MAJOR ARTICLE

The Role of *Staphylococcus aureus* Adhesins in the Pathogenesis of Ventricular Assist Device–Related Infections

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Ventricular assist devices (VADs) are an important form of therapy for end-stage congestive heart failure. However, infection of the VAD, which is often caused by *Staphylococcus aureus*, poses a major threat to survival. Using a novel in vitro binding assay with VAD membranes and a heterologous lactococcal system of expression, we identify 3 *S. aureus* proteins—clumping factor A (ClfA) and fibronectin binding proteins A and B (FnBPA and FnBPB) as the main factors involved in adherence to VAD polyurethane membranes. Adherence is greatly diminished by long implantation times, reflecting a change in topological features of the VAD membrane, and is primarily mediated by the FnBPA domains in the staphylococcal proteins. We also compare the adherence of *S. aureus* mutant strains and show that other staphylococcal components appear to be involved in adherence to VAD membranes. Finally, we demonstrate that ClfA, FnBPA, and FnBPB mediate bacterial infection of implanted murine intra-aortic polyurethane patches.

The ventricular assist device (VAD) is an important form of therapy for patients with congestive heart failure. Originally introduced as a bridge to cardiac transplantation, it is increasingly also used for "destination therapy" [1]. A serious limitation to the long-term use of VADs has been the high incidence of device-related infections, which occur in 28%–48% of patients [2–5]. These infections, which are often caused by *Staphylococcus aureus*, pose a major threat to survival, because eradication of the infection usually requires removal of the device [2, 6, 7].

Although the clinical features of VAD-related infections caused by *S. aureus* are well described, little is known about their pathogenetic processes [2, 5–7]. The microenvironment of the VAD surface is dynamic, with constant remodeling of the neointimal surface, beginning with platelet deposition and formation of a fibrin scaffold after which a variety of cell types attach and proliferate [8, 9]. As a result of this process, the bacterial–neointimal surface interactions change over time. Despite the increasing biological relevance of these interactions, the complex interplay of bacterial adhesins and the prosthetic neointimal surface has received limited attention. In particular, the contribution of proteins belonging to the family of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), which have been demonstrated to play a role in other *S. aureus* in-

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fections [10], to the development of VAD-related infections has not been previously investigated.

The overall goal of the present study was to characterize the interaction between *S. aureus* adhesins and the neointimal surface formed on textured membranes typified by pulsatile VADs. A novel in vitro binding assay combined with a heterologous protein expression system was developed to identify these adhesins. We provide the first information, to our knowledge, on the nature of *S. aureus*–VAD membrane interactions, including the topological features of the neointimal surface and the effect of implantation time on adherence.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Lactococcus lactis* strains MG1363 [11] and NZ9800 [12] were routinely grown at 30°C in medium composed of M17 medium (Difco) supplemented with 0.5% (wt/vol) glucose (GM17) with or without 1% agar. *S. aureus* strains SKM3 [13], LS-1 [14], LS-1 Δ*clfA,* LS-1 Δ*clfA clfB::ermC,* LS-1 *fnbA::tetK fnbB::ermC,* and LS-1 Δ*clfA clfB::ermC fnbA::tetK fnbB::ermC* were grown at 37°C in Todd-Hewitt broth (BD Biosciences) with or without 1% agar. A sortase A–deficient ($SrtA^-$) LS-1 strain was constructed by phage 85 transduction [15] of the *srtA::ermC* cassette from SKM3 into LS-1. Transductants were selected in erythromycin-containing plates (9.5 μmol/L) and screened for SrtA production by Western blotting with polyclonal antibodies to SrtA (gift from O. Schneewind, University of Chicago). The presence of *ermC* insertion in *srtA* was assessed by polymerase chain reaction (PCR) amplification of genomic DNA, as described elsewhere [13].

The majority of the plasmids that were used in the present study were described elsewhere (table 1). Strains expressing *sasI, sasF,* and *ebpS* in pKS80 were obtained using similar protocols for generation of *Nco*I restriction sites by PCR gene amplification and cloning into the *Nco*I site of the vector.

When necessary, culture medium was supplemented with the following antibiotics (Sigma): erythromycin (6.8 μmol/L for *L. lactis* and 9.5 μmol/L for *S. aureus*), chloramphenicol (30.9 μmol/L), tetracycline (5 nmol/L), or kanamycin (85.8 μmol/L). The nisin concentration used for expression in pNZ8037 was 3.5–4.0 ng/mL.

Construction of mutant strains. Mutants of strain LS-1 that were defective in 1 surface protein or more were constructed as follows. Initially a frameshift mutation *clfA5* (5 bases between positions 110 and 114 were replaced with a *Hin*dIII site) was isolated in each host by allele replacement using temperature-sensitive vector [15]. The *clfB::lacZ*[*ermC*] [19] mutation was transduced into the *clfA5* mutants. The linked *fnbA::* Tc^r *fnbB::ermC* [20] mutations were cotransduced into LS-1 and into the *clfA clfB::lacZ*[Em^r] mutants of both strains. Each transductant was validated by PCR, Southern hybridization, and Western blotting using specific antibodies recognizing the unique A domains of each protein.

Table 1. Plasmids used in the present study.

Plasmid	Protein encoded	Gene expression	Source
pKS80	None	N/A	[16]
pKS80::*clfA*	ClfA	Constitutive	[17]
pKS80::*clfB*	ClfB	Constitutive	[17]
pKSS80::*spa*	SpA	Constitutive	[17]
pKS80::*sdrC*	SdrC	Constitutive	[17]
pKS80::*sdrD*	SdrD	Constitutive	[17]
pKS80::*sdrE*	SdrE	Constitutive	[17]
pKS80::*sdrG*	SdrG	Constitutive	[16]
pKS80::*sasI*	SasI	Constitutive	Present study
pKS80::*sasF*	SasF	Constitutive	Present study
pKS80::*ebpS*	EbpS	Constitutive	Present study
pKS80::*eap*	Eap (Map)	Constitutive	[17]
pNZ8037	None	N/A	[18]
pNZ8037::*fnbA*	FnBPA	Nisin inducible	[15]
pNZ8037::*fnbB*	FnBPB	Nisin inducible	[15]

NOTE. Clf, clumping factor; Eap, extracellular adherence protein; EbpS, elastin-binding protein S; FnBP, fibronectin binding protein; Map, major histocompatibility complex class II analogous protein; N/A, not applicable; Sas, *Staphylococcus aureas* surface; Sdr, serine-aspartate repeat; SpA, staphylococcal protein A.

Antibodies. Anti–clumping factor (Clf) A is a murine monoclonal IgG1 antibody directed against the recombinant (r) A domain (anti-rAClfA; gift from John Vernachio, Inhibitex, Atlanta, GA). Anti-ClfB and the antibodies against the rA domains of fibronectin binding protein (FnBP) A (anti-rAFnBPA) and FnBPB (anti-rAFnBPB) have been described elsewhere [21, 22]. Isotypic control antibodies were murine or rabbit IgG1 (Sigma). $F(ab')_2$ fractions were prepared using either the ImmunoPure IgG1 Fab or $F(ab')_2$ Preparation Kit (Pierce Biotechnology), in accordance with the manufacturer's instructions.

Scanning electronic microscopy. VAD membranes were incubated with bacteria, washed in a similar manner as in VAD adhesion assays (described below), and subsequently processed as described elsewhere [23].

VAD explantation and processing. For the VAD explantation procedure, the inflow and outflow grafts were clamped and transected, the driveline was cut, and the device was removed en bloc from the preperitoneal VAD pocket. The VAD was immediately placed on a sterile table within the operating room. A HeartMate VAD explant kit (Thoratec Corporation) was used to unscrew the outer ring of the device, and this permitted separation of the 2 halves—the textured, sintered titanium side with inflow and outflow conduits and the textured polyurethane membrane with underlying pusher plate mechanism. Sterile gloves were worn when removing the membrane from the device, and the membrane was rinsed in sterile, cold PBS (Bio-Rad), fixed in 10% formaldehyde for 30 min at room temperature, and washed in PBS. The membrane was then

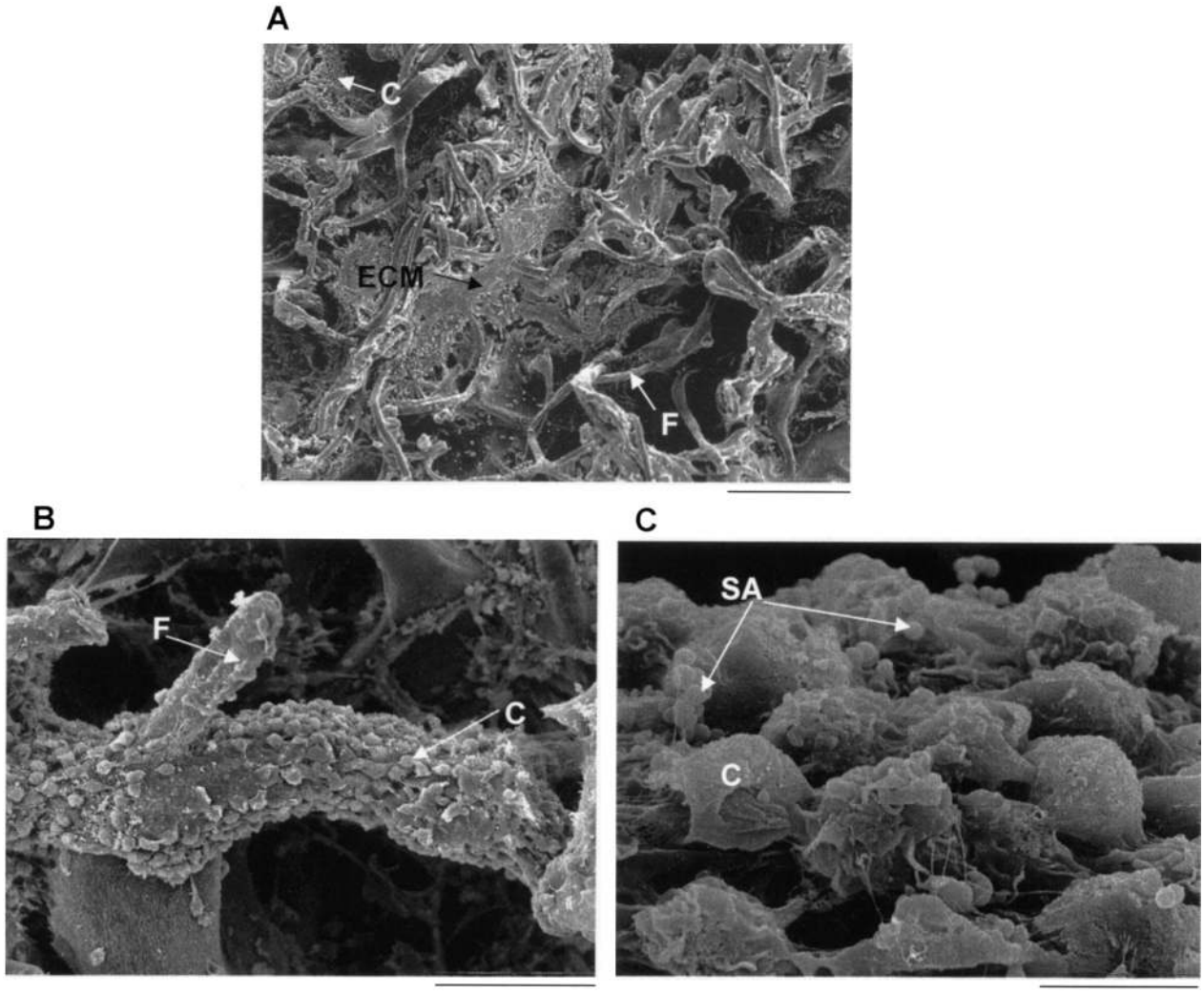

Figure 1. Morphological features of the neointimal lining of ventricular assist device (VAD) membranes. Shown are low-resolution *(A)* and high-resolution (*B* and *C*) scanning electron micrographs of the neointimal lining coating the textured membrane. The VAD was implanted for 39 days, and the neointimal surface has polyurethane fibers (F), host cellular components (C), and extracellular matrix (ECM) components. VAD membranes that were incubated with *Staphylococcus aureus* (SA) cells have bacteria bound to both ECM and cellular components. Scale bars, 200 μm *(A),* 50 μm *(B),* and 5 μm *(C).*

detached from its base; 9-mm-diameter disks were obtained with the use of a sterile cork borer and subsequently used in VAD adhesion assays.

Bacterial adherence to the VAD membrane. *L. lactis* or *S. aureus* cultures were grown to mid-log phase, harvested, and resuspended in cold PBS; next, concentrations were adjusted to an OD_{600} of 0.1, which corresponded to 2×10^{10}–7×10^{10} cfu/L, and then cultures were incubated with VAD disks for 1 h at 37°C. Membranes were transferred to 50-mL Falcon tubes and extensively washed with PBS. Viable adherent bacteria were then lifted off the membranes by use of 2 sequential methods of elution, so as to disrupt interactions with increasing levels of affinity. In the first method, bacteria were treated 3 times for 10 min with PBS supplemented with 1 mol/L NaCl at 37°C; in the second method, bacteria were incubated 2 times for 7 min with a solution of Trypsin-EDTA (1×; Gibco). Bacterial suspensions were then plated onto appropriate culture medium and incubated for 24 h.

Solid-phase assay of bacterial adherence. Adherence to solid-phase fibronectin (Fn) or fibrinogen (FnG) of *L. lactis* or *S. aureus* grown under the same conditions as for VAD adhesion assays was assessed using a modification of an assay described elsewhere [16]. When indicated, appropriate $F(ab')_2$ fragments were included in the assay. Adherent bacterial cells were lifted off the VAD membranes with 3 sequential 5-min incubations with a solution of Trypsin-EDTA (1×) at 37°C, plated onto appropriate culture medium, and incubated for 24 h.

Flow cytometry. Aliquots (1×10^8 cells) of bacterial sus-

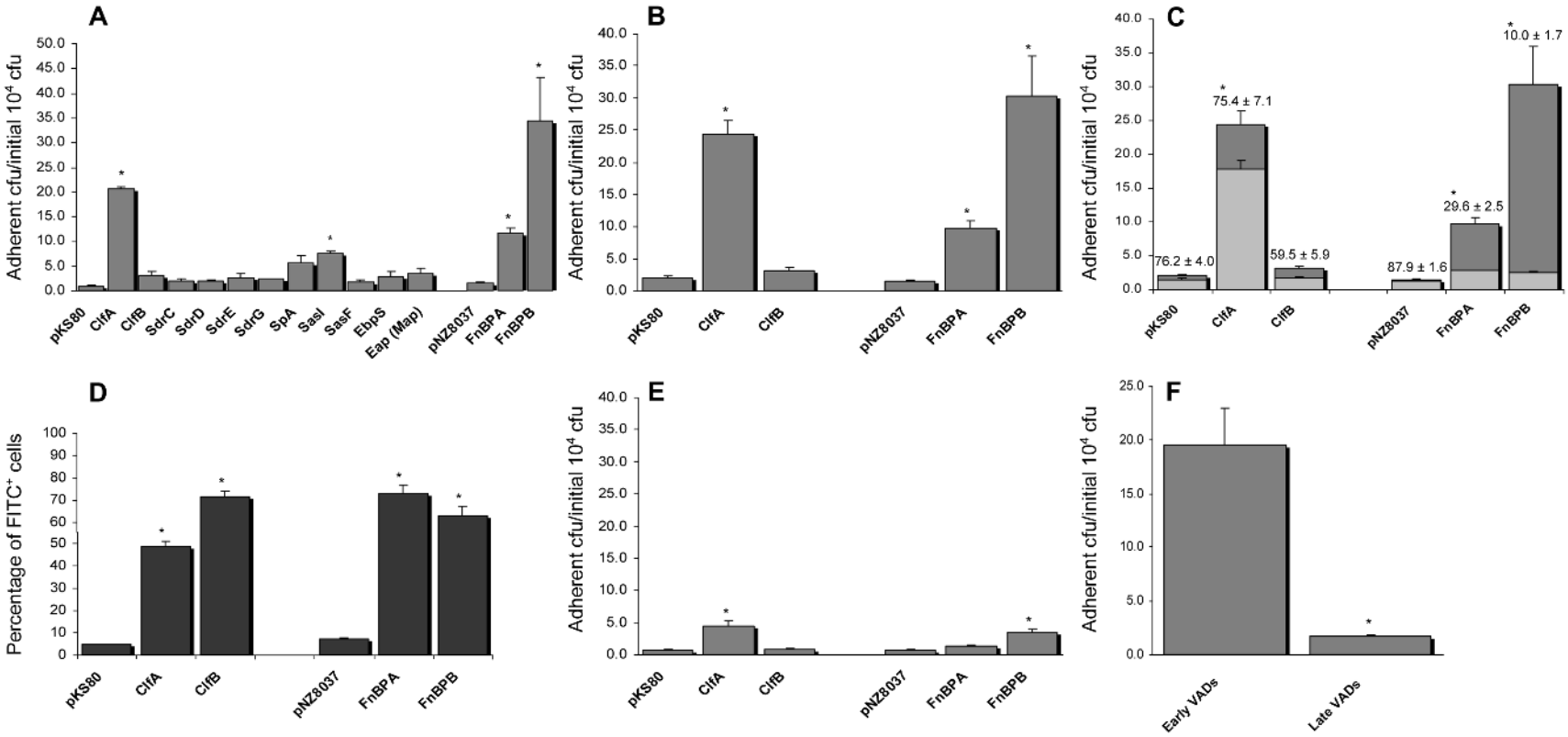

Figure 2. Adherence of *Staphylococcus aureus* surface proteins to ventricular assist device (VAD) membranes. *A,* Adherence of *Lactococcus lactis* strains expressing 12 *S. aureus* surface proteins to a VAD that had been implanted <6 months (early VAD). *$P < .05$, vs. control pKS80 or pNZ8037. *B,* Further analysis of the adherence of *L. lactis* strains expressing 4 *S. aureus* surface proteins to 4 different early VADs. *$P < .05$, vs. control pKS80 or pNZ8037. *C,* Differential elution of adherent *L. lactis* strains from early VADs after treatment with 1 mol/L NaCl *(light gray bars)* or Trypsin-EDTA *(dark gray bars).* Nos. above bars represent percentages of the total no. of adherent cells for each strain that were eluted with high-ionic-strength solution. The ratio of bacterial cells eluted from the VAD membrane by use of the 2 methods (NaCl vs. Trypsin-EDTA) for clumping factor (Clf) A vs. fibronectin binding protein (FnBP) A and FnBPB, as well as for FnBPA vs. FnBPB, were statistically different. *$P < .05$. *D,* Flow-cytometric results illustrating the presence of ClfA, ClfB, FnBPA, and FnBPB on the surface of *L. lactis* cells. *$P < .05$, vs. control pKS80 or pNZ8037. *E,* Adherence of *L. lactis* strains to VADs that had been implanted ⩾6 months (late VADs). *$P < .05$, vs. control pKS80 or pNZ8037. *F,* Adherence of *S. aureus* LS-1 to early VADs vs. late VADs. *$P < .05$. Data are the means ± SEs of at least 3 separate experiments. Statistical analyses were performed using analysis of variance. FITC, fluorescein isothiocyanate. Eap, extracellular adherence protein; Ebp, elastin-binding protein; Map, major histocompatibility complex class II analogous protein; Sas, *Staphylococcus aureas* surface; Sdr, serine-aspartate repeat; SpA, staphylococcal protein A.

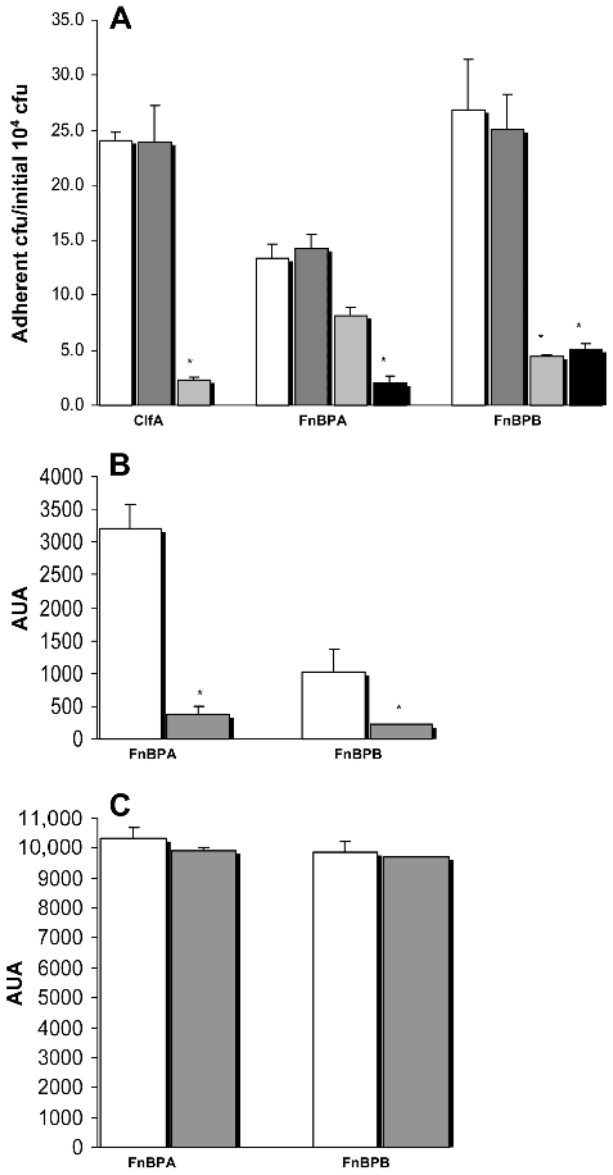

Figure 3. Effect of F(ab′)$_2$ fragments on adherence of clumping factor (Clf) A–, fibronectin binding protein (FnBP) A– and FnBPB-producing lactococci. *A,* Adherence of *Lactococcus lactis* strains to ventricular assist devices (VADs) that were implanted <6 months (early VADs). Shown are adherence in control experiments *(white bars),* after addition of isotypic control F(ab′)$_2$ (3.5 nmol/L; *dark gray bars*), and after addition of anti-A F(ab′)$_2$ (3.5 nmol/L, *light gray bars;* 7.0 nmol/L, *black bars*). *B* and *C,* Adherence of *L. lactis* strains to solid-phase fibrinogen (FnG; *B*) or fibronectin (Fn; *C*). Shown are adherence in control experiments *(white bars)* and after addition of anti-A F(ab′)$_2$ (3.5 nmol/L; *gray bars*). Adherence is expressed in arbitrary units of adherence (AUA). Data are the means ± SEs of at least 3 separate experiments. Statistical analyses were performed using analysis of variance. **P* < .05.

pensions used in VAD adhesion assays were incubated for 30 min with the appropriate antibody (0.33 μmol/L), washed once with PBS, and incubated for 30 min with the appropriate fluorescein isothiocyanate–conjugated secondary antibody (Sigma). Cells were subsequently washed with PBS and fixed with 1% formaldehyde for 10 min and then resuspended in 1 mL of PBS. Analysis was performed with a FACSCalibur flow cytometer using Cell Quest software (BD Biosciences).

Murine model of infection and histological analysis. For in vivo infection, 30 C57BL/6J mice were divided into groups of 5, and polyurethane patch implantation was performed using a modification of a recently described murine intra-aortic patch model [24]. Twenty-four hours after implantation, *L. lactis* cells were injected through the tail vein. When necessary, nisin was added to *L. lactis* cultures in a similar manner to that used in VAD adhesion assays. In such cases, nisin (12 μmol/L) was also added to the bacterial inocula as well as injected intraperitoneally into the mice at 4 and 8 h after bacterial inoculation (0.5 mL of 15 μmol/L nisin per mouse). A blood sample was obtained 1 h after inoculation, to confirm bacteremia. Twenty-four hours after inoculation, each mouse was anesthetized with an overdose of ketamine and xylazine. The abdomen was reopened, a blood sample was obtained from the inferior vena cava, and the polyurethane patch and the liver were excised and rinsed with PBS.

Liver and patch samples were homogenized in PBS (PRO 200 Homogenizer; PRO Scientific). Aliquots, as well as blood samples, were plated onto GM17 plates and incubated for 24 h at 30°C.

Intra-aortic patches from mice infected as described above were excised, molded in Optimum Cutting Temperature (Tissue-Tek), frozen, and stored at −80°C. Sections (8 μm) were Gram stained or Trichrom stained (Sigma) in accordance with the manufacturer's instructions. Micrographs were taken using a Zeiss Axioskop 50 microscope.

All procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at Columbia University. The study was reviewed and approved by the Columbia University Institutional Review Board.

Statistical analysis. Statistical significance was determined using analysis of variance. $P < .05$ was considered to be statistically significant.

RESULTS

Morphological features of the neointimal VAD membrane. The textured, polyurethane luminal surface of an implanted pulsatile VAD facilitates the formation of a "pseudoneointima," which reduces the potential for thromboembolic complications [8, 25]. However, this surface may also increase susceptibility to bacterial adhesion and, as a result, to VAD-related infections. We analyzed the neointimal surface of the VAD membrane by scanning electron microscopy. The textured membrane was covered by cellular and extracellular matrix (ECM) components (figure 1*A* and 1*B*). Incubation of the

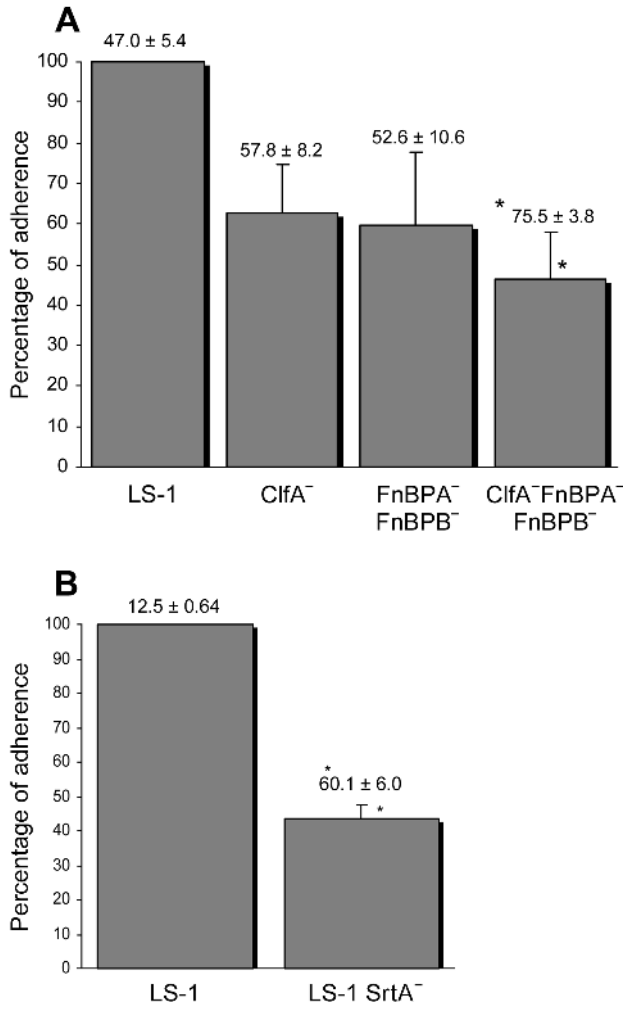

Figure 4. Adherence of *Staphylococcus aureus* mutant strains to membranes of ventricular assist devices (VADs) that were implanted for <6 months. *A,* Adherence of LS-1 and mutants defective in 1, 2, or 3 microbial surface components recognizing adhesive matrix molecules. *B,* Relative adherence of LS-1 defective in sortase A (SrtA) production, compared with the LS-1 wild-type strain. Nos. represent the percentage of adherent cells for each strain that were eluted with high-ionic-strength solution. Data are the means ± SEs of at least 3 separate experiments. Statistical analyses were performed using analysis of variance. *$P < .05$. Clf, clumping factor; FnBP, fibronectin binding protein.

membrane with *S. aureus* demonstrated that bacteria were adherent to both the matrix material and cells (figure 1*C*).

Mediation by ClfA, FnBPA, and FnBPB of bacterial adherence to the VAD membrane. Twelve of the *S. aureus* surface proteins considered to be the most likely to mediate adherence to explanted VADs were selected for the initial analysis (table 1). These proteins were heterologously produced in a nonadherent *L. lactis* strain. On the basis of preliminary adherence results, the VADs were classified by length of implantation and placed into 2 groups: early (<6 months) and late (≥6 months). Strains producing ClfA, FnBPA, and FnBPB were the most adherent to the VAD membrane, whereas strains producing protein A and SasI also showed increased adherence, compared with that in control strains (figure 2*A*).

We then analyzed in more detail the binding effects of ClfA, ClfB, FnBPA, and FnBPB with early VADs and confirmed our previous observation that ClfA, FnBPA, and FnBPB caused an increase in total adherence, whereas ClfB showed no difference in adherence, compared with that in control strains (figure 2*B*).

As was mentioned above, elution of adherent bacteria during the VAD adhesion assay was performed using 2 different sequential methods, to characterize in more detail these bacterial–neointimal surface interactions. Because we used this method, we were able to measure the relative number of adherent bacteria sequentially released from the VAD membrane by (1) high-ionic-strength solution, which was used to disrupt relatively weak interactions, and (2) enzymatic treatment directed against stronger adhesin-receptor adherent interactions. When this differential elution was analyzed, we observed that the majority of ClfA-producing adherent *L. lactis* cells were extracted using high-ionic-strength solution. In contrast, FnBPA-producing and FnBPB-producing bacteria were primarily eluted by the enzymatic treatment (figure 2*C*). These results strongly suggest that FnBPA-mediated and FnBPB-mediated contacts are stronger, having a significantly greater overall affinity than are those mediated by ClfA.

Flow cytometry was performed to document that differential expression of MSCRAMMs on the cell surface was not responsible for the lack of effect of ClfB-producing *L. lactis* cells (figure 2*D*). All tested MSCRAMMs were successfully exported onto the bacterial surface.

In addition, we observed that adherence of ClfA, FnBPA, and FnBPB to late VADs was significantly reduced, compared with that to early VADs (figure 2*E*). Similarly, none of the other previously tested MSCRAMMs caused a significant increase in adherence to late VADs (data not shown). *S. aureus* LS-1 adherence to late VADs was also reduced, compared with that to early VADs (figure 2*F*), which demonstrates that long implantation times drastically reduce the adherence properties of the neointimal surface.

Involvement of the A domains of ClfA, FnBPA, and FnBPB in adherence to the VAD membrane. The 3 major *S. aureus* proteins involved in adherence to the VAD membrane that were identified in this study are typical of MSCRAMM proteins. However, the 2 Fn-binding proteins contain 2 different ligand-binding domains: the amino-terminal A domain and the D repeat region, which is located closer to the carboxy-terminal. In addition, the FnG-binding protein ClfA possesses only an amino-terminal A ligand-binding domain that is homologous to those present in FnBPA and FnBPB [10].

Antibodies directed against regions of the MSCRAMM proteins were used to identify the ligand-binding domains involved

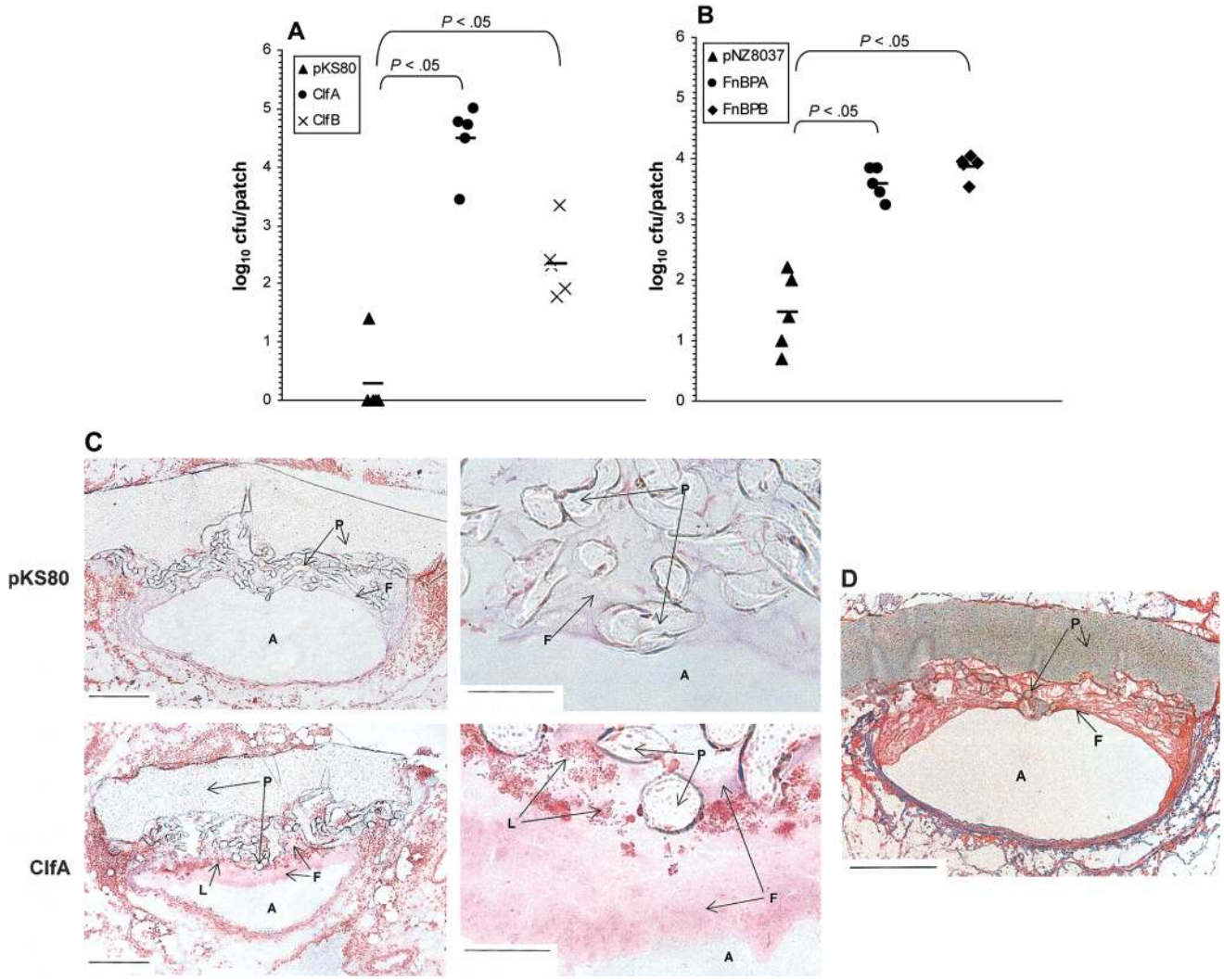

Figure 5. Effect of clumping factor (Clf) A, ClfB, fibronectin binding protein (FnBP) A, and FnBPB on infection in a murine intra-aortic polyurethane patch model. Bacterial densities in polyurethane patches infected with the *Lactococcus lactis* control pKS80, ClfA-producing *L. lactis,* or ClfB-producing *L. lactis (A),* as well as with the *L. lactis* control pNZ8037, FnBPA-producing *L. lactis,* or FnBPB-producing *L. lactis (B)* are indicated. Statistical means for each experimental group are represented by horizontal bars ($n = 5$ mice/group). Statistical analyses were performed using analysis of variance. *C,* Gram-stained sections of aortic polyurethane patches showing the presence of ClfA-producing *(lower panels) L. lactis* cells (L) or control (pKS80) *L. lactis* cells in fibrinogen (FnG)/fibrin deposits (F). Polyurethane fibers (P) and aortic lumen (A) are also indicated. Scale bars, 200 μm *(left panels)* and 50 μm *(right panels).* D, Trichrom-stained section of intra-aortic polyurethane patch showing FnG/fibrin deposits (F), polyurethane fibers (P), and aortic lumen (A). Scale bar, 200 μm.

in *S. aureus* adherence to the VAD membrane. Antibodies against the A domains of ClfA, FnBPA, and FnBPB were used. $F(ab')_2$ fragments were prepared to prevent Fc receptor–mediated adherence [9, 25].

Anti–rAClfA, anti–rAFnBPA, and anti–rAFnBPB caused a significant decrease in adherence of their respective *L. lactis* strains to the VAD membrane (figure 3*A*), suggesting that the FnG-binding A domain mediates the interaction. Both anti-A $F(ab')_2$ fragments significantly reduced the ability of FnBPA and FnBPB to bind FnG but not Fn in solid-phase assays (figure 3*B* and 3*C*).

The role of ClfA, FnBPA, FnBPB, and other* S. aureus *components in adherence to the VAD membrane. The findings from the heterologous lactococcal expression system were next extended to studies of *S. aureus*. The adherence of *S. aureus* LS-1–derived mutants that were defective in either ClfA; FnBPA and FnBPB; or ClfA, FnBPA, and FnBPB was investigated. Removal of either ClfA alone or of FnBPA and FnBPB did not elicit a significant decrease in adherence levels. Only the removal of all 3 MSCRAMMs that were previously identified as the primary adhesins yielded a significant decrease in adherence (figure 4*A*).

Table 2. Hepatic metastatic seeding of mice infected with different *Lactococcus lactis* strains.

Strain phenotype	Inoculum, cfu/mouse	Bacteria in liver, $\log_{10}$ cfu/g ± SE	P[a]
pKS80 control	5×10^7	2.35 ± 0.14	…
ClfA producing	5×10^7	3.90 ± 0.19	<.05
ClfB producing	5×10^7	3.58 ± 0.06	<.05
pNZ8037 control	7×10^7	3.72 ± 0.04	…
FnBPA producing	1.6×10^8	4.77 ± 0.04	<.05
FnBPB producing	8×10^7	4.65 ± 0.10	<.05

NOTE. Clf, clumping factor; FnBP, fibronectin binding protein.

[a] Compared with the control strain (analysis of variance).

To study the role that other MSCRAMMs play in adherence to the VAD membrane, we constructed an LS-1–derived mutant strain that was defective in the production of SrtA, the key enzyme responsible for anchoring proteins belonging to the MSCRAMM family into the staphylococcal peptidoglycan [13]. The adherence of LS-1 SrtA$^-$ was significantly reduced, compared with that of LS-1 (figure 4*B*). Interestingly, we also observed that the majority of LS-1 cells were successfully detached from the VAD membrane only after an enzymatic treatment, whereas a high-ionic-strength solution was sufficient to release most of the LS-1 SrtA$^-$ cells (figure 3*A* and 3*B*).

Facilitation by ClfA, ClfB, FnBPA, and FnBPB of* L. lactis *adherence to the VAD membrane in vivo. The in vitro data indicated that ClfA, FnBPA, and FnBPB were the major factors involved in adherence to the VAD membrane. To demonstrate the validity of the in vitro observations and to study the role exerted by these proteins under physiological conditions, we next used a recently developed murine model of intra-aortic polyurethane patch infection [24] to assess whether they facilitate adherence of *L. lactis* to the implanted patch. ClfA-producing lactococci caused a significant increase in the number of bacteria present on the polyurethane patch, compared with that caused by the control strain (figure 5*A*). To assess the in vivo role of FnBPA and FnBPB, we used lactococcal cells cultured and induced with nisin in a similar manner to that previously described for in vitro assays. In this way, FnBPA-producing and FnBPB-producing cells adhered to the polyurethane patch in significantly higher numbers than did the control pNZ8037 strain (figure 5*B*). Interestingly, we observed that ClfB-producing *L. lactis* cells also caused a significant increase in adherence to the polyurethane patch, although such adherence was markedly reduced with respect to that elicited by ClfA (figure 5*A*). Similarly, a significant difference in metastatic seeding of the liver was also demonstrated between the *L. lactis* strains producing the different staphylococcal adhesins and their control strains (table 2), although no lactococcal cells were found in the bloodstream of mice at the time of death (data not shown).

Gram staining of the infected polyurethane patch demonstrated that ClfA-producing *L. lactis* cells were adherent to the patch material and accumulated on the fibrin that immediately covers the polyurethane textured surface (figure 5*C* and 5*D*). Other areas adjoining the patch were free of bacteria. In contrast, no bacterial cells were detected when the control strain was used (figure 5*C*). These results demonstrate that ClfA, FnBPA, and FnBPB—the major adhesion factors that were identified in vitro—are independently sufficient to establish early infections in an in vivo setting.

DISCUSSION

This study is the first, to our knowledge, to investigate the role that bacterial factors play in the pathogenesis of VAD infections. The size of the device, immunocompromise in the patient, and the presence of a transcutaneous driveline all increase the risk of infection [3]. The most serious of these infections involve the luminal polyurethane or valvular surfaces of the device. The VAD illustrates the biologic complexity of understanding the pathogenesis of prosthetic device–related infections. The textured surface evolves over time, becomes increasingly covered with different host cells and matrix proteins, is subject to varying hemodynamic conditions, and is regularly exposed to circulating bacterial pathogens [8, 9, 25].

A novel screening system was used to assess the ability of staphylococcal surface proteins to adhere to the VAD membrane. The critical first step in the pathogenesis of these infections requires the adherence to and colonization of the device's surface, and this process is often mediated in *S. aureus* by adhesins belonging to the MSCRAMM family [10]. A heterologous lactococcal expression system was used to overcome the inherent redundancy found in *S. aureus* adhesins [10]. Despite the qualitative nature of our assay—which resulted from differences in the composition of the neointimal surface that were caused by varying lengths of VAD implantation, interpatient variability, and the use of 2 different expression systems [8, 9, 26, 27]—we found that ClfA, FnBPA, and FnBPB were the main MSCRAMMs that mediated bacterial adherence. Of interest, even though ClfB is structurally very similar to ClfA, it failed to mediate adherence in vitro.

FnBPA and FnBPB are closely related proteins that have been shown to bind to both FnG and Fn molecules [10, 28]. In the same way, both ClfA and ClfB, which belong to the serine-aspartate repeat family of proteins, share the ability to bind FnG molecules [10, 29, 30]. However, although ClfA binds to the δ chain, ClfB recognizes and adheres to the α chain of the molecule. FnBPA and FnBPB share with ClfA the capacity to recognize the FnG δ chain [10]. This difference in binding site might, in part, explain the inability of ClfB to adhere to the VAD membrane in vitro. Therefore, the large FnG/fibrin deposits in explanted VADs [8, 9, 26] and their affinity for ClfA,

FnBPA, and FnBPB make them the most likely ligands involved in *S. aureus* adherence. Previous studies have shown that FnG plays a role in the adherence of bacteria to intravascular catheters that are inserted over a short term [31] and hemodialysis tubing [32] and that fibrin plays a role in adherence to polyurethane surfaces [33]. More recently, other authors have identified *S. aureus* FnG binding activity as a key element in promoting early valve colonization in an in vivo rat endocarditis model [34].

Significant differences in the elution pattern between ClfA-, FnBPA-, and FnBPB-producing cells were detected, suggesting that the interaction of ClfA with the neointimal surface is substantially weaker than that of FnBPA or FnBPB. The ClfA-mediated interaction with FnG in vitro has been shown to be a high-affinity event [10]. However, in contrast to ClfA, which has a stoichiometric ratio of 1:1 with FnG, FnBPA and FnBPB mediate interactions with 2 molecules of Fn and have been shown to be able to form covalent cross-linking with fibrin and Fn [35, 36]. This, in addition to their ability to bind FnG, might result in a stronger overall interaction than that of ClfA, especially on the VAD membrane, where different types of receptors are most likely present. These differences in affinity for the host neointimal surface might play a significant role in the likelihood of a successful infection under physiological conditions. In this scenario, other factors, such as flow patterns and shear stress conditions in the bloodstream, may affect *S. aureus* adherence to the VAD membrane in the same way that they are known to affect *S. aureus* adherence to host components such as platelets and endothelial cells [37, 38]. Two other proteins, SasI and protein A, had a lesser effect on adherence to the VAD membrane. SasI expression is iron dependent [39], which might enhance its in vivo significance. Protein A mediates binding to von Willebrand factor [40], which is known to be present on the VAD neointima [8].

Our in vitro observations about MSCRAMM proteins were further validated using an in vivo model [24], in which mice implanted with intra-aortic polyurethane patches were infected with the ClfA-, FnBPA-, and FnBPB-producing *L. lactis* strains. The presence of staphylococcal proteins on the lactococcal surface conferred a survival advantage, even in environments known to be rich in phagocytes, such as the liver. Histological studies demonstrated that, at least in the ClfA cells, adherence occurred along the FnG-coated surface of the polyurethane patch.

These findings, therefore, support the validity of the in vitro adherence assays, as well as the hypothesis that FnG is the primary ECM component that is responsible for promoting *S. aureus* adherence. Interestingly, ClfB also caused an increase in adherence to the polyurethane patch, although this adherence was significantly lower than that caused by ClfA.

The inherent capacity of different *S. aureus* MSCRAMMs to bind the same matrix proteins complicated the study of adhesin-receptor interactions. However, once the major adhesins were identified, it was possible to assess the effect that *S. aureus* mutants lacking these proteins had on adherence. Mutants lacking ClfA or both FnBPA and FnBPB did not cause a significant reduction in adherence, whereas mutants lacking all 3 proteins caused a substantial decrease in adherence. The failure to completely inhibit adherence suggested the possibility that other factors contribute to binding, as has been observed in the ability of *S. aureus* to induce aggregation of human platelets [16]. This hypothesis was confirmed with the use of an SrtA-deficient *S. aureus* strain—a mutant defective in the production of the enzyme responsible for anchoring MSCRAMMs into the staphylococcal cell wall [13]. We observed a substantial reduction in the binding levels that accounted for 67% of the parental binding; this suggested that other factors not linked to SrtA activity and therefore not belonging to the MSCRAMM family are also involved in the process. However, the increasing elution of these less-adherent strains from the VAD membrane after treatment with a high-ionic-strength solution suggested a relatively weaker binding interaction than that occurring with the wild-type strain.

Another important observation was the failure of *S. aureus* adhesins to mediate the attachment to VADs that had lengthy implantation times. Similar observations were recently reported in a clinical study showing a significant decrease over time in the rate of bacterial infections in patients implanted with HeartMate VADs—the same device that was used in the present study [3, 5]. These observations probably reflect changes in the composition of the neointimal lining on the surface of textured cardiac assist devices [8, 9, 26, 27]. Over time, the neointima becomes increasingly covered with cellular components, and the amount of ECM that is exposed to the bloodstream is reduced [8, 41]. This change in the cellular surface may account for the reduced bacterial adherence to the VAD membrane. The effect of cellular deposition/proliferation on prosthetic devices [24] and their role in potentially reducing susceptibility to infection has not been previously examined.

Several antibodies were used to further identify those epitopes, among the ligand-binding domains of the MSCRAMM proteins, involved in adherence to the VAD membrane. Blocking of the A domains of ClfA, FnBPA, and FnBPB caused a significant reduction in adherence. This reduction suggests that these domains played the major role in adherence to the VAD membrane, most likely by recognizing FnG/fibrin in the neointima. The amount of anti–rAFnBPA and anti–rAFnBPB $F(ab')_2$ fragments required to elicit a similar effect was higher than that needed for anti–rAClfA, perhaps echoing differences in the amount of exported proteins onto the lactococcal surface, although possibly merely reflecting differences in the affinity of the $F(ab')_2$ fragments for their corresponding epitopes. Simi-

larly, it has also been recently shown that the Fn-binding A domains of ClfA and FnBPA appear to be responsible for the first stage in the pathogenesis of *S. aureus*–associated endocarditis [34].

The findings of the present study demonstrate that the first steps leading to the development of VAD infections involve numerous adhesin-receptor interactions that overlap, and this process makes the development of therapeutic strategies a complex and intricate task. To our knowledge, our results provide the first direct information on the identity of these major bacterial factors and the likely host surface constituents—fibrin/FnG deposits—that serve as the main receptor on the host neointimal surface. The in vitro data demonstrate that antibodies directed at specific domains of MSCRAMMs reduce early in vitro adherence to the VAD membrane. These observations may lead to future studies to assess the potential of these components as targets for the development of new strategies for treatment and prevention of these increasingly encountered infections. The dynamic nature of such surfaces calls for further studies of their composition, structure, and adherent properties for staphylococcal factors. Finally, a more profound understanding of the biological processes that lie subjacent to these processes of adherence to and colonization of these highly dynamic structures seems essential for the development of additional prophylactic and therapeutic targets.

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