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Fibrinogen and fibronectin binding cooperate for valve infection and invasion in *Staphylococcus aureus* experimental endocarditis

Yok-Ai Que, Jacques-Antoine Haefliger, Lionel Piroth, Patrice François, Eleonora Widmer, José M. Entenza, Bhanu Sinha, Mathias Herrmann, Patrick Francioli, Pierre Vaudaux, and Philippe Moreillon

The expression of *Staphylococcus aureus* adhesins in *Lactococcus lactis* identified clumping factor A (ClfA) and fibronectin-binding protein A (FnBPA) as critical for valve colonization in rats with experimental endocarditis. This study further analyzed their role in disease evolution. Infected animals were followed for 3 d. ClfA-positive lactococci successfully colonized damaged valves, but were spontaneously eradicated over 48 h. In contrast, FnBPA-positive lactococci progressively increased bacterial titers in vegetations and spleens. At imaging, ClfA-positive lactococci were restricted to the vegetations, whereas FnBPA-positive lactococci also invaded the adjacent endothelium. This reflected the capacity of FnBPA to trigger cell internalization in vitro. Because FnBPA carries both fibrinogen- and fibronectin-binding domains, we tested the role of these functionalities by deleting the fibrinogen-binding domain of FnBPA and supplementing it with the fibrinogen-binding domain of ClfA in cis or in trans. Deletion of the fibrinogen-binding domain of FnBPA did not alter fibronectin binding and cell internalization in vitro. However, it totally abrogated valve infectivity in vivo. This ability was restored in cis by inserting the fibrinogen-binding domain of ClfA into truncated FnBPA, and in trans by coexpressing full-length ClfA and truncated FnBPA on two separate plasmids. Thus, fibrinogen and fibronectin binding could cooperate for *S. aureus* valve colonization and endothelial invasion in vivo.
dissemination to distant organs. Using this model, we demonstrated that both fibrinogen and fibronectin binding were critical to initiate experimental endocarditis (18). Specifically, the *S. aureus* fibrinogen-binding protein A (clumping factor A [ClfA]; reference 19) and fibronectin-binding protein (FnBPA) A (20) were individually expressed in the less pathogenic *Lactococcus lactis*, and the recombinants were tested for augmented infectivity. Both determinants increased the capacity of lactococci to colonize damaged valves in rats with catheter-induced sterile vegetations by several orders of magnitude (18).

We further investigated the contribution of fibrinogen and fibronectin binding to disease progression. Although both ClfA and FnBPA promoted valve colonization, they also provoked very different disease evolutions. Rats infected with ClfA-positive lactococci tended to spontaneously sterilize their valves over time. In contrast, rats infected with FnBPA-positive lactococci developed progressive infections and suffered clinical signs of sepsis. This invasive phenotype correlated with the capacity of FnBPA-positive recombinants, but not ClfA-positive recombinants, to invade endothelial cells in vitro and in vivo.

Because FnBPA carries both fibronectin- and fibrinogen-binding domains (21), we further studied their individual importance first by deleting the fibrinogen-binding domain from FnBPA, in the form of a truncated protein, and then by supplementing it in cis and in trans with the fibrinogen-binding domain of ClfA. The deletion of the fibrinogen-binding domain of FnBPA did not decrease its ability to bind to fibronectin and trigger cell invasion in vitro. However, it totally abrogated its capacity to promote valve infection and invasion in vivo. This loss of function could be restored in cis by inserting the fibrinogen-binding domain of ClfA into the truncated FnBPA. Moreover, it could also be restored in trans by coexpressing truncated FnBPA with full-length ClfA, which does not confer invasion and persistence, as two separate proteins. Thus, fibrinogen and fibronectin binding could cooperate in vivo to promote valve colonization and invasion.

**RESULTS**

**Evolution of experimental endocarditis**

Groups of rats were challenged with 10 times the 80% infective dose (ID$_{80}$) of each of the test organisms (18). The dynamics of infection were followed over 3 d (Fig. 1). After 2 h, all the animals had similar vegetation bacterial titers irrespective of the infecting organism. This was in accordance with previous work in experimental endocarditis, indicating that a challenge with large inocula could overcome the differences because of bacterial adherence in early valve colonization (18, 22–25). Subsequently, the disease evolution varied depending on the organisms. Control lactococci carrying an empty vector were poorly infective and were progressively cleared from both the valves and the spleens. ClfA-positive lactococci were more prone to colonize the valves (18), but were also cleared from the animals. In sharp contrast, FnBPA-positive lactococci persisted and expanded at both anatomical sites, thus imitating the dynamics of experimental endocarditis induced by the well-characterized clinical isolate of *S. aureus* P8 (see Table I), depicted in Fig. 1. *L. lactis* ClfA (+) and *L. lactis* FnBPA (+) carried a vector expressing ClfA and FnBPA, respectively (references 18, 47). *S. aureus* P8 was used as a positive control. Rats infected with *L. lactis* FnBPA (+) worsened both vegetation bacterial titers and spleen infection (median [range] bacterial/gram of spleen = 3.04 [1.92–3.3], 1.8 [1.29–3.21], 1.92 [1.41–3.2], and 1.84 [1.08–3.21] at 12, 24, 48, and 72 h, respectively) over time. In contrast, rats infected with either *L. lactis* plp253 or *L. lactis* ClfA (+) underwent spontaneous clearing of the infection (* and **, P < 0.01).

**Immunohistology of cardiac valves**

The different disease evolutions induced by ClfA- and FnBPA-positive lactococci were not caused by differences in
growth rates in vitro or the loss of the expression vector in vivo (18). Moreover, immunohistology of the cardiac lesions indicated that both clfA and fnbA were expressed at the infected site (Fig. 2). However, ClfA-positive lactococci were strictly confined to the fibrin platelet vegetation meshwork (Fig. 2 A) and were absent from the neighboring endothelium (Fig. 2 B), whereas FnBPA-positive lactococci were detected both in the vegetation (Fig. 2 D) and colocalizing with the adjacent endothelium (Fig. 2 E). C and F were labeled with anti-von Willebrand primary antibodies to determine the presence of the endothelial layer. Fluorescence indicates the presence of bacteria (A, D, and E) and endothelial cells (C and F). Comparison between Gram’s stains and immunodetected bacteria indicated that all visible microorganisms expressed their recombinant gene (unpublished data; SMC, smooth muscle cells; L, vascular lumen).

Conversely, deleting the fnbA and fnbB genes from wild-type S. aureus Cowan I gave the reverse picture. Parent S. aureus Cowan I was detected both in the vegetation and the adjacent endothelium, whereas strain DU5883, an fnbA/ fnbB-deficient mutant of 8325-4 was confined to the vegetation (not depicted). Thus, the association between bacteria and the endothelium correlated with the expression of fibronectin-binding proteins on their surface.

**Imaging by electron microscopy (EM)**

Colocalization with the neighboring endothelium could be caused by surface-bound and/or internalized bacteria. To resolve this issue, valves infected with FnBPA-positive lactococci were scanned by light microscopy, and areas containing bacteria were processed for EM. Various stainings were used. Fig. 3 A (osmium staining) depicts an endothelial cell harboring both surface-attached and apparently internalized lactococci. Surface-attached lactococci displayed a thick, dark-stained outer shell, whereas lactococci in the cytoplasm were surrounded by a clear halo. This halo could result either from free space between noninternalized bacteria that...
were located within invaginations of the cell membrane, or from internalized bacteria that escaped staining of their outermost polyanionic layers (e.g., teichoic acids and/or exopolysaccharides). Fig. 3 B shows that the halos around intracellular lactococci could be stained with periodic acid (PAS) and were thus compatible with anionic polymers. Fig. 3 C shows mitochondria positioned in the vicinity of an internalized FnBPA-positive lactococcus at a higher magnification. Fig. 3 D is a tannic acid stain that helped determine whether the intracellular bacteria were surrounded by a membrane or not. No clear membrane structure was observed. Collectively, these observations indicate that the colocalization of the bacteria with the endothelial cells did not result from coincidental bacteria–endothelium superposition, but rather from genuine bacterial internalization. Moreover, the absence of a membrane lining around internalized bacteria suggests the possibility of membrane disruption after bacterial entry.

A similar analysis was performed with S. aureus Cowan I. In contrast to lactococci, which were mostly observed in intact endothelial cells, S. aureus generated a continuum from bacterial internalization to microbe-related cell lysis (Fig. 3, E–H). This most likely resulted from the expression of specific staphylococcus virulence factors, such as hemolysins.

A semiquantitative evaluation of internalization was assessed in animals killed between 12 and 48 h after inoculation. Two rats were used for each time point. Semithin sections from five to nine specimens from each animal’s valves were examined by light microscopy, and specimens containing bacteria were processed for EM by another investigator. Samples could not be formally blinded because the morphological characteristics of the bacteria allowed identification of the material. With FnBPA-positive lactococci, the proportion of valve samples demonstrating internalized bacteria progressively increased from 0% at 12 h to 4% at 24 h to 20% at 48 h. However, with S. aureus Cowan I, the proportion of positive blocs paradoxically decreased from 60% at 18 h to 12% at 24 h. Thus, although both organisms increased their vegetation bacterial titers over time (Fig. 1), internalization of FnBPA-positive lactococci increased from low to high numbers, and the internalization of bona fide staphylococci decreased from relatively high to low numbers. This is compatible with staphylococcal-induced endothelial destruction that was not apparent in lactococci-infected samples (Fig. 3, E–H).

Invasion of endothelial cells in vitro
The series of strains used in animals were further tested for their ability to invade cultured human umbilical vein endothelial cells (HUVECs) in vitro. Fig. 4 presents the results of internalization as tested by an antibiotic–lysostaphin protection assay (black bars) and by flow cytometry (gray bars), as well as by confocal microscopy (right). The production of fibrinogen-binding proteins was necessary and sufficient to mediate endothelial internalization of both recombinant lactococci and staphylococci. This supported the in vivo morphological observations.

Implication of the fibrinogen- and fibronectin-binding domains of FnBPA in infection
FnBPA was shown to bind both fibronectin and fibrinogen (21). The role of these two functionalities was studied in lactococci expressing either a truncated FnBPA, lacking its fibrinogen-binding domain, or the truncated FnBPA supplemented in cis or in trans with the fibrinogen-binding domain of ClfA (Fig. 5 A).

Deletion of the fibrinogen-binding domain of FnBPA abrogated binding to fibrinogen, but not binding to fibronectin and internalization into endothelial cells (Fig. 5 B). However, it completely abrogated its ability to promote cardiac valve infection and persistence in vivo. This loss of function was restored by supplementing the truncated FnBPA in cis with the fibrinogen-binding domain of ClfA (Fig. 5 B). In addition, it was also restored by supplementing the truncated FnBPA in trans, via the expression of whole ClfA on a separate plasmid. Fig. 6 depicts the dynamics of valve infection in rats inoculated with the cis- and trans-recombinants. The two supplemented constructs could engender a persistent and progressive infection. In contrast, lactococci expressing truncated FnBPA or ClfA alone were either noninfective or nonpersistent, respectively. Note that the trans-recombinant grew slower than its cis-counterpart on the valves. This correlated with a 10–20% slower growth rate of the cis-recombinant in vitro, which we attributed to the concurrent replication of two independent plasmids.

DISCUSSION
We previously showed that S. aureus ClfA and FnBPA promoted the colonization of damaged valves in rats with ex-
However, their role in disease progression was unknown. The present results indicate that the two factors conferred complementary features in the infection process. ClfA promoted early valve colonization, but not persistence, whereas FnBPA promoted both colonization and persistence, resulting in clinical symptoms reminiscent of genuine *S. aureus* infection. This difference in disease evolutions was related to the unique ability of FnBPA-producing lactococci to invade endothelial cells both in vitro and in vivo. FnBPA can trigger active internalization into a number of eukaryotic cells (6–12). In the present experiments, it allowed lactococci to expand not only in the vegetations, which are essentially devoid of host defense mechanisms (26, 27), but also in the spleen, which is notoriously rich in professional phagocytes. Thus, FnBPA must have afforded protection against these cellular host defenses. An intracellular location would provide such a protection. Lactococci overtly exposed to vegetation components would be the target of platelet microbicidal proteins (28), to which the lactococcus used in this study is susceptible (unpublished data). Likewise, lactococci trapped in the spleen would be eliminated by phagocyte-induced killing. Accordingly, both parent and ClfA-positive lactococci, which were not internalized in vitro or in vivo, were rapidly eradicated from the animals regardless of their abilities to initially colonize damaged endothelia. In contrast, FnBPA-producing lactococci, which were internalized, could survive and proliferate. Indeed, EM imaging consistently revealed the presence of dividing lactococci inside endothelial cells (Fig. 3, A–D), thus underlining the permissive nature of this particular environment. This is a salient example of host cell hijacking mediated by a highly specialized bacterial adhesin. It is reminiscent of *Listeria* species, in which the heterologous expression of *L. monocytogenes* Internalin A in nonpathogenic *L. innocua* conferred the ability to invade epithelial cells (29).

This observation raises multiple questions regarding these complex bacteria–host interactions. *S. aureus* carries several
adhesins that can mediate internalization into eukaryotic cells. These include FnBPA (6–12) and FnBPB (30), and the loosely surface-attached extracellular adherence protein (31). In contrast, FnBPs from coagulase-negative staphylococci do not promote cell internalization (32). The difference is supposed to result from structural variations between the two kinds of adhesins. Fibronectin bound on the surface of S. aureus forms multimers that promote interaction with integrins, whereas fibronectin bound on the surface of coagulase-negative staphylococci does not form such structures (32). Hence, internalization must be an important addition that allows successful infection by S. aureus.

Once internalized, S. aureus are equipped to lyse the host cells via quorum-sensing regulated production of hemolysins and toxins (33). Although only semiquantitative, the in vivo EM data presented here support this scenario (33, 34). Over time, the number of endothelial cells containing bacteria increased in rats infected with FnBPA-positive lactococci, whereas it decreased in rats infected with S. aureus. This correlated with S. aureus–mediated endothelial destruction, as observed by EM. Therefore, once inside the cells, S. aureus is not mere circumstantial parasites, but highly specialized pathogens that take advantage of the eukaryotic milieu to switch between hiding and dissemination strategies.

FnBPA is a multifunctional adhesin that affords binding to several matrix proteins, including fibronectin (domains B, C, and D; references 35–37), fibrinogen (domain A; reference 21), and elastin (38). Binding to fibronectin triggers internalization via a “zipper mechanism,” requiring eukaryotic cell-mediated processes (39). It is followed by profound changes in the endothelial cell metabolism, including the expression intercellular adhesion molecule (ICAM-1 and VCAM-1), and tissue factor (40). Eventually, the cells might undergo apoptosis (41). Binding to fibrinogen, on the other hand, is not required for internalization in vitro (35). Thus, the question arose as to whether the fibrinogen-binding domain (i.e., domain A) of FnBPA was required for valve infection in vivo, or whether fibronectin binding alone, afforded by domains B, C, and D, was sufficient for both valve colonization and invasion. The present results unequivocally show that FnBPA needed both binding capacities.

The fibronectin-binding property is partitioned into 11 binding motifs, which may independently bind to a series of consecutive F1 modules carried by the NH_{2}-terminal “N29” domain of fibronectin (20, 39, 42). The 11 binding motifs have varying affinities for their ligand. However, they are recruited to increase binding strength (39, 42–44). The present observation extends this notion to in vivo domain interactions. Binding to both fibrinogen and fibronectin was required for the sequential colonization and invasion of damaged valves. Moreover, the fibrinogen-binding domain of FnBPA could be supplemented both in cis and in trans by its homologous domain in ClfA. Thus, functional domains are interchangeable and can cooperate even when located on separate polypeptides. S. aureus is particularly well equipped in surface adhesins (45). Hence, associating them in various combinations may help target the organism to different anatomical sites. This may explain the notorious versatility of S. aureus infections (1), as well as the difficulty in studying staphylococcal pathogenesis using single-gene inactivated mutants.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The strains used in this study are described in Table I. L. lactis cremoris was grown at 30°C in M17 medium (Oxoid) supplemented with 0.5% glucose (46). S. aureus was

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<th>Table I. Bacterial strains and used in this study</th>
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<tr>
<td>Plasmid/strain</td>
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<tr>
<td>L. lactis cremoris 1363</td>
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<tr>
<td>L. lactis ClfA-positive</td>
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<tr>
<td>L. lactis FnBPA-positive</td>
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<tr>
<td>L. lactis LPXTG</td>
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<tr>
<td>L. lactis CD</td>
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<tr>
<td>L. lactis A-ClfA plus CD-FnPBA</td>
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<tr>
<td>L. lactis ClfA plus CD-FnPBA</td>
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<tr>
<td>S. aureus Newman</td>
</tr>
<tr>
<td>S. aureus Cowan I</td>
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<tr>
<td>S. aureus 8325–4</td>
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<td>S. aureus DU5883</td>
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ATCC, American Type Culture Collection.
Expression of staphylococcal adhesins in lactociocc and construction of truncated FnBPA proteins. The staphylococcal \( \delta f/4 \) and \( f/h \) genes were amplified from chromosomal DNA of \( S. aureus \) Newman and \( S. aureus \) 8325-4, respectively (Table I), ligated at the BamHI site of the pOr23 vec- tor, and expressed in \( L. lactis \) as described previously (18, 47). FnBPA con- tains domains binding both fibrinogen (region A) and fibronectin (regions B, C, and D; references 21, 35, 36). Deletion of internal binding domains took advantage of a previously described inverse PCR strategy (35). Plasmid pOr23-FnBPA, carrying the whole \( f/h \) gene, was used as a template (18). To delete regions A and B, encompassing the fibrinogen-binding region (21), a construct named “CD” was generated by using a backward (NH2- terminal) oriented primer starting at bp 228 of \( f/h \) (5′-TA CGA TCG GAA TCA AAT CCA ATT GAC TTT GAA-3′; Microsynth) and a for- ward (COOH-terminal oriented) primer starting at bp 193 of the gene (5′-GA CGA TCG TGC TGC AGC TTT GTC TTG T-3′). Both primers carried a PvuI (in bold) site at the 5′-end, permitting religation in new pOri-CD (Table I) that missed amino acid 37–605 (bp 229–1932) of FnBPA (Fig. 5). To delete the entire ABCD domains—encompassing both fibrinogen and fibronectin-binding regions (21, 35)—a construct was generated by using a backward (NH2-terminal oriented) primer starting at bp 228 (5′-TA CGA TCG CCG ACA CCA GAA GAT CCA AGT-3′) and a forward (COOH-terminal oriented) primer starting at bp 2761 of \( f/h \) (5′-GA CGA TCG TGC TGC AGC TTT GTC TTG T-3′). The resulting ABCD-FnBPA was deleted from amino acid 37 to 880 (bp 228– 2761; Fig. 5). Both truncated proteins contained their NH2-terminal leader and their COOH-terminal wall-anchoring sequences. Expand long tem- plate PCR system (Roche Diagnostics) was used according to the manufac- turer’s instructions to amplify the DNA-encoding parts of the \( f/h \) gene and the plasmid backbone. The following conditions were applied for 25 cycles: 95°C for 30 s, 60°C for 10 min. Amplified DNA was purified using the Quaquick PCR purification kit (Qiagen), digested with PvuI (Roche Diagnostics), and self-ligated with T4 DNA ligase. The plasmids were expanded in \( E. coli \) and transferred into \( L. lactis \) as previously described (18).

Cis- and trans-supplementation with the fibrinogen-binding domain of Cia. For cis-supplementation, a chimera protein containing the A do- main of Cia (bp 138–164 and amino acid 1–502; responsible for fibrinogen binding), and the C and D domains of FnBPA (bp 1933–2761 and amino acid 606–1018; responsible for fibronectin binding), as well as its wall- anchoring domain (bp 2761–3174), was constructed. The cloning strategy was as follows. The two primers 5′-TAG GAT CGG AAA ATA GTG TTA CGC AAT CTG TTA CGC AAT CCA ATT GAC TTT ACT TGAA-3′ and 5′-TAA GCC GGT TGT GTT TAC ACG TCA -3′ were used to amplify the A domain of Cia, and the two primers 5′-CAA GCC GGT AGT CAA ATG ACT TGG TAA-3′ and 5′-GCC GGT CGA GCC TTA CTT CTG TAT ATT GAA-3′ were used to amplify the C, D, W, and M domains of FnBPA (Microsynth). Restriction sites for BamHI, SufI, and Sall are shown in bold. Both fragments were cloned in pGEM-T-easy as instructed by the manu- facturer (Promega). After expansion in \( E. coli \) and extraction using standard techniques (48), the fragments were fused in pGEM-T-easy using the PvuI restriction sites, and the nucleotide sequence of the fusion protein was veri- fied. The new fragment encompassing the A domain of Cia fused to the C, D, W, and M domains of FnBPA was then cloned between the BamHI and Sall restriction sites of the pOr23 expression vector and transformed into \( L. lactis \) as described previously (18, 47).

For trans-supplementation, whole Cia and truncated FnBPA were ex- pressed simultaneously on separate plasmids. The second expression plasmid was constructed by replacing the erythromycin-resistant gene of pOr23-CD with a chloramphenicol-resistant gene. The erythromycin-resistant gene was removed by digestion with ScaI and Hpal. The chloramphenicol-resistant gene was amplified from plasmid pVE3702 (provided by Alexandra Gruss, Laboratoire de Génétique Appliquée, Institut de la Recherche Agronomique, Jouy-en-Josas, France). The forward primer (5′-TTT AGT ACT TTT AGG AGG CAT ATC AAA TGA AC-3′; Microsynth) started at bp 2011 and contained a ScaI site (indicated in bold). The backward primer (5′-AAT GTT AAC TAA AAA GTA CAG TCG GCA TTA TCT C-3′) started at codon 2706 and contained a Hpal site (indicated in bold). The ampiclon was double digested with ScaI and Hpal, and ligated into the ScaI–Hpal-digested pOr23-CD, generating pOr23cm-CD. This plasmid was transformed into \( L. lactis \) harboring pOr-Cia, which expresses Cia (Table I; references 18, 47). The transformants were selected on eryth- romycin- and chloramphenicol-containing plates. All 100% double- resistant transformants expressed both Cia and truncated FnBPA function- ally. The double transformant grew ~10-20% slower in liquid medium (containing the selective antibiotics) than either the parent or the single positive pOr-Cia or pOr-Ci-CD \( L. lactis \).

Antibodies. Anti-Cia \( F(ab)'_2 \) (19) were prepared as previously de- scribed. Peptides corresponding to the FnBPA residues 479–493 (IQQNK- FEYKEDTIKE) were conjugated to KLH and used to immune rabbits (Eurogentec). \( F(ab)'_2 \) fragments were further prepared from rabbit immune sera using the Immunopure \( F(ab)'_2 \) preparation kit (Pierce Chemical Co.) according to the manufacturer’s instructions. Anti–von Willebrand factor antibodies were obtained from Sigma-Aldrich.

Experimental endocarditis in rats. The permission for experimenta- tion on living animals regarding the present work was granted by the State Veterinary Office of the “Canton de Vaud” (permission 879.39). Sterile aortic vegetations were produced in female Wistar rats as described previ- ously (49). 24 h after catherization, groups of 8–10 rats were challenged intravenously with an inoculum 10 times greater than \( \times 10^6 \). Groups of an- imals were killed at various time points thereafter. Quantitative valve and spleen cultures were performed as previously described (18, 22, 24, 25). Lactociocc recombinants were plated on both erythromycin-containing and erythromycin-free agar to ascertain the presence of the expression vector encoding the staphylococcal genes (18, 47). When two expression plas- mids were used, bacteria were plated on agar containing both erythromycin and chloramphenicol. Bacterial densities in the vegetations were expressed as the mean of \( \log_{10} \) CFU/gram of tissue \( \pm SD \). The limit of detection was \( \pm 2 \log_{10} \) CFU/g of tissue. Rats with sterile valve cultures were consid- ered uninfected. Differences between mean vegetation bacterial densities were evaluated by one-way analysis of variance, and pair-wise differences between the means of groups were determined by the \( t \) test with the Bonferroni correction. Differences were considered important when \( P \leq 0.05 \) by use of two-tailed significance levels.

Immunohistochemistry. Rats with sterile aortic vegetations were inoc- ulated as described in the previous paragraph and killed 24 h later. Cardiac vegetation were excised and rapidly frozen in 2-methylbutane cooled in liquid nitrogen. Vegetations were then included in OCT medium (Leica Instruments) and cryostat sectioned at an 8-μm thickness. The sections were rinsed in PBS and incubated in buffer containing 3% BSA before an O/N incubation in the presence of anti-Cia \( F(ab)'_2 \) or anti–FnBPA \( F(ab)'_2 \) fragments diluted 1:200 in PBS. Primary antibodies were detected using goat anti–rabbit \( F(ab)'_2 \) immunoglobulins labeled with FITC (Pierce Chemical Co.). Sections were then rinsed in PBS, stained with Evan’s blue, and photographed under a microscope with film (Tmax 400; Kodak).

Electron microscopy. Inoculation and killing was performed as de- scribed in the Immunohistochemistry section. Small tissue fragments (1 mm) were fixed by immersion at 4°C for 2 h in 2% glutaraldehyde/0.1 M Sørensen phosphate buffer, washed, postfixed with 1% OsO4 at room tem-
temperature for 1 h, dehydrated through graded concentrations of acetone, and embedded in Epon-Araldite. Because endocarditis is a heterogeneous infection, semithin sections were first screened for the presence of bacteria. Ultrathin sections were further stained with 1% PAS for 30 min at room temperature, washed with bidistilled water, and dried for another 30 min. Tannic acid staining was used to assess the presence or absence of intracellular membranes (8).

Solid phase adhesion and invasion of cultured cells. Adherence of various L. lactis strains to fibronectin or fibroectin was conducted as previously described (24, 35, 47). In vitro cell internalization was assessed by several methods. First, an antibiotic protection assay was performed as previously described using HUVECs and live bacteria (50, 51). 2 μg/ml penicillin or 100 μg/ml gentamicin was used to kill noninternalized lactococci, and 20 mg/liter lysozyme was used to kill nonenuinterized staphylococci. Results were expressed as the percentage of surviving bacteria compared with S. aureus Cowan I (Table I). Second, the evaluation of invasion by flow cytometry was performed as previously described (8), with minor modifications. Cells were plated in 24 wells at 1.5 × 10^6 cells, resulting in an estimated multiplicity of infection of ~35:1, and fluorescence values were normalized to bacterial preparations of different species. Third, the confocal microscopy study was performed in certain experiments to more clearly identify internalization. Internalized FnBPA-positive lactococci were labeled with anti-FnBPA antibodies after permeabilization using standard techniques.

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