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Isolation and Characterization of Transposon Mutants of *Staphylococcus epidermidis* Deficient in Capsular Polysaccharide/Adhesin and Slime

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We used transposon (Tn) mutagenesis to study the role of capsular polysaccharide/adhesin (PS/A) and slime in adherence of *Staphylococcus epidermidis* to catheters. pLTV1, containing Tn*917*-LTV1, was transformed into *S. epidermidis* M187 by protoplast fusion with *S. aureus* RN4220(pLTV1), creating M187(pLTV1). Tn mutants were isolated following growth at 42°C; mutants deficient in PS/A and slime production were selected. PS/A- and slime-deficient Tn mutants had a 10-fold decrease in vitro in the initial phase of adherence to catheters, comparable to levels of strains that do not produce PS/A. Introduction of Tn*917*-LTV1-interrupted DNA from PS/A-deficient mutant M187sn3 into the parental strain via transformation of protoplasts yielded recipients with inserts identical to those of the Tn mutant that were PS/A and slime deficient. Chromosomal DNA flanking the Tn in mutant M187sn3 was cloned into *Escherichia coli*. The cloned DNA was found to hybridize to ~5-kb *Eco*RI fragments from the parental strain and from control Tn mutants that express parental levels of PS/A and to either ~9- or ~14-kb *Eco*RI fragments from other highly adherent, PS/A-producing strains. Mapping studies demonstrated that in the eight PS/A-deficient mutants that have been isolated, the Tn insertions all occur within a region of ~11.6 kb that is defined by three *Eco*RI sites. These results support previous findings indicating that in *S. epidermidis* PS/A is involved with in vitro adherence to plastic biomaterials and elaboration of PS/A is closely associated with slime production.

Staphylococcus epidermidis is a major component of normal skin flora and is virtually avirulent in the absence of foreign bodies. *S. epidermidis* has become an important nosocomial pathogen because of its ability to colonize and infect the prostheses and percutaneous catheters frequently used in contemporary medicine (1, 6, 14, 16, 25, 32). Bacteria that adhere to medical devices are rapidly enveloped in a thick biofilm (slime) (14, 17, 31, 36), which is believed to facilitate bacterial persistence by impeding antibiotic penetration and impairing host defenses (15, 21). However, slime is produced in vitro by only 30 to 60% of clinical isolates (10, 13, 20). Furthermore, slime production does not appear to correlate with the ability of *S. epidermidis* to cause experimental endocarditis (3); this may be due to the large contribution of host-derived components to biofilms that are deposited on biomaterials in vivo (4). In previous studies, we demonstrated that adherence to catheters in vitro correlates with elaboration of a capsular polysaccharide/adhesin (PS/A), which is produced by most clinical isolates (26, 34). In rabbits, antibodies to PS/A prevent bacteremia and endocarditis emanating from infected catheters (22, 34). However, the interrelationship of PS/A and slime and their role in pathogenesis have not been firmly established because of a lack of isogenic mutants that differ in the production of these factors.

In a number of bacterial species, transposon (Tn) mutagenesis has allowed modification of the expression of specific phenotypes so that their contributions to pathogenesis can be studied (23, 29, 35). However, molecular genetic studies of the roles of various surface components of *S. epidermidis* in adherence and infection have been hampered by the lack of a means for efficient introduction of DNA into pathogenic strains, which would enable Tn-mediated mutagenesis of chromosomal loci. To overcome this problem, protoplast fusion was investigated as a means to introduce temperature-sensitive plasmid pLTV1 into highly adherent strains of *S. epidermidis*. pLTV1 is a high-copy-number, 20.6-kb plasmid developed by Camilli et al. (5) (see Fig. 1) that contains a temperature-sensitive origin of replication for staphylococci (pE194ts) linked to tetracycline (Tc) resistance. It is stably maintained at 30°C. The actual transposable unit, Tn*917*-LTV1, is 13.7 kb and encodes erythromycin (Em) and chloramphenicol (Cm) resistances. Tn*917*-LTV1 has several other important properties: production of stable Tn insertions following growth at 42°C, rapid cloning of flanking DNA into *Escherichia coli* by using the ColE1 replicon within the Tn, and analysis of promoter functions of interrupted genes by using the promoterless *lacZ* gene contained within the Tn. This report describes the successful use of pLTV1 to create Tn mutants and to clone the chromosomal DNA flanking the Tn insertion from a highly adherent, PS/A- and slime-producing strain of *S. epidermidis*.

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MATERIALS AND METHODS

Bacterial strains and plasmids. Clinical isolates were obtained from patients with clear-cut diagnoses of *S. epider-*

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midis infection and were identified as *S. epidermidis* by using the Sceptor MIC/ID Panel (Becton Dickinson, Towson, Md.). pLTV1 (5) was maintained in *E. coli* HB101 (provided by P. Youngman, Athens, Ga.), and plasmid DNA was purified via isopycnic centrifugation through a cesium chloride gradient. *S. aureus* RN4220(pLTV1) was prepared by Jean Lee, Boston, Mass., by electroporation of pLTV1 into *S. aureus* RN4220, a restriction-deficient nitrosoguanidine mutant of *S. aureus* RN450 (provided by R. Novick, New York, N.Y.).

Media and reagents. *S. epidermidis* was maintained on Memphis agar (MA) (7) supplemented with 5% (wt/vol) mannitol. Tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.) supplemented with 0.5 M sucrose (TSB-sucrose) was used in the preparation and maintenance of protoplasts. Bacterial cells that survived protoplast fusions and transformations were recovered by use of modified DM3 medium prepared as follows: one liter of deionized water containing 0.75% agarose, 0.5 M sucrose, 0.05 M morpholine propanesulfonic acid (MOPS), 2.5 ml of 5 M NaOH, 50 g of mannitol, and 1 ml of bromcresol purple (16 mg/ml of ethanol) was autoclaved, cooled to 65°C, and then supplemented with 20 ml of sterile 1 M $MgCl_2$; 100 ml of a filter-sterilized solution containing 0.5% yeast extract, 0.5% Casamino Acids, 0.5% glucose, and 0.01% bovine serum albumin; and appropriate antibiotics. Inclusion of mannitol in the DM3 medium allowed differentiation of *S. epidermidis* and *S. aureus*, used as a donor strain in protoplast fusion experiments. RPMI 1640 (GIBCO, Grand Island, N.Y.) was supplemented with 1% (wt/vol) glucose and 10% (vol/vol) fetal bovine serum (Intergen, Purchase, N.Y.). Polyethylene glycol (molecular weight, 6,000 to 8,000; Sigma Chemical Co., St. Louis, Mo.) (40% wt/vol) was prepared in 2× SMM buffer (0.5 M sucrose, 0.02 M $MgCl_2$, 0.02 M maleate, pH 8.0) and filter sterilized. Antibiotics (Sigma) were used at the following concentrations: Em, induction at 0.006 μg/ml, selection at 1 μg/ml; Cm, induction at 0.5 μg/ml, selection at 5 μg/ml; Tc, 12.5 μg/ml; ampicillin (Am), 10 μg/ml. When the *cat* gene of the Tn is present in multiple copies in staphylococci, as with plasmid pLTV1, cells are resistant to >32 μg of Cm per ml; however, the presence of a single copy, i.e., following transposition of the Tn into the chromosome and loss of the plasmid, results in cells that are resistant to low levels of Cm (~8 μg of Cm per ml).

Phenotypic characterization. PS/A production by individual strains was measured by enzyme-linked immunosorbent assay inhibition (34). Wells of microtiter plates (Immulon 2; Dynatech Products, Chantilly, Va.) were coated with purified PS/A (2 μg/ml in 0.04 M phosphate buffer, pH 7.0). A 1:750 dilution of rabbit antiserum raised to purified PS/A was adsorbed for 1 h at 37°C and then for 18 h at 4°C with 10^9 CFU of heat-killed *S. epidermidis* strains harvested from tryptic soy agar plates per ml. Bacteria were removed from adsorbed serum by centrifugation, and the supernatants were added to the sensitized microtiter plates and incubated for 90 min at 37°C. After the plates were washed, goat antibodies to rabbit immunoglobulin G conjugated to alkaline phosphatase were added (1:1,000 dilution; Tago Inc., Burlingame, Calif.). The plates were incubated at 37°C for 60 min and washed, and a *para-o*-nitrophenyl phosphate substrate (Sigma) was added. Plates were read in a microtiter plate reader; PS/A elaboration was measured as the reduction in optical density achieved by the adsorbed serum compared with that of unabsorbed controls. When isogenic mutants were compared, the percent reduction achieved by the parental isolate was defined as a level of 100% PS/A production for the related strains.

Initial adherence to catheter tubing was measured by incubating 1.5-cm lengths of no. 2.8 French silastic catheter tubing (GESCO International Inc., San Antonio, Tex.) for 15 min at 22°C in TSB containing 10^5 CFU of *S. epidermidis* per ml (24, 28, 34). The inoculum was prepared from bacteria grown overnight in stationary TSB cultures. The organisms were sedimented by centrifugation and suspended in TSB, by using a 23-gauge needle to disperse the pellet gently, or the biofilm of slime-producing strains. The colonized catheters were washed for 1 min in phosphate-buffered saline with mixing by a stir bar at 60 rpm; CFU were quantified by rolling the catheters onto tryptic soy agar to dislodge adherent bacteria. Initial adherence data are presented as mean values of triplicate catheters from three experiments, i.e., nine replicate catheters.

Slime production, i.e., accumulation in vitro of bacterial microcolonies embedded in a biofilm (12), was quantified by measuring the optical density at 490 nm of the safranin-stained biofilms produced by inoculation of 10^5 to 10^6 CFU of *S. epidermidis* into 0.2 ml of TSB in flat-bottom 96-well tissue culture plates (Costar, Cambridge, Mass.), followed by growth for 18 h at 37°C. Slime production data are presented as mean values of triplicate wells from three experiments, i.e., nine replicate wells.

Polyacrylamide gel electrophoresis was used to analyze whole-cell lysates and extracellular protein production. Extracellular proteins were obtained by growing *S. epidermidis* overnight at 37°C on a dialysis membrane (molecular weight cutoff, 12,000) overlaid onto a tryptic soy agar plate. Cells and extracellular proteins were isolated by adding 100 μl of Tris-buffered saline (pH 7.2) to the membranes and scraping the growth into a microcentrifuge tube; the bacteria were separated from the extracellular proteins by centrifugation. Protein concentration was determined colorimetrically. Approximately 10 μl of packed, washed cells or 10 μg of protein was boiled for 3 min in reducing buffer and applied to an individual lane of a 10% polyacrylamide gel.

Protoplast transformation. Transformation was accomplished by a modification of the protoplast fusion protocol of Pattee (30). *S. epidermidis* recipients were grown in RPMI 1640 containing fetal bovine serum for 18 h at 30°C. Plasmid donor *S. aureus* RN4220(pLTV1) was grown similarly in RPMI medium containing 12.5 μg of Tc per ml. *S. epidermidis* recipients were treated with 5 U of lysostaphin and 35,000 U of lysozyme (Sigma) in TSB-sucrose for 3 h at 37°C; the plasmid donor was similarly treated for 45 min. Donor and recipient protoplasts were mixed together in 5 ml of TSB-sucrose, sedimented for 8 min at 1,000 × *g*, suspended in 0.2 ml of TSB-sucrose, and fused by gentle mixing for 90 s at room temperature in 1.8 ml of 40% polyethylene glycol. The fusion was stopped by addition of 10 ml of 2× SMM, and the cells were sedimented as described above. The cells were suspended in 2 ml of TSB-sucrose containing inductive levels of Em and Cm; incubated for 90 min at 30°C; then plated on DM3 containing selective levels of Tc, Em, Cm, and Am; and grown at 30°C. The *S. epidermidis* recipient strains produced β-lactamase, and although pLTV1 contains the β-lactamase gene (*bla*), the donor *S. aureus* RN4220(pLTV1) remained sensitive to Am; therefore, Am was used to select against growth of *S. aureus*.

Tn mutagenesis and enrichment for poorly adherent mutants. Transformants with autonomously replicating pLTV1 were grown in TSB containing Tc for 24 h at 30°C and then diluted 1:1,000 into 10-ml aliquots of TSB containing 1 μg of

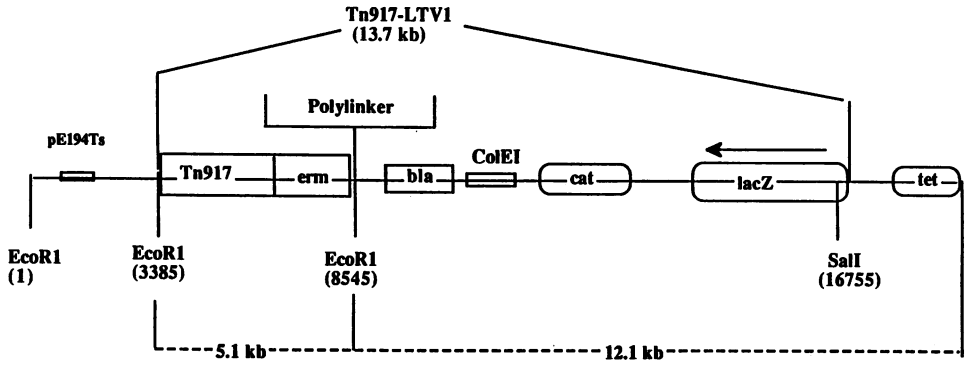

FIG. 1. Linear map of pLTV1 (features of Tn*917*-LTV1 are briefly discussed in the introduction).

Em per ml and 5 μg of Cm per ml which had been equilibrated at 42°C. These cultures were grown without shaking (statically) at 42°C for 24 h. To enrich for mutants poorly adherent to the culture vessel, and presumably poorly adherent to biomaterials, ~0.1 ml of the upper portion of the liquid culture was transferred into TSB containing 1 μg of Em per ml and 5 μg of Cm per ml and grown statically for 24 h at 37°C. This enrichment step was repeated twice. Following enrichment, aliquots of the growth were spread on MA containing 1 μg of Em per ml and 5 μg of Cm per ml, and plates were incubated at 37°C for 24 to 48 h. Individual colonies were tested for loss of slime production. Several colonies were isolated from the biofilm adherent to the culture vessel in order to have Tn-bearing, PS/A- and slime-expressing controls for subsequent experiments.

Genetic analysis. Genomic DNA was prepared from *S. epidermidis* after overnight growth at 30°C in RPMI 1640 supplemented with glucose and fetal bovine serum. Cells were treated with 5 U of lysostaphin and 35,000 U of lysozyme in 0.1 ml of 0.5 M sucrose for 3 h at 37°C and lysed at 65°C for 45 min by addition of 5 U of proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in 0.1 ml of lysis buffer (25 mM Tris-HCl, 50 mM glucose, 150 mM NaCl, 10 mM EDTA, 5% [wt/vol] sodium dodecyl sulfate). Proteins were removed from the DNA by phenol-chloroform extraction; the DNA was precipitated with cold ethanol and spooled onto glass rods.

DNA was incubated with restriction endonucleases (Boehringer) for 18 h at 37°C. Fragments were separated by agarose gel electrophoresis (0.65% agarose in Tris-acetate-EDTA buffer), vacuum transferred to Duralon membranes (Stratagene Cloning Systems, La Jolla, Calif.), and immobilized by UV cross-linking. Hybridizations were performed for 18 h at 65°C with the 5.1- and 12.1-kb *Eco*RI fragments of pLTV1, or plasmids containing DNA cloned from *S. epidermidis*, radiolabelled by nick translation (Boehringer) in the presence of [^{32}P]dCTP. Membranes were washed at high stringency (65°C), and hybridized DNA was visualized via autoradiography.

Back transformation and cloning of Tn-interrupted genes into *E. coli*. To ascertain that Tn interruption was responsible for the phenotypic changes observed in the mutants, back transformation was performed. Genomic DNA from Tn mutants was digested with *Spe*I, and fragments were separated by agarose gel electrophoresis. After visualization by staining with ethidium bromide, fragments of ~40 kb were cut out of the gel and purified by using the Gene Clean II kit (Bio 101 Inc., La Jolla, Calif.). Protoplasts of parental M187 cells were transformed with 1 μg of the ~40-kb *Spe*I fragments of DNA from the Tn mutants by using the protocol for protoplast fusion described above. The resulting transformants were selected on media containing Em and Cm and were characterized for PS/A and slime production as described above.

Cloning of DNA flanking the Tn insert in *S. epidermidis* M187sn3 was performed by transforming competent *E. coli* HB101 with 1 μg of the circularized ~10.5-kb *Eco*RI fragments of DNA from this mutant. The ~10.5-kb DNA fragments were isolated from an agarose gel and purified by using the Gene Clean II kit. These DNA fragments contained the portion of Tn*917*-LTV1 with the *E. coli* replicon and flanking genomic DNA and were circularized with T4 DNA ligase (Boehringer).

RESULTS

Transformation of *S. epidermidis* with pLTV1. Protoplasts of *S. epidermidis* were best obtained when cells were grown in RPMI 1640 with fetal bovine serum to reduce aggregation (28) and increase their sensitivity to lysostaphin (unpublished data). pLTV1 was transferred from *S. aureus* RN4220(pLTV1) to *S. epidermidis* M187 at low frequency. Eight transformants were recovered following fusion of 10^9 CFU of donors and recipients. Analysis of the plasmid DNA following restriction enzyme digestion and hybridization demonstrated that four of the transformants contained pLTV1-like plasmids that had rearrangements in the 12.1-kb *Eco*RI fragment of pLTV1 (Fig. 1). *S. epidermidis* RP62A, RP12, IE75, and M33 were also transformed at low efficiencies (one to nine transformants per fusion); however, all of those transformants contained pLTV1-like plasmids with rearrangements in the 12.1-kb *Eco*RI fragment.

Analysis of the plasmid DNA from one transformant of M187 demonstrated the presence of unrearranged pLTV1. Analysis of the phenotypic characteristics of this strain, M187(pLTV1), demonstrated that the transformant retained the wild-type phenotypes, as determined by the Sceptor MIC/ID system, and as indicated by PS/A and slime production and by measurements of the initial phase of adherence to catheter tubing (Table 1). Three other transformants of strain M187 containing unrearranged pLTV1 demonstrated a 50% reduction in slime production and were poorly adherent to catheters in vitro. This may have been a pleiotropic effect, associated with acquisition of pLTV1 and/or of chromosomal DNA of the *S. aureus* donor and was not studied further.

Production and characterization of Tn mutants. Cultures of M187(pLTV1) were grown at 30°C in the presence of Tc and

TABLE 1. Characterization of Tn*917*-LTV1 mutants of *S. epidermidis* M187

Strain	Relative PS/A production (%)[a]	Initial catheter adherence[b]	Accumulation (slime production)[c]
M187	100	189 ± 24	1.6
M187na[d]	5	4 ± 3	0.2
M187(pLTV1)	90	155 ± 39	1.4
M187sn1	16	4 ± 2	0.2
M187sn3	9	13 ± 4	0.2
M187sp2	108	176 ± 17	1.6
M187sp11	122	167 ± 23	1.6
M91[e]	0	3 ± 1	0.1

[a] Measured by enzyme-linked immunosorbent assay inhibition and compared with that of *S. epidermidis* M187 (i.e., 100%).
[b] Mean ± standard error of the mean number of adherent CFU per catheter after incubation in 10^5 CFU/ml for 15 min at 22°C.
[c] Optical density at 490 nm of a safranin-stained, 18-h biofilm.
[d] na, nonadherent phenotypic variant of *S. epidermidis* M187.
[e] Nonadherent, PS/A- and slime-deficient clinical isolate of *S. epidermidis*.

then transferred to media equilibrated to 42°C and containing Em and Cm to prevent plasmid replication and allow recovery of mutants in which Tn*917*-LTV1 had transposed into the chromosome. The rate of transposition was not determined, since the cultures were immediately enriched for poorly adherent Tn mutants by subculturing the upper, planktonic phase of static cultures that were grown at the nonpermissive temperature in the presence of the selective antibiotics Em and Cm. In addition, Tn mutagenesis was performed in a series of small volumes (10 ml) to facilitate the production of a number of independently derived mutants and therefore avoid a large number of clonally derived isolates that might be obtained from a single, large-scale mutagenesis experiment. After three rounds of enrichment for the poorly adherent cells, the cultures were inoculated onto MA containing 1 μg of Em per ml and 5 μg of Cm per ml; the resulting colonies were screened for slime production and loss of Tc resistance, which indicated transposition of Tn*917*-LTV1 into the genome and loss of autonomously replicating pLTV1. Colonies that retained Tc resistance were observed at a frequency of about 5%. In the few such colonies that were studied, it appeared that pLTV1 had lost temperature sensitivity, and the transposition event(s) causing their PS/A- and slime-deficient phenotypes was not studied further.

Slime-deficient mutants were readily isolated from 14 of 15 independent Tn mutagenesis enrichments. Following enrichment, 7 to 90% of the colonies tested were slime deficient. *S. epidermidis* has been reported to produce natural phenotypic variants deficient in slime production (8) that can be obtained by enrichment for the nonadherent cells growing in the upper portion of a broth culture. Such a naturally occurring slime-deficient variant of strain M187 (strain M187na) was isolated (Table 1), but 10 to 14 enrichment cycles were required to obtain this variant and its phenotype was unstable, rapidly reverting to the wild-type phenotype in the absence of further enrichment.

Eight slime-negative (sn) Tn mutants of M187 were produced; in each Tn mutant, a single copy of Tn*917*-LTV1 transposed into a unique site in the chromosome of M187 (Fig. 2). The sn mutants were characterized as being PS/A deficient and poorly adherent to silastic catheters; data are presented for Tn mutants M187sn1 and M187sn3 (Table 1); similar deficiencies occurred in the other six slime-deficient Tn mutants (data not shown). Fifteen slime-positive (sp)

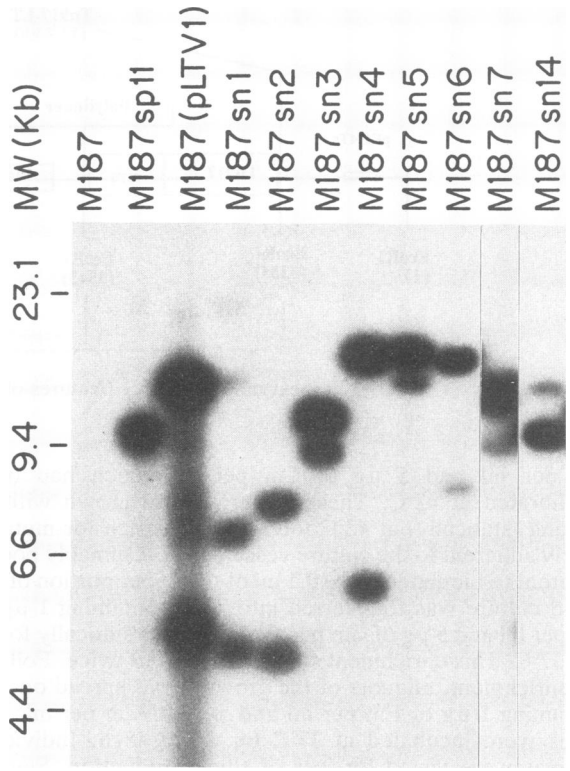

FIG. 2. Autoradiograph of a Southern blot of *Eco*RI-digested genomic DNAs of (i) *S. epidermidis* M187 transformed with autonomous pLTV1 [M187(pLTV1)] and (ii) Tn mutants resulting in the sn or sp phenotype. The blot was probed with ^{32}P-labelled fragments of pLTV1. Tn*917*-LTV1 contains a single *Eco*RI site; one Tn insertion would yield two fragments that hybridize with the probe (with M187sp11, both fragments are ~9.4 kb). MW, molecular size.

isolates containing single Tn inserts were isolated from the biofilms produced in the culture vessels during the enrichments for the sn mutants; they demonstrated that insertion of Tn*917*-LTV1 into the M187 chromosome at sites differing from those of the sn mutants did not affect PS/A or slime production or initial catheter adherence (Table 1). To determine whether the Tn insertion resulted in obvious loss of proteins, whole bacterial cells and extracellular protein preparations from the parental strain with autonomous pLTV1 and from sn and sp mutants were analyzed by using polyacrylamide gel electrophoresis. Such analysis did not reveal any apparent loss of proteins in any of the sn Tn mutants of *S. epidermidis* M187 (data not shown).

Cloning of sn mutant DNA into *E. coli*. Tn mutant *S. epidermidis* M187sn3 was selected for further study. Genomic DNA was digested with *Eco*RI, Southern blotted, and hybridized with pLTV1. Since Tn*917*-LTV1 contains a single *Eco*RI site in the polylinker, two bands of hybridization were expected. The ~8.2- and ~10.5-kb bands observed (Fig. 2) indicated that insertion of the 13.7-kb Tn*917*-LTV1 into an ~5-kb *Eco*RI fragment of the chromosome had occurred. *Eco*RI fragments of ~10.5 kb from M187sn3 hybridized with the 12.1-kb *Eco*RI fragments of pLTV1, i.e., the ~8.7-kb portion of Tn*917*-LTV1 that encodes the ColE1 replication functions and resistance to Am and Cm (Fig. 1). Therefore, ~1.8 kb of *S. epidermidis* DNA flanked the end of the Tn*917*-LTV1 insert on those ~10.5-kb *Eco*RI fragments. They were purified from an agarose gel and circular-

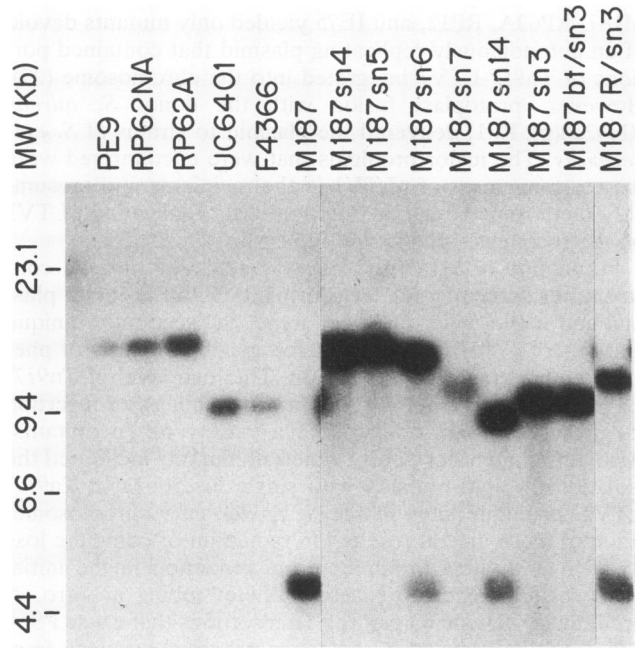

FIG. 3. Autoradiograph of a Southern blot of *Eco*RI-digested genomic DNA from *S. epidermidis* PE9, RP62A, RP62NA, UC6401, F4366, and M187 and its sn and sp Tn mutants. The back transformant M187bt/sn was made by transformation of DNA from strain M187sn3 into wild-type strain M187. The revertant M187R/sn3 was obtained from strain M187sn3 when Tn*917*-LTV1 spontaneously transposed to a different site in the chromosome. The blot was probed with ^{32}P-labelled pSN3E, which was obtained from M187sn3 and contained the ~8.7-kb portion of the Tn and ~1.8 kb of chromosomal *S. epidermidis* DNA flanking the Tn insertion. The Tn portion of pSN3E hybridized to the Tn in the mutants (Fig. 2), and the cloned M187sn3 portion hybridized to ~5-kb fragments from the wild type, from mutants M187sn6 and sn14, and from M187R/sn3. The cloned *S. epidermidis* DNA also hybridized to ~9- or ~14-kb *Eco*RI fragments from the other PS/A- and slime-producing strains. MW, molecular size.

ized with T4 DNA ligase. Competent *E. coli* was transformed with 1 μg of the ligated DNA and selected for Am and Cm resistances. The resulting plasmid, pSN3E, was used as a probe in Southern blot analysis of the parental strain and the Tn mutants. Owing to inclusion of ~8.7 kb of Tn*917*-LTV1, pSN3E hybridized to the identical Tn-containing *Eco*RI fragments of the sn mutants, as did the 12.1-kb *Eco*RI fragments of pLTV1 (Fig. 3). In addition, the ~1.8 kb of flanking DNA cloned from M187sn3 hybridized with ~5-kb *Eco*RI fragments from wild-type M187 and from sp Tn mutants with intact production of PS/A (data not shown), with DNAs from other PS/A- and slime-producing strains of *S. epidermidis*, with ~9-kb fragments from strains F4366 and UC6401, and with ~14-kb fragments from strains RP62A, RP62NA, and PE9. pSN3E was next digested with *Eco*RI and *Sal*I and the fragments were purified from an agarose gel; the ~1.8-kb fragments, containing the cloned *S. epidermidis* DNA and ~0.3 kb at the end of the Tn, were used to probe the same Southern blot and yielded a hybridization pattern the same as that shown in Fig. 3. This verified that the restriction fragment length polymorphisms were due to the cloned flanking DNA rather than homologies with portions of the Tn. The flanking DNA did not hybridize with DNAs from 12 PS/A- and slime-deficient strains (data not shown). The absence of the parental ~5-kb *Eco*RI fragments (Fig. 3) in *S. epidermidis* Tn mutants M187sn4, sn5, and sn7 and in the back transformant M187bt/sn3 (discussed below) indicated that the Tn was inserted in those strains in different sites within the same ~5-kb fragment, resulting in loss of PS/A and slime production.

Genetic mapping of Tn inserts in PS/A-deficient Tn mutants. A series of Southern blots was prepared with genomic DNAs from parental strain M187 and its sn mutants cut with *Hin*dIII, *Sal*I, or *Sma*I. The blots were probed with the *erm*-containing fragments (~5.1 kb) of pLTV1, stripped of the probe, and then probed with the *lacZ*-containing fragments (~12.1 kb). Approximate sizes of the bands that hybridized to the probes were calculated, and the Tn insertions were assigned locations on the preliminary map (Fig. 4) based upon the ~5-kb *Eco*RI fragments that appeared to contain the Tn insertions in M187sn3, sn7, sn4, and sn5. Fragments from strains M187sn7 and sn5 do not add up to the predicted ~5-kb fragment plus the ~13.7 kb provided by the Tn insertion. In the case of M187sn7, a rearrangement has occurred in the Tn in the region of *erm* and the polylinker. With M187sn5, the Tn appears intact and the inconsistency appears to be due to the proximity of the insertion to the *Eco*RI site; the location of that insertion relative to other Tn insertions has been verified by using other restriction enzymes. Mapping studies indicated that the Tn insertions in the other four sn mutants occurred in an adjacent ~6.6-kb *Eco*RI fragment. In both M187sn1 and sn2, large portions of the *lacZ*-containing fragments of Tn*917*-

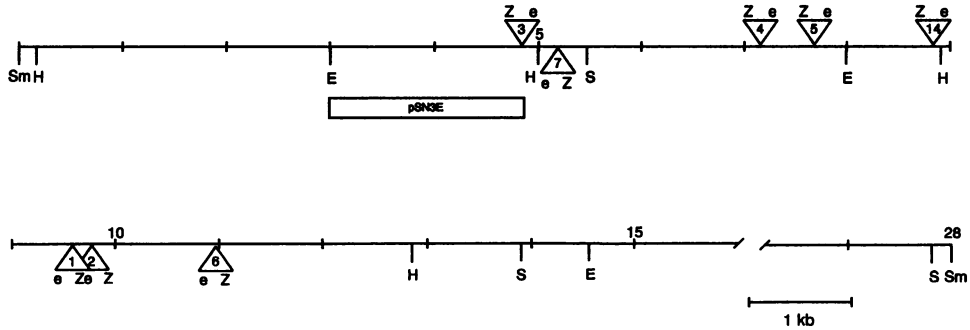

FIG. 4. Preliminary map of the ~28-kb *Sma*I fragment of the chromosome of *S. epidermidis* M187 that contains Tn*917*-LTV1 insertions associated with loss of PS/A and slime production. Sites of the Tn insertions are indicated by the triangles; Z and e indicate the orientation of the Tn insertion, i.e., *lacZ* and *erm* genes of the Tn. The region cloned in pSN3E is indicated below the map. Restriction sites: E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Sm, *Sma*I.

LTV1 have been lost. Those mutants were isolated by using the technique suggested by Camilli et al. (5), i.e., transposition at 42°C in medium containing only Em; that end of the Tn was lost without the selective pressure of Cm. Southern blots of *Sma*I-digested genomic DNA demonstrated that the Tn insertions in all eight of the sn mutants occurred within an ~28-kb *Sma*I fragment and confirmed the orientation and approximate locations of the Tn insertions in the genetic map.

Confirmation that deficiency in PS/A and slime production in sn mutants is Tn mediated. The Tn insertions in *S. epidermidis* M187sn3 and the other seven sn mutants of this strain were stable for 12 months of weekly subcultures on MA containing Em and Cm and throughout extensive passage in TSB in the absence of antibiotics. Nevertheless, attempts to obtain a revertant generated by excision of the Tn from its original insertion site were made. To detect reversion of the M187sn3 mutant to the parental PS/A- and slime-producing phenotype, the strain was grown in static TSB cultures without Em and Cm for 3 days at 37°C and cells adherent to the walls of the culture tubes were subcultured into fresh medium. This enrichment for the adherent phenotype was repeated six times, and finally, individual colonies were isolated and tested for slime production. From one of four such enrichments of M187sn3, a single revertant clone, designated *S. epidermidis* M187R/sn3, was isolated. It produced parental levels of PS/A and slime and exhibited a high level of initial adherence to silastic catheter tubing (data not shown). Southern blot analysis of M187R/sn3 (Fig. 3) demonstrated that the Tn had transposed from its original site in strain M187sn3 and the excision appeared to be exact, i.e., the ~5-kb *Eco*RI fragment that was lost owing to Tn interruption of the function(s) encoded within that fragment was restored.

Southern hybridization analysis revealed that Tn*917*-LTV1 lacked an *Spe*I site and in strain M187sn3, the Tn was located on *Spe*I fragments of ~40 kb. By using the protocol for protoplast fusion, parental strain M187 was transformed with 1 μg of ~40-kb *Spe*I fragments of DNA from strain M187sn3 that were purified from an agarose gel. All seven of the resulting Em- and Cm-resistant colonies were deficient in PS/A and slime production (data not shown) at levels comparable to those of the M187sn3 donor. Southern hybridization analysis of the back transformant M187bt/sn3 demonstrated that colonies transformed in this way had acquired Tn*917*-LTV1 via homologous recombination into the identical DNA fragments as in the M187sn3 DNA donor (Fig. 3). Similarly, when the ~40-kb *Spe*I fragments from another sn mutant, M187sn5, were used to transform parental strain M187, slime-deficient transformants that were resistant to Em and Cm and had a Southern hybridization pattern identical to that of the sn mutant donor were obtained (data not shown).

DISCUSSION

This report describes the successful use of Tn mutagenesis of *S. epidermidis* to generate derivatives deficient in the elaboration of both slime and PS/A. Grueter et al. (19) have previously described the introduction of Tn*917* from plasmid pTV1 into *S. epidermidis* via protoplast transformation, but none of those derivatives were characterized as to phenotypic changes. Electroporation has been used to introduce a 2.9-kb plasmid, pC194, which lacks transposable elements, into coagulase-negative staphylococci (2). Attempts in our laboratory to electroporate pLTV1 into *S. epidermidis* M187, RP62A, RP12, and IE75 yielded only mutants devoid of an autonomously replicating plasmid that contained portions of Tn*917*-LTV1 integrated into the chromosome (27). However, protoplast fusion with the donor *S. aureus* RN4220(pLTV1) delivered the plasmid to strains of *S. epidermidis*. The major problems that were encountered were the rearrangement of pLTV1 in the recipients and presumably pleiotropic effects of autonomously replicating pLTV1 on the recipient's phenotype.

Production of M187(pLTV1) was achieved through conscientious screening for transformants with the intact plasmid and with a wild-type phenotype. Subsequently, unique features of Tn*917*-LTV1 facilitated genetic analysis of phenotypes targeted by Tn insertion. The high level of Tn*917*-LTV1 transposase activity and its randomness of insertion (5) promoted the production of a variety of Tn mutants. Also, enrichment for poorly adherent mutants facilitated the isolation of eight mutants with single insertions of Tn*917*-LTV1 in unique sites in the *S. epidermidis* chromosome. Each of these inserts resulted in reduction or complete loss of PS/A and slime production and reduction in the initial phase of adherence to silastic catheter tubing in vitro. A preliminary genetic map of the Tn insertions that cause PS/A deficiency in strain M187 has been prepared by using fragments of pLTV1 and DNA cloned from M187sn3 to probe Southern blots. The *Eco*RI, *Hin*dIII, and *Sal*I sites have been mapped within an ~11.6-kb region defined by three *Eco*RI sites that contains all of the Tn insertions associated with PS/A deficiency in the M187 sn mutants.

The ColE1 replication functions and polylinker cloning sites engineered into Tn*917*-LTV1 (5) enabled the cloning of chromosomal DNA flanking the Tn insertion by simple circularization of restriction enzyme-digested genomic DNA from the mutants and transformation into competent *E. coli*. Use of this cloned DNA as a probe indicated that the gene(s) interrupted by Tn*917*-LTV1 in M187sn3 is present in other highly adherent strains of *S. epidermidis*; studies are in progress to assess the presence and restriction fragment length polymorphisms of those and other putative PS/A genes targeted by Tn insertion in a series of PS/A-proficient and -deficient clinical isolates of *S. epidermidis*.

The clustered nature of the Tn insertions that are responsible for PS/A deficiencies in M187 sn mutants and the isolation of back transformants and revertants of M187sn3 are strong proof that PS/A genes have been targeted by Tn-insertional inactivation. All of the sn mutants of M187 had reduced levels of PS/A production, of accumulation into biofilms (slime production), and of the initial phase of adherence to catheter materials, thereby suggesting a close relationship among these three phenotypic characteristics. This finding supports our previous data that PS/A-producing strains of *S. epidermidis* generally adhere better to plastic biomaterials than do PS/A-deficient strains (28, 34). We have not obtained mutants deficient in slime production but not in PS/A production, or vice versa. Since extracellular slime has been reported to be involved in interference with granulocyte function (21) and human cellular immune responses (18), as well as being a factor in foreign body colonization and infection (9, 11, 12), isogenic Tn mutants deficient in slime will be useful in further defining the role of this substance in pathogenesis. Studies are in progress to assess the virulence of PS/A- and slime-deficient isogenic mutants in animal models of catheter-related bacteremia (22) and endocarditis (33). The establishment of pLTV1 in M187 has provided a starting point for the genetic analysis of the

contributions of determinants of *S. epidermidis* to pathogenicity.

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