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Insertional Inactivation of a Chromosomal Locus That Modulates Expression of Potential Virulence Determinants in *Staphylococcus aureus*

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A single insertion of transposon Tn*551* into a unique chromosomal locus of *Staphylococcus aureus* ISP479C has resulted in a pleiotropic effect on the expression of both extracellular and cell wall proteins. In particular, the expression of cell wall protein A and clumping activity with fibrinogen were rendered undetectable in the mutant 1E3 compared with the parent. The secretion of α-hemolysin in mutant 1E3 was modestly increased. Southern blot and phenotypic analyses indicated that this locus is distinct from *agr*, *xpr*, and *sar*, three previously described global regulatory loci. Transduction experiments demonstrated that the genotype associated with mutant 1E3 could be transferred back into the parental strain ISP479C. The transductant 1E3-2 displayed a phenotypic profile similar to that of the original mutant. Northern (RNA) blot studies showed that this locus may be involved in modulating target genes at the mRNA level. In the rabbit endocarditis model, there was a significant decrease in both the infectivity rate and intravegetation bacterial density with mutant 1E3 compared with the parent at an inoculum of 10^3 CFU. Since protein A and the fibrinogen-binding protein(s) are major surface proteins that may mediate bacterial adhesion to host tissues, this locus may be an important genetic element involved in the expression of virulence determinants in *S. aureus*.

Staphylococcus aureus is a major human pathogen that has the capability to synthesize a plethora of extracellular and cell wall proteins, many of which are involved in pathogenesis (15). Investigations directed at defining the regulation of these virulence factors have led to the discovery of several global regulatory elements, including *agr*, *xpr*, and *sar* (8, 18, 33). Among these, the *agr* locus is the best described (18, 26). The *agr* system activates transcription of exoprotein genes while suppressing cell wall protein gene transcription (e.g., that of protein A) (36). The opposite regulation of two sets of genes by the same locus is a unique feature of the *agr* system. Consequently, *agr* mutants either do not synthesize exoproteins or produce them at a reduced rate, while the syntheses of surface proteins such as protein A and fibronectin-binding protein are upregulated (18, 36).

The *agr* locus has been cloned and involves at least five genes, *agrA*, *agrB*, *agrC*, *agrD*, and the *hld* (δ-hemolysin) gene. Sequence analysis indicates that it has features suggestive of a two-component regulatory system (24) in which *agrC* and *agrA* correspond to the signalling and transcription activation components, respectively (18). The *agr* locus is composed of two divergent transcripts designated RNAII and RNAIII. Recent experimental evidence suggests that the *agr* function is probably mediated via the RNAIII transcript (18, 26). However, the differential regulation of two sets of genes inherent in the complexity of the *agr* locus suggests that other global regulatory systems may be present to interact directly or indirectly with the *agr* system.

A regulatory locus designated *xpr* was recently identified by Smeltzer et al. (34). A Tn*551* insertion into this locus has resulted in reduced expression of exoproteins, including lipase, enterotoxin B, hemolysins, protease, and nuclease (30). Transcriptional studies have revealed that the *xpr* locus regulates exoproteins at the transcriptional level (17). Interestingly, the RNAIII transcript level was diminished in an *xpr* mutant compared with the parent (17). This finding and the similarities in exoprotein expression between *xpr* and *agr* mutants suggest that *xpr* and *agr* may behave as interactive regulatory genes (34).

We recently reported a third regulatory locus, called *sar*, that is also involved in the regulation of extracellular and cell wall proteins (8). In contrast to the case for *agr*, mutation in the *sar* locus results in decreased expression of both extracellular and cell wall proteins (13). One of the genes within the *sar* locus (named *sarA*) has recently been cloned (10). Additional phenotypic and transcriptional studies disclosed that the *sarA* gene is required for the optimal expression of RNAIII, the *agr* regulatory molecule (10).

In searching for a fibrinogen-binding-negative mutant, we discovered an additional genetic element that may be involved in the expression of cell wall protein synthesis. In particular, insertional inactivation of this locus by Tn*551* resulted in a lack of expression of both protein A and the clumping reaction with fibrinogen. Phenotypic and mapping studies indicated that this locus is distinct from *agr*, *xpr*, and *sar*. In the rabbit model of experimental endocarditis, the mutant 1E3 was less virulent than the parent.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phage. The bacterial strains and plasmids used in this study are listed in Table 1. Phage ϕ11 was used as a transducing phage for strain 1E3, an ISP479-derived mutant.

Media and antibiotics. Brain heart infusion (BHI), CYGP, and 0.3GL media

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Reference	Comment(s)
S. aureus strains		
ISP479	33	Derivative of 8325-4 that contains the thermosensitive plasmid pI258 as a Tn*551* delivery vehicle; plasmid pI258 carries several selectable markers [*blaI401 mer-14 repA36* Ω1(Tn*551*)]
ISP479C	34	ISP479 derivative in which the plasmid has been cured
DB	8	Clinical isolate that clumps with fibrinogen
1E3	This study	Derivative of 8325-4 with the specific mutation described in this paper
1E3-2	This study	ISP479C transductant with a mutation identical to that in 1E3
RN6911	36	Isogenic mutant of RN6390 with an *agr*::*tetM* mutation
B2	This study	Isogenic mutant of ISP479C with an *agr*::*tetM* mutation
S. epidermidis 6537	7	Isolate from a catheter
Plasmids or plasmid-containing strains		
pLTV1	3	Tn*917* derivative with an *Escherichia coli* selectable marker
RN7037		JM109 containing a pBluescript phagemid with an 800-bp *Hin*dIII-*Pst*I fragment of the protein A gene
DU5384	27	*E. coli* strain carrying a pBR322 plasmid with a 3-kb *Eco*RI-*Hin*dIII fragment of the α-hemolysin gene
pRN6735	36	Derivative of pC194 containing the pI258 *bla* promoter and 2/3 of the *blaZ* gene followed by the 1.5-kb *Mbo*I fragment of RNAIII lacking its promoter

were used for the growth of *S. aureus* (8, 25). Ampicillin and erythromycin were used at concentrations of 10 and 15 μg/ml, respectively. Cadmium nitrate was used at a concentration of 0.1 mM.

Transposon mutagenesis. The temperature-sensitive plasmid pI258 was used to deliver the transposon Tn*551* into the staphylococcal chromosome of strain ISP479. Briefly, ISP479 was grown overnight at 32°C in BHI containing ampicillin (10 μg/ml) and erythromycin (15 μg/ml). The overnight culture was diluted 1:500 in BHI containing 15 μg of erythromycin per ml, grown overnight at 43°C, and plated at 43°C on BHI agar with erythromycin to obtain mutants. These mutants were initially screened for the fibrinogen-binding-negative phenotype as previously described by us (8). Briefly, *S. aureus* cells ($\sim 2 \times 10^{10}$ CFU) were mixed with fibrinogen at a final concentration of 75 μg/ml at room temperature for 15 min. The supernatant containing nonclumping bacteria was plated on BHI agar with erythromycin at 43°C to obtain individual colonies. Each colony was further tested for the loss of the clumping reaction with fibrinogen in microtiter wells containing 200 μl of overnight bacterial culture. Strain DB and a *Staphylococcus epidermidis* isolate were used as positive and negative controls, respectively (8). Each colony in nonclumping wells was evaluated for sensitivity to cadmium (0.1 mM) and ampicillin (10 μg/ml) to ensure loss of the plasmid (19) and was further analyzed phenotypically.

Transduction. Phage ϕ11 was used to produce a phage lysate of mutant 1E3 as previously described (8). The lysate was filtered through a 0.2-μm-pore-size filter and used to infect parental strain ISP479C at a low multiplicity of infection (phage-to-recipient ratio of 1:10). Transductants were selected on 0.3GL agar with erythromycin (25). As a control, we also plated the phage lysate alone to eliminate the possibility of reisolating the donor strain. The ϕ11 lysate of RN6911 was used to infect strain ISP479C to obtain tetracycline-resistant transductants carrying the *agr* mutation.

Southern blot hybridization. Chromosomal DNAs from *S. aureus* strains were prepared from lysostaphin-lysed cells as described previously (8). DNA was digested with restriction enzymes (New England BioLabs, Beverly, Mass.), transferred onto a Hybond N^+ membrane (Amersham, Arlington Heights, Ill.), and hybridized with gel-purified probes labeled with ^{32}P by the random-priming method (Ready to Go Labeling Kit; Pharmacia Fine Chemicals, Piscataway, N.J.). After hybridization, the membrane was washed with SSPE (150 mM NaCl, 10 mM NaH_2PO4, 1 mM EDTA, pH 7.4) as previously described (8) and autoradiographed with an intensifying screen at −70°C.

To map the chromosomal location of the locus described here, clamped homogeneous electric field (CHEF) electrophoresis was performed as described by Smeltzer et al. (33). Chromosomal DNA was digested with *Sma*I in agarose plugs overnight and electrophoresed in 0.5× Tris-borate-EDTA in a CHEF apparatus (BioRad Laboratories, Richmond, Calif.), using previously published parameters (33). Following electrophoresis, the gel was depurinated. The DNA was transferred onto a Hybond N^+ membrane, hybridized with a gel-purified Tn*917* probe, washed, and autoradiographed as described previously (33).

Phenotypic characterization of the parent strain, mutant 1E3, and transductants. For phenotypic characterization, we assayed for the production of α-, β-, and δ-hemolysins on plain and cross-streaked sheep and rabbit erythrocyte agar, using specific indicator strains as standards as previously described (32). We also measured lipase production as determined on 1% Tween 20 agar plates (8). The fibrinogen and fibronectin binding capacities were evaluated by direct binding of ^{125}I-labeled fibrinogen and fibronectin, respectively, to *S. aureus* cells (10^9 CFU) (8, 16, 21).

The extracellular proteins of the parent strain, mutant 1E3, and its corresponding transductant harvested at stationary phase were concentrated 100-fold. The relative extracellular protein A content was detected by an immunoblot as described previously (6). Cell wall proteins were extracted from these strains with lysostaphin in 30% raffinose as previously described (6).

SDS-polyacrylamide gel electrophoresis and immunoblot analysis of extracellular and cell wall protein A. Proteins were separated in sodium dodecyl sulfate (SDS)–9% polyacrylamide gels (20). After electrophoresis, proteins were transferred onto nitrocellulose to assay for extracellular and cell wall-associated protein A as described previously (35). In brief, the blot was incubated with affinity-purified chicken anti-protein A antibody (Accurate Chemicals, Westbury, N.Y.) for 2 h at room temperature at a dilution of 1:3,000. The $F(ab)_2$ fragment of rabbit anti-chicken antibody conjugated to alkaline phosphatase (Jackson Immunoresearch, West Grove, Pa.) was then added (1:5,000 dilution). After incubation for 1 h at room temperature, the reactive bands were visualized as described by Blake et al. (1).

Northern (RNA) blot hybridization. Staphylococcal RNA was extracted by a new method developed in this laboratory (4). A 10-ml culture grown to late log or postexponential phase in CYGP was pelleted, resuspended in 1 ml of FastRNA reagent (BIO101, Vista, Calif.)–0.5 ml of 0.1-mm-diameter Zirconia/Silica beads, and shaken at 6,000 rpm in a high-speed reciprocating homogenizer (FastPREP apparatus; Savant Instrument, Farmingdale, N.Y.) for 20 s. After the addition of 200 μl of chloroform, the mixture was centrifuged at 12,000 × *g* for 10 min. The RNA in the upper aqueous layer was precipitated with isopropanol and resuspended in DEPC-treated water. The total cellular RNA yield was ≈0.5 to 1 mg, with an A_{260}/A_{280} ratio of 2.0. The integrity of the RNA species was verified with a formaldehyde gel.

Five micrograms of RNA from each bacterial strain was applied to a 1.5% agarose–0.66 M formaldehyde gel in MOPS (morpholinepropanesulfonic acid) running buffer as described previously (13). RNA was transferred to a Nytran nylon membrane by using the Turboblotter system (Schleicher & Schuell, Keene, N.H.). RNA on the membrane was cross-linked with UV light; hybridized with a ^{32}P-labeled gel-purified protein A, α-hemolysin, or RNAIII probe in 50% formamide at 42°C overnight; washed, and autoradiographed as described previously (13).

Animal model of endocarditis. Overnight bacterial cultures were harvested by centrifugation (2,000 × *g* for 10 min), washed twice in sterile normal saline, and resuspended in saline to an optical density of 1.6 at 620 nm (≈10^9 CFU/ml). This bacterial suspension was serially diluted in saline to ≈10^3 to 10^5 CFU/ml. Each dilution was confirmed by quantitative culture on blood agar plates. Endocarditis on the aortic valves of New Zealand White rabbits (2 to 2.5 kg) was induced by a modification of the method of Durack and Beeson (14) as described by us (12). In brief, rabbits were anesthetized by intramuscular injection of ketamine chloride (Aveco Inc., Fort Dodge, Iowa) at 35 mg/kg and xylazine (Mobay Corp., Shawnee, Kans.) at 1.5 mg/kg. Forty-eight hours after the introduction of a polyethylene catheter (inner diameter = 0.86 mm) to induce sterile thrombotic endocarditis, groups of ≈10 to 15 animals each were challenged intravenously with various inocula of different bacterial strains (10^3 to 10^5 CFU). This inoculum range encompasses the bacterial inocula causing endocarditis in most of the catheterized rabbits. The 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,

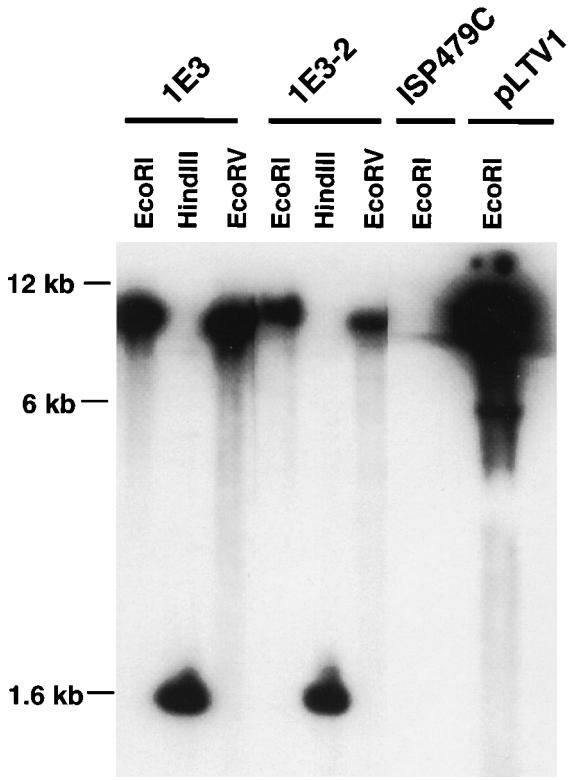

FIG. 1. Southern blot of chromosomal DNAs from strain ISP479C, mutant 1E3, and transductant 1E3-2. The blot was probed with a ^{32}P-labeled 1.5-kb *Hin*dIII fragment internal to transposon Tn*917*. Since there are no internal *Eco*RI and *Eco*RV sites within Tn*551*, we expect one hybridizing fragment from these digests. The plasmid pLTV1 served as a positive control.

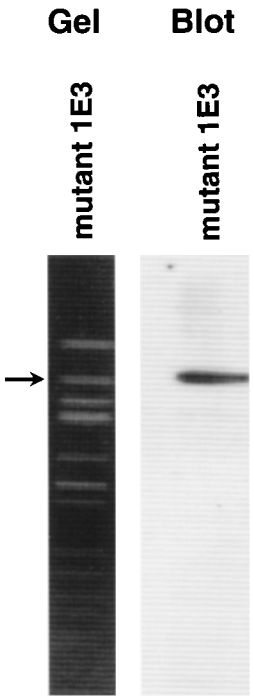

FIG. 2. Mapping of the specific mutation by CHEF electrophoresis. The band hybridizing with the Tn*917* probe (arrow) corresponds to the *Sma*I B fragment.

aortic valves and left ventricular vegetations were removed, pooled, weighed, homogenized in 0.5 ml of normal saline, and quantitatively cultured on plates with and without erythromycin. Animals with negative cultures of their undiluted vegetation homogenates were considered to have had no induction of endocarditis at a particular inoculum (12).

Statistics. Fisher's exact test was used to compare the rates of induction of endocarditis among strains. The mean bacterial vegetation densities 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 of ≤ 0.05 were considered significant.

RESULTS

Transposon mutagenesis and isolation of mutant 1E3. Using ISP479, which contains a thermosensitive plasmid, pI258, as a Tn*551* delivery vehicle (a gift of Peter Pattee, Iowa State University), we enriched for colonies that were cadmium and ampicillin sensitive but erythromycin resistant at 43°C and that did not clump in the presence of fibrinogen. One such colony, designated 1E3, was found. Since Tn*917* has significant homology with Tn*551* (23), we gel purified a 1.6-kb *Hin*dIII fragment of Tn*917* to probe a Southern blot of restriction digests of mutant 1E3 chromosomal DNA. As shown in Fig. 1, only one hybridizing fragment was found in both *Eco*RI and *Eco*RV digests, thus suggesting a single Tn*551* insertion into the staphylococcal chromosome.

Transduction was used to transfer the mutation from mutant 1E3 back into the plasmid-cured parental strain, ISP479C. Over 100 erythromycin-resistant transductants were obtained. One of these transductants, 1E3-2, was chosen for additional studies. Southern blot analysis of this transductant revealed single *Eco*RI and *Eco*RV fragments that hybridized to the Tn*917* probe (Fig. 1). These hybridizing fragments corresponded to the fragments seen with the original mutant, 1E3. These data implied that the Tn*551* insert in the transductant was identical to that in mutant 1E3.

To localize the Tn*551* chromosomal insertion, *Sma*I-digested chromosomal DNA of mutant 1E3 was resolved by CHEF electrophoresis, blotted onto a nylon membrane, and probed with transposon Tn*917*. The probe localized the Tn*551* insertion to the *Sma*I B fragment (Fig. 2), in accordance with the physical map of Pattee et al. (30). This chromosomal location differs from that of the structural gene of protein A and is distinct from those of *agr*, *xpr*, and *sar* (13, 30, 33).

Phenotypic characterization of the parent strain, mutant 1E3, and transductant 1E3-2. In growth studies, it was observed that the parent strain ISP479C and mutant 1E3 did not differ in their rates of growth under antibiotic-free conditions. Other phenotypic alterations in the mutants are shown in Table 2. Notably, the secretion of α-, β-, and δ-hemolysins was increased in the mutant as well as in the transductant 1E3-2 compared with the parent. However, the magnitude of the increase varied (e.g., a slight increase of δ-hemolysin). In contrast, the expression of lipase remained unchanged.

In assays for the determination of cell wall proteins, clumping activity with fibrinogen was conspicuously absent in the mutant. As expected, the binding of ^{125}I-labeled fibrinogen to mutant 1E3 and transductant 1E3-2 was markedly diminished compared with that to the parent (Table 2). Additional phenotypic analysis also disclosed a lack of cell wall protein A in the mutant and its corresponding transductant as assayed by an immunoblot of cell wall protein extracts probed with an affinity-purified chicken anti-protein A antibody (Fig. 3). The secretion of extracellular protein A in the mutant (and also in transductant 1E3-2) was not detectable in a comparison to that

TABLE 2. Phenotypic characterization of the mutation in strain 1E3[a]

Strain	Expression of[b]:						Fibrinogen-binding capacity[c]	Fibronectin-binding capacity[d]
	α-Hemolysin[e]	β-Hemolysin[f]	δ-Hemolysin	Lipase	Clumping activity with fibrinogen	Protein A[g] (cell wall)		
ISP479C	−	+		+	++	++	6,045 ± 46	4,465 ± 34
Mutant 1E3	+	++	+	+	−	−	3,543 ± 237	4,586 ± 23
Transductant 1E3-2	+	++	+	+	−	−	3,155 ± 37	4,961 ± 155

[a] The phenotypes were characterized as described previously by us (8).
[b] ++, strong expression; +, moderate expression; +/−, very weak expression; −, no expression.
[c] Data are presented as counts per minute of ^{125}I-fibrinogen bound to 10^9 CFU ± standard error of the mean. Mutant 1E3 bound significantly less radiolabeled fibrinogen than the parent ($P \leq 0.001$ by the Student *t* test).
[d] Data are presented as counts per minute of ^{125}I-fibronectin bound to 10^9 CFU ± standard error of the mean.
[e] Determined by the zone of hemolysis on cross-streaked rabbit erythrocyte agar plates. Results were also confirmed on immunoblots by probing the extracellular fluid with anti-α-hemolysin antibody.
[f] Determined by the zone of hemolysis on cross-streaked rabbit erythrocyte agar plates.
[g] The presence of protein A in the cell wall extracts (8) was assayed on immunoblots with affinity-purified chicken anti-protein A antibody.

in the parent (data not shown). In contrast, the expression of fibronectin-binding capacity was not significantly altered in the mutant and in the transductant 1E3-2 (Table 2).

Since the expression of α-hemolysin was upregulated in the mutant, we proceeded to evaluate whether α-hemolysin was modulated at the transcriptional level. Northern blot analysis indicated that the mRNA level for the α-hemolysin transcript was enhanced in mutant 1E3 compared with the parent ISP479C (Fig. 4). In concordance with results reported by Vandenesch et al. (36), the transcription of the α-hemolysin gene was maximal during the postexponential phase (optical density at 650 nm = 1.7) for both strains. In contrast to that of α-hemolysin, the expression of protein A was decreased in mutant 1E3 (Fig. 3). A Northern blot probed with a protein A probe disclosed that protein A mRNA was not detectable in mutant 1E3 (Fig. 5), whereas the positive control, the isogenic *agr* mutant B2, demonstrated an expected upregulation in protein A transcription (28, 36). Since a functioning *agr* locus normally represses the transcription of protein A while activating α-hemolysin transcription, we also explored the level of RNAIII, the *agr* regulatory molecule, in mutant 1E3. As displayed in Fig. 6, the RNAIII level was increased in mutant 1E3 compared with that of the parent.

Animal model of endocarditis. In pilot studies, it was determined that the 50% infective dose for parental strain ISP479C was between 10^3 and 10^4 CFU. The rates of induction of endocarditis with the parent strain ISP479C and mutant 1E3 were thus evaluated at various challenge inocula of between

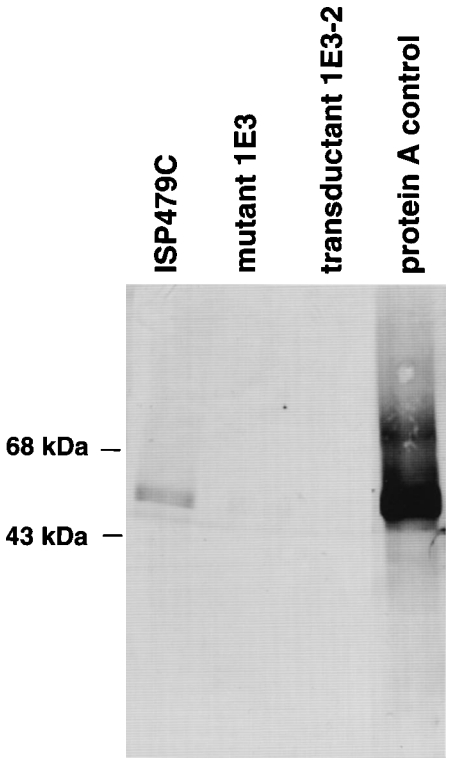

FIG. 3. Immunoblot of cell wall protein extracts of ISP479C, mutant 1E3, and the corresponding transductant. Equivalent volumes of cell wall extracts (0.5 μl) harvested from matched numbers of *S. aureus* cells were applied to each lane. The blot was developed with an affinity-purified chicken anti-protein A antibody (1:3,000 dilution) followed by an alkaline phosphatase conjugate of a rabbit anti-chicken antibody (1:5,000 dilution) and an appropriate substrate (1, 6). Purified Cowan protein A (Calbiochem, La Jolla, Calif.) was used as a positive control.

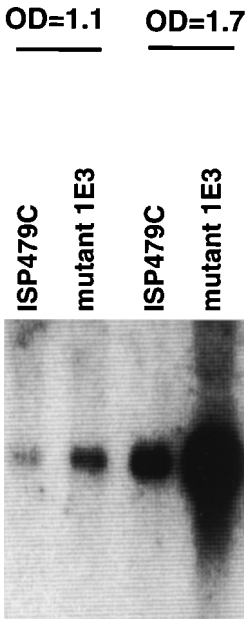

FIG. 4. Northern blot of α-hemolysin transcripts of parental strain ISP479C and mutant 1E3. The size of the transcript was ≈1.8 kb. The optical densities (OD_{650}) of 1.1 and 1.7 correspond to late exponential and postexponential phases, respectively.

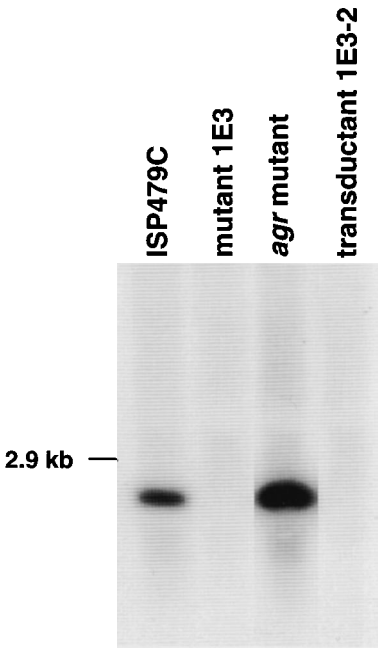

FIG. 5. Northern blot of protein A transcripts in parent strain ISP479C, mutant 1E3, and transductant 1E3-2. The *agr* mutant of ISP479C, strain B2, was included as a positive control. The protein A transcript is ≈1.9 kb. The marked position (2.9 kb) corresponds to the 23S ribosomal RNA band.

10^3 and 10^5 CFU (Table 3). At the lowest challenge inoculum (10^3 CFU), strain ISP479C had a significantly higher induction rate than the isogenic mutant (60 versus 0%; $P < 0.01$) (Table 3). In these two groups of animals, bacterial vegetation densities of the parental strain were significantly higher than those of mutant 1E3 at an inoculum of 10^3 CFU (Table 3). At higher challenge inocula (10^4 and 10^5 CFU), the induction rates did not significantly differ for these two strains. Likewise, the mean bacterial vegetation densities for these two strains were similar at the higher inocula (10^4 and 10^5 CFU) (Table 3). Notably, the number of 1E3 colonies from infected vegetations plated on erythromycin-containing plates was similar to that found on antibiotic-free medium, suggesting maintenance of the transposon Tn*551* upon in vivo passage. With a *Hin*dIII fragment of pLTV1 as a probe, additional Southern blot analysis of chromosomal DNAs of bacterial colonies isolated directly from vegetations also revealed hybridization patterns identical to that of the infecting strain isolated before animal passage (data not shown). These data suggested that the mutation remained stable in animal passage.

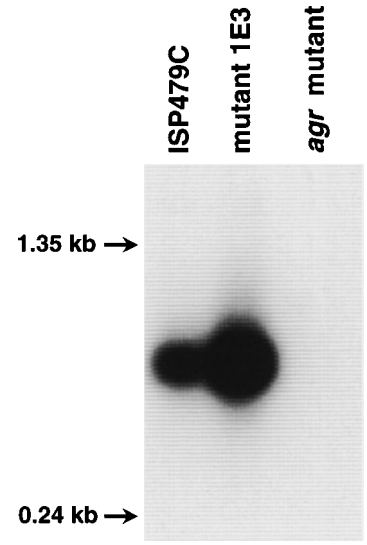

FIG. 6. Northern blot of RNAIII transcripts in parent strain ISP479C and mutant 1E3. The isogenic *agr* mutant B2 was used as a negative control.

TABLE 3. Induction of endocarditis in a rabbit model with parent strain ISP479C and mutant 1E3[a]

Challenge inoculum (CFU)	No. of animals with endocarditis/total no. tested (% with endocarditis) with strain:		Mean $\log_{10}$ CFU/g of vegetations ± SD	
	ISP479C	1E3	ISP479C	1E3
10^3	12/18 (66)	0/10 (0)[b]	4.60 ± 3.40	0[c]
10^4	11/17 (65)	5/9 (55)	5.50 ± 2.70	6.30 ± 3.40
10^5	13/15 (87)	6/9 (66)	6.60 ± 4.40	5.60 ± 2.50

[a] Bacteria were injected into the marginal ear veins at 48 h postcatheterization. All rabbits were sacrificed for morphological examinations and quantitative valvular cultures 48 h after bacterial challenge. Except as indicated, differences in results for strains ISP479C and 1E3 were not significant by Fisher's exact test.
[b] Significantly different from result for strain ISP479C ($P < 0.01$ by Fisher's exact test).
[c] Significantly different from result for strain ISP479C.

DISCUSSION

A single insertion of Tn*551* into a unique locus of the *S. aureus* chromosome in strain ISP479C has resulted in a pleiotropic effect on the expression of a number of extracellular and cell wall proteins in mutant 1E3. Upon transduction of the mutation back into parental strain ISP479C, the same phenotypic alterations were found in the transductant, thus suggesting that the transposon insertion site in the mutant was linked to the phenotypic changes observed (Table 2). Southern blot analysis confirmed that mutant 1E3 and the corresponding transductant had an identical mutation, which was subsequently localized to the *Sma*I B fragment (30). This area of the staphylococcal chromosome is distinct from all known regulatory loci previously described for *S. aureus*. Although a modest increase in α-hemolysin production as a consequence of the mutation was observed, the most remarkable phenotypic alterations were the complete absence of protein A and a lack of fibrinogen binding activity as evidenced by both a poor clumping reaction and a diminished ability to bind radiolabeled fibrinogen. On the basis of these observations, it would appear that this locus is an important genetic element that is involved in the expression of potential cell wall virulence determinants, including protein A (29, 31) and other proteins implicated in the clumping reaction (7, 9, 15).

In addition to genetic mapping data, detailed phenotypic analyses also revealed that this mutation is distinct from other pleiotropic mutations previously described (i.e., *agr*, *xpr*, and *sar*). For instance, the production of hemolysins is mostly downregulated in *agr*, *xpr*, and *sar* mutants (13, 18, 34). In contrast, the secretion of hemolysins in mutant 1E3 is either modestly increased (α-hemolysin) or slightly augmented (δ-hemolysin) (Table 2). With the exception of the fibronectin-binding protein(s), the expression of cell wall-associated proteins (e.g., protein A and fibrinogen-clumping factor) is markedly decreased in mutant 1E3 and its corresponding transductant (Table 2). This pattern of cell wall protein expres-

sion contrasts with that of *sar* mutants, in which both fibronectin- and fibrinogen-binding capacities are decreased, while the protein A content is slightly increased (13). In contrast to the case for mutant 1E3, cell wall proteins such as protein A and fibronectin-binding proteins are usually overexpressed in *agr* mutants. The most unusual phenotypic consequence in mutant 1E3 is the relative absence of cell wall-associated protein A (Fig. 5). The production of extracellular protein A, like that of its cell wall counterpart, was also markedly reduced in mutant 1E3 compared with the parent (unpublished data).

It should be mentioned that residual fibrinogen binding activity remained detectable by radioisotopic analysis in mutant 1E3 despite a negative clumping reaction with fibrinogen (Table 2). This finding may be explained by the observation that *S. aureus* probably possesses several fibrinogen-binding proteins, only one of which exhibits clumping activity with fibrinogen. Notably, two research groups have recently cloned and sequenced two different fibrinogen-binding protein genes that do not appear to have sequence similarity with each other (2, 22). More recently, we have sequenced another fibrinogen-binding protein that had a high degree of homology with coagulase (11). On the basis of phenotypic analysis, the fibrinogen-binding protein (also designated clumping factor) described by McDevitt and Foster is likely to be solely responsible for the clumping phenomenon with fibrinogen (22). Although clumping factor production appears to be altered as a result of the mutation described in the present paper, the influence of this mutation on the synthesis of other fibrinogen-binding proteins was not determined in this study.

Because cell wall protein A was conspicuously absent in mutant 1E3, we also explored the protein A transcript level in mutant 1E3 by Northern blot analysis. Our data suggested that the mutation likely affects the protein A gene at the mRNA level. However, the possibility that this mutation may affect transcript stability together with translational control cannot be entirely ruled out. Whether this mutation affects other cell wall protein genes in a similar fashion will be of clinical interest.

Since this mutation led to decreased expression of cell wall protein A and fibrinogen-binding protein, two putative virulence determinants in *S. aureus*, we evaluated the role of this locus in the induction of endocarditis in the rabbit model (12, 14). Our data showed that mutant 1E3 had a significantly lesser capacity than the parent to induce endocarditis only at the lowest inoculum (10^3 CFU). This induction rate contrasts with that found in our prior animal studies, in which *sar* mutants had significantly lower infection rates than the parent at inocula of both 10^3 and 10^4 CFU (5, 12). Given our earlier observations that *S. aureus* may have a wide array of adhesins and extracellular toxins to facilitate bacterial adherence and propagation within valvular tissues (5), we hypothesize that the lower induction rate in *sar* mutants may be attributable to a combination of reduced synthesis of both extracellular and cell wall proteins (5, 13). In the case of mutant 1E3, it is conceivable that a reduction in the synthesis of cell wall proteins such as protein A and fibrinogen-binding proteins alone may have contributed to a higher induction rate than those of the *sar* mutants. Another interesting feature of the animal studies is a lack of a clear-cut dose response between inocula and induction rates. In our previous animal studies, we have shown that an *agr* mutant derived from strain RN6390 also revealed a reduction in virulence only at the inoculum of 10^3 CFU and not at higher levels (5). This may be explained by the presence of a critical threshold above which valvular infections can be established even in the presence of intact host defense mechanisms.

It is evident from these studies that the locus inactivated in mutant 1E3 is involved in the expression of virulence determinants in *S. aureus*. On the basis of phenotypic analysis, it appears that the effect of the mutation in 1E3 (exoproteins increased and cell wall proteins decreased) is opposite to that found in an *agr* mutant (exoproteins decreased and cell wall proteins increased). The finding that the RNAIII level in mutant 1E3 was higher than that in the parent seems to corroborate the results of the phenotypic studies. Whether the alteration in RNAIII was a direct or indirect effect of the mutation in mutant 1E3 is not clear. Future analysis of the DNA sequence and transcriptional studies of the affected genes should shed light on this issue.

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