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Characterization of Tn*917* Insertion Mutants of *Staphylococcus epidermidis* Affected in Biofilm Formation

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Biofilm formation is thought to result from the concerted action of primary attachment to a specific surface and accumulation in multilayered cell clusters. Here we describe the isolation and characterization of transposon (Tn*917*) mutants of *Staphylococcus epidermidis* O-47 which were biofilm negative in the polystyrene microtiter plate assay. Among 5,000 Tn*917* insertion mutants, 4 biofilm-negative mutants were isolated. Each mutant carried one copy of Tn*917*. The mutants were divided into two phenotypic classes: class A (mut1 and mut1a) and class B (mut2 and mut2a). Mutants of phenotypic class A lacked four cell surface proteins, were less hydrophobic, and were affected in primary attachment to polystyrene, but were still able to form multilayered cell clusters. They were able to form a biofilm on a glass surface, a trait that was even more pronounced than in the wild-type strain O-47. Loss of several surface proteins might have led to the reduced surface hydrophobicity by unmasking hydrophilic structures, thus favoring primary attachment to a glass surface and leading to subsequent biofilm formation. Mutants of phenotype class B were able to attach to polystyrene but were unable to form multilayered cell clusters, had unchanged cell surface proteins and hydrophobicity, and were unable to form a biofilm on a glass surface. mut1 and mut2 could be complemented by wild-type DNA fragments containing the Tn*917* insertion sites of mut1 and mut2, respectively. The complemented biofilm-positive clone mut1(pRC20) produced a 60-kDa protein which is postulated to function as the adhesin for binding to plastic. The traits of binding to polystyrene and the ability to form multilayered cell clusters are phenotypically and genetically distinct.

Staphylococcus epidermidis is a ubiquitous inhabitant of human skin and mucous membranes that rarely causes infections in normal hosts (12, 25). The increase in the number of infections by these organisms has been correlated with the growing number of immunocompromised patients in hospitals and with the widespread medical use of prosthetic and indwelling devices (e.g., intravascular catheters, cerebrospinal fluid shunts, prosthetic cardiac valves, artificial cardiac pacemakers, and orthopedic appliances) (5). This selective virulence suggests the existence of bacterial factors that enable *S. epidermidis* to opportunistically infect foreign bodies. One of these factors could be a preferential ability to adhere to and colonize the smooth surfaces of medical devices. Indeed, scanning electron microscopic investigations of *S. epidermidis*-infected polymer devices have shown that multilayered cell clusters of staphylococci are embedded in a thick matrix of a slime substance (7, 21–23) which is composed of a mixture of teichoic acid and protein (10) or of extracellular polysaccharides (3). The totality of the accumulated bacteria and the extracellular slime on a solid surface is referred to as biofilm. Whether slime production is an important virulence factor has been a matter of debate for years (5, 11, 20). Biofilm formation is suggested to proceed in two phases: primary attachment of *S. epidermidis* cells to a polymer surface is followed by accumulation in multilayered cell clusters on the polymer surface, which requires cell aggregation (intercellular adhesion).

The question of whether attachment to a polymer surface involves a protein (30) or a polysaccharide (17, 31) is also a matter of debate. It was shown that a transposon mutant of *S. epidermidis* which was deficient in capsular polysaccharide/adhesin and slime was also affected in the initial phase of adherence to catheters (17). It has been assumed that the cell surface hydrophobicity is important for the binding to plastic (9, 14, 19).

In a genetic approach, two types of biofilm-negative *S. epidermidis* mutants affected in the formation of multilayered cell clusters have been obtained. One mutant, obtained by transposon mutagenesis of *S. epidermidis* 9142, lacks a hexosamine-containing polysaccharide as an intercellular adhesin (15). The other mutant, obtained by mitomycin mutagenesis of *S. epidermidis* RP62A, lacks two extracellular proteins (115 and 29 kDa) (29). A correlation between hemagglutination and biofilm formation on polystyrene has also been described (28). Since the mechanisms of biofilm formation are not fully understood, we isolated a number of biofilm-negative transposon insertion mutants of the clinical isolate *S. epidermidis* O-47. We characterized two genetically distinct classes of mutants affected either in primary attachment or in intercellular adhesion.

MATERIALS AND METHODS

Bacterial strains and media. The biofilm-forming strain *S. epidermidis* O-47 was isolated from a patient at the Institut für Medizinische Mikrobiologie und Hygiene, Universität zu Köln. This strain was used to isolate biofilm-negative transposon insertion mutants. *S. epidermidis* RP62A was described by Christensen et al. (4). The staphylococci were cultivated in tryptic soy broth (TSB) (Difco) or in BM medium (8) at 37°C, unless otherwise stated. An overnight culture of O-47 reached on the average an optical density at 578 nm (OD_{578}) of between 8.0 and 9.0 (an OD_{578} of 0.1 corresponds to 10^8 CFU/ml). TS agar (TSA) plates contained 1.4% agar.

DNA isolation, plasmids, transformation, and Southern hybridization. Chromosomal staphylococcal DNA was prepared according to the procedure of Marmur (16). Plasmids pTV1ts (32), pT181mcs (1), pCA44 (27), and pRB473 (2)

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were prepared from *Staphylococcus carnosus* by using lysostaphin (0.4 U/OD_{578} unit) to digest the peptidoglycan and using the cleared-lysate method (18) or by using NUCLEOBOND (Macherey-Nagel GmbH, Düren, Germany) according to the protocol supplied by the manufacturer. *S. epidermidis* O-47 and *S. carnosus* were transformed by protoplast transformation (8).

For Southern hybridization, chromosomal DNA was digested with *Eco*RI or *Bam*HI and separated on an agarose gel. For the transfer of DNA to nylon filters, Hybond-N 0.45-μm-pore-size filters (Amersham, Amersham, Great Britain) were used. Southern blotting was performed using a vacuum blot apparatus (VacuGene; Pharmacia LKB, Bromma, Sweden), as recommended by the supplier. A 1.8-kb *Bgl*II fragment of Tn*917* was used as a DNA probe. Labeling of the fragment and DNA hybridization were performed according to the protocol supplied with the DIG DNA labeling and detection kit (Boehringer, Mannheim, Germany).

Transposon mutagenesis. *S. epidermidis* O-47 harboring pTV1ts::Tn*917* (32) was grown overnight in BM containing chloramphenicol (cm; 10 μg/ml; resistance encoded by pTV1ts) at 30°C. The bacterial culture was then diluted 1:1,000 in BM. Cells of this dilution were spread on BM agar plates containing erythromycin (EM; 20 μg/ml; resistance encoded by Tn*917*) and incubated at 45°C for 36 h. Transposon insertion mutants were subcultivated on BM agar plates containing EM (10 μg/ml).

Cloning of mut1- and mut2-complementing wild-type DNA fragments. Tn*917*-containing *Eco*RI fragments were cloned directly in *S. carnosus* by using pT181mcs as a vector and selecting for EM-resistant (10 μg/ml) transformants. By using the Tn*917* flanking region of mut1 as a DNA probe, a hybridizing 16-kb *Pst*I fragment of the wild-type DNA was ligated in pT181mcs and transformed in *S. carnosus*. The isolated plasmid was used to complement mut1. The vector pRB473 was used to construct plasmid pRC20, which contains a 2.7-kb *Hin*dIII fragment of the 16-kb *Pst*I fragment. In a similar way a mut2-complementing wild-type DNA fragment was cloned by using the vector pCA44.

Quantitative assay of biofilm formation on polystyrene and glass. Biofilm-positive *S. epidermidis* strains form a macroscopically visible biofilm firmly attached to the surfaces of various plastic materials. For quantitation of the biofilm-forming capacity, a test similar to those described previously for biofilm production was used (6, 24). *S. epidermidis* strains were cultivated overnight in TSB supplemented with 0.25% glucose. The culture was diluted 1:200 in TSB-glucose, and 200 μl of this cell suspension was used to inoculate sterile, 96-well polystyrene microtiter plates (Greiner, Frickenhausen, Germany). After cultivation for 24 h at 37°C, the wells were gently washed twice with 200 μl of sterile phosphate-buffered saline (PBS). The plates were air dried, and the remaining surface-adsorbed cells of the individual wells were stained with 0.1% safranin (Serva) for 30 s. Absorbance was measured with a Micro-ELISA Autoreader (Titertek Multiscan) at 490 nm. A well, to which sterile TSB lacking cells was added, served as a control; the value for this well was subtracted from the experimental readings. Each assay was performed in triplicate.

Determination of biofilm formation on a glass surface was carried out essentially in the same way, except that glass tubes were used instead of microtiter plates and 5 ml of TSB was inoculated instead of 200 μl.

Initial adherence on polystyrene surface. Cultures were aerobically grown in TSB to the early stationary phase. Cell suspensions were adjusted with PBS to an OD_{578} of 0.1. A 10-ml aliquot of each cell suspension was added to a polystyrene petri dish (Sarstedt) and incubated for 30 min at 37°C. Petri dishes were washed five times with 5 ml of PBS, and adhered bacterial cells were observed by microscopy, photographed, and counted; the number of adhered cells per square centimeter was determined.

Bacterial hydrophobicity assay. Bacterial adherence to xylene was determined in a xylene-water system as described by Rosenberg et al. (26). Cells were cultivated to early stationary phase in TSB and washed once in PBS buffer. The OD_{578} was adjusted to 1.0 in the same buffer. Analytical grade *p*-xylene (Merck AG, Darmstadt, Germany) was added to 1.2-ml cell suspensions (final *p*-xylene concentration was 4, 8, and 20%) in sterile glass or plastic tubes. Glass and plastic tubes revealed comparable results. The tubes were mixed on a mixer for 60 s. After phase separation, the optical density of the aqueous phase was determined. The percentage of bacteria that adhered to xylene was calculated by dividing the difference between the OD_{578} values of the suspensions before and after mixing with xylene by the original OD_{578} value before mixing and multiplying the result by 100% (19).

SDS-PAGE, protein isolation, and sequencing. Surface proteins were isolated by cultivating the staphylococcal strains on a sterile dialysis membrane laid on TSA. The plates were inoculated with 1 ml of an overnight culture (precultivated on TSA) adjusted in PBS buffer to an OD_{578} of 1.0. After 24 h of cultivation, cells were rinsed from the dialysis membrane and washed once in PBS. The cell pellet was resuspended in 1 volume of Laemmli sample buffer (13) and boiled for 5 min. After centrifugation, 30 μl of the supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% separation gel, 4.5% stacking gel). Proteins were stained with Coomassie brilliant blue R250 (0.1%; Serva).

For protein sequencing the surface proteins were separated by SDS-PAGE as described above and blotted onto a polyvinylidene difluoride membrane. After staining with Coomassie blue, the 60-kDa proteins of the wild type and mut1(pRC20) were cut out and the N-terminal amino acid sequences were determined on an Applied Biosystems 477A gas phase protein sequencer.

RESULTS

Transposon mutagenesis. In the quantitative assay for biofilm formation, the clinical biofilm-positive isolate *S. epidermidis* O-47 and strain RP62A gave similar results: only the biofilm-forming capacity of RP62A was more pronounced. A disadvantage of RP62A was both its resistance to DNA transformation and its plasmid (pEpi62)-encoded resistance to erythromycin, kanamycin, streptomycin, and penicillin (29); we were unsuccessful in curing the 29-kb pEpi62. In contrast to strain RP62A, strain O-47 was sensitive to these antibiotics and carried no plasmid, and we were able to introduce plasmids into strain O-47 by protoplast transformation (8). For these reasons strain O-47 was chosen for further genetic analyses of biofilm formation.

For transposon mutagenesis we introduced the transposon Tn*917*-carrying plasmid pTV1ts into strain O-47. Transposon insertion mutants were selected by cultivation at 45°C in the presence of EM. Approximately 90% of the Em^r clones were Cm^s, indicating successful transposition of Tn*917* into the chromosome and loss of the plasmid. The transposition frequency was on the average 6×10^{-4}. Approximately 5,000 Em^r Cm^s colonies were tested for loss of biofilm formation by the quantitative assay.

Four biofilm-negative mutants designated mut1, mut1a, mut2, and mut2a were identified. Southern blot analysis with Tn*917*-specific DNA as a probe and *Eco*RI- or *Bam*HI-restricted chromosomal DNA of the various mutants indicated that each mutant carried one copy of Tn*917*.

Based on phenotypic characterization, two classes, A and B, were distinguished: transposon mutants mut1 and mut1a belonged to phenotype class A, and mut2 and mut2a belonged to phenotype class B. All mutants within each class showed identical or at least very similar characteristics in all traits tested (see below). Southern blot analysis revealed that Tn*917* was inserted at different loci in mut1 and mut2. A restriction map of Tn*917* insertion sites in the chromosomes of mut1, mut2, and mut2a with the corresponding flanking DNA sequences in mut2 and mut2a is shown in Fig. 1. The Tn*917* insertion sites are linked in mut2 and mut2a. In mut1, transposition was accompanied by an 8-kb deletion of chromosomal DNA.

Complementation of mut1 and mut2 by wild-type DNA. In order to confirm that the biofilm-negative phenotype of the mutants is due to the Tn*917* insertion, we cloned from the wild-type strain 16- and 5-kb DNA fragments, including the transposon insertion sites of mut1 and mut2, respectively, in *Staphylococcus carnosus*. The 16-kb fragment encoded on plasmid pT181mcs was able to complement mut1 for biofilm formation. The same plasmid was also able to complement mut1a, although transposon insertion in mut1a occurred at a different locus, indicating that a spontaneous secondary mutation must have led to the altered phenotype of mut1a. The 16-kb fragment was subcloned in pRB473. A 2.7-kb *Hin*dIII subfragment was the smallest fragment which was able to complement mut1 for biofilm production; the plasmid was designated pRC20. The 5-kb DNA fragment encoded on plasmid pCA44 was able to complement mut2 and mut2a. The transposon insertion sites of mut2 and mut2a are located on different *Eco*RI fragments but on the same *Hin*dIII DNA fragment (Fig. 1). Southern blot analysis revealed that the two complementing DNA fragments shared no homology and were unlinked. For further characterization, one mutant of each phenotypic class (mut1 from class A and mut2 from class B) was studied.

Characterization of mut1 and mut2. (i) Determination of primary attachment to polystyrene. In order to distinguish between mutants defective in primary attachment and those

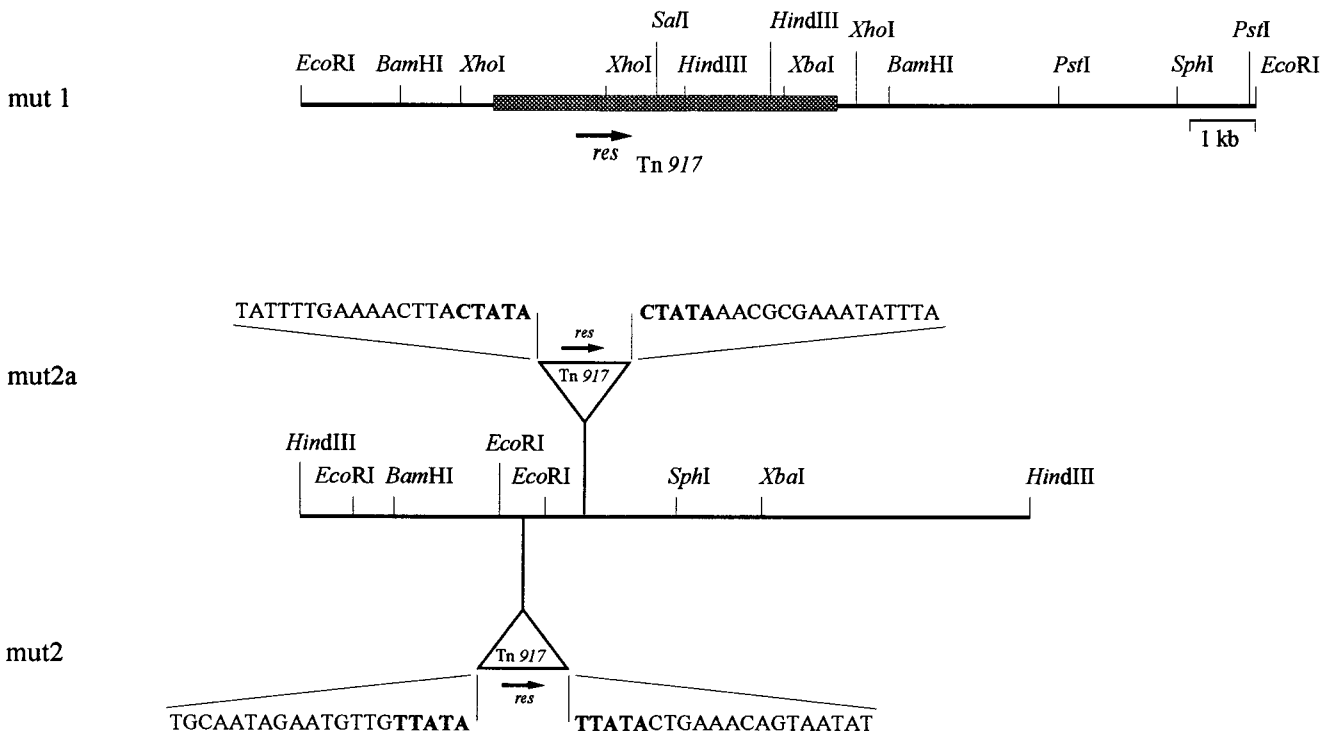

FIG. 1. Tn*917* insertion sites in the chromosomes of mut1, mut2, and mut2a. In mut1 the Tn*917* insertion was accompanied by an 8-kb DNA deletion. In mut2 and mut2a the flanking DNA sequences of Tn*917* are indicated; transpositions were accompanied by 5-bp duplications, indicated in bold letters. *res*, resolvase gene; arrow, direction of transcription of *res*.

defective in accumulation, the capacity for primary attachment to polystyrene was determined. Diluted cell suspensions were incubated for 30 min in polystyrene petri dishes, and after a washing procedure, the number of attached cells was determined by microscopic evaluation (Fig. 2). Strain O-47 (20.5×10^3 cells per cm^2) and mut2 (22.6×10^3 cells per cm^2) showed no significant differences in their capacity to bind to polystyrene. With mut1, however, the number of adhered cells (0.4×10^3 cells per cm^2) was 50 times lower, indicating that mut1 was defective in primary attachment to polystyrene.

(ii) Biofilm formation on polystyrene. In the quantitative biofilm assay, primary attachment and accumulation in multilayered cell clusters on a surface, which together lead to biofilm formation, can be determined. After normal growth in the microtiter plates, differences in the appearance of the cell cultures were apparent. While strain O-47 formed an adhesive film of bacteria, mut2 appeared only to bind to the plastic surface (Fig. 3A). In agreement with results of the primary attachment assay, mut1 did not have any affinity for the polystyrene surface; the cell pellet formed after overnight cultivation was completely removed when the culture was poured out. The results of the quantitative assay (Fig. 3B) agreed with the above observations. After overnight cultivation, strain O-47 formed a thick cell layer firmly attached to the surface of

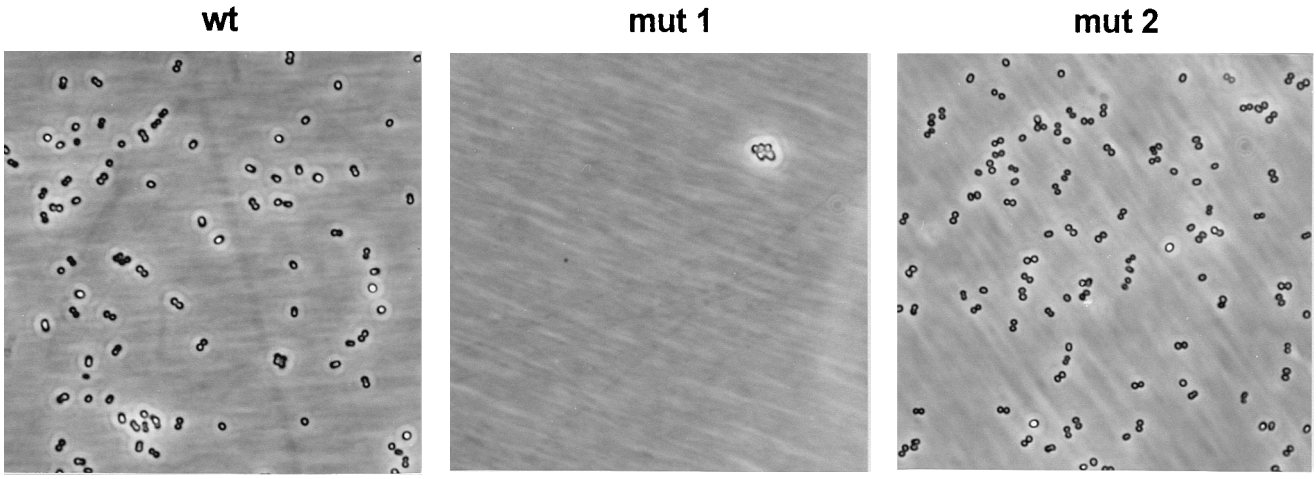

FIG. 2. Phase-contrast micrographs of adhered cells on polystyrene petri dishes. *S. epidermidis* O-47 (wt), mut1, and mut2 are shown.

FIG. 3. (A) Growth of *S. epidermidis* O-47 and its biofilm-negative mutants in TSB in polystyrene microtiter plates; (B) quantitative assay of biofilm formation. Lanes: 1, *S. epidermidis* O-47; 2, mut1; 3, mut2.

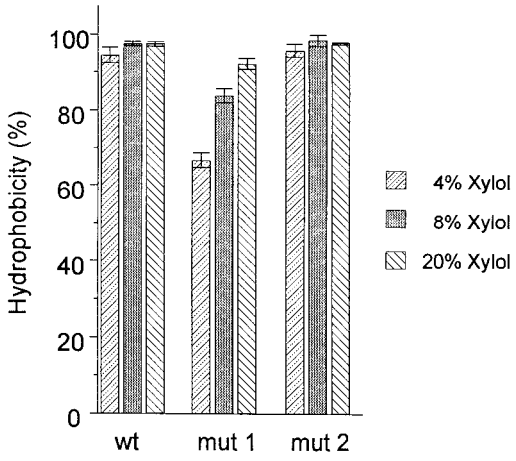

FIG. 4. Surface hydrophobicity of *S. epidermidis* O-47 (wt), mut1, and mut2.

polystyrene microtiter plates. After several washing steps and staining of attached cells of the control strains O-47 and RP62A (data not shown) with safranin, A_{490} values between 1.5 and 1.9 were measured. mut1 revealed no detectable biofilm-forming capacity in this assay; the A_{490} value was below 0.03, confirming that mut1 is defective in mediating primary attachment to polystyrene. mut2 exhibited a reduced A_{490} value of 0.2 to 0.3; however, this value was 10-fold higher than that for mut1 and indicates that mut2 cells attach to the plastic surface but are not capable of forming multilayered cell clusters and of accumulation.

(iii) Hydrophobicity of cells. Pascual et al. (19) have demonstrated that the hydrophobicity of adhesion-positive staphylococci is higher than that of adhesion-negative strains. In the hydrophobicity assay (Fig. 4), strain O-47 and mut2 had the same hydrophobicity values. mut1, on the other hand, was significantly less hydrophobic or more hydrophilic.

(iv) Cell aggregation (intercellular adhesion). Cell aggregation indicates the capacity of strains to express intercellular adhesion and to accumulate in multilayered cell clusters. Phase-contrast microscopic observations of overnight-grown cultures showed clear differences in cell arrangement (Fig. 5). Strain O-47 formed cell clusters; however, pairs, tetrads, and short chains of three or four cells were also visible. In the mut1 culture, much larger cell aggregates were present and individual cells and pairs of cells were barely detectable. In contrast to strain O-47 and mut1, mut2 showed no cell aggregation. Only single cells or pairs of cells were detectable, indicating that mut2 lacked the capacity for formation of multilayered cell clusters and for intercellular adhesion.

(v) Cell-surface-associated proteins. Surface-associated proteins were detected by boiling the cell suspension in Laemmli sample buffer and analyzing the supernatant by SDS-PAGE after centrifugation (Fig. 6). Strain O-47 and mut2 showed essentially the same protein pattern. Six proteins of 120, 60, 52, 45, 38, and 29 kDa were predominant. In mut1, however, only the 29-kDa protein was present at a concentration similar to that in strain O-47; the amount of the 52-kDa protein was decreased, or it was mashed by another protein of similar size. The other four proteins were not detectable. In the presence of the complementing plasmid, pRC20, mut1 produced the 60-kDa protein, indicating that this protein mediates primary attachment to plastic surfaces. The protein was isolated from O-47 and mut1(pRC20) to determine the N-terminal amino acid sequence; both proteins started with the following sequence: Val-Ser-Ser-Gln-Lys-Thr-Ser-Ser-Leu.

(vi) Biofilm formation on a glass surface. The assay for biofilm formation on a glass surface (Fig. 7) was carried out essentially as described for the polystyrene microtiter plates, except that glass tubes were used. Strain O-47 and mut1 formed a biofilm on the glass surface, with the biofilm formation of mut1 more pronounced than that of strain O-47. mut2 was unable to form a biofilm on a glass surface.

DISCUSSION

It is assumed that coagulase-negative staphylococci form biofilms on prosthetic devices in two steps: (i) attachment of the bacterial cells to the polymer surface, which occurs in only a few minutes, and (ii) accumulation in multilayered cell clusters in a growth-dependent process. The aim of this study was to analyze biofilm-negative mutants of *S. epidermidis* O-47, obtained by transposon mutagenesis. We found two classes of mutants: class A mutants were defective in attachment to a polystyrene surface; class B mutants were able to bind to the plastic surface, but they were unable to carry out the second step of biofilm formation, accumulation in multilayered cell

FIG. 7. Biofilm formation on a glass surface. *S. epidermidis* O-47 (wt), mut1, and mut2 are shown.

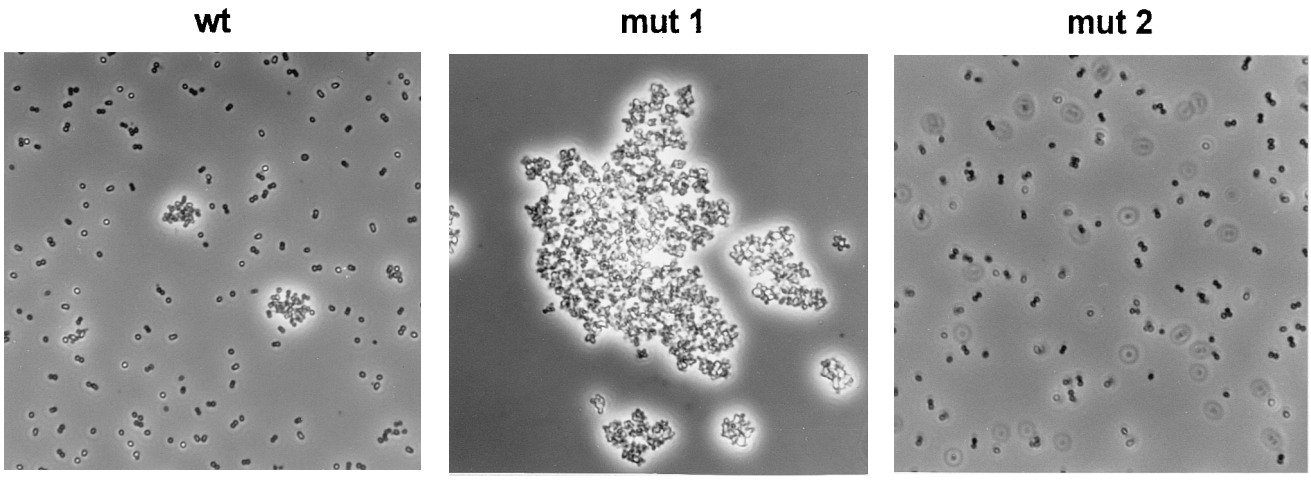

FIG. 5. Phase-contrast micrographs of overnight-grown cells in TSB of *S. epidermidis* O-47 (wt), mut1, and mut2.

clusters. Since it is known that the altered phenotype of transposon insertion mutants is not necessarily coupled with the transposon insertion, because of the occurrence of secondary spontaneous mutants, we have cloned wild-type DNA fragments containing the Tn*917* insertion sites of the mutants mut1 and mut2. Since the cloned 16- and 5-kb DNA fragments of the wild-type strain were able to complement mut1 and mut2, respectively, evidence is provided that the phenotype of these mutants is due to Tn*917* insertion. The Tn*917* insertion sites in mut1 and mut2 were located on different *Bam*HI fragments on the chromosome, indicating that the genes for primary attachment and intercellular adhesion are unlinked and represent genetically distinct chromosomal loci, as shown in the restriction map of the Tn*917* insertion sites (Fig. 1).

mut1, representing phenotype class A, was unable to form a biofilm on a polystyrene surface. The number of mut1 cells adhered to a polystyrene surface was 50-fold less than those of the wild type and mut2. This phenotype occurred concomitantly with other characteristics, such as decreased hydrophobicity and the lack of four surface proteins. In the biofilm-positive clone, mut1(pRC20), the predominant surface proteins in O-47 of 120, 52, 45, and 38 kDa were still missing; only the 60-kDa protein was expressed, indicating that this protein is sufficient to mediate attachment to the polystyrene surface.

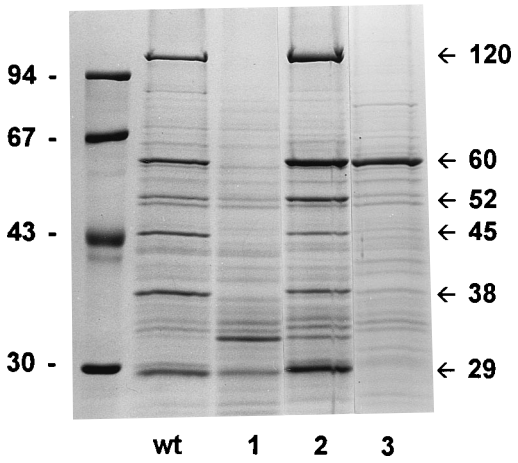

FIG. 6. SDS-PAGE of cell surface proteins of *S. epidermidis* O-47 (wt), mut1 (lane 1), mut2 (lane 2), and mut1 complemented with pRC20 (plasmid pRB473 containing a 2.7-kb insert of wild-type DNA) (lane 3). Marker proteins are shown on the left; in the right margin the sizes (kilodaltons) of predominant surface proteins in O-47 are indicated.

Other groups have also provided indications that cell-wall-associated proteins mediate initial adhesion of *S. epidermidis* to a plastic surface (9, 30). Timmerman et al. (30) identified a 220-kDa proteinaceous adhesin in *S. epidermidis* 354 that mediates attachment to polystyrene. A protein of this size was, however, not missing in mut1.

mut1 was still able to form a biofilm on glass, a trait which was even more pronounced than with strain O-47. We assume that with the loss of the four surface proteins, hydrophilic structures of the cell wall became unmasked, making the cell surface more hydrophilic. Since glass is hydrophilic in character, a hydrophilic interaction in the adhesion of cells to a glass surface is anticipated. The ability to form large cell clusters and the capacity for intercellular adhesion were not impaired in mut1; therefore, biofilm formation on a glass surface was possible and even more pronounced than in strain O-47. Perhaps the absence of the cell surface proteins leads to an increased accessibility of an intercellular adhesin, which is probably identical to the compound mediating increased adherence to glass.

The loss of four or five surface proteins in mut1 suggests that the Tn*917* insertion affected the expression of a series of proteins. This effect can be explained by the 8-kb DNA deletion at the site of Tn*917* insertion in mut1 accompanying transposition.

mut2 represents phenotype class B. This mutant was able to attach to polystyrene but was unable to form large cell clusters and to accumulate on a polymer surface. Its cell surface hydrophobicity was similar to that of the wild type. Mack et al. (15) described a biofilm-negative transposon insertion mutant that exhibits a phenotype similar to that of mut2. They could demonstrate that intercellular adhesion correlates with the presence of a polysaccharide antigen composed of a hexosamine polymer, which was not detectable in the biofilm-negative mutant. The polysaccharide antigen is probably also missing in mut2.

The two classes of transposon insertion mutants described here are the basis for genetically characterizing the compounds involved in primary attachment and intercellular adhesion. The identification of the involved genes encoded on the cloned

fragments will provide further clues as to the mechanism of biofilm formation in *S. epidermidis.*

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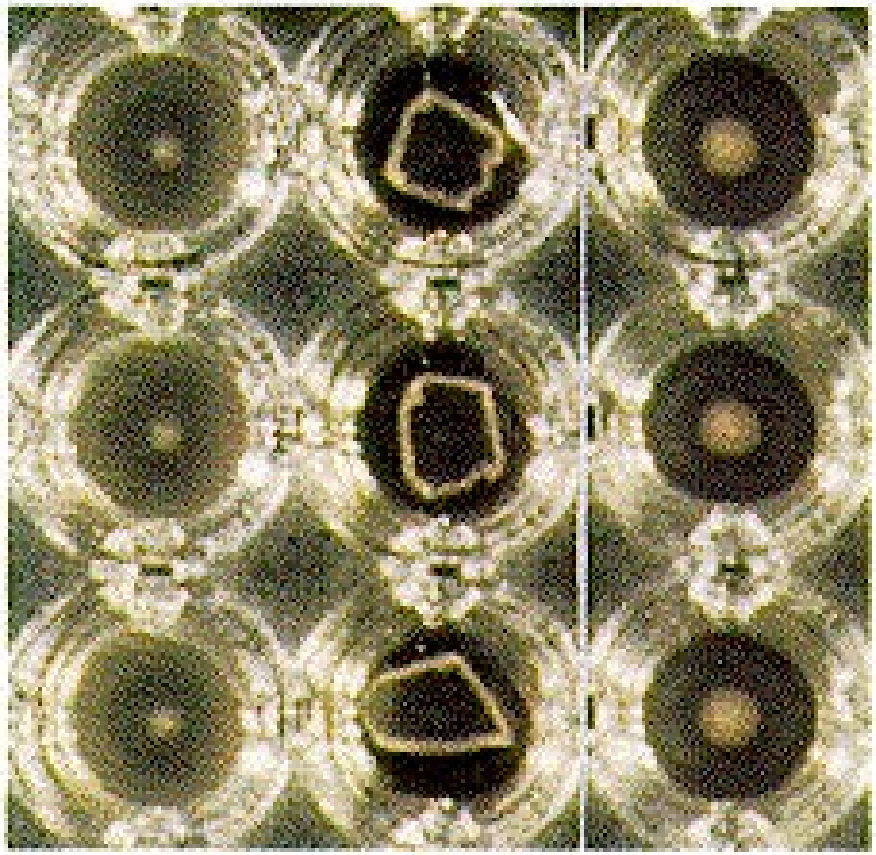
A



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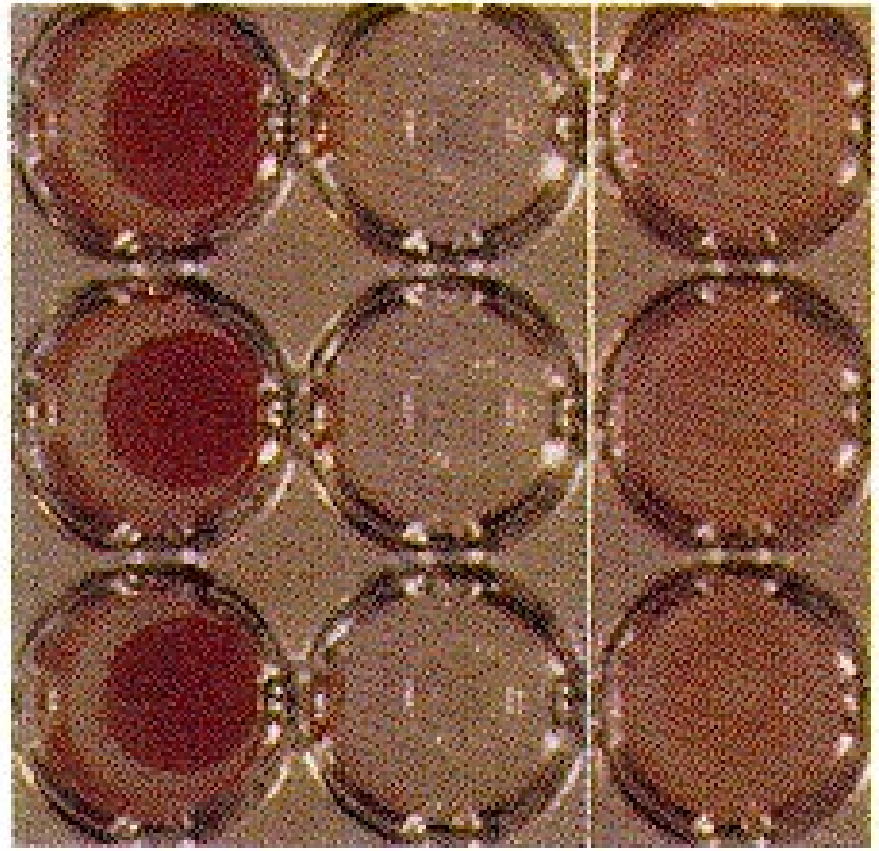

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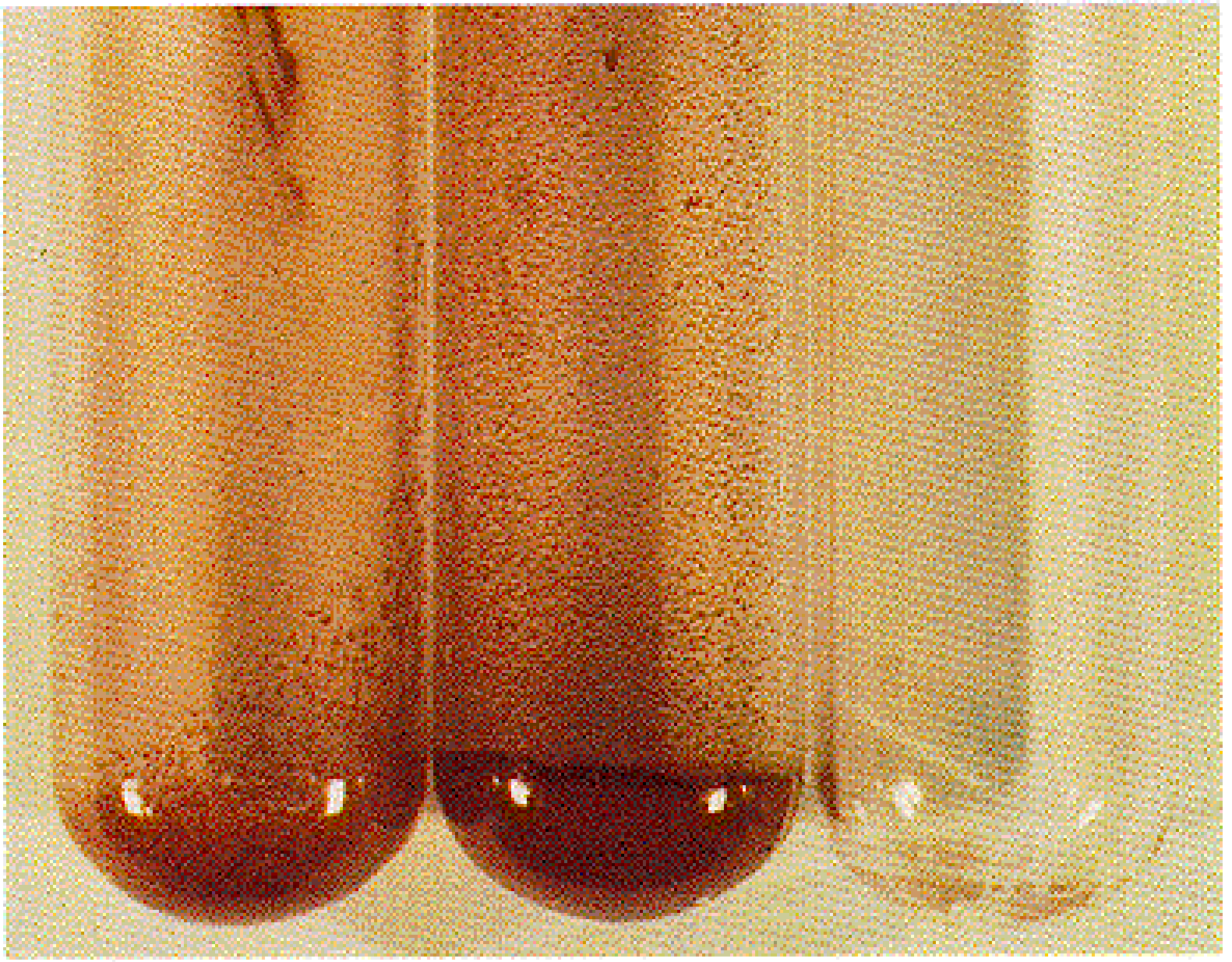
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