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Role of the *sar* Locus of *Staphylococcus aureus* in Induction of Endocarditis in Rabbits

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A regulatory locus on the *Staphylococcus aureus* chromosome, designated *sar*, is involved in the expression of cell wall proteins, some of which are potentially important in the pathogenesis of endocarditis. For instance, mutant 11D2 (*sar*::Tn*917*LTV1) was found to bind substantially less to matrix proteins (i.e., fibrinogen and fibronectin) than parent strain DB. Remarkably, these two strains did not differ in other phenotypes considered important in the initiation of endocarditis (e.g., binding to platelets and resistance to platelet-derived microbicidal proteins). The isogenic pair were compared for pathogenicity in a rabbit endocarditis model. There were significant differences in infectivity rates between the two strains (71 and 88% for DB versus 17 and 42% for mutant 11D2 at inocula of 10^3 and 10^4 CFU, respectively). In early adherence studies, parent DB adhered substantially better than the mutant to valvular vegetations at an inoculum of 10^6 CFU ($P = 0.05$). Southern blot analysis of colonies indicated that the location of the Tn*917*LTV1 insert in mutant 11D2 remained stable after animal passage. In vitro adherence assays revealed that mutant 11D2 was less adherent to cultured human endothelium than parent DB. These studies suggest that the *sar* locus is involved in the initial adherence of *S. aureus* to the fibrin-platelet-endothelium matrix on damaged valvular endothelium.

Staphylococcus aureus continues to be a major pathogen in both acute and subacute infective endocarditis (2, 17). Despite the use of newer antimicrobial agents to treat these infections, the morbidity and mortality rates remain high (17). Permanent valvular damage often remains in patients who survive and thereby predisposes them to recurrent endocarditis and other cardiovascular complications (e.g., congestive heart failure). In addition, the recent emergence of increased antibiotic resistance among staphylococcal strains has made many currently available antibiotics ineffective and is beginning to pose major public health problems (11, 27). Thus, there is an urgent need for alternative approaches in the treatment of serious *S. aureus* infections.

One effective mechanism for counteracting the emergence of antibiotic resistance among *S. aureus* strains is the development of safe and effective vaccines with cell wall immunogens. However, experimental data justifying a direct vaccine strategy are lacking to date. Alternatively, knowledge of the genetic control apparatus, gene products, and their phenotypic consequences on virulence determinants in *S. aureus* may allow the rational design of novel antimicrobial agents to control this microorganism (26).

It has been shown that *S. aureus* possesses at least three distinct regulatory elements. One well-described regulatory locus, *agr* (29), controls the production of extracellular and cell wall proteins involved in *S. aureus* virulence (e.g., alpha and beta toxins, toxic shock syndrome toxin 1, coagulase, protein A, and staphylococcal enterotoxins B and C). A second locus, designated *xpr*, which was recently identified by Tn*551* insertional mutagenesis, also appears to regulate exoprotein synthesis (35). A mutation in either locus reduces animal pathogenicity in a murine sepsis model when the organism is tested intraperitoneally at a very high inoculum (10^9 CFU) (35). Using plasmid pLTV1 as a transposon delivery vehicle for Tn*917*LTV1, we identified a third locus on the *S. aureus* chromosome, designated *sar*, for staphylococcal accessory regulator, that is involved in the global regulation of extracellular and cell wall proteins (6). Detailed phenotypic characterization and Southern blot analysis with Tn*917* and *agr* probes indicated that this locus is distinct from *agr* and *xpr* (6, 31, 35). The location of the Tn*917*LTV1 insert in the staphylococcal chromosome has been mapped to the *Sma*I-D fragment, a region distinct from *agr* and *xpr* (9). Inactivation of the *sar* locus as a result of a single Tn*917*LTV1 insertion into the staphylococcal chromosome has resulted in the reduced expression of selected cell wall proteins in the mutant (6). Some of these cell wall proteins (e.g., fibrinogen- and fibronectin-binding proteins) have been shown to be important staphylococcal adhesins for human endothelium and appear to play a role in initiating endovascular infection in experimental animal systems (8, 24, 33).

To evaluate the in vivo biological significance of this locus in endocarditis, the prototypic endovascular infection, both *sar* mutant 11D2 and wild-type parent DB were evaluated for their ability to induce infection in a rabbit model of catheter-induced endocarditis (16, 30). At low challenge inocula (10^3 and 10^4 CFU) that mimic the level of bacteremia in human endocarditis, the *sar* mutant appeared to be less virulent than the parent. Early adherence studies conducted at 30 min after intravenous (i.v.) bacterial injection revealed that the parent strain adhered substantially better to valvular vegetations than the isogenic *sar* mutant strain. The results of this study suggest that an intact *sar* locus probably plays a role in initial adherence in the pathogenesis of endocarditis.

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MATERIALS AND METHODS

Preparation of the bacterial strains. Parent strain DB, a wild-type *S. aureus* blood isolate (3, 4), and strain 11D2, an erythromycin-resistant *sar* mutant derived from DB and containing a *sar*::Tn*917*LTV1 mutation, have been described elsewhere (6). Both strains were grown in brain heart infusion broth (Difco, Detroit, Mich.) at 37°C overnight with rotation (30 rpm). In preliminary studies, it was determined that the growth rates of strains DB and 11D2 in the absence of antibiotics were almost identical over a 24-h period. The inclusion of 50% rabbit serum in the growth medium (to mimic in vivo conditions) resulted in growth curves that were equivalent to those observed with medium alone for both strains. For some experiments with *sar* mutant 11D2, erythromycin (10 μg/ml) was added to the overnight growth medium. The overnight culture was harvested by centrifugation (2,000 × *g* for 10 min), washed twice in sterile normal saline solution (NSS), and resuspended in NSS to an optical density of 1.6 at 620 nm (≈10^9 CFU/ml). This bacterial suspension was serially diluted in NSS to ≈10^3 to 10^5 CFU/ml. Each dilution was confirmed by quantitative culturing on brain heart infusion agar plates.

Comparisons of strains DB and 11D2 for other phenotypic characteristics involved in endocarditis pathogenesis. To determine whether the isogenic pair differed in other phenotypic traits likely to be important in endocarditis pathogenesis, we studied the comparative in vitro abilities of strains DB and 11D2 to (i) adhere to and aggregate platelets (22, 34) and (ii) resist the bactericidal action of platelet-derived microbicidal proteins (PMP) (40).

(i) Bacterial binding to platelets. Two assays of direct bacterial adherence to platelets were carried out. In the first method, platelet ghosts (≈2.5 × 10^8/ml) were obtained from rabbit platelet-rich plasma (PRP) by allowing platelets to outdate for 5 to 8 days at 4°C as described by Herzberg et al. (22). Platelet ghosts were pelleted by centrifugation for 10 min at 2,000 × *g*, washed twice in 0.02 M Tris-HCl with 1% EDTA (pH 7.25) and then with phosphate-buffered saline (PBS), resuspended to an optical density at 620 nm of 0.85 in PBS (~5 × 10^8 platelets per ml), and stored at 4°C. The responsiveness of these senescent platelet ghosts was verified by calcium stimulation, which reconstituted ADP-induced aggregation in platelet-free plasma. Equal volumes (100 μl) of *S. aureus* DB or 11D2 along with platelet ghosts were mixed in V-well microtiter plates (Corning Glass Works, Corning, N.Y.) to achieve a final *S. aureus*/platelet ghost ratio of 10:1. Wells containing suspensions of either bacteria or platelets alone served as controls. Following incubation for 30 min at 4°C, the plates were centrifuged at 55 × *g* for 5 min at 4°C to pellet only bacterium-platelet complexes. Supernatants in 100-μl aliquots from individual wells were diluted 1:20 with PBS and assayed spectrophotometrically at an optical density at 700 nm. The percentage of bacterium-platelet adherence was calculated as previously described (22). Each experiment was repeated at least three times.

In the second method, we measured bacterium-platelet adherence by fluorescence-activated cell sorting (FACS). In brief, rabbit whole blood was collected in polypropylene tubes containing sodium citrate (5:1) and 1 μg of prostaglandin E_1 (Sigma) per ml to mitigate platelet activation. Platelets were harvested by centrifugation, washed as described above, and labeled for 1 h with 25 μg of 5-(4,6-dichloro-triazinyl)-aminofluorescein (DTAF; Molecular Probes, Eugene, Oreg.) per ml in PBS (pH 7.0). Similarly, bacterial cells grown overnight were washed in Tris-EDTA-NaCl buffer (0.05 M Tris-HCl with 0.1 M NaCl and 0.02 M EDTA [pH 7.2]) and labeled for 3 h with Hoechst 33342 dye (25 μg/ml; Polysciences, Warrington, Pa.), a DNA-binding fluorochrome. Adherence studies were performed at 20°C by mixing bacteria and platelets in Tyrode's salts solution (Sigma) at a bacterium/platelet ratio of 10:1. One minute after mixing, the suspensions were analyzed with a FACS IV or a FACStar^{Plus} cytofluorograph (each from Becton-Dickinson, San Jose, Calif.) and predetermined combinations of argon lasers, excitatory wavelengths, and appropriate filters (40). The percentage of bacteria adhering to platelets was determined as previously described (40).

For FACS studies examining the effects of thrombin activation on platelet binding, rabbit blood was collected (except that prostaglandin E_1 was omitted) and labeled with DTAF as described above. One minute prior to testing by FACS, DTAF-labeled platelets were exposed to thrombin (1 U/ml; Sigma); previous studies confirmed that this concentration of thrombin induces platelet activation, as evidenced by serotonin secretion (10).

(ii) Platelet aggregation. The relative abilities of strains DB and 11D2 to aggregate platelets was determined by platelet aggregometry as previously described with a dual-channel platelet aggregometer (Peyton Associates, Buffalo, N.Y.) attached to a chart recorder (38). *S. aureus* cells in PBS were added to prewarmed (37°C) PRP in a siliconized microcuvette to achieve a final bacterium/platelet ratio of 10:1 (38) and stirred at 900 rpm. Light transmission was then recorded until platelet aggregation was complete, as indicated by chart recorder equilibrium (40). Control platelet aggregation was produced by the addition of 25 μl of 2 × 10^{-5} M ADP reagent (Sigma) to 250 μl of PRP samples.

(iii) Susceptibility to PMP. PMP were prepared from thrombin-stimulated PRP preparations and assayed for bioactivity (in units per milliliter) as previously described (39). PMP were added to bacterial suspensions of DB or 11D2 in low-level protein-binding microtiter plates (Corning) to achieve a final PMP concentration of either 100 or 200 U/ml and a final bacterial inoculum of ~10^3 CFU/ml. Following 2 h of incubation at 37°C, 20-μl aliquots were processed for quantitative culturing as described previously (39). The percentage of bacterial survival was calculated for each *S. aureus* strain. *Bacillus subtilis* (ATCC 6633), a highly PMP-susceptible strain (13), served as a positive control for PMP bioactivity, while suspensions of strains DB and 11D2 in minimal essential medium without PMP served as negative controls.

Animal model of endocarditis. Endocarditis on the aortic valve of New Zealand White rabbits (2 to 2.5 kg) was induced by a modification of the method of Durack and Beeson (16). In brief, rabbits were anesthetized by intramuscular injection of ketamine chloride (Aveco Inc., Fort Dodge, Iowa) at 35 mg/kg of body weight xylazine (Mobay Corp., Shawnee, Kans.) at 1.5 mg/kg. A polyethylene catheter with an internal diameter of 0.86 mm (Becton Dickinson, Cockeysville, Md.) was introduced into the left ventricle via the right carotid artery to produce sterile thrombotic vegetations on the aortic valve. Correct placement was denoted by catheter pulsation. To induce endocarditis, groups of about 10 to 15 animals each were challenged i.v. at 48 h postcatheterization with various inocula of either strain DB or strain 11D2. Catheters remained in place until animals were sacrificed by lethal injection of sodium pentobarbital (100 mg/kg) at 48 h after bacterial challenge. Animals with macroscopic valvular vegetations and proper catheter placement were analyzed for data in this study. At the time of sacrifice, aortic valve and left ventricular vegetations from individual animals were removed, pooled, weighed, homogenized in 0.5 ml of NSS, and quantitatively

cultured in either antibiotic-free medium (for strain DB) or erythromycin-containing medium (for strain 11D2). To detect possible reversion of the *sar* genotype as a result of transposon loss during animal passage, vegetation homogenates from animals infected with mutant 11D2 were also plated on antibiotic-free medium. Animals with negative cultures of undiluted vegetation homogenates were considered to have had no induction of endocarditis at a particular inoculum.

To determine the relative abilities of strains DB and 11D2 to adhere to sterile endocardial vegetations early after i.v. challenge, separate groups of six to eight rabbits each were challenged i.v. at 48 h after catheterization with 10^4, 10^5, or 10^6 CFU of either strain. At 30 min after bacterial injection, groups of animals were sacrificed, after which the hearts were opened and all visible left-sided vegetations from each animal were excised and pooled. Each vegetation pool was washed gently with PBS to remove any surface blood and nonadherent bacterial cells, placed in 0.5 ml of PBS, and homogenized in a tissue grinder. To account for any bacterial cells that might have adhered to the tissue grinder or tube during homogenization, the apparatus were washed with 1 ml of PBS. The vegetation homogenate and the washes from each animal were quantitatively cultured on either erythromycin-containing (for strain 11D2) or antibiotic-free (for strain DB) brain heart infusion agar by the method of Dankert (12).

To ensure that any observed differences in valvular adherence between strains DB and 11D2 were not due to a disparity in the levels of early bacteremia, quantitative blood culturing was done for samples obtained at 1 and 30 min after i.v. inoculation with DB or 11D2 at 10^4, 10^5, and 10^6 CFU.

Southern blot hybridization. To ensure retention of the Tn*917*LTV1 insertion at the *sar* locus after animal passage, Southern blot hybridization was performed with randomly primed samples of gel-purified *Hin*dIII fragments of Tn*917* (6) as probes (18, 25). In brief, chromosomal DNA from 11D2 colonies isolated from valvular vegetations was prepared as previous described (6). Following digestion with restriction enzymes, chromosomal fragments were resolved on a 0.7% TBE gel and transferred to a Hybond-N^+ membrane (Amersham, Arlington Heights, Ill.) according to the manufacturer's instructions (25). DNA probes were labeled with ^{32}P ([α-^{32}P]dCTP; Amersham) by use of random-primer DNA labeling kits (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The membrane was then hybridized with the labeled DNA probes at 65°C overnight and washed twice with 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]) containing 0.1% sodium dodecyl sulfate (SDS) at room temperature for 10 min each time and once with 1× SSPE containing 0.1% SDS at 65°C for 15 min. The membrane was subjected to autoradiography with an intensifying screen at −70°C.

Assay for adherence to cultured HUVEC. The adherence of strains DB and 11D2 to confluent monolayers of human umbilical vein endothelial cells (HUVEC) was assayed in 96-well microtiter plates as previously described (7, 8). HUVEC were cultured in medium 199 (M199) supplemented with 16% fetal calf serum, 4% human serum, endothelial cell growth factor, and heparin as described previously (8). For assays involving cytokine-stimulated endothelium, the culture medium (200 μl per well) in quadruplicate wells was aspirated 4 h prior to the assays and replaced with an equal volume of either culture medium containing recombinant human tumor necrosis factor alpha (TNF-α) at 250 U/ml (Chiron Corp., Emeryville, Calif.) or culture medium alone as a control (7). After determination of the number of endothelial cells in each well (8), the monolayers were washed twice with M199, lightly fixed with 0.05% glutaraldehyde in M199 for 3 min, and washed twice with M199 again to remove residual glutaraldehyde. Monolayers fixed in this manner did not detach during the ensuing washing procedure and retained bacterium-binding capacity approaching 50 to 75% that of untreated controls (8). The fixed HUVEC were then preincubated with 35 μl of plasma for 30 min at 37°C with rotation (200 rpm) and rinsed twice with M199 to remove excess plasma components. Parent DB or mutant 11D2 labeled with [*methyl*-^3H]thymidine as described previously (5) (≈10^7 CFU in 35 μl of M199) was added to monolayers in quadruplicate wells. A tritium-labeled *Staphylococcus epidermidis* isolate, strain 6937 (8), was used as a negative control. After incubation for 1 h at 37°C with agitation (200 rpm) in a microplate shaker (Dynatech, Chantilly, Va.), the monolayers were washed three times with M199 and lysed with 200 μl of 2.5% SDS in 0.2 M NaOH. Portions of the lysate were transferred to scintillation vials, neutralized with 0.05 M acetic acid, and counted for radioactivity. The number of adherent bacteria was derived from a standard curve of CFU versus radioactivity (5).

Statistical analysis. The Fisher exact test was used to compare the rates of induction of endocarditis by the two challenge strains. The relative abilities of the isogenic strains to adhere to platelets in vitro and to endocardial vegetations in vivo were statistically compared by the two-tailed Wilcoxon rank-sum test. Levels of bacteremia achievable by each strain were similarly compared. The Student *t* test was used to compare the adherence of strains DB and 11D2 to HUVEC.

RESULTS

Comparisons of strain DB and *sar* mutant strain 11D2 for phenotypic characteristics involved in endocarditis pathogenesis. In previous studies, we demonstrated that strain DB has a higher capacity than strain 11D2 to bind in vitro to extracellular matrix proteins, such as fibrinogen and fibronectin (6). We also compared in the isogenic pair other phenotypic traits that have been postulated to be important in the pathogenesis of endocarditis, including the ability to adhere to and aggregate platelets and resistance to the bactericidal action of PMP (22, 39).

For strains DB and 11D2, the abilities to aggregate platelets in PRP were equivalent. The times required to complete platelet aggregation in PRP were essentially identical (≈3 min; data not shown). Analysis of platelet binding by FACS demonstrated no significant differences between the two strains in the percentages of bacteria bound, irrespective of whether platelets were resting or thrombin activated prior to being mixed with bacteria: for nonactivated and thrombin-activated platelets (11 samples each), the percentages of bacteria bound (mean ± standard error of the mean) were 72 ± 16.8 and 68 ± 16 for strain DB and 56 ± 30.1 and 73 ± 13.5 for strain 11D2, respectively. Similarly, strains DB and 11D2 exhibited equivalent levels of adherence to platelets in the microtiter assay (≈30% of the initial inoculum).

We and others have shown that the ability of viridans group streptococci and staphylococci to induce bacterial endocarditis may be substantially influenced by the innate resistance of different strains to the killing action of PMP (12, 38). Strains DB and 11D2 exhibited very similar susceptibilities to the killing action of PMP. After exposure to PMP at 100 and 200 U/ml for 120 min, the mean levels of bacterial survival were 71 and 40%, respectively, for strain DB, and 66 and 44%, respectively, for strain 11D2. The susceptibilities of the two strains to PMP at 100 and 200 U/ml were also nearly identical following overnight growth of the strains in the presence of

TABLE 1. Induction of endocarditis in a rabbit model with parent DB and *sar* mutant 11D2[a]

Expt	Challenge inoculum (CFU)	No. of animals with endocarditis/total no. tested with strain:		*P* value
		DB	11D2	
1	10^3	7/9	2/12	0.008
	10^4	8/9	5/12	0.037
	10^5	12/12	10/12	NS
2	10^3	3/5	0/12	0.015

[a] At 48 h postcatheterization, bacteria were injected directly into the marginal ear veins. All rabbits were sacrificed for morphological examination and valvular cultures at 48 h after bacterial challenge. In experiments 1 and 2, the strains were grown in erythromycin-containing and antibiotic-free media, respectively. *P* values were determined by the Fisher exact test. NS, not significant.

50% rabbit serum (data not shown). The addition of erythromycin to the growth medium prior to the assay with PMP did not affect the rate of survival of strain 11D2.

Induction of endocarditis. In pilot studies, we determined that the 90% infective dose of parent DB in the rabbit endocarditis model was between 10^4 and 10^5 CFU. The rates of induction of endocarditis with parent DB and *sar* mutant 11D2 (grown in the presence of erythromycin) were then evaluated at various challenge inocula between 10^3 and 10^5 CFU, thereby encompassing the 90% infective dose of the parent. At the two smaller challenge inocula ($\approx 10^3$ and 10^4 CFU), strain DB caused substantially higher rates of induction of endocarditis than strain 11D2 (71 and 88% versus 17 and 42% [$P \leq 0.008$ and $P \leq 0.037$], respectively) (Table 1, experiment 1). In these two groups of animals, bacterial densities in culture-positive vegetations were higher with strain DB than with strain 11D2 (the mean $\log_{10}$ CFU per gram of tissue were 3.41 versus 2.6 and 4.79 versus 3.9 at inocula of 10^3 and 10^4 CFU for strains DB and 11D2, respectively), although these differences did not reach statistical significance. At the higher challenge inoculum ($\approx 10^5$ CFU), both strains induced endocarditis in nearly all catheterized animals. Notably, the number of 11D2 colonies from vegetation homogenates plated on erythromycin-containing medium was similar to that found on antibiotic-free medium, suggesting the preservation of transposon-mediated antibiotic resistance during animal passage.

To control for any possible effects of prechallenge antibiotic exposure on the subsequent ability of mutant 11D2 to induce experimental endocarditis, we also challenged two parallel groups of catheterized animals in a separate experiment with 10^3 CFU of strains DB and 11D2 grown in erythromycin-free medium. Pilot studies in our laboratory revealed that a single passage of 11D2 in antibiotic-free medium did not result in the loss of the *sar* genotype (unpublished observation). As shown in Table 1, experiment 2, a higher rate of induction was seen with parent DB than with mutant 11D2. These data are similar to those obtained with strain 11D2 grown in erythromycin-containing medium. Thus, the lower rates of endocarditis induction with 11D2 at smaller challenge inocula were not related to the presence of erythromycin in the growth medium.

Determination of early adherence to sterile vegetations. Figure 1 shows the absolute number of colonies of strain DB versus strain 11D2 adherent to aortic valve vegetations at 30 min after i.v. challenges with 10^4, 10^5, or 10^6 CFU. At the smallest inoculum (10^4 CFU), the number of adherent bacteria for either strain was too low to make a meaningful comparison. However, at the larger inocula (10^5 and 10^6 CFU), the level of

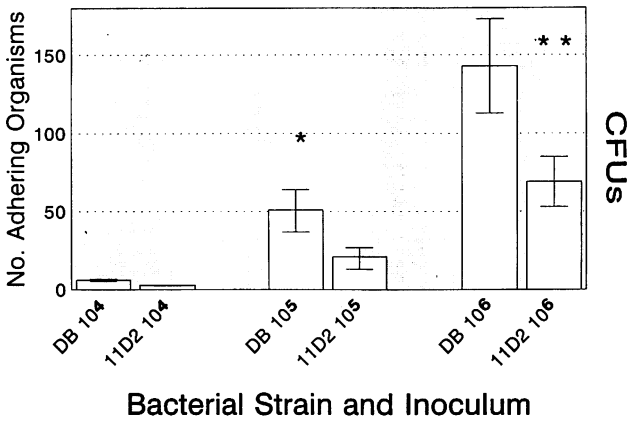

FIG. 1. Relative levels of adherence of strains DB and 11D2 to aortic valve vegetations, given as the total number of organisms adhering at 30 min after i.v. challenge. Each bar represents the mean (± standard error of the mean) value for six to eight animals in each bacterial strain group. *, $P = 0.06$; **, $P = 0.05$.

adherence of strain DB was higher than that of strain 11D2. For example, at 10^6 CFU, a mean of 143 colonies of DB adhered to valvular vegetations, compared with 69 colonies of 11D2 ($P = 0.05$).

To ensure that the differences in early valvular adherence were not related to the level of bacteremia following i.v. challenge with strain DB or 11D2, quantitative blood culturing was done at 1 and 30 min after i.v. challenge. No significant differences in the frequency or quantitative counts of postchallenge bacteremia were observed between the two groups at 1 min after i.v. challenge (Fig. 2). At 30 min after i.v. challenge, the extent or frequency of bacteremia also did not differ between the two groups. For instance, at 10^4 CFU, all blood cultures were sterile at 30 min postchallenge. At 10^5 CFU, 8 of 12 and 5 of 12 blood cultures were positive for strains DB and 11D2, respectively ($P = 0.20$), while the quantitative counts in the animals in these groups were invariably <4 CFU/ml. At 10^6 CFU, most blood cultures were positive; however, the quantitative counts were low and did not differ significantly between

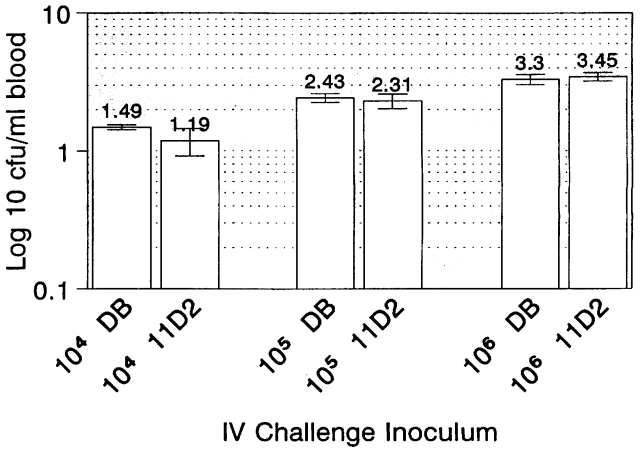

FIG. 2. Levels of bacteremia at 1 min after i.v. challenge in the various bacterial strain groups. Each bar represents the mean (± standard error of the mean) value for six to eight animals per bacterial strain group.

TABLE 2. Adherence of strain DB and *sar* strain 11D2 to normal and TNF-α-stimulated cultured HUVEC[a]

Expt	Pretreatment of HUVEC	No. of CFU[b] of the following strain bound/ HUVEC:	
		DB	11D2
1	M199	2.0 ± 0.2	1.1 ± 0.2
	Plasma	129 ± 9.0	36[c] ± 5.0
2	M199 (stimulation with TNF-α)	4.6 ± 0.2	3.3 ± 0.4
	Plasma (stimulation with TNF-α)	131 ± 27	49[d] ± 18

[a] Following mild fixation, endothelial cell monolayers in microtiter wells were preincubated with either M199 or human plasma, washed, and incubated with tritium-labeled bacteria. After incubation, the endothelial cells were washed with M199. The number of adherent bacteria was determined from a standard curve of CFU versus radioactivity.

[b] Mean ± standard error of the mean (n = 4).

[c] Statistically significant in comparison with the adherence of strain DB to plasma-treated endothelium ($P < 0.001$).

[d] Statistically significant in comparison with the adherence of strain DB to TNF-α-stimulated endothelium ($P < 0.049$).

the two challenge groups, ranging from 1 to 5 CFU/ml of blood.

Adherence to cultured HUVEC. To confirm the in vivo finding that the lower rates of endocarditis induction with *sar* mutant 11D2 were probably attributable to decreased valvular adherence, bacterial adherence assays with cultured HUVEC were also performed. The results demonstrated that in the presence of plasma, *sar* mutant 11D2 was markedly less adherent to cultured HUVEC than parent DB, while the difference was not significant in the M199 control (Table 2, experiment 1). In a separate experiment (Table 2, experiment 2), the attachment of mutant 11D2 to HUVEC that had been stimulated with recombinant human TNF-α was also diminished in the presence of plasma when compared with that of parent DB, thus implying that the initial level of adherence to valvular endothelium may be reduced in the *sar* mutant.

Southern blot analysis of 11D2 colonies isolated from valvular vegetations. To verify the stability of the Tn*917*LTV1 insertion in mutant 11D2 after animal passage, chromosomal DNA of 11D2 colonies isolated from valvular tissues was digested with either *Eco*RI or *Nco*I and probed with a ^{32}P-labeled 1.5-kb *Hin*dIII fragment internal to transposon Tn*917* (6). As this *Hin*dIII fragment encompassed a single internal *Nco*I site within the Tn*917* region of transposon Tn*917*LTV1, it resulted in two hybridizing *Nco*I fragments (Fig. 3A) (6). The 6-kb hybridizing *Nco*I fragment is internal to transposon Tn*917*LTV1, while the larger fragment is composed of both the transposon and a flanking chromosomal sequence (6). As expected, the presence of a unique *Eco*RI site within the transposon (but outside the probe) yielded a single hybridizing *Eco*RI fragment that comprises part of the transposon and an adjacent chromosomal fragment (Fig. 3B). Notably, these hybridizing fragments corresponded to those of 11D2 before the in vivo challenge. These results indicated that the location of the Tn*917*LTV1 insertion in the host chromosome remained unchanged after animal passage. As a negative control, restriction fragments from parent DB did not hybridize to the Tn*917* probe.

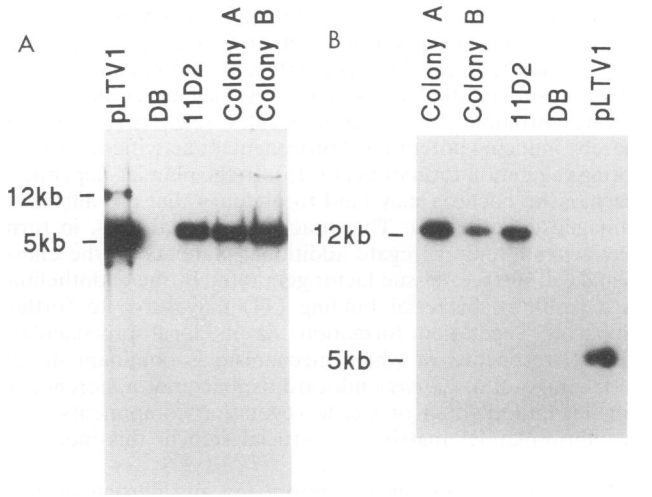

FIG. 3. Southern blots of chromosomal DNA of parent DB, mutant 11D2, and colonies isolated from valvular vegetations. The DNA was digested with *Nco*I (A) or *Eco*RI (B) and probed with a ^{32}P-labeled 1.5-kb *Hin*dIII fragment internal to transposon Tn*917*. The plasmid pLTV1 control was digested with *Eco*RI in both of these blots. Four other colonies were also analyzed, and identical hybridization results were obtained (data not shown).

DISCUSSION

Microbial pathogenicity is a complex process that involves the products of many genes in a series of pathogenic steps. Many of these gene products are virulence factors that are regulated by global regulatory systems. The *sar* and *agr* loci in *S. aureus* are examples of two such regulatory systems (6, 23). However, the multifactorial nature of pathogenicity implies that targeting a single pleiotropic regulator of virulence factors for the development of new therapeutic modalities may be more encompassing and revealing than studying a specific virulence determinant. The recent finding that the *sar* locus controls the expression of several potential virulence determinants in *S. aureus*, including fibrinogen- and fibronectin-binding proteins (6), implies that it may be possible to target a regulatory locus for therapeutic intervention. However, the correlation of in vitro observations with experimental infections needs to be confirmed to justify such an approach. The present study was thus designed to examine the role of the *sar* locus in the pathogenesis of infective endocarditis, the prototypic endovascular infection.

Several interesting observations emanated from the induction study. Our results indicated that the functional integrity of the *sar* locus appears to be important in the induction of endocarditis, as evidenced by the diminished ability of the *sar* mutant strain to induce endovascular infections at the two smaller challenge inocula (≈10^3 and 10^4 CFU) in comparison with the parent strain. In addition, we confirmed a dose-response relationship between inoculum size and rates of induction of experimental endocarditis in our evaluation of the *sar* mutant (Table 2). More importantly, the challenge inocula at which the *sar* mutant exhibited diminished virulence are consistent with the level of bacteremia often seen in human endocarditis (32). Because of this finding and the similarity in pathology between the rabbit model (16, 30) and subacute human endocarditis, it is likely that our results for the role of the *sar* locus in the induction of rabbit endocarditis may be applicable to similar infections in humans.

The process of induction of endocarditis may occur via two interfacing pathogenic mechanisms, one being platelet independent and the other being platelet dependent. In the proposed platelet-independent mechanism, *S. aureus* cells ad-

hering directly to valvular endothelium are phagocytized by endothelial cells, in which they remain in a nonreplicative but viable state (21, 28, 36). The infected endothelial cells or adjacent subendothelial stromal cells may be stimulated to secrete thrombin-generating molecules (e.g., tissue factor), thereby inducing potent local procoagulant activities and facilitating vegetation formation (14, 15). In the platelet-dependent mechanism, bacteria may bind to platelets that are affixed to damaged endothelium. The attached bacterial cells, in turn, may activate and aggregate additional platelets on the endothelial cell surface. Tissue factor generated by the endothelium as a result of bacterial binding (14) may serve to further propagate vegetation formation via its local procoagulant effect. Irrespective of which mechanism is dominant in the initial stage of *S. aureus* endocarditis, bacterial adherence to valvular endothelium or sterile vegetation components (e.g., the fibrin-platelet matrix) is a crucial step in this induction process.

As matrix proteins such as fibrinogen and fibronectin may mediate bacterial adhesion to the fibrin-platelet matrix found on damaged endothelium (8, 24, 33), the possibility that perturbation of early valve adherence occurs as a result of the *sar* mutation seems reasonable to explore, since the level of binding of mutant 11D2 in vitro to these matrix proteins was substantially lower than that of the parent (6). Our adherence studies demonstrated that *sar* mutant 11D2 was less adherent to valvular vegetations early after an i.v. challenge than parental DB. The difference in adherence between parent DB and mutant 11D2 did not reach statistical significance until a larger inoculum (10^6 CFU) was used. This result is probably attributable to the finding that at smaller inocula (10^4 and 10^5 CFU), the number of adherent organisms was too small to achieve meaningful statistical comparisons. Notably, the difference in early vegetation adherence between strains DB and 11D2 could not be explained by a difference in the extent of bacteremia at both 1 min (Fig. 2) and 30 min after bacterial challenge. The enhanced ability of *sar*$^+$ parent DB (as opposed to *sar* mutant 11D2) to bind to fibrinogen and fibronectin (6) and TNF-α-activated endothelium in vitro (Table 2, experiment 2) appears to support the concept that a functional *sar* locus is important in the intrinsic binding of *S. aureus* to the fibrin-platelet matrix found on damaged valvular endothelium.

In comparing the data in Table 2 and Fig. 1, it is evident that very few adhering microorganisms (less than 10) are necessary for the effective induction of endocarditis during early bacteremia. For instance, at inocula of 10^3 and 10^4 CFU, less than 10 CFU of the parent strain were found to adhere, while the induction rates at these inocula were quite high (seven of nine and eight of nine rabbits, respectively) (see Results). As the level of bacteremia in human endocarditis is often in the range of 10^2 to 10^3 CFU/ml (32), our data seem to suggest that a malfunctioning *sar* locus is likely to further reduce the already low number of adherent microorganisms, thereby leading to a significant reduction in the rates of endocarditis induction (see Results).

To further delineate the role of the *sar* locus in the induction of endocarditis, we also evaluated other potentially important phenotypic characteristics of the isogenic strain pair with regard to endocarditis pathogenesis, including growth kinetics in the presence of serum proteins, platelet surface binding, platelet aggregation, and susceptibility to PMP. Interestingly, each of these phenotypic traits was found to be virtually identical between the *sar*$^+$ DB strain and the *sar* 11D2 strain. Nevertheless, the role of differential binding of the two strains to platelets on valvular vegetations cannot be entirely ruled out because *sar*$^+$ strain DB, with intact fibrinogen- and fibronectin-binding regions, can readily attach to platelets via fibrinogen bound to the GpIIb/IIIa receptor (21).

It should be noted that although a mutation in the *sar* locus resulted in a substantial diminution of both valvular adherence and the induction of endocarditis, binding of the *sar* mutant to sterile vegetations was not completely eliminated. This result suggests that the induction process is likely to be multifactorial and may require as-yet-unidentified ligand-receptor interactions. Alternatively, additional regulatory loci may send counterregulatory signals to promote adherence via fibrinogen and fibronectin. In particular, the phenotypic properties of intact *agr* (23) and *xpr* (35) loci in the presence of a *sar* mutation are not known. Studies of these properties are presently being pursued in our laboratory.

Although previous studies showed that the in vitro growth rates of strains DB and 11D2 were comparable in the absence of antibiotics (6), the lack of statistically significant differences in bacterial vegetation densities between culture-positive animals in each group at 48 h after bacterial challenge did not provide discriminatory data on the role of the *sar* locus in intravegetation bacterial proliferation following initial adherence. The reason is that factors that influence postinduction growth rates within cardiac vegetations are likely to be complex and multifactorial and involve not only microbial evasion strategies (e.g., hemolysins and leukocidins) (37) but also host defense mechanisms (including platelet- and neutrophil-released factors) (1, 38).

Other than antimicrobial prophylaxis for selected groups of surgical patients, no successful strategy has been developed for the prevention of *S. aureus* endocarditis. In a study done by Greenberg et al., whole-cell-induced *S. aureus* antibody did not prevent or modify any stage in the development of rabbit endocarditis caused by the homologous *S. aureus* strain (20). Similarly, no protective efficacy was observed with anti-protein A antibody against an *S. aureus* bacteremic infection in an infant rat model (19). To our knowledge, this is the first study that implicates an *S. aureus* regulatory locus in the induction of endocarditis in a rabbit model. Our data also seem to indicate that a functional *sar* locus is implicated in the initiation of bacterial adherence to damaged valvular tissues during staphylococcal bacteremia. Work is currently under way to construct a more specific site-directed deletion mutation within the *sar* locus to further dissect the role of *sar* in the endocarditis model. As adherence always precedes tissue colonization and invasion, a detailed understanding of the *sar* gene product and its mechanism of control will allow us to target specific areas in regulatory pathways which may be amenable to therapeutic interventions in our quest to control this virulent pathogen.

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