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Research Article

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Diminished Virulence of a *sar*⁻/*agr*⁻ Mutant of *Staphylococcus aureus* in the Rabbit Model of Endocarditis

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Abstract

Microbial pathogenicity in *Staphylococcus aureus* is a complex process involving a number of virulence genes that are regulated by global regulatory systems including *sar* and *agr*. To evaluate the roles of these two loci in virulence, we constructed *sar*⁻/*agr*⁻ mutants of strains RN6390 and RN450 and compared their phenotypic profiles to the corresponding single *sar*⁻ and *agr*⁻ mutants and parents. The secretion of all hemolysins was absent in the *sar*⁻/*agr*⁻ mutants while residual β -hemolysin activity remained in single *agr*⁻ mutants. The fibronectin binding capacity was significantly diminished in both single *sar*⁻ mutants and double mutants when compared with parents while the reduction in fibrinogen binding capacity in the double mutants was modest. In the rabbit endocarditis model, there was a significant decrease in both infectivity rates and intravegetation bacterial densities with the double mutant as compared to the parent (RN6390) at 10^3 – 10^6 CFU inocula despite comparable levels of early bacteremia among various challenge groups. Notably, fewer bacteria in the double mutant group adhered to valvular vegetations at 30 min after challenge (10^6 CFU) than the parent group. These studies suggest that both the *sar* and *agr* loci are involved in initial valvular adherence, intravegetation persistence and multiplication of *S. aureus* in endocarditis. (*J. Clin. Invest.* 1994. 94:1815–1822.) Key words: *Staphylococcus aureus* • regulation of gene expression • endocarditis • bacterial adhesion • staphylococcal infections

Introduction

Septicemia due to *Staphylococcus aureus* is often the consequence of a local infection that has gained access to the bloodstream (1). Once the bacteria enter the bloodstream, patients are at risk of developing endocarditis and other life-threatening metastatic complications (1). Although the use of newer antimicrobial agents has initially controlled some of these infections, the recent emergence of multiple-drug resistance in *S. aureus*

has made many currently available antimicrobial agents (including fluoroquinolones) ineffective, thereby posing an important public health problem (2, 3). Thus, there is an urgent need for alternative approaches in the treatment of *S. aureus* infections.

One potentially effective mechanism for dealing with the emergence of antibiotic resistance among *S. aureus* strains is the development of safe and effective vaccines. However, experimental data justifying a direct vaccine strategy are presently lacking. An alternative approach is to consider targeting regulatory loci that are involved in the control and expression of potential virulence determinants (e.g., hemolysins and fibrinogen and fibronectin binding proteins) (4–7). From this perspective, an understanding of the genetic control apparatus and their regulatory pathways may allow the design of novel antimicrobial or pharmacologic agents to control this virulent pathogen (8).

Postexponential phase expression in bacteria is generally governed by global regulatory systems in which a common regulator controls the activities of several unlinked genes (9, 10). Many exoproteins normally synthesized and secreted during the postexponential phase in *S. aureus* are virulence factors (4). In contrast, synthesis of a number of surface proteins (e.g., protein A, coagulase, and fibronectin binding proteins) that clearly play a role in staphylococcal infections is repressed postexponentially (4, 9). The regulation of virulence determinants and other exoprotein genes in *S. aureus* involves at least three global regulatory systems including *agr*, *xpr*, and *sar* (9, 11, 12). The *agr* locus controls production of extracellular and cell wall proteins that appear to play a role in virulence (e.g., hemolysins, coagulase, and protein A). The *xpr* locus, like that of *agr*, also positively regulates exoprotein synthesis (11). Smeltzer et al. (11) reported that two sets of *agr*⁻ and *xpr*⁻ mutants with different genetic backgrounds were less lethal than their corresponding parents in a mouse peritonitis model; however, a very high challenging inoculum (10^9 – 10^{10} CFU) was required for lethality in this study.

We recently identified by Tn917LTV1 insertional mutagenesis an additional regulatory locus, designated *sar*, in *S. aureus* (12). Inactivation of this locus has led to decreased expression of several exoproteins (hemolysins) and cell wall proteins (fibrinogen and fibronectin binding protein) that are potential virulence determinants in experimental infections (4, 12). In a rabbit endocarditis model, we have previously shown that there was a significant decrease in infectivity rates in a *sar*⁻ mutant at 10^3 – 10^4 CFU inocula when compared to the parental strain (13).

To evaluate the contributory roles of both *sar* and *agr* loci in staphylococcal virulence, we have constructed *sar*⁻/*agr*⁻ mutants of RN6390 and RN450 and compared their in vitro properties to the wild-type parent and single isogenic mutants. In

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studies with RN6390-related strains in the rabbit endocarditis model, there was a significant reduction in infectivity rates across a broad range of challenging inocula (10^3 – 10^6 CFU) with the double mutant when compared to the corresponding *sar*⁺/*agr*⁺ (parent) strain. These results suggest that both loci are involved in the initiation and persistence of *S. aureus* infection in the fibrin-platelet-endothelial matrix in endocardial vegetations.

Methods

Bacterial strains, plasmids, DNA probes, and phage. The bacterial strains, plasmids and DNA probes used in this study are listed in Table I. Phage ϕ 11 was used as a transducing phage for RN6390 and RN450.

Media and antibiotics. CYGP, 0.3GL, and Brain-Heart-Infusion (Difco Laboratories Inc., Detroit, MI) were used for the growth of bacteria (12). Antibiotics were used at the following concentrations: erythromycin at 10 μ g/ml and tetracycline at 5 μ g/ml.

Transduction. Phage ϕ 11 was used to prepare lysates of strain RN6911 (14) which is an *agr*⁻ mutant of parental strain RN6390 (12). The phage lysate was used to infect *sar*⁻ mutants R and A (15) derived from strains RN6390 and RN450, respectively, at a low multiplicity of infection (phage to recipient ratio = 1:10). Transductants carrying the double mutations were selected on 0.3 GL agar containing both erythromycin and tetracycline. The ϕ 11 lysate of RN6911 was also used to infect strain RN450 to obtain tet^r transductants carrying the *agr* mutation alone.

Phenotypic characterization. For phenotypic characterization, the measurements included α ; β ; and δ -hemolysin production on sheep and rabbit erythrocyte agar as previously described (16), lipase production as measured on 1% Tween 20 agar plates (12), fibronectin binding capacity as assayed by ¹²⁵I-labeled fibronectin binding (17), and fibrinogen binding capacity as determined by ¹²⁵I-labeled fibrinogen binding (18).

To determine whether the double mutant differed from either the parent or single isogenic mutants in other phenotypic traits likely to be important in endocarditis pathogenesis, we studied the comparative in vitro ability of these strains to: (a) adhere to platelets as measured by flow cytometry (19, 20); and (b) resist the bactericidal action of platelet microbicidal protein (PMP)¹ (21).

For platelet adherence assays, rabbit whole blood was collected into polypropylene tubes containing sodium citrate (5:1) and 1 μ g/ml prostaglandin E₁ (PGE₁) (Sigma Chemical Co., St. Louis, MO) to mitigate platelet activation. Platelets were isolated by centrifugation (150 g for 10 min) and labeled externally with P2 (2.5 μ g/ml), an FITC-conjugated monoclonal antibody against CD41 (AMAC, Westbrook, ME), or internally labeled with 5 μ M 5-chloromethylfluorescein diacetate (CMFDA; Molecular Probes, Eugene, OR). After 1 h of incubation, the platelets were washed and suspended in Tyrode's solution as previously described (22). To label bacteria, overnight cultures of each strain 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 μ g/ml; Polysciences, Warrington, PA), a DNA-binding fluorochrome (22). Adherence studies were performed at 20°C by mixing bacteria and platelets in Tyrode's salts solution (Sigma Chemical Co.) at a bacterial/platelet ratio of 10:1. One minute after mixing, the suspensions were analyzed using a FACStar^{Plus} (Becton Dickinson, San Jose, CA) cytofluorograph, using predetermined combinations of argon lasers, excitatory wavelengths, and appropriate filters (19, 22). The percentage of bacteria adhering to platelets was determined as previously described (19).

For the second assay, PMP was prepared from thrombin-stimulated platelet-rich plasma and assayed for bioactivity (U/ml) as previously

described (21). PMP was added to washed bacterial suspensions in low protein-binding microtiter plates (Corning Glassworks, Corning, NY) to achieve a final PMP concentration of 100 U/ml and a final bacterial inoculum of $\sim 10^3$ CFU/ml. After 2 h of incubation at 37°C, 20- μ l aliquots were processed for quantitative culture as described previously (21). The percent bacterial survival was calculated for each *S. aureus* strain. *Bacillus subtilis* (ATCC 6633), a highly PMP-susceptible strain, served as a positive control for PMP bioactivity while wells containing the organism plus minimal essential medium (Irvine Scientific, Santa Ana, CA) alone were used as negative controls (23).

Southern blot hybridization. *S. aureus* DNA was prepared from lysostaphin-lysed cells as described (12). Chromosomal DNA was digested with selected restriction enzymes (New England BioLabs, Beverly, MA), transferred onto Hybond N⁺ membrane (Amersham, Arlington Heights, IL) and hybridized at 65°C with probes labeled with ³²P by the random-primed method (Ready to Go Labeling Kit; Pharmacia Fine Chemicals, Piscataway, NJ) or the nick translation method (Nick Translation Kit; Boehringer Mannheim, Indianapolis, IN). After hybridization, the membrane was washed with SSPE (10 mM NaH₂PO₄, 150 mM NaCl, 1 mM EDTA, pH 7.4) according to the membrane manufacturer's instructions (12) and autoradiographed with an intensifying screen at -70°C.

Northern blot hybridization. *S. aureus* RNA was prepared using a modification of the method of Kornblum et al. (24) as previously described (15). For Northern blots, equal volumes (7.5 μ l) of RNA samples extracted from an equivalent number of bacterial cells were electrophoresed through a 1.5% agarose-0.66 M formaldehyde gel in MOPS running buffer as described (15). The intensity of the 23 S and 16 S ribosomal RNA bands stained with ethidium bromide was verified to be equivalent among all samples before transfer. RNA was transferred to Hybond N (Amersham), cross-linked with UV light, allowed to hybridize with a ³²P-labeled gel purified DNA probes (25) in 50% formamide at 42°C overnight, washed, and autoradiographed as described (15).

Animal model of endocarditis. All bacterial strains were grown in brain-heart infusion broth at 37°C overnight with rotation (30 rpm) supplemented with appropriate antibiotics if necessary. In preliminary studies, it was determined that the growth rates of parental and mutant strains were almost identical both in the presence and absence of antibiotics over a 24-h period. In our previous study with a *sar*⁻ mutant (13), we established that incorporation of antibiotics into the overnight growth media did not influence the ability of the strain to induce experimental endocarditis. Overnight cultures were harvested by centrifugation (2,000 g for 10 min), washed twice in sterile normal saline and resuspended in saline to an OD_{620nm} of 1.6 ($\sim 1 \times 10^9$ CFU/ml). This bacterial suspension was serially diluted in saline to $\sim 10^3$ – 10^6 CFU/ml. Each dilution was confirmed by quantitative culture on blood agar plates.

Endocarditis on the aortic valve of New Zealand White rabbits (2–2.5 kg) was induced by a modification of the method of Durack and Beeson (26). In brief, rabbits were anesthetized by intramuscular injection of ketamine chloride (Aveco Inc., Fort Dodge, IA) at 35 mg/kg and xylazine (Mobyay Corp., Shawnee, KS) at 1.5 mg/kg. A polyethylene catheter with an internal diameter of 0.86 mm (Becton Dickinson, Cokesville, MD) was introduced into the left ventricle via the right carotid artery to produce sterile thrombotic endocarditis. To induce endocarditis, groups of ~ 10 – 15 animals each were challenged intravenously at 48 h after catheterization with various inocula of different bacterial strains (10^3 – 10^6 CFU). 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 time of sacrifice, aortic valves and left ventricular vegetations were removed, pooled, weighed, homogenized in 0.5 ml of normal saline and quantitatively cultured in antibiotic-free medium (for all strains), erythromycin-containing medium (for mutant R and I), tetracycline-containing medium (for RN6911 and mutant I) or medium containing both antibiotics (double mutant). Animals with negative cultures of

1. Abbreviation used in this paper: PMP, platelet microbicidal protein.

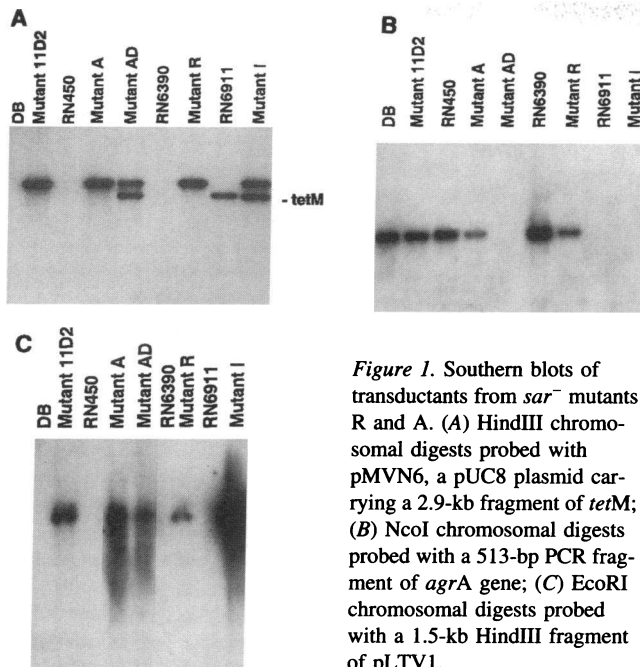


Figure 1. Southern blots of transductants from *sar*⁻ mutants R and A. (A) HindIII chromosomal digests probed with pMVN6, a pUC8 plasmid carrying a 2.9-kb fragment of *tetM*; (B) NcoI chromosomal digests probed with a 513-bp PCR fragment of *agrA* gene; (C) EcoRI chromosomal digests probed with a 1.5-kb HindIII fragment of pLTV1.

their undiluted vegetation homogenates were considered to have had no induction of endocarditis at a particular inoculum (13).

To determine the relative ability of different bacterial strains to adhere to sterile endocardial vegetations early after intravenous challenge, separate groups of 6–8 catheterized rabbits each were challenged intravenously at 48 h after catheterization with 10⁴, 10⁵, or 10⁶ CFU of either the parent or mutant strains. Thirty minutes after bacterial injection, all animals were sacrificed, their hearts opened, and all visible left-sided vegetations from each animal 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 together with the wash from each individual animal were quantitatively cultured into antibiotic-free as well as antibiotic-selective brain–heart infusion agar based on the infecting strain.

To assure that any observed differences in valvular adherence and endocarditis induction rates among strains were not due to a disparity in the level of early bacteremias, quantitative blood cultures were obtained in a separate study at 1-min post-iv inoculations with each bacterial strain at 10³–10⁶ CFU.

Statistical analysis. The Fisher's exact test was used to compare the induction rates of endocarditis among strains. The mean bacterial vegetation densities and early adherence were evaluated by the Kruskal-Wallis analysis of variance for nonparametric data with Tukey post-hoc analysis for multiple comparisons. The Student *t* test was used to compare the binding of radiolabeled fibrinogen and fibronectin to different isolates. *P* values ≤ 0.05 were considered significant.

Results

Transduction of the *agr*⁻ genotype into *sar*⁻ mutants R and A. Transduction was used to transfer the *agr*⁻ genotype from *agr* mutant RN6911 into *sar*⁻ mutants R and A derived from RN6390 and RN450, respectively. Southern blots of chromosomal DNA from transductants (mutants AD and I) digested with HindIII, EcoRI, or NcoI revealed a hybridization pattern consistent with the transfer of the *agr*⁻ genotype (Fig. 1). More

specifically, the double *sar*⁻/*agr*⁻ mutants hybridized with *tetM* but not the *agr* probe (Fig. 1, A and B). Notably, two bands hybridized to the probe that comprises of *tetM* cloned into pUC8. The upper band represented hybridization of the pUC8 probe with the *E. coli*-derived portion of inserted transposon Tn917LTV1 while the lower hybridizing band corresponded to that of *tetM* (Fig. 1 A). This pattern of hybridization of the double mutants with *tetM* and *agr* probes was similar to that of the *agr* mutant RN6911 in which the entire *agr* locus had been replaced by the *tetM* gene (Fig. 1, A and B). To verify the maintenance of the Tn917LTV1 insertion in the double mutants, Southern blots of chromosomal DNA from these transductants digested with EcoRI was performed. Our results indicated that a single EcoRI fragment from these double mutants hybridized with a 1.5-kb HindIII fragment of Tn917 as a probe (Fig. 1 C). This hybridizing pattern is analogous to that seen with original *sar*⁻ mutant 11D2, thus suggesting that the location of the Tn917LTV1 insert in these double mutants was identical to that of mutant 11D2 (Fig. 1 C).

We have also transduced the *agr*⁻ genotype from RN6911 into RN450 to obtain *sar*⁺/*agr*⁻ mutants. At least 10 *tet*^R transductants were found one of which (mutant D) is listed in Table I. The results of Southern blot studies of these transductants with *tetM* and *agr* probes were similar to that of RN6911 (data not shown), thus implying displacement of the *agr* locus by the *tetM* gene in these transductants.

Northern blot analysis of the *sar*⁺/*agr*⁻ and *sar*⁻/*agr*⁻ mutants with both *agrA* and *hld* probes confirmed that transcription of these genes did not occur in these strains (data not shown). Similarly, no transcripts were detected in the *sar*⁻/*agr*⁺ and *sar*⁻/*agr*⁻ strains when total cellular RNA immobilized on nylon membranes were probed with the *sarA* gene probe (data not shown).

Phenotypic characterization of *sar*⁻/*agr*⁻ mutants in comparison to *sar*⁻/*agr*⁺, *sar*⁺/*agr*⁻, and *sar*⁺/*agr*⁺ (parent) strains. Two sets of mutants were evaluated for phenotypic alterations (Table II). Compared with parental strain RN6390, no hemolysins (α , β , or δ) were detected in the corresponding *sar*⁻/*agr*⁻ mutant. In contrast, a small but detectable amount of β -hemolysin was secreted by both single *sar*⁻ and *agr*⁻ mutants (mutant R and RN6911, respectively). In assays with RN450-related strains, the secretion of β hemolysin was absent in both *sar*⁻/*agr*⁻ and *sar*⁻/*agr*⁺ mutants but present in the *sar*⁺/*agr*⁻ mutant. Northern blot analysis of one set of strains (RN6390, RN6911, *sar*⁻ mutant R and double mutant I) with α - and β -hemolysin probes revealed almost complete abolition of these exoprotein gene transcripts in the double mutant (Fig. 2, A and B). In concordance with our previous findings (15) and that of Vandesdesh et al. (14), a significant downregulation of these transcripts in the *sar*⁻/*agr*⁺ (mutant R) and *sar*⁺/*agr*⁻ (RN6911) mutants was also observed.

The effect of these mutations on cell-bound proteins was also studied. The fibronectin binding capacity was significantly reduced in both double mutants and single *sar*⁻ mutants (Table II) when compared with their respective parents. Similar to the results reported by Kornblum et al. (9), the binding of radiolabeled fibronectin was increased in both *sar*⁺/*agr*⁻ mutants. The ability to bind fibrinogen was diminished in both double mutants, but the magnitude of the reduction, albeit statistically significant, was more substantial in mutant AD than mutant I. Consistent with our previous studies (15), there was a modest decline in the ability to bind fibrinogen as a result of the *sar*

Table I. Bacterial Strains, Plasmids, and DNA Probes

Types	References	Comments
Bacterial strains		
<i>S. aureus</i>		
DB	12	A wild-type blood isolate that is the parent of the original <i>sar</i> ⁻ mutant 11D2.
11D2	12	An isogenic mutant of strain DB with a <i>sar</i> ::Tn917LTV1 mutation
RN6390	40	A prototypic strain that maintains its hemolytic pattern when propagated on sheep erythrocyte agar.
Mutant R	15	An isogenic mutant of RN6390 carrying a <i>sar</i> ::Tn917LTV1 mutation.
RN6911	14	An isogenic mutant of RN6390 with an <i>agr</i> :: <i>getM</i> mutation.
Mutant I	This study	A derivative of RN6390 carrying both <i>sar</i> ::Tn917LTV1 and <i>agr</i> :: <i>tetM</i> mutations.
RN450	40	A prototypic strain, which is a derivative of NCTC 8325 cured of prophages, secretes β hemolysin but not α hemolysin.
Mutant A	15	An isogenic mutant of RN450 with a <i>sar</i> ::Tn917LTV1 mutation.
Mutant D	This study	An isogenic mutant of RN450 with a <i>agr</i> :: <i>tetM</i> mutation.
Mutant AD	This study	A double mutant of RN450 with both <i>sar</i> ::Tn917LTV1 and <i>agr</i> :: <i>tetM</i> mutations.
<i>E. coli</i> with plasmids		
DU5384	41	An <i>E. coli</i> strain carrying a pBR322 plasmid with a 3-kb EcoRI–HindIII fragment of the α -hemolysin gene to be gel-purified to be used as a probe.
RN6929	42	JM109 containing a pBluescript plasmid with a 2.2-kb ClaI fragment of the β -hemolysin gene which was gel-purified to be used as a probe.
DNA probes		
pLTV1	12, 43	A derivative of Tn917 carrying a <i>E. coli</i> replicon. A 1.5-kb HindIII gel-purified fragment was used as a probe.
pALC4	31	A shuttle plasmid (pSPT181) containing a 732-bp PCR fragment of <i>sarA</i> gene of RN6390.
pMVN6	44	A pUC8 plasmid carrying a 2.9-kb BamHI fragment of <i>tetM</i> .
<i>hld</i> probe	This study	A 511-bp PCR fragment of the RNIII from nucleotides 1265–1775 based on published sequence (9).
<i>agrA</i> probe	This study	A 513-bp PCR fragment of the <i>agrA</i> gene from nucleotides 3830–4342 (9).

Table II. Phenotypic Characterization of RN6390, RN450, and Their Corresponding *sar/agr* Mutants

Phenotypes*	Strains							
	RN6390 <i>sar</i> ⁺ <i>agr</i> ⁺	RN6911 <i>sar</i> ⁺ <i>agr</i> ⁻	Mutant R <i>sar</i> ⁻ <i>agr</i> ⁺	Mutant I <i>sar</i> ⁻ <i>agr</i> ⁻	RN450 <i>sar</i> ⁺ <i>agr</i> ⁺	Mutant D <i>sar</i> ⁺ <i>agr</i> ⁻	Mutant A <i>sar</i> ⁻ <i>agr</i> ⁺	Mutant AD <i>sar</i> ⁻ <i>agr</i> ⁻
α -Hemolysin	+++ [‡]	–	–	–	–	–	–	–
β -Hemolysin	++	+	+	–	+	+	–	–
δ -Hemolysin	++	–	–	–	+/-	–	–	–
Lipase	+	–	++	–	–	–	–	–
Fibronectin binding protein [§]	3314±35	4059±117	2636±80	2809±60	15221±671	26852±691	4874±192	4909±611
Fibrinogen binding protein	5694±70	5427±314	5156±94	5118±118	6139±119	5326±103	3463±449	3094±97
Susceptibility to PMP (% survival)	0%	0%	0%	1.3%				
Platelet binding by FACS (% bound) [¶]	61.7±39.7	57.3±42	69.1±26.6	72.8±26.1				

* ++, strong producer; +, moderate producer; +/-, very weak producer; –, non producer. [‡] The expression of α -hemolysin in these strains was also confirmed by immunoblots of extracellular proteins probed with affinity purified anti- α -hemolysin antibody (Toxin Technology, Sarasota, FL). [§] Data are presented as cpm of ¹²⁵I-fibronectin bound to 10⁹ CFU. Values are given as mean±SEM (n = 4). The reduction in fibronectin binding of double mutants I and AD in comparison with the respective parental strains is statistically significant (P ≤ 0.009 and 0.001 for mutant I and AD, respectively; t test). The augmentation in fibronectin binding as a result of the *agr* mutation alone (RN6911 and mutant D) was also significant when compared to the respective parents (P ≤ 0.004 and 0.001 for RN6911 and mutant D, respectively; t test). ^{||} Data are given as cpm of ¹²⁵I-fibrinogen bound to 10⁹ CFU and are reported as the mean±SEM (n = 4). The binding of radiolabeled fibrinogen to double mutants I and AD was lower than that of the respective parental strain (P ≤ 0.023 and 0.0001 for mutants I and AD, respectively; t test). Similarly, *sar*⁻ mutant A bound less radiolabeled fibrinogen than parent strain RN450 (P ≤ 0.008). [¶] Platelets labeled with P2 or CMFDA were bound comparably by all strains.

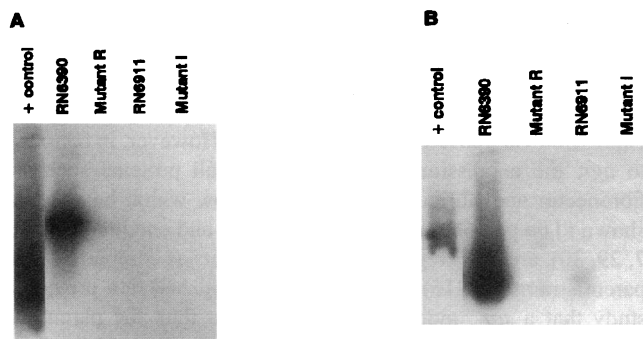


Figure 2. Northern blots of α - (A) and β - (B) hemolysin transcripts of parental strain (RN6390) and the corresponding *sarlagr* mutants. The positive control in A is a pBR322 plasmid carrying a 3-kb EcoRI–HindIII fragment of the α -hemolysin gene (41). In B, the positive control is a pBluescript plasmid with a 2.2-kb ClaI fragment of the β -hemolysin gene (42).

mutation in both strains (see mutants R and A in Table II). However, the effect of the *agr* mutation alone on fibrinogen binding capacity was more variable, with one resulting in a slight reduction (mutant D) while the other one remaining unchanged (RN6911).

In preliminary studies, it was determined that strain RN6390 was virulent in the animal endocarditis model (see below). Based on this finding, we evaluated other phenotypic traits in RN6390 and its corresponding mutants that are likely to be important in the pathogenesis of endocarditis, including susceptibility to PMP (19) and the ability to bind platelets (27, 28). As seen in Table II, all strains were very susceptible to the killing action of PMP. After exposure to PMP at 100 U/ml for 120 min, the mean survival among these strains was between 0 and 1.3%. Likewise, the ability to bind platelets as assayed by FACS was similar among all strains studied (Table II).

Animal model of endocarditis. In pilot studies, it was determined that the ID₉₀ for parental strain RN6390 was between 10³ and 10⁴ CFU. The induction rates for endocarditis with parent RN6390 and its corresponding mutants were evaluated at various intravenous challenge inocula between 10³ and 10⁶ CFU, thereby encompassing the ID₉₀ for the parental strain (Table III). At all four challenge inocula, the *sar*⁻/*agr*⁻ double mutant caused substantially lower induction rates of endocarditis than *sar*⁺/*agr*⁺ parent (RN6390) (0, 0, 28, and 50% vs 90, 70, 100, and 100% at 10³–10⁶ CFU with *P* ≤ 0.00006, 0.0015, 0.002, and 0.03, respectively). Similar to our previously reported data on parent strain DB and its isogenic *sar*⁻/*agr*⁺ mutant (13), intravenous challenge with the *sar*⁻/*agr*⁺ mutant R also resulted in reduced endocarditis induction rates as compared with the parent strain at the two lower challenge inocula (0 and 11% vs 90 and 70% at 10³–10⁴ CFU with *P* values ≤ 0.0001 and 0.015, respectively). In contrast, the difference in induction rates between the *sar*⁺/*agr*⁻ (RN6911) strain and its *sar*⁺/*agr*⁺ parent (RN6390) was only significant at the lowest inoculum (40 vs 90% at 10³ CFU; *P* ≤ 0.03). Notably, there were no statistical differences between the induction rates of *sar*⁻/*agr*⁺ versus *sar*⁺/*agr*⁻ mutants at any challenge inocula.

The vegetation bacterial densities in different animal groups were parallel-plated onto antibiotic-containing as well as antibiotic-free media. Our results indicated that there were no differences in quantitative recovery of bacteria between these two

Table III. The Induction of Endocarditis in a Rabbit Model with *sar*⁺/*agr*⁺ (RN6390), *sar*⁺/*agr*⁻, *sar*⁻/*agr*⁺, and *sar*⁻/*agr*⁻ Strains

	Strains			
	RN6390 (<i>sar</i> ⁺ / <i>agr</i> ⁺)	RN6911 (<i>sar</i> ⁺ / <i>agr</i> ⁻)	Mutant R (<i>sar</i> ⁻ / <i>agr</i> ⁺)	Mutant I (<i>sar</i> ⁻ / <i>agr</i> ⁻)
10 ³ CFU	9/10	4/10*	0/10 [‡]	0/10 [§]
10 ⁴ CFU	7/10	4/11	1/9 [‡]	0/10 [§]
10 ⁵ CFU	11/11	8/10	10/11	2/7 [§]
10 ⁶ CFU	9/9	8/11	9/12	4/8 [§]

At 48 h after catheterization, 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. Values are given as number with endocarditis/total number in the group. * Statistically significantly when compared with *sar*⁺/*agr*⁺ parental strain (*P* ≤ 0.03; Fisher Exact test). [‡] When compared with the parental strain, induction rates are significant with the *P* values ≤ 0.0001 and 0.015 at 10³ and 10⁴ inocula, respectively. [§] Induction rates are significantly lower than that of the parental strain at all inocula (*P* values of 0.00006, 0.0015, 0.002, and 0.03 at 10³–10⁶ CFU, respectively).

plating methods. As shown in Fig. 3, animals challenged with the double *sar*⁻/*agr*⁻ mutant had significantly lower vegetation densities as compared with the *sar*⁺/*agr*⁺ parent at all challenge inocula. At the two higher inocula (10⁵–10⁶), challenge with the *sar*⁻/*agr*⁺ strain yielded lower bacterial densities in aortic vegetations than the *sar*⁺/*agr*⁺ parent. Although the mean bacterial vegetation densities achievable with the *sar*⁺/*agr*⁻ mutant were lower than the parent at higher challenge inocula (10⁵–10⁶ CFU), they did not reach statistical significance.

To verify the stability of the *sar* and *agr* mutations in mutant strains after animal passage, chromosomal DNA of *sar*⁺/*agr*⁺ (parent), *sar*⁺/*agr*⁻, *sar*⁻/*agr*⁺, and *sar*⁻/*agr*⁻ colonies isolated

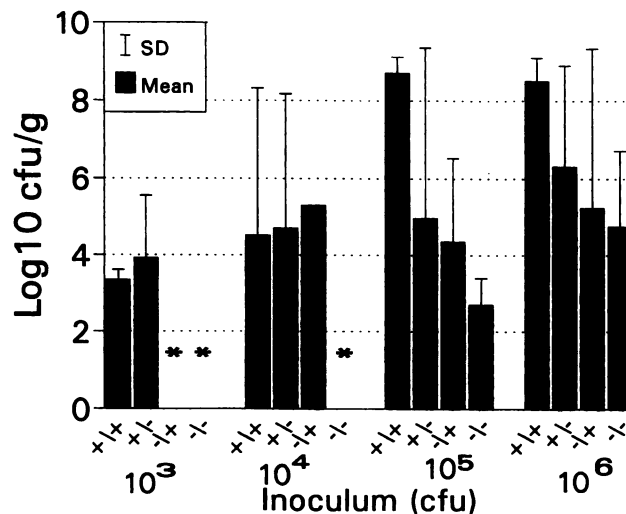


Figure 3. Bacterial vegetation densities of parental strain RN6390 vs three *sarlagr* mutants. Values are given as mean values ± SD obtained from 8–10 animals in each group (*sar*⁺/*agr*⁺, *sar*⁺/*agr*⁻, *sar*⁻/*agr*⁺, and *sar*⁻/*agr*⁻). The analyzed statistical data are as follows: 10⁵ CFU +/+ vs -/- (*P* ≤ 0.0001), +/+ vs -/+ (*P* ≤ 0.05); 10⁶ CFU +/+ vs -/- (*P* ≤ 0.05), +/+ vs -/+ (*P* ≤ 0.05). * The vegetation cultures in these animal groups were negative.

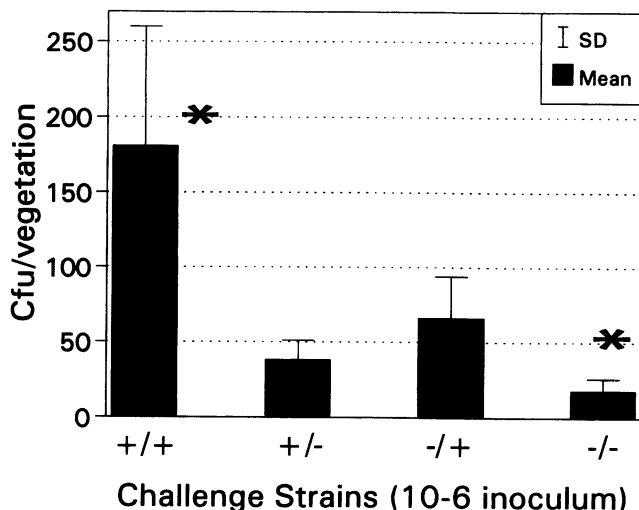


Figure 4. Relative bacterial adherence of parent (RN6390) vs three *sar/ agr* mutants to aortic valvular vegetations. These are presented as total number of organisms adhering at 30 min post-iv challenge. Values are given as mean \pm SD obtained from 6–8 animals in each bacterial strain group. *The *P* value in early valvular adherence between *sar*⁺/*agr*⁺ parent and *sar*⁻/*agr*⁻ double mutant is < 0.01.

from cultured vegetations (at least six each) were digested with EcoRI, NcoI, or HindIII and probed with ³²P-labeled *agrA*, *hld*, *tetM*, *Tn917*, and *sarA* probes. In each instance, the colonies harvested from the vegetations had hybridization patterns identical to those of the respective infecting strains isolated before animal passage (data not shown as they are similar to those in Fig. 1). These data indicated that the mutations remained stable in vivo.

Determination of early adherence to sterile valvular vegetations. To determine if the above disparities in endocarditis induction rates were attributable to differences in early valvular adherence of bacteria, we sacrificed groups of catheterized animals 30 min after intravenous challenge to determine the total number of CFU binding to valvular vegetations. At lower inocula (10³–10⁵), the number of adherent bacteria for all strains was too low to make a meaningful comparison. However, at a higher challenge inoculum (10⁶ CFU), the adherence of the parental strain was significantly higher than that of the double mutant (181 \pm 79 vs 18 \pm 8 CFU/vegetations, respectively; *P* \leq 0.01) (Fig. 4). Notably, the adherence of single mutants was also less than the parent strain, but these differences did not reach statistical significance (e.g., *P* \leq 0.14 between *sar*⁺/*agr*⁻ and *sar*⁺/*agr*⁺ strains).

To confirm that differences in early valve adherence were not related to the level of bacteremia following intravenous challenge, quantitative blood cultures were obtained to assay for comparative bacterial clearance. Because our previous observations had confirmed that very few colonies (1–5 CFU) could be isolated on blood culture at 30 min post-iv challenge (13), only blood cultures at 1 min post-iv challenge were obtained. At each of the tested inoculum (10³–10⁶ CFU), there were no significant differences in the mean quantitative counts of post-challenge bacteremias among various groups at 1 min after intravenous challenge.

Discussion

Microbial pathogenicity is a complex process that involves the products of a number of genes many of which are virulence

determinants controlled by global regulatory systems. The *agr* and *sar* loci in *S. aureus* are examples of two such regulatory systems (9, 12). Northern blot analyses suggested that the *sar* locus, like that of *agr* (14), positively regulates exoprotein gene expression via transcriptional control (15). However, in contrast to *agr*, the expression of selected cell wall proteins such as fibronectin and fibrinogen binding proteins, which have been shown to be potential adhesins to catheters and endothelium (5, 7, 29, 30), are downregulated in *sar*⁻ mutants as compared with parent strains (12, 15). We have also established in a previous study that a *sar*⁻ mutant was less virulent than the parent at low challenge inocula (10³ and 10⁴ CFU) in the endocarditis model (13). Because of the counter-regulatory effects of the same set of cell-wall protein genes by *sar* and *agr* loci, the current study was undertaken to examine the in vitro phenotypic interactions as well as the net in vivo effects of these two loci in a prototypical endovascular infection model (experimental endocarditis).

Our detailed examination of the phenotypic characterization of *sar* and *agr* mutants has yielded interesting insights into the control of exoprotein expression. As expected from previous studies (9, 15), the secretion of hemolysins was generally decreased in both *sar*⁺/*agr*⁻ and *sar*⁻/*agr*⁺ mutants derived from RN6390. Based on our recent observation that the optimal transcription of RNAIII, which is the *agr* regulatory molecule that controls exoprotein gene transcriptions (9), is dependent on an intact *sar* locus (31), we speculate that the *sar* locus likely regulates exoprotein gene expression via transcriptional control of RNAIII. However, additional phenotypic analysis of the double *sar*⁻/*agr*⁻ mutant (mutant I in Table I) suggested that the *sar* locus may also affect exoprotein genes independently of *agr*. This is predicated upon the finding that a double mutant does not secrete β -hemolysin while single isogenic mutants do. Additionally, the mild augmentation of lipase production in *sar*⁻/*agr*⁺ strain (mutant R) in comparison with the parent (RN6390) was completely abolished in the double mutant I.

Although the fibronectin binding capacity was increased in *sar*⁺/*agr*⁻ mutants (RN6911 and mutant D in Table II) as predicted (9), this capacity was reduced in the double mutants (mutants I and AD) and single *sar*⁻ mutants (mutants R and A) as compared to their respective parents. However, the ability to bind radiolabeled fibrinogen remained essentially unchanged in *sar*⁺/*agr*⁻ mutants. In contrast, the fibrinogen-binding capacity of both double mutants decreased to a level similar to that of *sar*⁻/*agr*⁺ strains, although the magnitude of the decrease was much smaller in double mutant I. As a single mutation in the *sar* or *agr* locus yields opposite effects in cell-wall protein expression, these experimental observations on the double mutants provide two potential insights into regulatory control of cell-wall proteins. First, in distinction to the exoprotein control in which the *sar* and *agr* loci are possibly elements operating under the same regulatory pathway, these data suggested that the mechanisms of cell-wall protein control by these two loci are likely to be different from that of exoprotein genes. Second, two theoretical possibilities can account for the cell wall protein profile of the double mutant. One possibility is that both *sar* and *agr* loci function within the same regulatory pathway in which the *agr* and *sar* gene product(s) act as the repressor and the activator, respectively, for cell wall protein target sequences. In the case of a double mutant, a lack of a repressor (i.e., *agr*⁻) does not result in an overexpression of cell-wall proteins in the absence of an activator (i.e., *sar*⁻) of target sequences. The

second possibility is that the *sar* and *agr* loci control the expression of cell wall proteins via different regulatory mechanisms such that a malfunctioning *sar* locus, irrespective of the functionality of the *agr* locus, will lead to downregulation of cell wall proteins.

As inactivation of both *sar* and *agr* loci resulted in reduced production of extracellular and cell wall proteins that are putative virulence determinants in *S. aureus* (5, 7), we evaluated the net in vivo effect of a double mutant in a rabbit model of endocarditis. Our data showed that the double mutant had a lower capacity to induce endocarditis as compared to the parent strain at all challenge inocula (Table III). Moreover, the vegetation bacterial densities were markedly decreased in the double mutant in comparison with the parent strain at all challenge levels. In contrast, the *sar*⁻/*agr*⁺ mutant induced endocarditis at a significantly lower rate than the parent strain only at the two lower challenge inocula (10³–10⁴ CFU). This induction rate is in concordance with our previous studies in which a different single *sar*⁻ mutant (strain 11D2) had a lower induction rate than the isogenic parent only at such challenge inocula (13). Interestingly, the induction rate for *sar*⁺/*agr*⁻ strain differed from the parent only at the 10³ CFU inoculum. Although the induction rates for single *sar* mutant were statistically equivalent to those of the parent strain at the higher challenge inocula (10⁵–10⁶ CFU), the mean vegetation bacterial densities were substantially lower in the *sar* mutant group, but not in the *agr* mutant group, when compared to the parental group at these inocula (Fig. 3).

Differences in the ability of the double mutant and parental strains to induce endocarditis as described here may be related to potential perturbations in either of the two critical events in the pathogenesis of endocarditis: initial adherence to sterile vegetation and/or subsequent intravegetation bacterial propagation (26, 32). Within the matrices of the valvular vegetations, fibrinogen, fibronectin, vitronectin, platelets, and released platelet proteins (e.g., thrombospondin) are commonly deposited (5, 7, 32). In addition, underlying extracellular matrix proteins, such as collagen and laminin, may also be exposed as a result of prior endothelial damage (33, 34). During the initial phase of bacteremia, a combination of impaired fibronectin and fibrinogen binding may conceivably contribute to a reduction in the number of initial adherence events at the vegetation surface for the double mutant. Notably, this diminished capacity to bind fibronectin and fibrinogen in the double mutant is likely attributable to a mutation in the *sar* locus (Table II). This concept is consistent with the experimental observation that at higher inocula (10⁵–10⁶ CFU), the bacterial vegetation densities achievable by the single *sar* mutant, but not the single *agr* mutant, were significantly lower than the parent despite comparable induction rates (Fig. 3). Nevertheless, the possibility that additional staphylococcal surface receptors such as those for collagen, laminin and thrombospondin (35–37) may be involved in adhesion cannot be entirely ruled out. Our data also seem to minimize the role of platelets in accounting for the differences in early adherence between the parent and mutant strains since they bound equally well to platelets, and were highly susceptible to platelet-microbicidal-protein-induced lethality in vitro.

After initial adherence to the vegetation surface, the eventual establishment of infection is still dependent on the ability of the microorganism to proliferate within the vegetations. As noted in Fig. 3, both the single *sar* mutant and the double mutant

achieved lower intravegetation bacterial densities than the parent. Whether this reduction in intravegetation bacterial densities is due to a diminution in cytotoxin secretion (e.g., hemolysins) and/or a decline in initial adhesion observed in these mutants is not resolved in this study. Nevertheless, α -hemolysin, a pore-forming cellular cytotoxin, has been shown to exhibit lethality for platelets and endothelial cells (38, 39), two important components of the vegetation in endocarditis (33). It is conceivable that the absence of α hemolysin in a mutant strain may adversely affect its ability to persist within vegetation. Because very few, if any, leukocytes are found within infected aortic vegetations in both experimental and human endocarditis (33), any dissimilarity in cellular immune response between the parent vs the mutant strains is unlikely to explain the observed differences in induction rates or intravegetation bacterial densities. As these two regulatory loci control the synthesis of a variety of extracellular and cell wall proteins, our data would seem to support the multifactorial nature involved in the pathogenesis of *S. aureus* endocarditis.

Considerable efforts have been spent previously to single out a specific virulence factor in the pathogenesis of *S. aureus* endocarditis and other related intravascular infections. However, these experimental approaches have not been successful in explaining the range of this organism's ability to induce and propagate infections at a challenging inoculum found in similar human infections (4, 7). For example, in experiments involving inactivation of the fibronectin binding protein alone, the contributory role of this protein in endocarditis virulence is modest at best even at a fairly high challenge inoculum (10⁶ CFU) (7). Based on these investigations, it appears that *S. aureus* may have a wide arsenal of adhesins and toxins to facilitate adherence and propagation within human valvular tissues. In this study, we have shown that the *sar* and *agr* loci of *S. aureus* are important regulatory elements in the expression of virulence determinants necessary for the induction and propagation of intravegetation growth in the animal model. Hence, it may be meaningful to target these regulatory loci for the development of novel antimicrobial agents (e.g., designing a synthetic analog to block activation of virulence factors by the native *sar* gene products).

One of the genes (*sarA*) within the *sar* locus has recently been cloned (31). Sequence analysis revealed an open reading frame of 372 bp with a predicted mol wt of 14,718 D. Additional studies indicated that this gene is necessary for the optimal transcription of RNAlII (31). In this current study, there is additional evidence to suggest that both *agr*-dependent and *agr*-independent pathways are probably operational in the regulation of virulence determinants in *S. aureus*. To our knowledge, this is the first study that implicates both regulatory loci (i.e., *sar* and *agr*) in early bacterial adherence, induction, and intravegetation persistence in the pathogenesis of endocarditis. A detailed understanding of the entire *sar* locus and its mode of regulation will provide insight into the molecular mechanisms of staphylococcal pathogenesis and may uncover specific targets which may be amenable to therapeutic intervention in *S. aureus* infections.

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