISA was 0.03  $\mu\text{g/ml}$ , a concentration that is too low to support bacterial adhesion. Furthermore chicken antibodies diluted 1 in 5 caused a 60% reduction in adherence of P1 to immobilized fibronectin but did not inhibit adherence to immobilized  $\kappa$ -elastin (data not shown).

**Localization of the Elastin Binding Domain in FnBPA and FnBPB**—To determine whether the fibronectin binding regions of FnBPA were involved in the elastin binding phenotype, the ability of soluble fibronectin to inhibit elastin binding was assessed. Cells from an early exponential phase culture of strain P1 *fnbA fnbB* (pFNBA4) were preincubated with soluble human fibronectin (100  $\mu\text{g/ml}$ ). The ability of cells to bind to immobilized fibronectin and elastin then was tested. Preincubation of cells with soluble fibronectin resulted in a 76% inhibition of binding to immobilized fibronectin (Fig. 5). Conversely, preincubation with soluble fibronectin did not inhibit binding to immobilized human aortic elastin.

The ability of polyclonal anti-FnBPA A domain antibodies to block P1 *fnbA fnbB* (pFNBA4) cells from binding to immobilized human aortic elastin was tested. Cells were preincubated with anti-rFnBPA<sub>37-544</sub> antibodies, which recognize region A of FnBPA, or anti-rEbpS<sub>1-267</sub> antibodies, which recognize the N terminus of EbpS (18). The anti-rFnBPA<sub>37-544</sub> antibodies inhibited FnBPA-mediated adherence to elastin in a dose-dependent manner with the highest concentration of inhibiting antibody (100  $\mu\text{g/ml}$ ) resulting in 87% inhibition (Fig. 6). The same range of concentrations of the control anti-rEbpS<sub>1-267</sub> antibodies did not inhibit elastin binding. This implies that the elastin binding domain of FnBPA is located within region A.

To obtain further information regarding the regions of FnBPA and FnBPB that promote bacterial adherence to immo-

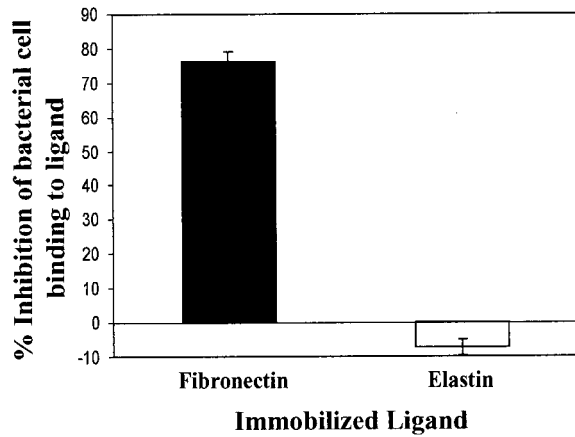


FIG. 5. Inhibition of *S. aureus* binding to immobilized fibronectin and elastin by preincubation of cells with human fibronectin. Cells of *S. aureus* strain P1 *fnbA fnbB* (pFNBA4) were preincubated in human fibronectin (100  $\mu\text{g/ml}$ ), and then their ability to bind to immobilized human fibronectin (5  $\mu\text{g/ml}$ ) and human aortic elastin (10  $\mu\text{g/ml}$ ) was tested. The values are expressed as percentages of a control lacking inhibitor.

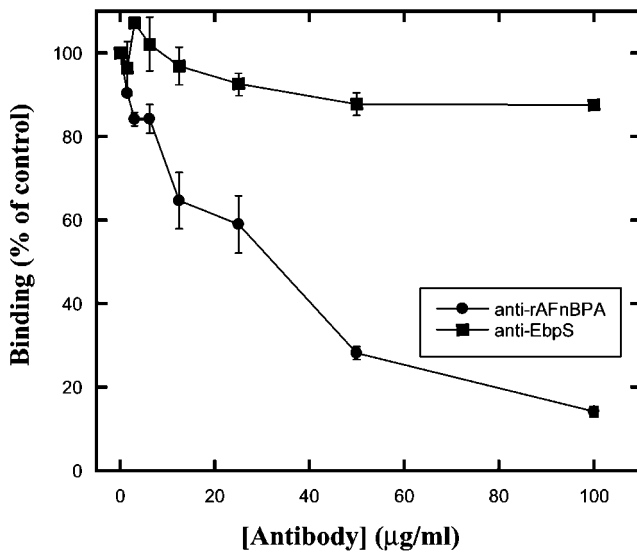


FIG. 6. Inhibition of cells of *S. aureus* strain P1 *fnbA fnbB* (pFNBA4) binding to immobilized human aortic elastin (10  $\mu\text{g/ml}$ ) by preincubation with anti-rFnBPA<sub>37-544</sub> or anti-rEbpS<sub>1-267</sub> antibodies. The values are expressed as percentages of a control lacking inhibitor.

bilized elastin, the ability of the recombinant proteins rFnBPA<sub>37-544</sub>, rFnBPB<sub>37-540</sub>, and rFnBPA<sub>745-878</sub> to bind to immobilized elastin and inhibit adherence of *S. aureus* cells to immobilized elastin was tested. Recombinant truncated derivatives of FnBPA and FnBPB were expressed in *E. coli* as N-terminal His<sub>6</sub> fusion proteins and purified by nickel-chelate affinity chromatography. rFnBPA<sub>37-544</sub> comprised region A of FnBPA, rFnBPB<sub>37-540</sub> comprised region A of FnBPB, and rFnBPA<sub>745-878</sub> corresponded to the D repeats (D1–D4) of FnBPA (Fig. 1).

For the inhibition study, increasing concentrations of rFnBPA<sub>37-544</sub>, rFnBPB<sub>37-540</sub>, rFnBPA<sub>745-878</sub>, and protein A were tested for their ability to block adherence of *S. aureus* P1 *fnbA fnbB* (pFNBA4) cells to elastin-coated wells. The presence of the recombinant proteins, rFnBPA<sub>37-544</sub> and rFnBPB<sub>37-540</sub>, inhibited the binding of P1 *fnbA fnbB* (pFNBA4) to immobilized elastin in a dose-dependent manner, indicating that the recombinant A regions of both FnBPA and FnBPB bound to immo-

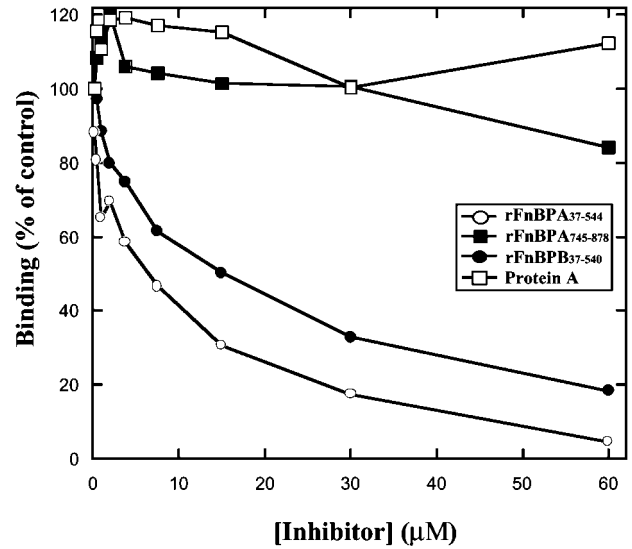


FIG. 7. Inhibition of *S. aureus* binding to immobilized elastin peptides by preincubation with recombinant FnBP truncates. 10  $\mu\text{g/ml}$  of human aortic elastin peptides were coated onto the wells of a microtiter plate. Wells were preincubated with increasing concentrations of rFnBPA<sub>37-544</sub>, rFnBPB<sub>37-540</sub>, rFnBPA<sub>745-878</sub>, and protein A. After incubation, the ability of cells from an early exponential phase culture of *S. aureus* strain P1 *fnbA fnbB* (pFNBA4) to bind to the immobilized elastin was tested. The values are expressed as percentages of a control lacking inhibitor. This figure is representative of the results of three independent experiments performed in duplicate.

bilized elastin. Protein A and rFnBPA<sub>745-878</sub> did not inhibit bacterial adherence (Fig. 7).

Recombinant rFnBPA<sub>37-544</sub> and rFnBPB<sub>37-540</sub> then were tested for their ability to bind to immobilized elastin. Human aortic elastin (20  $\mu\text{g/ml}$ ) or fibrinogen (10  $\mu\text{g/ml}$ ) was coated onto the wells of a microtiter plate. Increasing concentrations of rFnBPA<sub>37-544</sub> and rFnBPB<sub>37-540</sub> were incubated in the wells. rClfA<sub>40-559</sub> was added as a positive control for fibrinogen binding. Bound protein was detected with polyclonal antibodies recognizing the A regions of FnBPA, FnBPB, or ClfA as appropriate. Fig. 8, A and B, reveals dose-dependent and saturable binding of both FnBP recombinant proteins to immobilized elastin and fibrinogen. rFnBPB<sub>37-540</sub> appears to have a greater affinity for elastin than rFnBPA<sub>37-544</sub> as reflected in the  $K_d$  values of 0.123 and 0.542  $\mu\text{M}$ , respectively. This is not due to a discrepancy in antibody potency, because both anti-rFnBPA<sub>37-544</sub> and anti-rFnBPB<sub>37-540</sub> antibodies proved to be equally sensitive in detecting their respective recombinant FnBP proteins in an ELISA assay (data not shown). rFnBPB<sub>37-540</sub> is also shown to have a higher binding specificity for fibrinogen compared with rFnBPA<sub>37-544</sub> with  $K_d$  values of 0.00054 and 0.027  $\mu\text{M}$ , respectively (Fig. 8B). Moreover, the affinity of rFnBPB<sub>37-540</sub> for fibrinogen is demonstrated to be higher than that of rClfA<sub>40-559</sub>, one of the major *S. aureus* fibrinogen-binding proteins, which shows a  $K_d$  value of 0.014  $\mu\text{M}$ . Recombinant FnBPB protein had to be diluted >10-fold than the FnBPA recombinant protein to achieve a dose-dependent fibrinogen binding curve (Fig. 8B, inset).

To demonstrate that the FnBP-elastin interaction was specific, inhibition studies were carried out using soluble elastin peptides. Fig. 8C shows that soluble elastin peptides inhibited both recombinant FnBP proteins from binding to immobilized elastin in a dose-dependent manner. These data indicate the FnBP binding to immobilized elastin peptides is a specific receptor-ligand interaction.

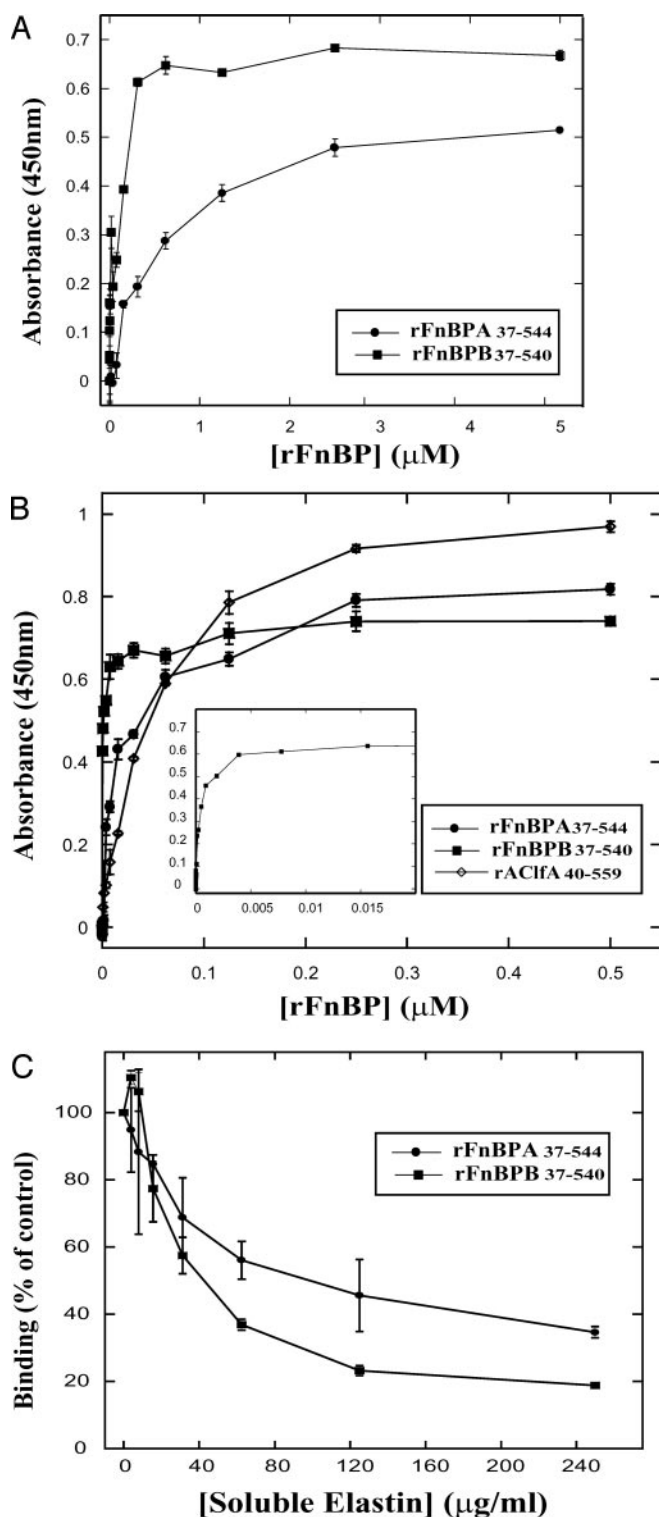


FIG. 8. A, binding of recombinant FnBP truncates to immobilized elastin. Microtiter wells were coated with 20  $\mu\text{g/ml}$  of human aortic elastin. Increasing concentrations of rFnBPA<sub>37-544</sub> (●) and rFnBPB<sub>37-540</sub> (■) were incubated in the wells for 1 h at room temperature. Bound protein was detected with polyclonal anti-rFnBPA<sub>37-544</sub> or anti-rFnBPB<sub>37-540</sub>. Values represent the mean of triplicate wells. B, binding of recombinant FnBP truncates to immobilized fibrinogen. Microtiter wells were coated with 10  $\mu\text{g/ml}$  of human fibrinogen. Increasing concentrations of rFnBPA<sub>37-544</sub> (●) and rFnBPB<sub>37-540</sub> (■) and rClfA<sub>40-559</sub> (□) were incubated in the wells for 1 h at room temperature. Bound protein was detected with polyclonal anti-rFnBPA<sub>37-544</sub>, anti-rFnBPB<sub>37-540</sub>, or anti-rClfA<sub>40-559</sub> antibodies. *Inset graph* represents rFnBPB<sub>37-540</sub> binding to lower concentrations of immobilized fibrinogen. The same axis labels apply to both graphs. Values represent the mean of triplicate wells. C, inhibition of recombinant FnBP truncates binding to immobilized

## DISCUSSION

An important factor in bacterial pathogenesis is the ability of the invading organism to colonize host tissue. *S. aureus* possesses on its cell surface a number of proteins, collectively called MSCRAMMs, that promote the binding of the organism to components of the host extracellular matrix and play an important role in bacterial virulence. *S. aureus* causes a number of diseases of elastin-rich tissue such as lung and heart valves, and for this reason, the interaction of the organism with elastin was examined. Recent studies identified the EbpS as a surface protein that promotes the binding of soluble elastin and tropoelastin to *S. aureus* cells (17, 18). It was proposed that EbpS could behave like other matrix-binding proteins of *S. aureus* and promote the adherence of the organism to elastin-rich tissue and facilitate bacterial colonization. An ELISA-based assay was developed to study the adherence of *S. aureus* to immobilized elastin. It was found that several *S. aureus* strains adhered in a growth phase-dependent manner but that adherence was independent of EbpS since there was no detectable reduction with a strain defective in EbpS expression.

Adherence only occurred with cells from early exponential phase cultures. This indicated that the surface factor responsible was either expressed only during the early exponential phase of growth or was occluded by another surface component such as capsular polysaccharide during the later phase of growth.

The ability of *S. aureus* strain P1 to adhere to immobilized elastin peptides was lost when the organism did not express the fibronectin-binding proteins, FnBPA and FnBPB. The elastin-binding phenotype was complemented by expression of FnBPA or FnBPB from plasmids. FnBPB complemented elastin binding to the same level as strain P1, whereas FnBPA complemented elastin binding to a lower level than the wild-type strain. This difference in the levels of elastin binding exhibited by the proteins could be attributed to protein affinity or expression levels. These data indicate that the fibronectin-binding proteins of *S. aureus* are the proteins responsible for bacterial adherence to immobilized elastin peptides. Fibronectin-binding proteins are known to be expressed predominantly in the exponential phase of growth. This is consistent with the expression of the elastin binding phenotype. The differences in elastin binding activity between the strains may be attributed to differences in the levels of FnBP expression. Strains P1 and Cowan express high levels of FnBPs (45) and promote strong binding to immobilized fibronectin and elastin, whereas strain 8325-4 expresses low levels of FnBPs (46) and binds weakly to immobilized elastin peptides. Strain Newman binds weakly to immobilized fibronectin (21) and elastin.

If the fibronectin-binding proteins are responsible for the colonization of elastin-rich tissue in the human, they must bind to elastin prepared from human tissue. Strain P1 adhered to bovine elastin and to two forms of human elastin in a dose-dependent manner. Adherence to human elastins was also FnBP-dependent.

The identification of the FnBPs as the proteins responsible for the adherence of cells to immobilized elastin peptides raised the question of the purity of the elastin preparations used in adherence assays. No fibronectin or fibrinogen was detectable

elastin by soluble elastin peptides. Microtiter wells were coated with 50  $\mu\text{g/ml}$  of human aortic elastin. rFnBPA<sub>37-544</sub> (0.1  $\mu\text{M}$ , ●) and rFnBPB<sub>37-540</sub> (0.07  $\mu\text{M}$ , ■) were preincubated for 1 h at room temperature with increasing concentrations of soluble elastin peptides (0–250  $\mu\text{g/ml}$ ). After incubation in the wells for 1 h at room temperature, bound protein was detected using polyclonal anti-rFnBPA<sub>37-544</sub> or anti-rFnBPB<sub>37-540</sub> antibodies. Values represent the mean of triplicate wells and are expressed as the percentages of a control lacking an inhibitor.

in the elastin preparations by Western blotting. Soluble fibronectin inhibited the binding of FnBPA-expressing *S. aureus* to immobilized fibronectin but not to immobilized elastin peptides. If adherence to immobilized elastin was the result of fibronectin contamination, a reduction in binding would have occurred because of blocking of the fibronectin binding domains on the FnBPA protein. On the contrary, no inhibition of elastin binding was observed. FnBPA has also been shown to bind fibrinogen at the same site on the  $\gamma$ -chain peptide as ClfA (20). Adherence to elastin was completely abolished in the absence of FnBPA and FnBPB from the bacterial cell surface. Adherence could not be attributed to fibrinogen contamination, because the fibrinogen binding MSCRAMMs, ClfA and ClfB, are expressed during early exponential phase of growth (2, 3). Together, these results demonstrate that FnBP binding to elastin is specific and not an artifact of fibronectin or fibrinogen contamination.

The inability of soluble fibronectin to inhibit adherence of *S. aureus* to elastin indicated that the fibronectin binding domains on FnBPA were not involved in elastin binding. To identify the regions of the fibronectin-binding proteins responsible, inhibition studies and direct binding assays were performed using antibodies and recombinant truncates of FnBP proteins. Antibodies raised against rFnBPA<sub>37-544</sub> (region A of FnBPA) inhibited the adherence of *S. aureus* cells expressing FnBPA to immobilized human aortic elastin. This suggested that the N-terminal A region of FnBPA is involved in elastin binding. Inhibition studies and direct binding assays using recombinant A regions of both FnBPA (rFnBPA<sub>37-544</sub>) and FnBPB (rFnBPB<sub>37-540</sub>) confirmed that elastin binding is a property of the A region. Recombinant region A proteins inhibited FnBPA-mediated bacterial cell adherence in a dose-dependent manner, whereas the recombinant FnBPA D repeats had no effect. It is interesting to note that recombinant region A of FnBPB inhibited FnBPA-mediated bacterial adherence. This finding suggests that FnBPA and FnBPB bind to the same or very closely located sites in elastin. Both of the recombinant region A proteins bound directly to immobilized elastin in a dose-dependent and saturable manner. Binding was inhibited by preincubation with soluble elastin peptides, which is indicative of a specific receptor-ligand interaction. FnBPB appears to have a higher affinity for elastin than the FnBPA protein as detected in the direct binding of recombinant FnBP proteins to immobilized elastin. This increased specificity of FnBPB for elastin was also observed in bacterial binding assays; however, different levels of protein expression may also be a contributing factor.

To coat the wells of microtiter plates, elastin peptides were dried onto the plastic surface under a UV lamp. This method has been used to study the effects of immobilized elastin peptides on the elastin receptors of mammalian cells (47). To ensure that peptides retained epitopes specific to elastin, the ability of anti-human aortic elastin antibodies to recognize immobilized human aortic elastin was assessed by ELISA. These antibodies bound in a dose-dependent manner to the immobilized antigen. This finding suggests that the overall structure of immobilized elastin peptides was not grossly affected by drying under UV light and that the protein retained many of the epitopes in the soluble form of the antigen. Therefore, the adherence assay used in these studies is an effective and reliable method of studying the interactions of *S. aureus* with mammalian elastin.

Adherence to elastin may have a role to play in staphylococcal diseases of elastin-rich tissue such as heart valves and lung. Numerous *in vivo* studies have underlined the role of the fibronectin-binding proteins in infective endocarditis. In a rat

model of endocarditis, Kuypers and Proctor (48) show that a low fibronectin-binding mutant of *S. aureus* had significantly reduced adherence to traumatized heart valves. Que *et al.* (49) demonstrate that FnBPA expressed on the surface of *Lactococcus lactis* is sufficient to produce endocarditis in rats with catheter-induced aortic vegetations. *S. aureus* expresses a number of surface proteins that recognize mammalian ECM proteins, such as fibrinogen, fibronectin, collagen, and von Willebrand factor, that could promote adhesion to the vegetation. MSCRAMMs might also aid bacterial persistence at the site of infection and promote the shedding of bacteria into the blood stream (50). In a study of experimental endocarditis, Hienz *et al.* (51) suggest that the *S. aureus* collagen adhesin, Cna, plays a limited role in initial attachment to traumatized aortic valves but is important in bacterial persistence within a vegetative lesion. Perhaps the fibronectin-binding proteins could perform a similar function by binding to the exposed elastin fibers of the damaged heart valve.

FnBPs have also been implicated in staphylococcal pneumonia. Mongodin *et al.* (52) demonstrate that strains of *S. aureus* isolated from the airway secretions of patients suffering from nosocomial pneumonia exhibited elevated levels of FnBP expression and fibronectin binding compared with strains from cystic fibrosis patients. Since elastin is highly expressed in the lung parenchyma, perhaps FnBP-mediated elastin binding contributes to the ability of *S. aureus* to colonize injured lungs and persist within the respiratory tract.

The ability to bind to more than one ligand is an emerging theme among surface proteins of *S. aureus*. Clumping factor B binds to cytokeratin 10 as well as to fibrinogen (53), and protein A binds to von Willebrand factor as well as to immunoglobulin G (5). Here, we show that both fibronectin-binding proteins A and B can bind to elastin as well as to fibrinogen (20) and fibronectin (19). This multifunctionality most probably reflects the importance of these proteins for disease pathogenesis in expanding the options available for colonizing different tissues and evading host defenses.

We are currently attempting to localize the elastin binding domain within the FnBPA A domain. We propose to determine whether elastin binds to the same domain in FnBPA as the  $\gamma$ -chain fibrinogen peptide in ClfA binds to a groove between domain N1 and N2, probably by the "dock-latch-lock" mechanism (54). We also wish to localize the binding domain for FnBP in elastin. This will be attempted by methods similar to those that were used to locate the EbpS binding domain within truncates of the soluble precursor tropoelastin (41).

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**The N-terminal A Domain of Fibronectin-binding Proteins A and B Promotes Adhesion of *Staphylococcus aureus* to Elastin**

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*J. Biol. Chem.* 2004, 279:38433-38440.

doi: 10.1074/jbc.M402122200 originally published online July 2, 2004

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Access the most updated version of this article at doi: [10.1074/jbc.M402122200](https://doi.org/10.1074/jbc.M402122200)

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