The Fibrinogen- and Fibronectin-Binding Domains of *Staphylococcus aureus* Fibronectin-Binding Protein A Synergistically Promote Endothelial Invasion and Experimental Endocarditis[▽][†]

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Staphylococcus aureus experimental endocarditis relies on sequential fibrinogen binding (for valve colonization) and fibronectin binding (for endothelial invasion) conferred by peptidoglycan-attached adhesins. Fibronectin-binding protein A (FnBPA) reconciles these two properties—as well as elastin binding—and promotes experimental endocarditis by itself. Here we attempted to delineate the minimal subdomain of FnBPA responsible for fibrinogen and fibronectin binding, cell invasion, and in vivo endocarditis. A large library of truncated constructs of FnBPA was expressed in *Lactococcus lactis* and tested in vitro and in animals. A 127-amino-acid subdomain spanning the hinge of the FnBPA fibrinogen-binding and fibronectin-binding regions appeared necessary and sufficient to confer the sum of these properties. Competition with synthetic peptides could not delineate specific fibrinogen- and fibronectin-binding sites, suggesting that dual binding arose from protein folding, irrespective of clearly defined binding domains. Moreover, coexpressing the 127-amino-acid subdomain with remote domains of FnBPA further increased fibrinogen binding by ≥10 times, confirming the importance of domain interactions for binding efficacy. In animals, fibrinogen binding (but not fibronectin binding) was significantly associated with endocarditis induction, whereas both fibrinogen binding and fibronectin binding were associated with disease severity. Moreover, fibrinogen binding also combined with fibronectin binding to synergize the invasion of cultured cell lines significantly, a feature correlating with endocarditis severity. Thus, while fibrinogen binding and fibronectin binding were believed to act sequentially in colonization and invasion, they appeared unexpectedly intertwined in terms of both functional anatomy and pathogenicity (in endocarditis). This unforeseen FnBPA subtlety might bear importance for the development of antiadhesin strategies.

Staphylococcus aureus is a major pathogen responsible for a wide variety of diseases, including skin and soft-tissue infections, bacteremia, osteomyelitis, and endocarditis (11). The first step in infection is host tissue colonization, which is mediated by a wealth of surface-bound adhesins, also referred to as MSCRAMMs (for microbial surface components recognizing adhesive matrix molecules) (15). Important staphylococcal MSCRAMMs were shown to bind matrix proteins as diverse as fibrinogen (13, 29), fibronectin (24), elastin (1, 20), collagen (15), and keratin (14). Moreover, the MSCRAMMs binding fibronectin, i.e., fibronectin-binding protein A (FnBPA) and FnBPB, were also shown to trigger active internalization into eukaryotic cells in vitro and in vivo (16, 19, 25).

Recent experiments demonstrated that binding to fibrinogen and binding to fibronectin via clumping factor A (ClfA) and

FnBPA were essential to promote experimental endocarditis in rats (17, 19). The individual contributions of these properties were assessed by expressing truncated and chimeric ClfA and FnBPA in poorly pathogenic *Lactococcus lactis* (19). Fibrinogen binding promoted early valve colonization but not persistence and invasion. In contrast, fibronectin binding was unable to initiate infection but mediated aortic cell invasion and microbial persistence once the valve had been colonized. Thus, the two adherence functions were required for progressive infection.

In fact, FnBPA is a multifunctional adhesin reunifying both fibrinogen- and fibronectin-binding capacities (22, 24, 29), as well as elastin binding (20). As a result, it can promote valve colonization and invasion by itself. However, whether these functions are partially redundant, additive, or synergistic for infection is not known. FnBPA has a symmetric functional anatomy (Fig. 1A). Its N-terminal part (called the A domain) is responsible for fibrinogen (29) and elastin binding (9, 20), whereas its C-terminal part (called B-DuC-D) binds to fibronectin (23). This traditional organization was recently refined into further subdomains which might cooperate with each other for binding (23). Residues in the N-terminal domain of the protein are predicted to fold into three subdo-
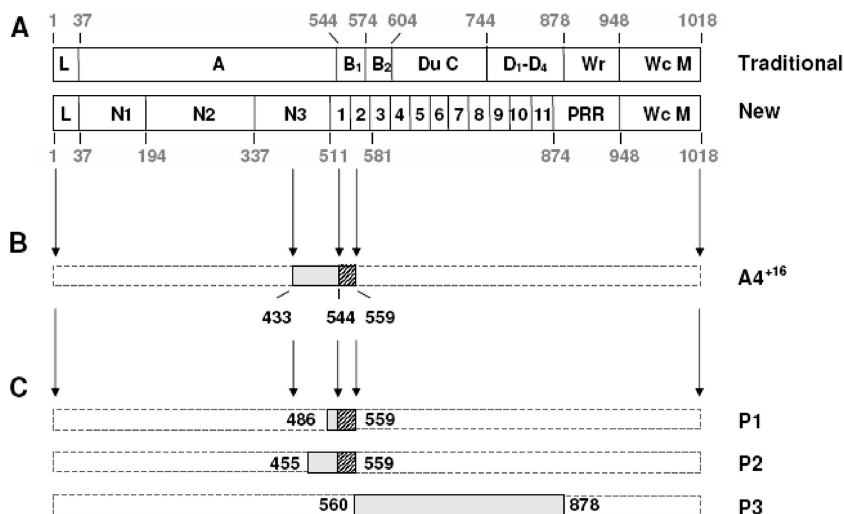
FIG. 1. Schematic representation of FnBPA and major derived peptides used in the experiments described here. (A) Traditional (24) and new (23) layouts of the protein's domains from its N- to its C-terminal end (left and right sides, respectively). Major regions include the leader sequence (L), the fibrinogen- and elastin-binding domains (A), the fibronectin-binding repeats (B-DuC-D), the wall-spanning and -anchoring domains (Wr and Wc), and the membrane-spanning sequence (M). These regions are further divided into functional subdomains in the new layout of the protein. (B) Minimal FnBPA fragment (A4^{+16}) reconciling all of its major properties involved in endocarditis pathogenesis, including fibrinogen and fibronectin binding, triggering of cell internalization, and colonizing of damaged valves. (C) Synthetic peptides used in competition assays against FnBPA and A4^{+16}. Amino acid numbering is displayed around the schemes.

mains (N1, N2, and N3), with N2 and N3 being involved in fibrinogen and elastin binding (9). Symmetrically, the C-terminal residues are responsible for fibronectin binding and are organized in 11 tandem repeats, each interacting with so-called type 1 modules of fibronectin through a tandem beta-zipper mechanism (23).

Here we attempted to delineate the minimal region of FnBPA that is required for valve infection. A large library of *L. lactis* strains expressing different FnBPA domains was constructed and tested for in vitro and in vivo adherence and infectivity. A 127-amino-acid fragment which spanned part of fibrinogen-binding module N3, as well as the first fibronectin-binding tandem repeat, could bind both fibrinogen and fibronectin and was necessary and sufficient for the invasion of cultured cell lines in vitro and valve infection in animals. Domain shuffling and competition assays with synthetic peptides also indicated that physically distant regions of FnBPA could cooperate for its binding. Moreover, the dynamics of infection and a mathematical model of cell internalization showed that fibrinogen binding was likely to be the first step in in vivo infection and acted synergistically with fibronectin binding to trigger further cell invasion. This dual role of fibrinogen binding in disease induction and progression may provide an argument for making it a priority target in antiadherence strategies.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. lactis* subsp. *cremoris* 1363 and *Escherichia coli* XL1-Blue (Stratagene) were used for cloning experiments by conventional and already described methods (18, 21). *L. lactis* was grown at 30°C in M17 medium (Oxoid) supplemented with 0.5% glucose. *E. coli* was grown at 37°C in Luria-Bertani medium. *S. aureus* Cowan and 8325-4 were grown at 37°C in Mueller-Hinton or tryptic soy broth (Difco). Whenever appropriate, antibiotics were added to the media at the following concentrations: erythromycin, 5 μg/ml for *L. lactis* and 500 μg/ml for *E. coli*; ampicillin, 75 μg/ml for *E. coli*. Bacterial stocks were kept at −70°C in liquid medium supplemented with 10% (vol/vol) glycerol.

DNA transformation and cloning experiments. Plasmids expressing fragments of the *S. aureus fnbA* gene were generated from pOri23-*fnbA* (17) by an inverse PCR strategy described by Massey et al. (12). The Expand Long Template PCR system (Roche Diagnostics) was used as recommended in the manufacturer's instructions. The amplification conditions were 18 cycles of 95°C for 30 s, 60°C for 30 s, and 68°C for 10 min. Amplicons were purified with a QIAquick PCR purification kit (Qiagen), digested with NcoI or PvuI, respectively, and self-ligated with T4 DNA ligase.

The maps of native FnBPA and its truncated derivatives expressed in lactococci are depicted in Fig. 1 and 2. For the primers selected to amplify the various domains or FnBPA subdomains, see Table S1 in the supplemental material. To facilitate amplicon religation, an NcoI or a PvuI restriction site was incorporated at the 5′ end of each primer. The constructs were named according to the traditional nomenclature of FnBPA domains. Domains Du and C were always together and were hence referred to as C for simplicity.

In order to overlap the hinge between the described A and B domains of FnBPA, an extended A domain (named A^{+16}) was generated which included the traditional A domain plus the first 16 N-terminal amino acids of the B domain. In symmetry, a B$^{−16}$CD construct was generated which included the B domain minus its first 16 N-terminal amino acids, followed by the whole Du-C and D domains. To further dissect the functional regions of A^{+16}, this domain was subdivided into four equivalent portions of ca. 130 amino acids each (named A1, A2, A3, and A4^{+16}) which were expressed either individually or as fusions with other subdomains of FnBPA (Fig. 2).

The various constructs in pOri23 were propagated in *E. coli* XL1 (21), purified with the Wizard Plus Midipreps DNA purification system (Promega), and electroporated into *L. lactis* as described previously (18).

Detection of surface-expressed recombinant proteins. The presence of the recombinant proteins expressed in *L. lactis* was determined by two different methods, Western immunoblotting and liquid chromatography coupled with mass spectrometry (LC-MS). Western immunoblotting was performed as previously described (7, 10), with antibodies recognizing residues 479 to 493 (IQNN KFEYKEDTIKE) of FnBPA (17). LC-MS can assess the correct expression of staphylococcal proteins in the cell wall of *L. lactis* recombinant bacteria, as well as the presence or absence of the desired internal fragments (A. Panchaud, E. Widmer, M. Kussmann, P. Moreillon, and M. Affolter, submitted for publication). Briefly, cells grown to an OD$_{600}$ of 0.4 were harvested by centrifugation and resuspended in 40 ml phosphate-buffered saline (PBS), before the addition of 40 ml 8% boiling sodium dodecyl sulfate. The suspension was further boiled for 15 min. Boiled cells were washed several times and then resuspended in 1 ml water. Cell lysis was performed with a FastPrep apparatus (Qbiogene, Morgan Irvine, CA) with glass beads by three cycles of spinning for 45 s at 6.5 m/s. After
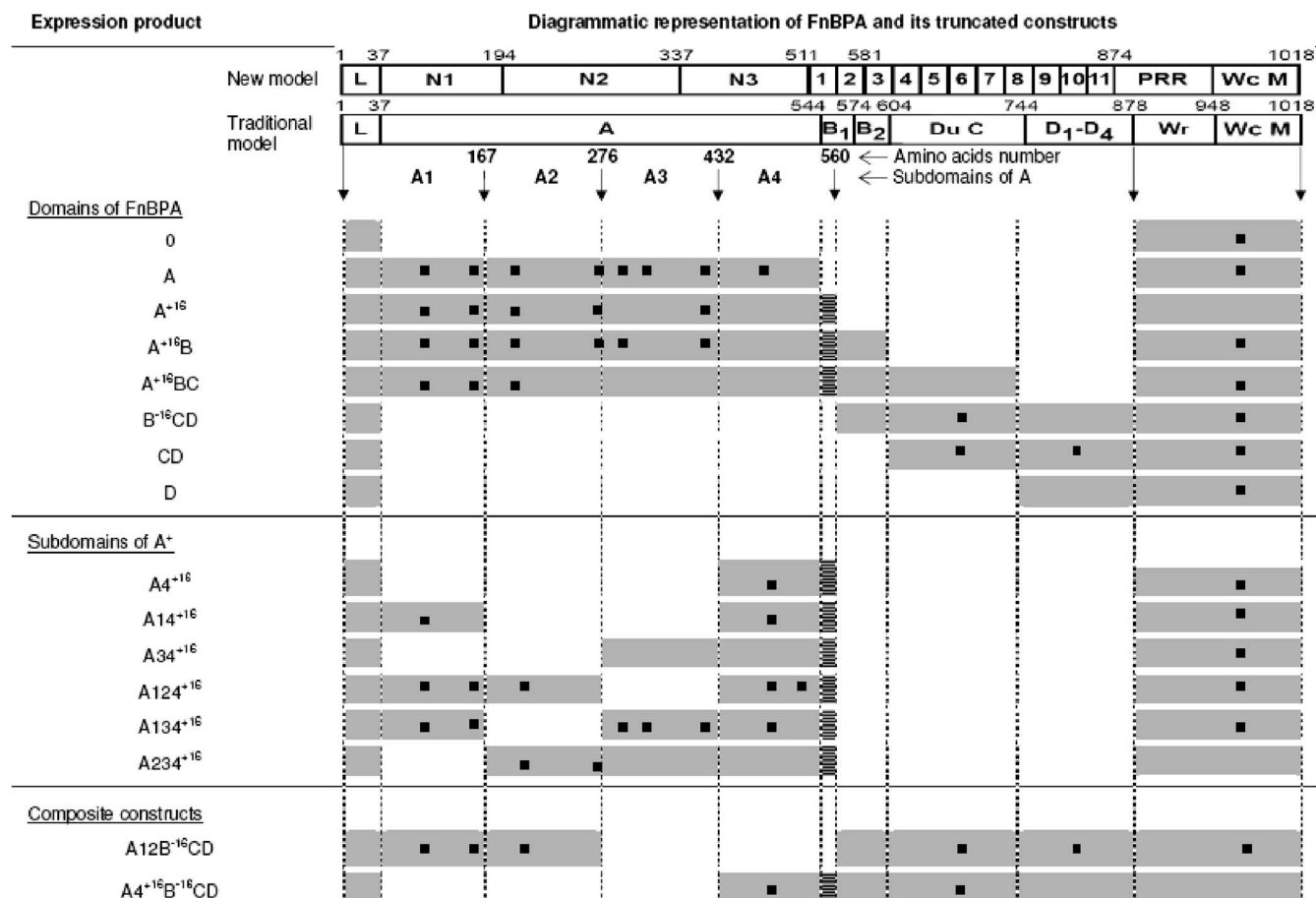
FIG. 2. Recombinant FnBPA products expressed in lactococci and validation by LC-MS. FnBPA is represented at the top in its new and traditional views (see also Fig. 1). L, leader sequence; Wr and Wc, cell wall-spanning and -anchoring regions; M, membrane-spanning sequence. The different constructs expressed in *L. lactis* are shown below. Grey rectangles indicate the presence of the corresponding part of FnBPA, whereas a missing rectangle indicates that this part was removed during gene construction. Black squares represent the peptides identified by LC-MS, confirming the presence of the proteins on the cell surface.

ultracentrifugation, the pellet containing the cell walls was resuspended in 1.5 ml Milli-Q water, dried with a SpeedVac, and resuspended in 100 μl of 100 mM ammonium bicarbonate (NH_4HCO_3) at pH 7.8.

Proteins were reduced for 30 min at 50°C with 10 μl 45 mM 1,4-dithio-DL-threitol (Fluka, Buchs, Switzerland) and further alkylated for 60 min at room temperature in the dark with 10 μl of 100 mM iodoacetamide (Fluka). The resultant protein mixture was digested overnight at 37°C with trypsin (Promega, Madison, WI) at an enzyme-protein ratio of 1:50 (wt/wt). The tryptic peptides were cleaned on Sep-Pak tC18 cartridges (Waters, Milford, CT) and eluted with 2 × 1 ml 80% acetonitrile in two separate 1.5-ml centrifuge tubes. Peptides were diluted by adding 190 μl 0.1% formic acid and stored at −20°C until MS processing. The resulting peptides were separated by LC on a 5 to 50% acetonitrile–0.1% formic acid gradient and analyzed on an HCTultra ion-trap mass spectrometer (Bruker, Bremen, Germany) coupled on line to an Ultimate 3000 high-performance liquid chromatography system (Dionex, Sunnyvale, CA) equipped with an analytical Magic C_{18} reversed-phase column (100 by 0.075 mm, 5 μm; Spectronex, Basel, Switzerland) by gas phase fractionation. Proteins were identified by comparison to a database consisting of the *L. lactis* proteome plus the expected staphylococcal protein with Mascot (Matrix Science, London, England) (see Table S2 in the supplemental material).

In vitro adherence assays. In vitro adherence to immobilized fibrinogen or fibronectin was assessed as previously described (2, 18), with 96-well plates (Nunc-Immuno Plate, MaxiSorp Surface; Nalge Nunc International) coated with serial twofold dilutions of purified fibrinogen or fibronectin (starting with 500 μg/ml). Each experiment was repeated on three or more independent occasions. Adherence titers were defined as the inverse of the highest dilution of ligand demonstrating adherence by optical density.

Competition experiments. Three peptides were used as competitors in adherence assays (Fig. 1C). P1 (corresponding to amino acids 486 to 559 of FnBPA) and P2 (corresponding to amino acids 455 to 559 of FnBPA) were synthesized in vitro. P3 (corresponding to fragments 560 to 880, i.e., B^{-16}CD) was expressed in *E. coli* with the six-histidine tag expression vector pQE-30 of Qiagen. Briefly, the B^{-16}CD fragment of FnBPA (corresponding to nucleotides 1798 to 2760) amplified by PCR (forward primer, 5′-AG**GGATCC**GATGGTGGAGGTGGATA-3′; reverse primer, 5′-AG**CCCGGG**TGGCGTTGGTGGCACGATTGGAG) was cloned into pQE-30 between the BamHI and SmaI restriction sites (in bold) and propagated in *E. coli*. Recombinant proteins were expressed and purified by Ni^{2+} chelate chromatography by following the recommendations of the manufacturer.

For competition experiments, the wells of the microtiter plates were coated with constant ligand concentrations of 125 μg/ml fibrinogen and 31.25 μg/ml fibronectin. Coated plates were first incubated for 2 h at 37°C with serial twofold dilutions of either competitor (starting with 32 μg/ml) and washed twice with PBS before the addition of 5 × 10^7 CFU of the recombinant bacteria. Experiments were repeated on three or more independent occasions. Results were expressed as a percentage of the adhesion achieved without a competitor.

Bacterial internalization into cultured cell lines. In vitro bacterial internalization into bovine aortic endothelial cells (BAEC) was assessed with a previously described antibiotic protection assay (19, 25). Confluent endothelial cells were incubated with 10^5 CFU of bacteria for 90 min at 37°C and washed three times with PBS before the addition of 100 μg/ml gentamicin to kill noninternalized bacteria. Cells were then washed three times with PBS and osmotically lysed by the addition of 1 ml of H_2O. Lysates were finally plated for colony counts.
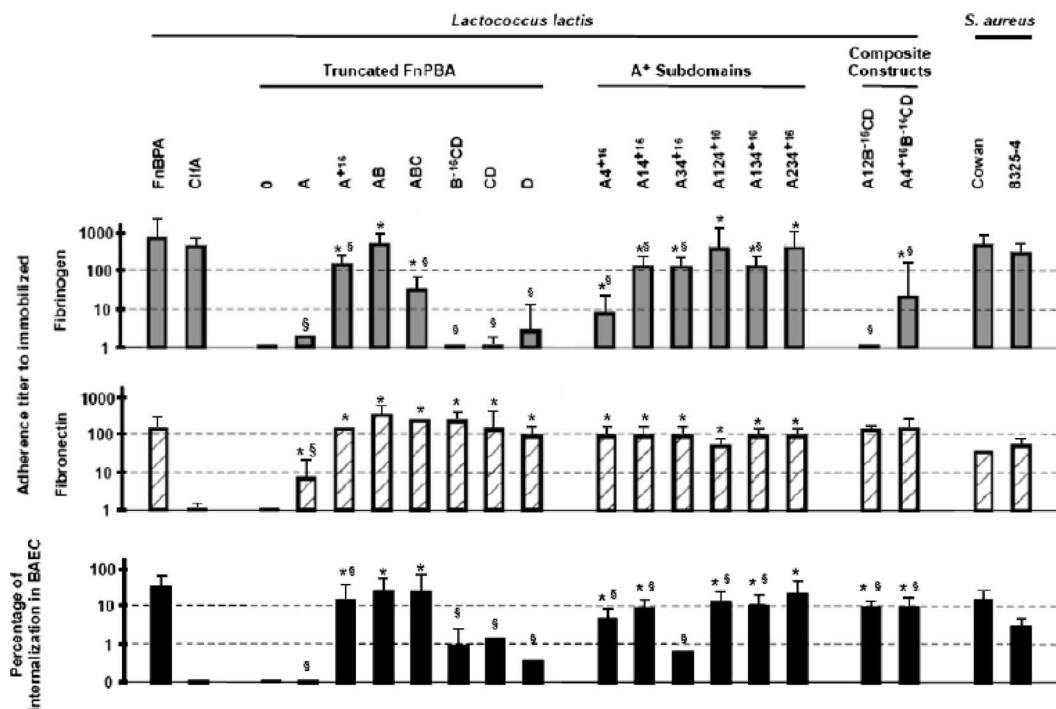
FIG. 3. In vitro adherence to immobilized fibrinogen and fibronectin and internalization into cultured endothelial cells. *L. lactis* expressing various FnBPA fragments were tested first for in vitro adherence to immobilized fibrinogen (upper panel) and fibronectin (middle panel) and second for internalization into cultured BAEC (lower panel). *S. aureus* Cowan and 8325-4 were used as wild-type controls, *L. lactis* 0 (i.e., expressing only the signal sequence and cell wall-anchoring domain) served as a negative control, and *L. lactis* FnBPA+ was used as a positive control. Binding titers were defined as the inverse of the highest dilution of ligand demonstrating adherence by optical density. Internalization results are expressed as the percentage of surviving bacteria compared to the initial inoculum. All data represent the median of three or more separate experiments. Intra- and interrun variations were ≤20%. For all *L. lactis* strains expressing truncated FnBPA or A^{+16} subdomains, significant differences (analysis of variance and Fisher's post hoc PLSD test, $P < 0.05$) from *L. lactis* 0 (∗) and *L. lactis* FnBPA+ (§) are indicated.

Results are expressed as the percentage of surviving bacteria compared with the inoculum.

Experimental endocarditis in rats. Sterile aortic vegetations were produced in female Wistar rats as previously described (5). Twenty-four hours after catheterization, groups of 8 to 10 rats were challenged intravenously with 7.5 $\log_{10}$ CFU, an inoculum lower than the minimal inoculum of the parent *L. lactis* subsp. *cremoris* strain infecting ≥90% of the animals (90% infective dose or ID_{90}) (17). Recombinants producing endocarditis at this low inoculum demonstrated increased infectivity compared to the parent. Rats were killed 24 h later. Quantitative valve and spleen cultures were prepared as previously described (17). Bacterial densities in the vegetations were expressed as the mean $\log_{10}$ number of CFU per gram of tissue. The limit of detection was ≥2 $\log_{10}$ CFU/g of tissue. Rats with sterile valve cultures were considered uninfected.

Statistical analysis. Chi-square and nonparametric Wilcoxon tests were used for comparison wherever appropriate. Fibronectin- and fibrinogen-binding titers and cell internalization percentages of the different truncated parts of FnBPA were first globally compared by using analysis of variance, and then post hoc tests (Fisher's PLSD test) were used, allowing direct comparisons between *L. lactis* expressing truncated parts of FnBPA with (i) *L. lactis* expressing all of FnBPA (positive control) and (ii) *L. lactis* 0 expressing only the signal sequence and cell wall-anchoring domain (negative control). The association between the different domains and subdomains of FnBPA and infection of valves or spleen, evaluated as a qualitative variable, was adjusted for by using logistic regression with backward stepping. The association between fibronectin or fibrinogen binding with BAEC internalization, evaluated as a continuous variable, was first tested by using simple regression and then adjusted for by using multiple linear regression with backward stepping. The association between fibronectin- or fibrinogen-binding and bacterial titers in vegetations and spleens was also first evaluated by using simple regression and then adjusted for by using multiple linear regression with backward stepping. Final models included all variables significantly and independently associated with the dependent variables, including adjustments

for possible variations in inoculum size. For all tests, a P value of <0.05 was considered significant.

RESULTS

***L. lactis* constructs expressing various FnBPA fragments.** Figure 2 presents all of the FnBPA constructs expressed in *L. lactis*. Full expression and anchoring of all of the constructs in the cell wall of the surrogate bacteria were confirmed both by ligand-blotting experiments (data not shown) and by LC-MS. For each construct tested, Fig. 2 shows the positions of the different fragments recognized by LC-MS (black squares). These results confirm that all recombinant lactococci expressed the desired FnBPA fragments on their surface.

In vitro adherence and cell internalization. Figure 3 shows that (from left to right and top to bottom) (i) FnBPA-expressing lactococci could bind both fibrinogen and fibronectin, as well as trigger cell internalization, whereas ClfA-expressing recombinants bound only fibrinogen, as expected from previous results (19); (ii) truncated forms of FnBPA could bind both fibrinogen and fibronectin, as well as trigger cell internalization, only if they contained the extended A^{+16} domain depicted in Fig. 2; (iii) the minimal subdomain of A^{+16} that could confer all of these three properties was $A4^{+16}$ (depicted in Fig. 1 and 2), yet combining it with other subdomains of A (i.e., A1, A2, and A3) increased its fibrinogen-binding capacity by up to
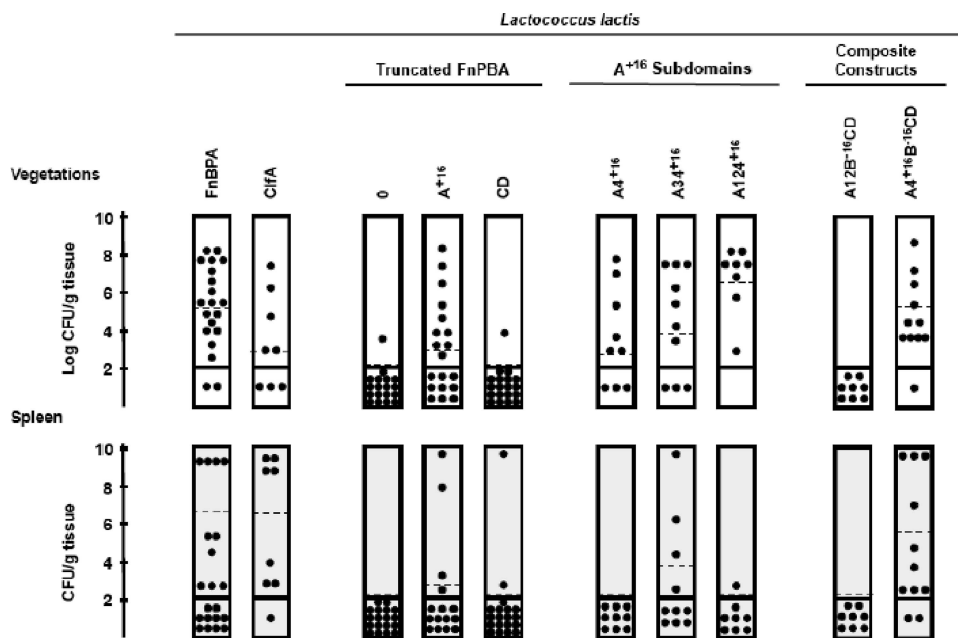
FIG. 4. Results of experimental endocarditis. Groups of 8 to 10 animals with catheter-induced aortic vegetations were challenged with a bacterial inoculum (ca. 7.5 log$_{10}$ CFU) below the expected ID$_{90}$ of the *L. lactis* parent strain and killed 24 h later. Each dot indicates the bacterial density in the vegetations (upper panel) or the spleen (lower panel) of one single animal. Dashed lines represent mean bacterial concentrations. Bacterial densities in the tissues were expressed as the mean log$_{10}$ number of CFU per gram of tissue (vegetations) or the mean number of CFU per gram of tissue (spleen). The limit of detection was $\geq$2 log$_{10}$ CFU/g of tissue. Rats with sterile cultures were considered uninfected.

10 times; and (iv) deletion of minimal A4^{+16} from FnBPA ablated fibrinogen binding (see composite construct A12B^{-16}CD), while adding it restored fibrinogen binding (see composite construct A4^{+16}B^{-16}CD). Thus, A4^{+16} was the minimal active subdomain conferring all three FnBPA properties, and various other subdomains of the protein could cooperate with it for ligand binding.

Capacity of the different FnBPA domains to induce experimental endocarditis. The various constructs were then tested in vivo for the capacity to induce endocarditis. Groups of rats with catheter-induced aortic vegetations were challenged with 7.5 log$_{10}$ CFU of the different *Lactococcus* mutants, a number below the expected ID$_{90}$ of the parent *Lactococcus* strain (Fig. 4). Only recombinants expressing whole FnBPA, domain A^{+16}, subdomain A4^{+16}, or any other constructs containing subdomain A4^{+16} were able to induce infective endocarditis ($P <$ 0.01). Indeed, *L. lactis* expressing subdomain A4^{+16} alone or in combination infected 59/77 rats (76.6%), in contrast to those missing this specific domain (2/45 or 4.4% of the animals, $P <$ 0.0001, chi-square test). A multivariate analysis further confirmed that A4^{+16} was the only FnBPA subdomain significantly associated with endocarditis (odds ratio = 23; 95% confidence interval, 8.7 to 62; $P <$ 0.0001).

Relationships among fibrinogen and fibronectin binding, endocarditis induction, endocarditis severity, and invasion of cultured cell lines. We further tested whether the magnitude of in vitro binding to fibrinogen or fibronectin (Fig. 3) correlated with the in vivo infectivity of the recombinants (Fig. 4). Only in vitro binding avidity for immobilized fibrinogen, but not for fibronectin, was statistically significantly associated with the in vivo induction of endocarditis (odds ratio per difference in

dilution titer = 1.7; 95% confidence interval, 1.4 to 2.0; $P <$ 0.0001, logistic regression). This confirmed that fibrinogen binding was strongly associated with endocarditis induction, whereas binding to fibronectin was not (19).

We next evaluated the importance of fibrinogen and fibronectin binding for disease severity, as assessed by vegetation bacterial titers. In contrast to endocarditis induction, for which fibrinogen binding was the only associated factor, disease severity correlated with both fibrinogen and fibronectin binding (coefficients of 0.428 $\pm$ 0.044 and 0.208 $\pm$ 0.081, $P <$ 0.0001 and $P =$ 0.01, respectively).

Since we previously showed that the capacity to invade endothelial cells in vitro was associated with progressive experimental endocarditis in animals (19), we further asked whether fibrinogen and fibronectin binding could synergize the invasion of cultured cell lines. Figure 3 confirms that fibronectin binding was a prerequisite for cell internalization. In addition, however, when fibrinogen binding was simultaneously present with fibronectin binding, it significantly promoted the rate of internalization compared to fibronectin binding alone (16.4% versus 4.6%, respectively; $P <$ 0.0001, Wilcoxon test). Thus, at a given fibronectin-binding titer, the avidity for fibrinogen synergistically enhanced cell invasion (Fig. 5). This synergism was further confirmed by multivariate analyses where the intensities of fibrinogen binding and fibrinogen binding also correlated with the percentage of internalization into cultured endothelial cells (coefficients of 1.438 $\pm$ 0.228 and 1.734 $\pm$ 0.160, respectively; $P <$ 0.0001). Hence, the fibrinogen-binding property of FnBPA was not only required for infection initiation but also combined with fibronectin binding to synergize bacte-
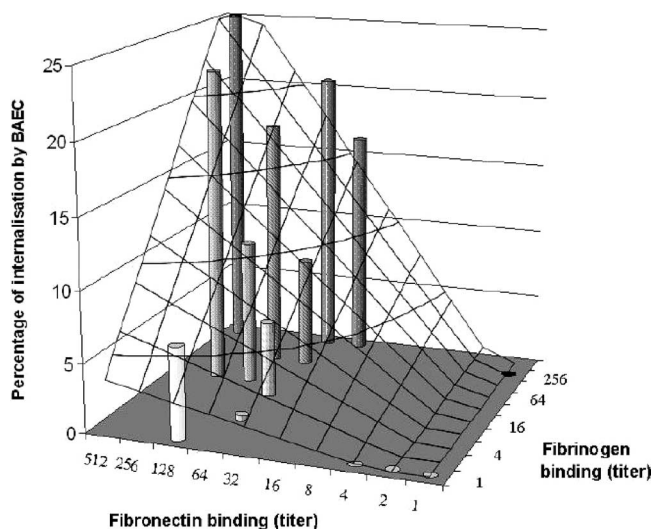
FIG. 5. Synergy between fibrinogen and fibronectin binding for internalization into endothelial cells. The graph represents the predicted percentage of in vitro bacterial internalization into BAEC (vertical axis and latticework) according to the fibrinogen- and fibronectin-binding titers (horizontal axes). This representation was drawn from experimental data (cylinders) by multiple linear regression. It shows that at a given fibronectin-binding titer, the avidity for fibrinogen synergistically and dramatically enhanced internalization.

rial uptake by endothelial cells, a feature correlating with disease severity in vivo (19).

Attempt to outcompete $A4^{+16}$ and FnBPA binding with synthetic peptides. Because the minimal $A4^{+16}$ subdomain conferred three major FnBPA properties involved in experimental endocarditis (i.e., fibrinogen binding, fibronectin binding, and endothelial cell invasion), we sought to determine whether synthetic peptides derived from it could compete with in vitro binding by itself or by whole FnBPA. Figure 1C maps competing peptides P1 and P2, encompassing parts of $A4^{+16}$, and control peptide P3, encompassing fibronectin-binding domains $B^{-16}CD$. Figure 6 depicts the results. Regarding fibrinogen binding, only peptide P2, which spanned almost the entire length of $A4^{+16}$, demonstrated some competition with $A4^{+16}$, but not with whole FnBPA. Control peptide P3 also interfered marginally with fibrinogen binding, although it contained only fibronectin-binding repeats. This might relate to the fact that the last 16 amino acids of $A4^{+16}$ also overlapped one fibronectin-binding repeat, which itself also appeared indispensable to confer both fibrinogen and fibronectin binding on the fragment (Fig. 1 and 4). Thus, some kind of cross-interference could have occurred at this level.

With regard to fibronectin binding, control peptide P3 could clearly compete for binding by $A4^{+16}$ but competed only partially with binding by whole FnBPA. In contrast, P1 and P2 had
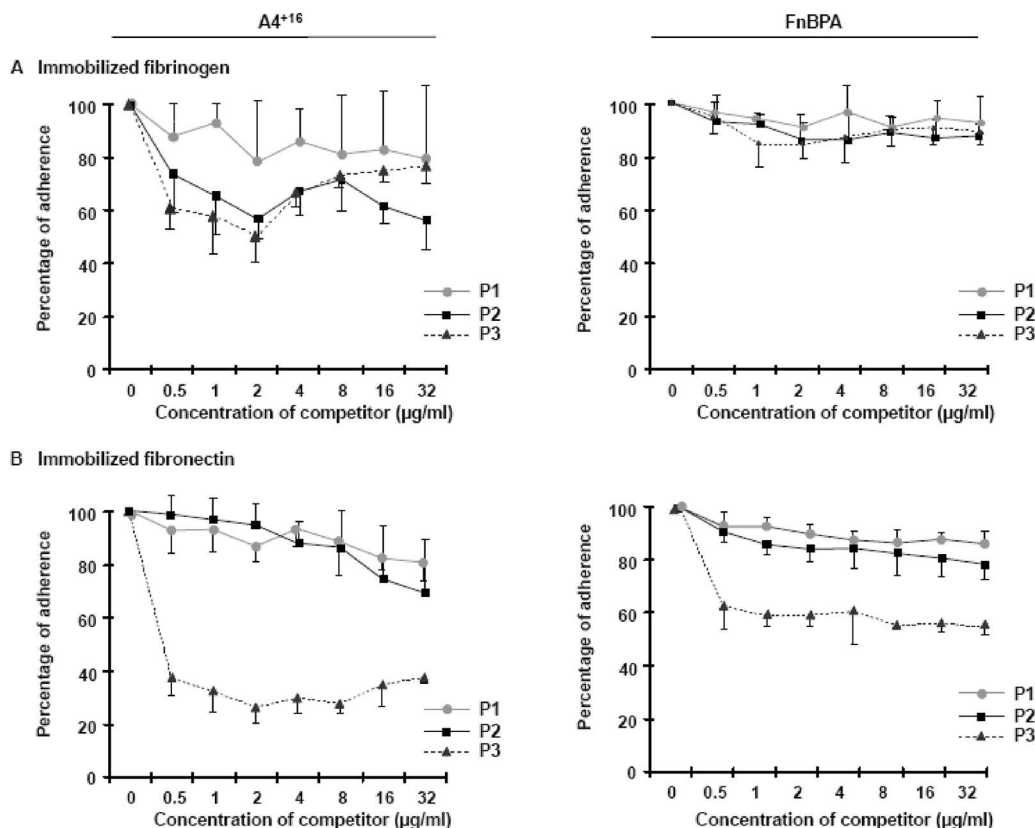

FIG. 6. Competition experiments. Synthetic peptides P1, P2, and P3, corresponding to either parts of the $A4^{+16}$ fragment (P1 [●] and P2 [■]) or the $B^{-16}CD$ fragment (P3 [▲]) (for precise localizations, see Fig. 1), were used to compete for the adhesion of either $A4^{+16}$ (left panels) or FnBPA (right panels) to immobilized fibrinogen (A) or fibronectin (B). Coated plates were first incubated with serial twofold dilutions of either competitor (starting at 32 μg/ml) before the addition of 5×10^7 CFU of the recombinant bacteria. Experiments were done at least in triplicate. Results are expressed with the standard deviation as a percentage of the adhesion achieved without a competitor.

no effect at the concentrations used in this study. Hence, competition under these conditions was only partial and less pronounced against whole FnBPA than against A4^{+16}. This stresses the potential importance of the FnBPA domain redundancy.

DISCUSSION

Using ClfA and FnBPA as model proteins, we previously showed that fibrinogen binding is necessary and sufficient for endocarditis induction but not sufficient for disease progression and that fibronectin binding is necessary and sufficient for cell invasion and disease progression but not sufficient for disease induction (17, 19). This suggested a two-step disease process in which the two binding functions cooperate sequentially.

The present report shows that the system is more intertwined than originally thought—at least for FnBPA—in terms of both functional anatomy and pathogenic mechanisms. First, at the level of functional anatomy, the smallest subdomain of FnBPA reunifying all of its relevant functions in endocarditis pathogenesis was A4^{+16}. This was in agreement with current knowledge on the fibronectin binding of FnBPA, since this subdomain encompasses 1 of the 11 fibronectin-binding repeats of FnBPA, which has been shown to be sufficient for fibronectin binding. Conversely, this was totally unexpected for fibrinogen binding since A4^{+16} spanned only part of the fibrinogen-binding N3 module and since we failed to observe significant fibrinogen binding when the A domain (encompassing the N1, N2, and N3 modules) was expressed alone, by contrast with previous studies (3, 8, 29). Adding only 16 amino acids (544 to 559) to domain A or A4 (leading to A^{+16} or A4^{+16}, respectively) dramatically increased the binding affinity for both fibrinogen and fibronectin, whereas peptides containing the same 16 amino acids (e.g., P1 and P2 in Fig. 1C and 6) could barely outcompete such binding. Likewise, binding of A4^{+16} to fibrinogen was enhanced ≥10 times when the fragment was associated with upstream portions of FnBPA (e.g., A1 to A3), which did not efficiently bind fibrinogen by themselves (for instance, see domain A4^{+16} versus domain A34^{+16} in Fig. 3). Eventually, fibrinogen binding was affected to some degree by synthetic peptide B^{-16}CD, which carried fibronectin-binding repeats that did not bind to fibrinogen. Such subtle functional folding was recently suggested from in silico and in vitro data indicating that FnBPA binds fibrinogen via a dock-lock-latch mechanism of binding (8). Indeed, whereas isolated modules N2 and N3 were not able to bind fibrinogen, C-terminal residues of domain N3, when associated with N2, are likely to undergo a conformational change to bind adjacent to domain N2, trapping the fibrinogen in its groove between N2 and N3 and locking it in place. Neither the N-terminal N1 domain nor amino acids 512 to 544 were needed for this binding. Since domain A4^{+16} does not encompass the N2 module and the N-terminal part of N3, it is likely that another mechanism is involved. Moreover, its specific regions for fibrinogen and fibronectin binding could not be separated by competition with synthetic peptides, suggesting that some kind of folding of the fragment afforded its dual capacity, irrespective of clear boundaries between individual binding domains.

Thus, this functional versatility may be partly related to the location of the protein; i.e., the in vivo conformation of surface-bound FnBPA may be different from that of unbound recombinant proteins. It is also compatible with the induced-fitness dynamics of FnBPA binding, where binding modules are disordered in the absence of fibronectin and undergo a disordered-to-ordered transition upon ligand binding (6). Moreover, the present results identify a region of dual binding at the hinge of the major fibrinogen- and fibronectin-binding regions of FnBPA. This provides a functional rationale for the redundant structure of FnBPA, which might have evolved a minimal (necessary and sufficient) intertwined core A4^{+16} reinforced by multiple binding modules. This redundancy may not only increase but also guarantee binding abilities in the case of blockage of some of these modules.

Second, at the level of pathogenic mechanisms, the primary role of fibrinogen binding in the induction of experimental endocarditis was statistically confirmed (17, 19). Indeed, platelet activation is an important initial step in the pathogenesis of infectious endocarditis, via a fibrinogen bridge between FnBPA and the platelet integrin GPIIb/IIIa. Since it has been shown that platelets in vitro can also be similarly activated by FnBPA fibronectin-binding domains only (3), it appears that the risk of in vivo endocarditis induction is mainly correlated with the avidity of fibrinogen binding. On the other hand, there is a shared responsibility of fibrinogen and fibronectin binding in disease severity. The synergistic effect of fibrinogen and fibronectin binding was clearly apparent in endothelial cell invasion, a property that is associated with in vivo invasion and persistence (17, 19). This synergy was unexpected, because fibronectin binding was previously shown to be sufficient (and necessary) for internalization into eukaryotic cells (25, 26). A contribution of fibrinogen binding in this situation had never been studied previously in *S. aureus*. However, it has already been shown in other pathogens such as *Streptococcus agalactiae* that fibrinogen binding may promote adherence, leading to the invasion of endothelial cells (4, 28). Thus, while fibronectin binding is sufficient for attachment to and internalization into eukaryotic cells, fibrinogen binding could enhance both steps, either by favoring attachment or by stabilizing the structure of the various fibronectin-binding motifs.

Staphylococcal FnBPA thus appears as a sophisticated multifunctional adhesin whose structure results from the association of various domains that are responsible for virulence. These domains act both sequentially (fibrinogen binding for disease initiation and fibronectin binding for endothelial cell invasion and disease progression) and synergistically, with fibrinogen binding enhancing endothelial cell bacterium uptake triggered by fibronectin binding. Moreover, fibrinogen binding is likely to operate at an early step in the infection process, whereas fibronectin binding, while pivotal in the pathogenic process of infectious endocarditis, is not the primum movens of valve infection but acts subsequently by triggering cell invasion.

Finally, the present observations also carry two practical messages. First, blocking the binding functions of FnBPA with peptides or antibodies targeting only part of it is likely to fail or to succeed only partially. This finds a concrete demonstration both in the present competition experiments (Fig. 6) and in previous work showing that antibodies directed against FnBPA could not block adherence and even increased it by stabilizing the fibronectin-FnBPA complex (27). Thus, the protein should

be targeted extensively to ensure binding inhibition. Second, if one binding function were to be a priority target, then it would fibrinogen binding because it is directly responsible for disease initiation and also combines synergistically with fibronectin binding for further progression and persistence. While this assumption awaits experimental confirmation, it will be critical to test whether other fibrinogen-binding proteins of *S. aureus* also produce the same synergism with fibronectin binding.

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