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# Characterization of Transposon Mutants of Biofilm-Producing *Staphylococcus epidermidis* Impaired in the Accumulative Phase of Biofilm Production: Genetic Identification of a Hexosamine-Containing Polysaccharide Intercellular Adhesin

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**The primary attachment to polymer surfaces followed by accumulation in multilayered cell clusters leads to production of *Staphylococcus epidermidis* biofilms, which are thought to contribute to virulence in biomaterial-related infections. We isolated Tn*917* transposon mutants of biofilm-producing *S. epidermidis* 13-1, which were completely biofilm negative. In pulsed-field gel electrophoresis no obvious deletions of the mutants were noted. The Tn*917* insertions of mutants M10 and M11 were located on different *Eco*RI fragments but on identical 60-kb *Sma*I and 17-kb *Bam*HI chromosomal fragments. Linkage of transposon insertions of mutants M10 and M11 with the altered phenotype was demonstrated by phage transduction, whereas the several other mutants apparently represented spontaneous variants. In a primary attachment assay with polystyrene spheres, no significant difference between any of the mutants and the wild type could be detected. Cell clustering as an indication of intercellular adhesion, which is a prerequisite for accumulation in multilayered cell clusters, was not detected with any mutant. These results demonstrate that the mutants were impaired in the accumulative phase of biofilm production. Mutants M10 and M11 did not produce detectable amounts of a specific polysaccharide antigen (D. Mack, N. Siemssen, and R. Laufs, Infect. Immun. 60:2048–2057, 1992), whereas substantially reduced amounts of antigen were produced by the spontaneous variants. Hexosamine was determined as the major specific component of the antigen enriched by gel filtration of biofilm-producing *S. epidermidis* 1457 because almost no hexosamine was detected in material prepared from the isogenic biofilm-negative transductant 1457-M11, which differentiates the antigen from other *S. epidermidis* polysaccharide components. Our results provide direct genetic evidence for a function of the antigen in the accumulative phase of biofilm production by *S. epidermidis* by mediating intercellular adhesion.**

In recent years coagulase-negative staphylococci, mostly *Staphylococcus epidermidis*, have emerged as one of the most frequent causes of nosocomial sepsis and of infections associated with implanted biomaterials such as intravascular catheters, peritoneal dialysis catheters, cerebrospinal fluid shunts, prosthetic heart valves, and prosthetic joints, resulting in substantial morbidity and mortality (23, 39, 45).

Coagulase-negative staphylococci were shown by scanning electron microscopy to colonize intravascular catheters by forming large adherent biofilms composed of multilayered cell clusters embedded in an amorphous extracellular material, which is composed of exopolysaccharides referred to as slime or glycocalyx (9, 11, 22, 38, 43). Most strikingly, the majority of staphylococcal cells in these biofilms have no direct contact with the polymer surface, indicating that the cells have to express intercellular adhesion to reside in the adherent biofilm. In vitro a proportion of coagulase-negative staphylococcal strains are able to produce a macroscopically visible adherent biofilm on test tubes or tissue culture plates with a morphology in scanning electron micrographs very similar to that of strains isolated from infected intravascular catheters (9, 10, 30, 48). This phenotype is often referred to as slime production; however, as the term "slime" is applied to different phenomena by different authors, we refer to these strains as biofilm producers, distinguishing them from biofilm-negative strains (2, 9, 29, 44). Formation of a macroscopically visible biofilm on a polymer surface by coagulase-negative staphylococci proceeds in two phases: rapid primary attachment to the polymer surface followed by accumulation in multilayered cell clusters on the polymer surface, which requires the expression of intercellular adhesion. These properties leading to biofilm production will be differentiated when appropriate. The importance of biofilm production for virulence has been supported by several clinical studies (9, 12, 14, 16, 19, 31, 59).

Numerous studies indicated that almost all *S. epidermidis* strains are able to attach to polymer surfaces albeit with quantitative differences between strains (20, 26, 42, 53). It is postulated that primary attachment of *S. epidermidis* to polymer surfaces is mediated by nonspecific van der Waals and hydrophobic interactions (11). A polysaccharide adhesin (PS/A) which probably mediates primary attachment to silicon catheter surfaces was purified from *S. epidermidis* RP62A (53). However, PS/A expression alone is not sufficient for biofilm production by *S. epidermidis*, because almost half of the PS/A-producing strains were biofilm negative (41).

Apparently, additional factors differentiate biofilm-producing *S. epidermidis* strains from those which are biofilm negative. With reference to the suggested role of *S. epidermidis* biofilm production in pathogenicity, it is especially important to iden-

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TABLE 1. Bacterial strains used in this study

| Strain | Plasmid(s) | Antibiotic resistance | Reference(s) |
|---|---|---|---|
| *S. epidermidis* 9142 | | Ofl$^r$, Pen$^r$ | 36 |
| *S. epidermidis* 23 | pTV1ts | Em$^r$, Cm$^r$ | 25, 60 |
| *S. aureus* WBG4883 | pWBG636 | Gm$^r$ | 56 |
| *S. epidermidis* 13-1 | pTV1ts, pWBG636 | Ofl$^r$, Pen$^s$, Em$^r$, Cm$^r$, Gm$^r$ | This study |
| Mutants M1, M2, M4, M5, M7, M8, M9, M10, M11, and M14 | pWBG636 | Ofl$^r$, Pen$^s$, Em$^r$, Cm$^s$, Gm$^r$ | This study |
| *S. epidermidis* 1457 | | | 36 |
| *S. epidermidis* 1457-M11 | | Em$^r$ | This study |

tify molecules which are functionally involved in accumulation of biofilm-producing *S. epidermidis* strains in multilayered cell clusters on a polymer surface. We recently described a polysaccharide antigen specific for biofilm-producing *S. epidermidis* strains, which is most probably functionally related to intercellular adhesion (36).

In the present study we used *Enterococcus faecalis* transposon Tn*917* to isolate isogenic mutants of the biofilm-producing *S. epidermidis* 13-1 with a completely biofilm-negative phenotype. We provide genetic evidence for the function of the specific antigen of biofilm-producing *S. epidermidis* as a polysaccharide intercellular adhesin in the accumulative phase of biofilm production. The isolated isogenic mutants will prove useful in elucidating the role of the accumulative phase of biofilm production of *S. epidermidis* in the pathogenesis of biomaterial associated infections.

(Part of this work will appear in the doctoral theses of M.N. and A.K., Universitätskrankenhaus Eppendorf, Hamburg, Germany.)

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and phages.** The strongly biofilm-producing *S. epidermidis* 9142 has been described previously (36). Strain 9142 was typeable with *S. epidermidis* phages 48, 71, 82, and 155 (13). Phage typing was performed by V. T. Rosdahl, Statens Seruminstitut, Copenhagen, Denmark. *S. epidermidis* 13-1 is a transconjugant of strain 9142 carrying plasmid pTV1ts and was the wild type used in the transposon mutagenesis experiments described in this study (see below). *S. epidermidis* 23 transformed by protoplast transformation with plasmid pTV1ts, which carries the *E. faecalis* transposon Tn*917* (60), was kindly provided by L. Grüter, Hamburg University, Hamburg, Germany (25). *S. aureus* WBG4883 carrying the conjugative plasmid pWBG636 was kindly provided by W. B. Grubb, Curtin University of Technology, Perth, Australia (56, 57). The relevant plasmids and antibiotic resistance markers of these strains are listed in Table 1. *S. epidermidis* 1457, 5179, and 9896, and *S. aureus* Cowan 1 have been described previously (36). *S. epidermidis* phage 48 and its propagating strain *S. epidermidis* 48 were kindly provided by V. T. Rosdahl (13, 46).

**Mobilization of pTV1ts into *S. epidermidis* 9142 by coconjugation.** *S. epidermidis* 23 carrying plasmid pTV1ts and *S. aureus* WBG4883 were mated on membrane filters as described previously (54). Donor and recipient strains were grown in brain heart infusion (BHI) broth (Oxoid, Basingstoke, England) overnight at 30°C with shaking, and 3 ml of recipient and 1 ml of donor cultures were filtered onto 0.45-μm-pore-size nitrocellulose filters. These were incubated on BHI agar at 30°C for 20 h. Bacterial growth was plated on peptone yeast (PY) agar (1.0% peptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, 1.5% agar [pH 7.5]) while selecting for pTV1ts (chloramphenicol, 10 μg/ml; erythromycin, 10 μg/ml) and for pWBG636 (gentamicin, 8 μg/ml) at 30°C. Transconjugants were purified on selective PY agar plates and were mated with *S. epidermidis* 9142 on membrane filters. Bacteria were plated on PY agar selecting for pTV1ts with chloramphenicol and erythromycin and for *S. epidermidis* 9142 with 2 μg of ofloxacin per ml at 30°C. A transconjugant clone, *S. epidermidis* 13-1, was used for further experiments. pTV1ts of *S. epidermidis* 13-1 displayed the expected restriction fragment pattern (data not shown) and was fully functional.

**Transposon mutagenesis.** *S. epidermidis* 13-1 was grown in PY broth containing 10 μg of chloramphenicol and 10 μg erythromycin per ml at 30°C overnight with shaking. The culture was diluted 1:100 into PY broth containing 2.5 μg of erythromycin per ml. After 5 h of incubation at 30°C, dilutions were plated on BHI agar containing 20 μg of erythromycin per ml. Plates were incubated for 36 h at 45.5°C, transposon mutants were subcultured onto BHI agar containing 20 μg of erythromycin per ml at 45.5°C, and the resulting clones were tested for a biofilm-negative phenotype.

**Phage transduction.** For propagation of phages, NB-2+ broth (nutrient broth no. 2 [Oxoid] containing 0.04% $CaCl_2$) was used for broth cultures and ST agar (2% nutrient broth no. 2, 0.5% NaCl, 0.04% $CaCl_2$, 1.5% agar) was used for plaque titer determinations (15). *S. epidermidis* phage 48 was adapted to *S. epidermidis* 9142 by plaque purification. High-titer phage stocks (ca. $5 \times 10^9$ PFU/ml) for transduction experiments were grown by the soft-agar technique (50) and sterilized by passage through a 0.2-μm-pore-size filter (Sartorius, Göttingen, Germany).

For phage transduction a procedure described by Kayser et al. (32) was used with modifications. As a recipient in transduction experiments, *S. epidermidis* 9142 or 1457 was subcultured on blood agar at 37°C for 18 h. Bacteria were suspended to a concentration of $0.5 \times 10^{10}$ to $1.0 \times 10^{10}$ CFU/ml in NB-2+ broth. For transduction of transposon Tn*917* insertions, lysates grown on the individual transposon mutants were UV irradiated until the number of PFU was reduced by 90%. The recipients were infected with the lysate at a multiplicity of infection of less than 1 PFU per bacterial cell and incubated for 30 min at 37°C. Phage absorption was stopped by adding 1 volume of BHI broth containing 40 mM sodium citrate, and after centrifugation at 4°C bacterial cells were also washed twice in BHI broth containing 20 mM sodium citrate. Finally, recipient cells were suspended in 3 ml of the same medium and were incubated with shaking for 3 h at 37°C. Then recipient cells were mixed with BHI soft agar containing 20 mM sodium citrate and 10 μg of erythromycin per ml and were plated on BHI agar containing 10 μg of erythromycin per ml. Transductants were isolated after incubation at 37°C for 24 to 48 h. Transducing plasmid pTV1ts recipient cells were plated in BHI soft agar immediately after the cells had been washed, and incubation was carried out at 30°C. Transduction frequencies

are expressed as transductants per PFU of the non-UV-irradiated phage lysate.

**DNA isolation.** For isolation of chromosomal DNA, bacteria were grown in PY broth with selection as appropriate. Cells were collected by centrifugation and were incubated at 37°C for 3 to 5 h in 3 ml of 2.5 M NaCl–50 mM Tris-HCl–50 mM EDTA (pH 7.0) containing 75 U of lysostaphin (Sigma, Munich, Germany) and 25 μg of RNase (Boehringer, Mannheim, Germany). Lysis was effected by addition of 3 ml of 7% sodium dodecyl sulfate. After 30 min on ice the mixture was extracted with 7 ml of chloroform-isoamyl alcohol (24:1). After phase separation the upper phase was mixed with 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol. Precipitating DNA was hooked onto a Pasteur pipette and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). Plasmid minipreparations were obtained by the method of Townsend et al. (55).

**Pulsed-field gel electrophoresis.** Pulsed-field gel electrophoresis was performed essentially as described by using *Sma*I for cleavage of DNA and a CHEF-DR II system (Bio-Rad, Munich, Germany) for analysis of resulting fragments (49). Phage lambda DNA concatemers were used as molecular weight markers.

**Southern hybridization.** Chromosomal DNA was digested with the restriction enzymes *Eco*RI and *Bam*HI under conditions suggested by the manufacturer (Pharmacia, Freiburg, Germany). DNA fragments were separated on 0.5% agarose gels in Tris-borate buffer (47). After being stained with ethidium bromide, DNA fragments were transferred by alkaline capillary blotting onto Zeta-Probe nylon membranes (Bio-Rad). Plasmid pTV1ts was labeled with [$^{32}$P]dCTP with an oligopriming DNA labeling kit as suggested by the manufacturer (Pharmacia) and hybridized at 65°C overnight under conditions suggested for Zeta-Probe membranes (1 mM EDTA, 0.5 M $NaH_2PO_4$, 7% sodium dodecyl sulfate [pH 7.2]). After being washed, the blots were exposed to Kodak X-Omat X-ray films.

**Adherence assay for quantitation of biofilm production by *S. epidermidis* strains.** Biofilm production by *S. epidermidis* strains resulting in a macroscopically visible biofilm firmly attached to wells of tissue culture plates (Nunc, Roskilde, Denmark) was determined as described previously (10, 36). This assay measures primary attachment and accumulation of multilayered cell clusters on the plastic surface, which in concert lead to biofilm production (9). Briefly, after growth in Trypticase soy broth (TSB; Becton Dickinson, Cockeysville, Md.) for 20 h at 37°C, plates were gently washed four times with phosphate-buffered saline and adherent bacterial cells were fixed with Bouin's fixative followed by staining with gentian violet. The optical densities at 570 nm ($OD_{570}$) of stained adherent bacterial biofilms were measured in an automatic spectrophotometer (Behring, Marburg, Germany). Strains were tested in quadruplicate. Biofilm-producing strains were defined to have a mean $OD_{570}$ of greater than 0.1 (36).

**Measurement of primary attachment of *S. epidermidis* to polystyrene.** Primary attachment of *S. epidermidis* strains to polystyrene surfaces was assayed essentially as described by Timmerman et al. (52) with modifications. *S. epidermidis* strains were grown in TSB for 18 h at 37°C with shaking. Bacteria (about $7 \times 10^6$ CFU/ml in phosphate-buffered saline) were incubated with polystyrene spheres (diameter, 6 mm; Precision Plastic Ball Co., Chicago, Ill.) in a total volume of 0.5 ml in 20-well microtiter plates (Abbott, Wiesbaden-Delkenheim, Germany) for 30 min at 37°C. The inoculum was aspirated, and the polystyrene spheres were washed with 0.5 ml of phosphate-buffered saline, transferred into glass tubes, and given four additional washes with 5 ml of phosphate-buffered saline. Bacteria attached to the polystyrene surface were released into 2 ml of phosphate-buffered saline by sonication at 35 kHz and 120 W (Sonorex TK 52 H, Bandelin Electronics, Berlin, Germany) for 2 min and were quantitated by colony counts. Results are given as means ± standard deviations of three polystyrene spheres in three independent experiments. Control experiments established that more than 95% of measured attached bacteria were released only after sonication.

**Preparation of bacterial extracts and quantitation of specific antigen.** Antigen extracts of *S. epidermidis* grown on tissue culture dishes in TSB were prepared by sonication as described previously (36). Absorbed rabbit antiserum specific for the specific antigen of biofilm-producing *S. epidermidis* strains has been described previously (36). Specific antigen in bacterial extracts was quantitated by a specific coagglutination assay (36). *S. aureus* Cowan 1 was sensitized with the absorbed specific antiserum. Aliquots (5 μl) of serial twofold dilutions of bacterial extracts in phosphate-buffered saline were mixed with 25 μl of coagglutination reagent on microscope slides. Agglutination was evaluated after 3 min in bright light against a dark background. The antigen titers were defined as the highest dilution displaying positive coagglutination.

**Isolation of the specific antigen by gel filtration.** For preparative isolation of the antigen, *S. epidermidis* 1457 or 1457-M11 was grown for 22 h at 37°C with shaking at 100 rpm/min in 900 ml of $TSB_{dia}$, prepared by dialysis of 100 ml of 10-fold-concentrated TSB against 900 ml of water. Biofilm production by $TSB_{dia}$ and TSB was comparable (data not shown). Bacterial cells were collected by centrifugation and were suspended in 20 ml of phosphate-buffered saline. The antigen was extracted by sonicating the cells four times for 30 s on ice. Cells were removed by centrifugation at 6,000 rpm in a Beckman JA 17 rotor at 4°C, and extracts were clarified by centrifugation for 60 min at 12,000 rpm in the same rotor. The extracts were filter sterilized, dialyzed against 2 liters of phosphate-buffered saline overnight, concentrated by using Centriprep 10 (Amicon, Witten, Germany), and applied to a 1.6- by 100-cm Sephadex G-200 (Pharmacia) column equilibrated with phosphate-buffered saline. Fractions of 1.5 ml were collected, and the specific antigen concentration was determined by coagglutination. The void and inclusion volumes of the columns were determined by using 2-MDa blue dextran (Pharmacia) and $K_3Fe(CN)_6$.

**Colorimetric assays.** Hexose was determined by the phenol-sulfuric acid assay with glucose as the standard (18). Hexosamine was determined by the 3-methyl-2-benzothiazolone hydrazone hydrochloride method with *N*-acetylglucosamine as the standard (33). Ketoses were determined by the phenol-boric acid-sulfuric acid assay with fructose as the standard (5). Uronic acids were determined by the alkaline *m*-phenylphenol method with glucuronic acid-γ-lactone as the standard (3). Pentose was determined by the ferric-orcinol assay with xylose as the standard (5). Phosphate was determined by the method of Ames (1) after extensive dialysis of samples against 50 mM Tris-HCl (pH 7.5) with $KH_2PO_4$ as the standard. Protein content was determined by the method of Bradford (4) with bovine serum albumin as the standard.

## RESULTS

**Mobilization of plasmid pTV1ts.** Because protoplast transformation or electroporation of the biofilm-producing *S. epidermidis* 9142 was unsuccessful, we made use of the property of the conjugative *S. aureus* plasmid pWBG636 to mobilize other nonconjugative plasmids during conjugation (56, 57). Plasmid

TABLE 2. Properties of Tn*917* transposon mutants of biofilm-producing *S. epidermidis* 13-1

| Strain | Biofilm production ($OD_{570}$)[a] by: | | Transduction frequency[b] (transductants/PFU) | Primary attachment[c] ($10^4$ CFU/sphere) | Concn of specific antigen[d] (reciprocal titer) |
|---|---|---|---|---|---|
| | Tn*917* mutant | Transductant[b] | | | |
| 9142 (wt) | 2.50 | NA[e] | NA | ND[e] | 512 |
| 13-1 | 2.50 | 2.50 | $6.0 \times 10^{-6}$ | 9.20 ± 3.48 | 1,024 |
| M1 | 0.05 | 2.50 | $3.0 \times 10^{-6}$ | 7.39 ± 0.94 | 8 |
| M2 | 0.06 | 2.50 | $9.6 \times 10^{-6}$ | 7.16 ± 0.85 | 16 |
| M4 | 0.11 | 2.50 | $1.5 \times 10^{-6}$ | 6.02 ± 1.29 | 64 |
| M5 | 0.09 | 2.50 | $6.1 \times 10^{-8}$ | 6.42 ± 0.92 | 8 |
| M7 | 0.05 | 2.50 | $2.7 \times 10^{-6}$ | 6.82 ± 1.20 | 8 |
| M8 | 0.06 | 2.50 | $5.0 \times 10^{-6}$ | 8.08 ± 1.43 | 2 |
| M9 | 0.03 | 2.50 | $5.2 \times 10^{-6}$ | 6.01 ± 1.16 | 1 |
| M10 | 0.04 | 0.05 | $3.6 \times 10^{-7}$ | 7.63 ± 1.35 | 0 |
| M11 | 0.04 | 0.04 | $2.3 \times 10^{-7}$ | 8.32 ± 1.40 | 0 |
| M14 | 2.50 | 2.50 | $5.6 \times 10^{-7}$ | 10.20 ± 2.84 | 1,024 |

[a] Biofilm production by Tn*917* mutants and the wild-type strain 13-1 was determined as described in Materials and Methods.
[b] Transposon mutations were transduced into wild-type *S. epidermidis* 9142, and biofilm production was determined as described in Materials and Methods. Transduction frequencies are given as transductants per PFU of nonirradiated phage stocks.
[c] Primary attachment of transposon Tn*917* mutants and wild-type strain 13-1 to polystyrene spheres after 30 min at 37°C. Results are means and standard deviations of nine determinations for each strain.
[d] Concentration of the specific antigen in bacterial extracts of strains grown on plastic cell culture dishes in TSB was determined by coagglutination as described in Materials and Methods.
[e] NA, not applicable; ND, not done.

pTV1ts was mobilized into the biofilm-producing *S. epidermidis* 9142 by filter mating as described in Materials and Methods. One transconjugant, *S. epidermidis* 13-1, was used for further transposon mutagenesis experiments. *S. epidermidis* 13-1 had an identical biotype and expressed the specific antigen of biofilm-producing *S. epidermidis* at similar concentrations to those of *S. epidermidis* 9142 and was also strongly biofilm producing (Table 2). The antibiotic resistance profile of *S. epidermidis* 13-1 differed from that of *S. epidermidis* 9142 as apparently a penicillinase plasmid was eliminated during conjugation and the additional erythromycin and chloramphenicol resistance of plasmid pTV1ts and gentamicin resistance of pWBG636 were observed (Table 1).

**Transposon mutagenesis.** We made use of plasmid pTV1ts, which carries the *E. faecalis* transposon Tn*917* and has a temperature-sensitive replicon. At the nonpermissive temperature of 45.5°C, pTV1ts is eliminated, and, by using the erythromycin resistance determinant of Tn*917*, mutants with Tn*917* transposed into the chromosome can be selected (25, 60). More than 6,000 transposon mutants from two separate experiments were screened for a biofilm-negative phenotype by using the assay described in Materials and Methods. The rate of transposition of Tn*917* was about $4.6 \times 10^{-3}$, and 91% of transposon mutants tested had eliminated the chloramphenicol resistance determinant of the plasmid portion of pTV1ts. Nine Tn*917* insertion mutants which exhibited completely biofilm-negative phenotypes were isolated (Table 2). All mutants had the expected antibiotic resistance profile and had a biotype identical to that of *S. epidermidis* 13-1. The observed phenotypic change was stable for at least 18 passages on blood agar without selection.

Southern blot analysis of *Eco*RI-digested chromosomal DNA of the transposon mutants with $^{32}$P-labeled pTV1ts as a probe showed single hybridizing fragments (Fig. 1). Since Tn*917* has no *Eco*RI site, this indicates that all mutants had single insertions of Tn*917* (60). To prove linkage of the respective transposon insertions with the observed phenotypic changes, we established a generalized transduction system by using *S. epidermidis* phage 48 (13). By using biofilm-producing *S. epidermidis* 9142 as the recipient, this method allowed isolation of transductants of all the respective transposon insertions investigated. Southern blot analysis established that Tn*917* was integrated in identical *Eco*RI fragments in the isolated transposon Tn*917* mutants when compared with their respective transductants (Fig. 1).

With mutants M10 and M11, *Eco*RI fragments of 6.8 and 5.8 kb hybridized with the pTV1ts probe (Fig. 1). Taking into account the 5.3 kb of the Tn*917* sequence, the transposon inserted into individual *Eco*RI fragments of about 1.3 and 0.5 kb. Transductants of Tn*917* mutants M10 and M11 showed the identical biofilm-negative phenotype to that of the respective mutants (Table 2), and therefore the respective transposon insertions are linked to the observed alteration of biofilm production. More than 20 independent transductants from two separate transduction experiments displayed identical biofilm-negative phenotypes, excluding the possibility of accidental cotransduction of the transposon insertions and another unlinked DNA fragment carrying the mutations responsible for the phenotypic change.

Mutants M10 and M11 and *S. epidermidis* 13-1 were compared by pulsed-field gel electrophoresis. Identical fragment patterns were observed after digestion of DNA by *Sma*I in ethidium bromide-stained gels except for decreased mobility of a fragment of 60 kb (Fig. 2A), which could indicate insertion of

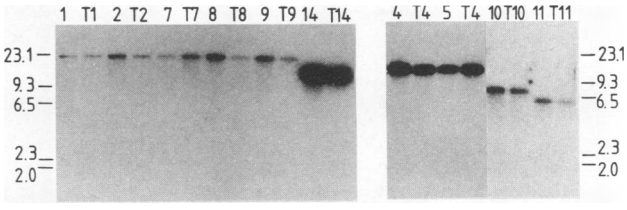


FIG. 1. Chromosomal DNA of transposon mutants M1, M2, M7, M8, M9, M14, M4, M5, M10, and M11 and of the corresponding transductants (T) was digested with *Eco*RI. Fragments were separated on a 0.5% agarose gel. An autoradiogram is shown after Southern transfer and hybridization with $^{32}$P-labeled pTV1ts.

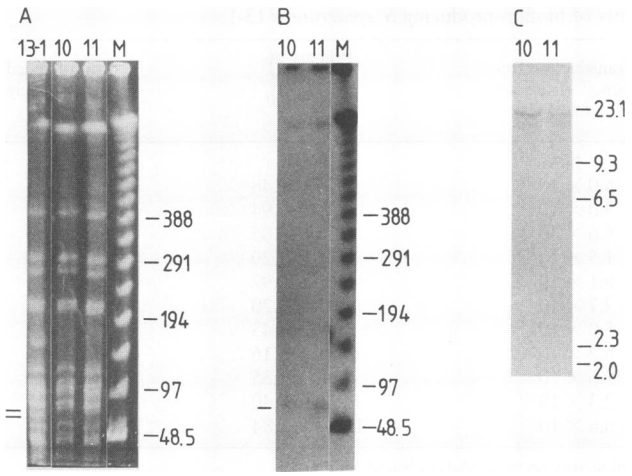


FIG. 2. (A) DNA of *S. epidermidis* 13-1 and transposon mutants M10 and M11 was digested by *Sma*I. Fragments were separated by pulsed-field gel electrophoresis. A photograph of the ethidium bromide-stained gel is shown. A 60-kb fragment with decreased mobility in mutants M10 and M11 is marked by lines. Phage lambda DNA is used as marker starting with the monomer and concatemers with increments of 48.5 kb. (B) DNA of mutants M10 and M11 was digested by *Sma*I. Fragments were separated by pulsed-field gel electrophoresis. An autoradiogram after Southern transfer and hybridization with $^{32}$P-labeled pTV1ts is shown. The probe was stripped, and the blot was rehybridized with $^{32}$P-labeled lambda DNA, indicating the monomer and concatemers with increments of 48.5 kb (lane M). (C) Chromosomal DNA of mutants M10 and M11 was digested with *Bam*HI. An autoradiogram after Southern transfer and hybridization with $^{32}$P-labeled pTV1ts is shown.

Tn*917* into this fragment. This was confirmed by Southern blot hybridization with $^{32}$P-labeled pTV1ts as a probe, because identical 65-kb hybridizing fragments were observed (Fig. 2B). The largest 600- to 700-kb *Sma*I fragment, which also gives a signal, probably represents an intermediate containing the target sequences after incomplete cleavage of *Sma*I sites. After digestion of chromosomal DNA of these two mutants by *Bam*HI, hybridization with identical 22.4-kb fragments was detected (Fig. 2C). When considering the 5.3 kb of Tn*917*, this indicates that the individual Tn*917* insertions of mutants M10 and M11, which are located on different small *Eco*RI fragments, are clustered in a region of about 17 kb.

Identical 21.4- and 12.4-kb *Eco*RI fragments hybridized with mutants M1, M2, M7, M8, and M9 and mutants M4 and M5, respectively (Fig. 1). However, in contrast to mutants M10 and M11, all transductants of transposon Tn*917* mutants M1, M2, M7, M8, M9, M4, and M5 tested for biofilm production were indistinguishable from the wild-type *S. epidermidis* 9142 (Table 2). The respective transposon insertions apparently were not involved in the observed change of biofilm production, and therefore these mutants represent spontaneous variants. In pulsed-field gel electrophoresis of *Sma*I-digested DNA, no significant differences between profiles of the mutants and *S. epidermidis* 13-1 were detected, (data not shown), indicating that no obvious deletions occurred with these mutants.

M14 had a single Tn*917* insertion without any observed phenotypic change and was used as a control in phenotypic characterization (Fig. 1; Table 2).

**Characterization of the phenotype of biofilm-negative transposon mutants.** Interference with either primary attachment to a polymer surface by *S. epidermidis* or accumulation in multilayered cell clusters on the polymer surface, which in concert lead to biofilm production by *S. epidermidis*, results in an alteration in expression of this phenotype. We therefore investigated which of the two phases relevant to biofilm production was affected in the isolated mutants.

Primary attachment of *S. epidermidis* 13-1 and the isolated transposon mutants to the surface of polystyrene spheres was determined by using the assay of Timmerman et al. (52). A mean of $9.2 \times 10^4$ CFU of wild-type *S. epidermidis* 13-1 was attached to the surface of polystyrene spheres after 30 min at 37°C (Table 2). Only minor differences of attached bacterial cell counts within a range of 83 to 110% of attached wild-type *S. epidermidis* 13-1 cells were observed for the biofilm-negative Tn*917* mutants M10 and M11 and for the biofilm-producing control M14 (Table 2). For the spontaneous variants, numbers of attached bacteria varied between 65 and 88% of attached wild-type bacteria (Table 2).

In a previous study we demonstrated that significant proportions of cells of biofilm-producing *S. epidermidis* strains are located in large cell clusters, indicating the ability for intercellular adhesion. In contrast, no such cell clusters were observed with biofilm-negative *S. epidermidis* strains (36).

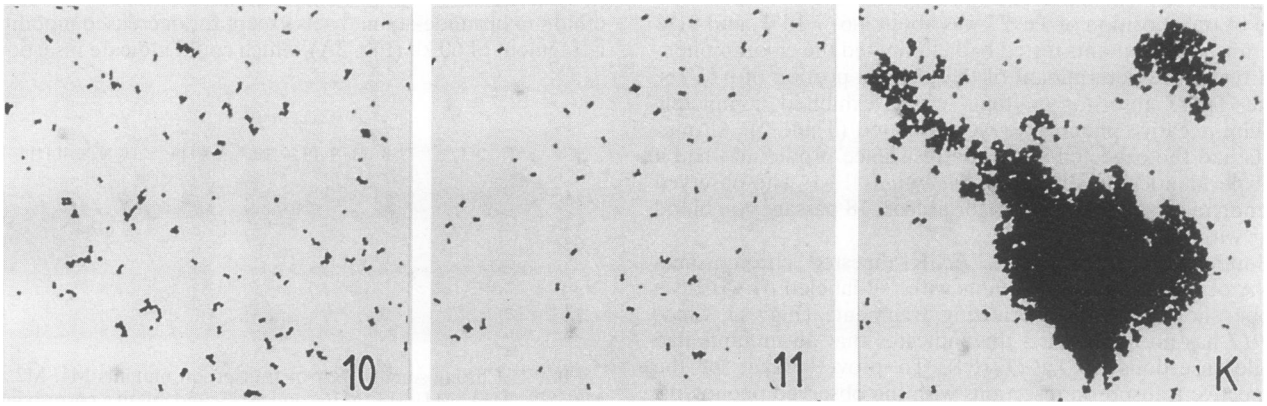


FIG. 3. Transposon mutants M10 (panel 10) and M11 (panel 11) and wild-type *S. epidermidis* 13-1 (panel K) were grown in TSB in plastic tissue culture dishes for 22 h. Medium was aspirated, cells were scraped into phosphate-buffered saline, and appropriate dilutions were applied to microscope slides. Representative microphotographs of gram-stained preparations are shown.

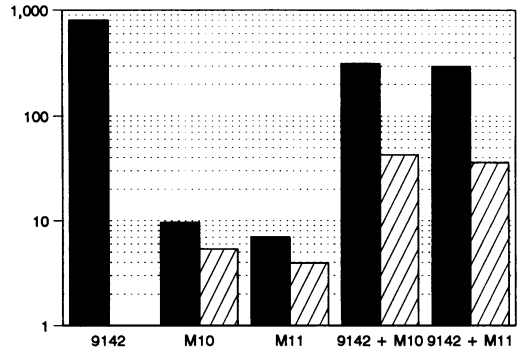


FIG. 4. *S. epidermidis* 9142, M10, and M11 grown in TSB were diluted 1:200 in TSB and were then incubated on 6-cm Nunc tissue culture plates overnight at 37°C either alone or in mixed culture. The medium was aspirated; plates were thoroughly washed four times with phosphate-buffered saline; adherent cells were scraped into phosphate-buffered saline, vortexed, and sonicated for 2 min; and appropriate dilutions were plated on TSB (■) agar and on TSB agar containing 10 μg of erythromycin per ml (▨). Results are the mean CFU ($10^6$) per tissue culture plate of two plates each of a representative experiment.

To evaluate cell cluster formation of the isolated Tn*917* transposon mutants, bacterial cells grown in TSB in plastic tissue culture dishes were scraped from the surface and were applied to microscope slides. Large cell clusters were observed with biofilm-producing wild-type *S. epidermidis* 13-1, whereas cell clustering could not be demonstrated with the biofilm-negative transposon insertion mutants M10 and M11 (Fig. 3) or the other biofilm-negative spontaneous variants (results not shown). Apparently, Tn*917* insertion mutants M10 and M11 and the spontaneous variants are unable to pursue intercellular adhesion.

**Specific antigen expression of biofilm-negative transposon mutants.** We have previously presented indirect biochemical evidence that a polysaccharide antigen specific for biofilm-producing *S. epidermidis* is functionally related to intercellular adhesion of biofilm-producing *S. epidermidis* in large cell clusters (36). We therefore investigated if, concomitant with impaired cell clustering, transposon Tn*917* mutants M10 and M11 and the other biofilm-negative spontaneous variants expressed reduced amounts of the cell-associated specific antigen. Bacterial cell concentration measured as $OD_{578}$ of cells grown in TSB did not vary significantly among the different strains investigated (data not shown). Similar concentrations of the antigen were determined by coagglutination in extracts of *S. epidermidis* 9142, 13-1, and the biofilm-producing control M14 (Table 2). In contrast, no coagglutinating activity could be detected in bacterial extracts of the biofilm-negative Tn*917* mutants M10 and M11 (Table 2). In extracts of the spontaneous variants specific antigen could be detected but in substantially reduced amounts (Table 2).

**Cointegration of transposon mutants into biofilm.** Apparently, lack of production of the specific antigen resulted in abolished intercellular adhesion of the transposon mutants M10 and M11. To establish that these mutants would still accumulate into a biofilm if the specific antigen was produced by a biofilm-producing *S. epidermidis* strain during cocultivation, we cocultivated mutants M10 and M11 with the wild-type *S. epidermidis* 9142 on tissue culture plates. The plates were washed; the adherent biofilm was scraped from the surface, vortexed, and sonicated; and the cells were quantitated by colony counts. Mutants M10 and M11 were specifically quantitated by being plated in parallel on agar plates containing 10 μg of erythromycin per ml. There was about an 80-fold difference in the number of cells scraped from the surface of tissue culture plates of biofilm-producing *S. epidermidis* 9142 and those of the biofilm-negative Tn*917* mutants M10 and M11 grown alone (Fig. 4). A ca. twofold reduction of biofilm-adherent cells was detected when *S. epidermidis* 9142 was cocultivated with M10 or M11 (Fig. 4). However, compared with mutants M10 and M11 grown on tissue culture plates alone, an eight- to ninefold increase in the number of erythromycin-resistant biofilm-integrated cells was detected after cocultivation with *S. epidermidis* 9142 (Fig. 4); after correction for the plating efficiency of mutants M10 and M11 on agar plates containing erythromycin, this represented about 25% of the total number of cells in the biofilm.

TABLE 3. Carbohydrate composition of crude extracts and of Sephadex G-200-purified specific antigen of *S. epidermidis* 1457 and the isogenic Tn*917* mutant 1457-M11

| Component | Amt of carbohydrate in strain: | | | |
|---|---|---|---|---|
| | 1457 | | 1457-M11 | |
| | Crude extract | Sephadex G-200 | Crude extract | Sephadex G-200 |
| Hexoses[a] | 1,425 | 821 | 597 | 273 |
| Hexosamines[a] | 10,750 | 6,865 | 398 | 12 |
| Uronic acid[a] | 142 | 26 | 0 | 0 |
| Ketoses[a] | 207 | 185 | 143 | 0 |
| Pentoses[a] | 467 | 189 | 154 | 47 |
| Phosphate[a] | 1,423 | 447 | 701 | 184 |
| Protein[a] | 5,541 | 613 | 3,762 | 180 |
| Specific antigen[b] | 16,384 | 8,192 | 0 | 0 |

[a] Values given are micrograms of the respective compound per liter of bacterial culture grown in $TSB_{dia}$ ($OD_{578}$, 0.7).
[b] Reciprocal titers were determined in parallel by coagglutination.

**Sugar composition of the specific antigen.** Several polysaccharide components of *S. epidermidis* slime have been described (8, 28, 44, 53). Therefore, it was important to differentiate the antigen detected by our absorbed antiserum from

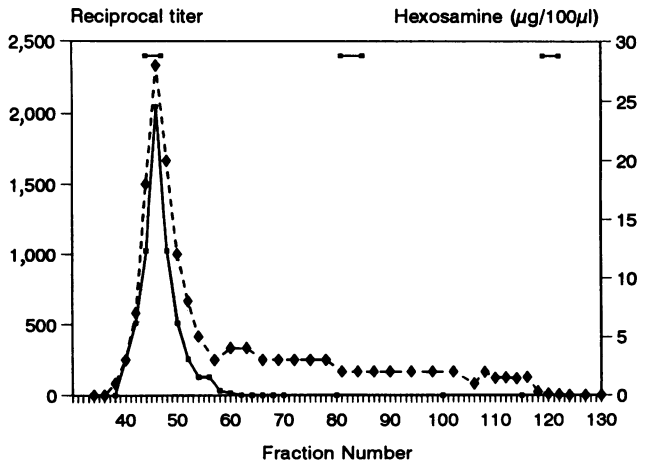


FIG. 5. Crude extract containing the specific antigen of *S. epidermidis* 1457 was applied to a Sephadex G-200 column and was eluted with phosphate-buffered saline. The antigen titer was determined in fractions by coagglutination (■). The hexosamine content of fractions was determined by colorimetric assay (◆). Positions of elution of 2-MDa blue dextran, dextran 10, and $K_3Fe(CN)_6$, respectively, are indicated at the top (■——■).

these components. Specific antigen of biofilm-producing *S. epidermidis* 1457 was prepared in $TSB_{dia}$ medium to exclude contamination with medium components. The main component of the crude extract was hexosamine followed by protein, hexose, and phosphate (Table 3). The antigen was partially purified by gel filtration on Sephadex G-200. It eluted in a single peak near the void volume of the column (Fig. 5). A parallel peak was observed when the fractions were assayed for hexosamine content (Fig. 5). The peak fractions were pooled, concentrated, and assayed for sugar content. Protein was substantially removed by the purification step, and hexosamine was the main component of the enriched antigen preparation (Table 3).

To evaluate which of the detected components are specific for the antigen, the transposon mutation of mutant M11 was introduced into *S. epidermidis* 1457 by transduction with *S. epidermidis* phage 48. The isolated transductant had the expected antibiotic resistance profile and a phenotype identical to that of mutant M11; i.e., 1457-M11 was completely biofilm negative, it did not express cell clusters (data not shown), and specific antigen production was completely abolished (Table 3). When chromosomal DNA of 1457-M11 was analyzed by Southern blotting with $^{32}$P-labeled pTV1ts as a probe, hybridization of identical *Eco*RI fragments was detected in 1457-M11 and M11 (data not shown).

Extracts of *S. epidermidis* 1457-M11 were prepared in parallel with *S. epidermidis* 1457 under identical conditions. No specific antigen could be detected. Already in the crude extract only 3.7% of the hexosamine content of the wild-type *S. epidermidis* 1457 was determined, whereas much smaller differences were detected with the other components (Table 3). The crude extract of *S. epidermidis* 1457-M11 was also fractionated by using Sephadex G-200. The sugar components of the pooled fractions corresponding to the fractions of the *S. epidermidis* 1457 antigen extract pooled were determined. Almost no hexosamine was detected in the material of the biofilm-negative isogenic Tn*917* insertion mutant 1457-M11, whereas 30 to 40% of the respective hexose, protein, and phosphate content was detected (Table 3). This identifies amino sugars as the main, specific components of the specific antigen of biofilm-producing *S. epidermidis*.

## DISCUSSION

In the present study we used transposon mutagenesis with *E. faecalis* transposon Tn*917* carried by plasmid pTV1ts to isolate Tn*917* insertion mutants of biofilm-producing *S. epidermidis* 13-1 with a completely biofilm-negative phenotype.

Introduction of plasmid pTV1ts into the wild-type *S. epidermidis* 9142 by protoplast transformation or electroporation failed, so we mobilized pTV1ts into *S. epidermidis* 9142 by using the conjugative *S. aureus* plasmid pWBG636 (56). Thomas and Archer (51) used the conjugative *S. aureus* plasmid pGO1 in a similar approach to mobilize recombinant derivatives of plasmid pC221 into different *S. epidermidis* strains; however, mobilization of functional plasmid pTV1ts was unsuccessful because of rearrangements. Mobilization of other plasmids of interest into biofilm-producing *S. epidermidis* by the conjugative *S. aureus* plasmid pWBG636 could be a rewarding alternative if more direct approaches such as protoplast transformation or electroporation fail.

It is essential in any transposon mutagenesis experiment to show linkage of the transposon insertions with the observed phenotype changes (21). This is especially important in the analysis of biofilm-producing *S. epidermidis* mutants with a biofilm-negative phenotype, because biofilm production is due to phenotypic change by phase variation and biofilm-negative variants may appear with a significant frequency (6, 7).

We established a generalized phage transduction system by using *S. epidermidis* phage 48, which allowed transduction of plasmid pTV1ts and the isolated transposon insertions back into the wild-type *S. epidermidis* 9142. By using this method, linkage of the isolated transposon insertions of mutants M10 and M11 and the respective phenotype changes could be demonstrated, whereas with several other biofilm-negative isolates with Tn*917* insertions no linkage of the transposon insertions was detected, and these mutants were thus identified as spontaneous variants with silent Tn*917* insertions. In addition, phage 48 successfully transduced plasmid pTV1ts and the transposon Tn*917* insertion of mutant M11 into the unrelated biofilm-producing *S. epidermidis* 1457. Apparently, generalized phage transduction has great potential for the genetic manipulation of clinical isolates of biofilm-producing *S. epidermidis* strains for use in pathogenicity studies.

Interestingly, the Tn*917* insertions of M10 and M11 were located on two different small *Eco*RI fragments (Fig. 1); however, these fragments were clustered on identical 60-kb *Sma*I and 17-kb *Bam*HI fragments (Fig. 2), which most probably excludes the possibility that significant deletions of chromosomal DNA occurred in conjunction with Tn*917* insertion. In addition, these results could indicate that the two insertions of M10 and M11 linked with the biofilm-negative phenotype are located in an operon, which could represent synthetic genes for the specific polysaccharide antigen (see below). However, it cannot be ruled out at present that the transposon insertions in mutants M10 and M11 are located in a single gene with an internal *Eco*RI site.

Biofilm production as detected by the assay used in the present study results from the concerted action of primary attachment to the polymer surface together with accumulation on the polymer surface in multiple cell layers, which requires competence for intercellular adhesion. Interference with any of these properties will probably lead to a biofilm-negative phenotype. Competence for primary attachment to polystyrene spheres was very similar for the wild-type, biofilm-producing *S. epidermidis* 13-1, the control M14, the biofilm-negative Tn*917* mutants M10 and M11, and the other spontaneous variants (Table 2). It seems highly unlikely that the marginal differences observed result in the completely biofilm-negative phenotypes. However, mutants M10 and M11 and the spontaneous variants were severely impaired in intercellular adhesion, as formation of the characteristic large cell clusters typical for biofilm-producing *S. epidermidis* strains (36) were completely abolished (Fig. 3). Apparently, the biofilm-negative phenotypes of the Tn*917* mutants M10 and M11 and the spontaneous variants are very similar as a result of defective accumulative growth on the polymer surface.

However, significant differences were detected between the Tn*917* mutants M10 and M11 and the other spontaneous variants as the antigen specific for biofilm-producing *S. epidermidis* (36) could not be detected with M10 and M11 (Table 2). In contrast, compared with *S. epidermidis* 13-1, significantly lower but detectable antigen titers were observed in extracts of the spontaneous variants (Table 2); these were comparable to titers expressed by biofilm-producing *S. epidermidis* strains grown in TSB lacking glucose, which express a biofilm-negative phenotype in that medium (36). This could result from inactivation of the biosynthetic genes responsible for the synthesis of the specific antigen by the Tn*917* insertions in mutants M10 and M11, whereas point mutations leading to residual enzymatic activity could be responsible for the observed leaky phenotypes of the spontaneous variants.

Apparently, transposon mutants M10 and M11 are still efficiently integrated into a biofilm adhering to the surface of a tissue culture plate when the specific antigen is synthesized during cocultivation of the mutants with biofilm-producing *S. epidermidis* 9142 (Fig. 4). This demonstrates that the Tn*917* insertions of mutants M10 and M11 did not result in alteration of cell surface properties leading to significant changes of interaction of the bacterial cells with the specific antigen. Therefore the observed lack of cell clustering results from abolished production of the specific antigen acting as a polysaccharide intercellular adhesin. The cell surface components interacting with the antigen remain to be identified in future studies.

It has been shown that the presence of albumin or serum in the incubation medium leads to a biofilm-negative phenotype of biofilm-producing *S. epidermidis* strains in the tube test and to significantly reduced primary attachment of staphylococcal cells to polymer surfaces (44, 58). When several biofilm-producing *S. epidermidis* strains were grown in TSB containing 3% bovine serum albumin, the cells still produced a cohesive biofilm with cell clustering identical to that obtained with controls grown in TSB; however, the biofilm no longer adhered to the surface of the tissue culture plates, resulting in an overall biofilm-negative phenotype (data not shown). Therefore, the accumulation of staphylococcal cells in a biofilm mediated by intercellular adhesion, which functionally involves the specific antigen, may be also functional in vivo in the presence of albumin and serum, irrespective of which of the different mechanisms leading to primary attachment of *S. epidermidis* to polymer surfaces is actually operative (20, 26, 42, 52, 53, 58).

Several polysaccharide components of *S. epidermidis* which are major constituents of *S. epidermidis* slime or glycocalyx or have been proposed to be functionally significant for biofilm production have been described. Therefore, the antigen specific for biofilm-producing *S. epidermidis* strains detected by our absorbed antiserum (36) had to be differentiated from those components.

We identified hexosamine as the major specific component of the antigen enriched by gel filtration of biofilm-producing *S. epidermidis* 1457 because almost no hexosamine was detected in material prepared from the isogenic biofilm-negative transductant 1457-M11 (Table 3). Minor amounts of hexose, phosphate, and protein, which may represent minor contaminants at that stage of purification, were detected in both extracts.

A high-molecular-weight polysaccharide extracellular slime substance of *S. epidermidis* consisting mainly of galactose and mannose was proposed to be functionally involved in attachment or accumulation of *S. epidermidis* on polymer surfaces (34, 44). However, this material was shown by others to consist primarily of polysaccharide contaminants derived from agar (17, 27).

Hussain et al. (28) found that the major high-molecular-weight slime component of biofilm-producing *S. epidermidis* RP62A grown in a chemically defined medium had a composition similar to that of a purified cell wall teichoic acid. However, similar amounts of teichoic acid were produced by biofilm-negative *S. epidermidis* strains, which argues against a significant functional role for teichoic acid in biofilm production (28, 29). The low level of phosphate and hexose detected in our antigen preparations eluted from Sephadex G-200 argues against the conclusion that the antigen detected by our absorbed antiserum was teichoic acid.

The reported composition of purified PS/A containing 54% hexoses and 20% amino sugars biochemically differentiates PS/A from the antigen detected by our absorbed antiserum (53). In addition, most clinical isolates of *S. epidermidis* were reported to be PS/A positive; however, no association of PS/A expression with the ability to accumulate in multiple layers on polymer surfaces leading to biofilm production was observed (41). In contrast, biofilm production as detected by the assay described in Materials and Methods was significantly associated with a positive coagglutination reaction of cell suspensions of more than 200 coagulase-negative staphylococcal isolates when our specific absorbed antiserum was used ($P < 0.001$) (37).

A slime-associated antigen (SAA) was proposed to be associated with biofilm production by *S. epidermidis* strains (8). This assumption is based mainly on the observation that SAA was not detected in extracts of a biofilm-negative morphology variant H4A of biofilm-producing *S. epidermidis* RP62A and an acriflavin-induced biofilm-negative mutant HAM892 of this strain but, on the other hand, rests on rather weak evidence because only a single biofilm-negative *S. epidermidis* strain was investigated.

Semipurified SAA enriched by Sephadex G-200 chromatography was reported to contain 64% hexose and only 0.91% hexosamine as determined by a colorimetric assay (8). Contents of 59% glucose and about 7% *N*-acetylglucosamine and *N*-acetylgalactosamine were determined by gas-liquid chromatography. However, as the composition of a similar extract of the biofilm-negative phase variant H4A or mutant HAM892 was not determined, it is not at all clear that the determined sugars are specific components of SAA. It is well known that extracts prepared from *S. epidermidis* grown on agar can be grossly contaminated with high-molecular-weight polysaccharide compounds derived from agar or medium (17, 27). As the antiserum specific for SAA and an SAA preparation were not available to us, we could not directly analyze our specific antigen preparations for SAA content. If the antigen detected by our absorbed antiserum was related to SAA, the main specific carbohydrate component of the antigen detected by our antiserum, hexosamine, would be significantly different from the reported SAA composition (8). In extracts of *S. epidermidis* RP62A and 1457 prepared as described by Christensen et al. (8), the specific antigen detected by our antiserum was not detected at all or only at a very low titer even after removal of eventually inhibitory low-molecular-weight components by gel filtration, providing further evidence against a relationship of the specific antigen to SAA (35).

Recently, biofilm-negative transposon insertion mutants of the biofilm-producing *S. epidermidis* M187 were isolated by using a derivative of transposon Tn*917*, Tn*917*-LTV1 (40). The presence of several transposon insertions clustered in an 11-kb region of a 28-kb *Sma*I chromosomal fragment led to a biofilm-negative phenotype. In contrast to mutants M10 and M11 (described in this study), the isolated mutants were severely impaired in the primary attachment phase of biofilm production because only 2.5 to 8% of mutant cells were attached to silicone catheters compared with those of the wild type (40). Consistent with that observation, the mutants synthesized a ca. 90% reduced amount of PS/A (40). These results confirm the epidemiologic observation that production of PS/A is a prerequisite for biofilm production by most *S. epidermidis* clinical isolates (41).

Grüter et al. (24) reported the isolation of a transposon Tn*917* mutant and a spontaneous phase variant of a biofilm-producing *S. epidermidis* strain with a biofilm-negative phenotype, which both showed lack of accumulative growth. However, because linkage of the transposon insertion and the phenotype change was not demonstrated, these results remain inconclusive.

Taken together, our results show that accumulation of *S.*

*epidermidis* in a biofilm can be genetically separated from the primary attachment to a polymer surface. As demonstrated by the complete lack of cell cluster formation and of production of the specific antigen of biofilm-producing *S. epidermidis* detected by our antiserum by transposon mutants M10 and M11, this antigen acts as a polysaccharide intercellular adhesin, which directly confirms our previous indirect biochemical evidence for a functional relationship between the antigen and intercellular adhesion of biofilm-producing *S. epidermidis* (36).

We have succeeded in transducing the Tn*917* insertion of mutant M11 into two unrelated biofilm-producing *S. epidermidis* strains, 9142 and 1457. This pair of isogenic mutants can now be used to establish the role of the accumulative phase of biofilm production in the pathogenicity of *S. epidermidis* in polymer-associated infections. Analysis of the genes interrupted by Tn*917* in mutants M10 and M11 will lead to a more thorough understanding of synthesis of the antigen and its function.

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## REFERENCES

1. **Ames, B. N.** 1966. Assay of inorganic phosphorus, total phosphorus, and phosphates. Methods Enzymol. **8:**115–118.
2. **Bayston, R., and J. Rogers.** 1990. Production of extra-cellular slime by *Staphylococcus epidermidis* during stationary phase of growth: its association with adherence to implantable devices. J. Clin. Pathol. **43:**866–870.
3. **Blumenkrantz, N., and G. Asboe-Hansen.** 1973. New method for quantitative determination of uronic acids. Anal. Biochem. **54:**484–489.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
5. **Chaplin, M. F.** 1986. Monosaccharides, p. 1–36. *In* M. F. Chaplin and J. F. Kennedy (ed.), Carbohydrate analysis. A practical approach. IRL Press, Oxford.
6. **Christensen, G. D., L. M. Baddour, B. M. Madison, J. T. Parisi, S. N. Abraham, D. L. Hasty, J. H. Lawrence, J. A. Joseph, and W. A. Simpson.** 1990. Colonial morphology of staphylococci on memphis agar: phase variation of slime production, resistance to β-lactam antibiotics, and virulence. J. Infect. Dis. **161:**1153–1169.
7. **Christensen, G. D., L. M. Baddour, and W. A. Simpson.** 1987. Phenotypic variation of *Staphylococcus epidermidis* slime production in vitro and in vivo. Infect. Immun. **55:**2870–2877.
8. **Christensen, G. D., L. P. Barker, T. P. Mawhinney, L. M. Baddour, and W. A. Simpson.** 1990. Identification of an antigenic marker of slime production for *Staphylococcus epidermidis*. Infect. Immun. **58:**2906–2911.
9. **Christensen, G. D., W. A. Simpson, A. L. Bisno, and E. H. Beachey.** 1982. Adherence of slime-producing *Staphylococcus epidermidis* to smooth surfaces. Infect. Immun. **37:**318–326.
10. **Christensen, G. D., W. A. Simpson, J. J. Younger, L. M. Baddour, F. F. Barrett, D. M. Melton, and E. H. Beachey.** 1985. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J. Clin. Microbiol. **22:**996–1006.
11. **Costerton, J. W., K.-J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie.** 1987. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. **41:**435–464.
12. **Davenport, D. S., R. M. Massanari, M. A. Pfaller, M. J. Bale, S. A. Streed, and W. J. Hierholzer, Jr.** 1986. Usefulness of a test for slime production as a marker for clinically significant infections with coagulase-negative staphylococci. J. Infect. Dis. **153:**332–339.
13. **Dean, B. A., R. E. O. Williams, F. Hall, and J. Corse.** 1973. Phage typing of coagulase-negative staphylococci and micrococci. J. Hyg. Camb. **71:**261–270.
14. **Deighton, M. A., and B. Balkau.** 1990. Adherence measured by microtiter assay as a virulence marker for *Staphylococcus epidermidis* infections. J. Clin. Microbiol. **28:**2442–2447.
15. **deSaxe, M. J., and C. M. Notley.** 1978. Experiences with the typing of coagulase-negative staphylococci and micrococci. Zentralbl. Bakteriol. Mikrobiol. Hyg. 1 Abt. Orig. A **241:**46–59.
16. **Diaz-Mitoma, F., G. K. M. Harding, D. J. Hoban, R. S. Roberts, and D. E. Low.** 1987. Clinical significance of a test for slime production in ventriculoperitoneal shunt infections caused by coagulase-negative staphylococci. J. Infect. Dis. **156:**555–560.
17. **Drewry, D. T., L. Galbraith, B. J. Wilkinson, and S. G. Wilkinson.** 1990. Staphylococcal slime: a cautionary tale. J. Clin. Microbiol. **28:**1292–1296.
18. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Reabers, and F. Smith.** 1956. Colorimetric method for the determination of sugars and related substances. Anal. Chem. **28:**350–356.
19. **Dunne, W., M. D. B. Nelson, and M. J. Chusid.** 1987. Epidemiological markers of pediatric infections caused by coagulase-negative staphylococci. Pediatr. Infect. Dis. J. **6:**1031–1035.
20. **Espersen, F., B. J. Wilkinson, B. Gahrn-Hansen, V. T. Rosdahl, and I. Clemmensen.** 1990. Attachment of staphylococci to silicone catheters in vitro. APMIS **98:**471–478.
21. **Foster, T. J.** 1991. Potential for vaccination against infections caused by *Staphylococcus aureus*. Vaccine **9:**221–227.
22. **Franson, T. R., N. K. Sheth, H. D. Rose, and P. G. Sohnle.** 1984. Scanning electron microscopy of bacteria adherent to intravascular catheters. J. Clin. Microbiol. **20:**500–505.
23. **Goldmann, D. A., and G. B. Pier.** 1993. Pathogenesis of infections related to intravascular catheterization. Clin. Microbiol. Rev. **6:**176–192.
24. **Grüter, L., H. Feucht, M. Mempel, and R. Laufs.** 1993. Construction of a slime-negative transposon mutant in *Staphylococcus epidermidis* using the *Enterococcus faecalis* transposon Tn917. Microbiol. Immunol. **37:**35–40.
25. **Grüter, L., O. König, and R. Laufs.** 1991. Transposon mutagenesis in *Staphylococcus epidermidis* using the *Enterococcus faecalis* transposon Tn917. FEMS Microbiol. Lett. **82:**215–218.
26. **Hogt, A. H., J. Dankert, C. E. Hulstaert, and J. Feijen.** 1986. Cell surface characteristics of coagulase-negative staphylococci and their adherence to fluorinated poly(ethylenepropylene). Infect. Immun. **51:**294–301.
27. **Hussain, M., J. G. M. Hastings, and P. J. White.** 1991. Isolation and composition of the extracellular slime made by coagulase negative staphylococci in a chemically defined medium. J. Infect. Dis. **163:**534–541.
28. **Hussain, M., J. G. M. Hastings, and P. J. White.** 1992. Comparison of cell-wall teichoic acid with high-molecular-weight extracellular slime material from *Staphylococcus epidermidis*. J. Med. Microbiol. **37:**368–375.
29. **Hussain, M., M. H. Wilcox, and P. J. White.** 1993. The slime of coagulase-negative staphylococci: biochemistry and relation to adherence. FEMS Microbiol. Rev. **104:**191–208.
30. **Hussain, M., M. H. Wilcox, P. J. White, M. K. Faulkner, and R. C. Spencer.** 1992. Importance of medium and atmosphere type to both slime production and adherence by coagulase-negative staphylococci. J. Hosp. Infect. **20:**173–184.
31. **Ishak, M. A., D. G. M. Gröschel, G. L. Mandell, and R. P. Wenzel.** 1985. Association of slime with pathogenicity of coagulase-negative staphylococci causing nosocomial septicemia. J. Clin. Microbiol. **22:**1025–1029.
32. **Kayser, F. H., J. Wüst, and P. Corrodi.** 1972. Transduction and elimination of resistance determinants in methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **2:**217–223.
33. **Lane-Smith, R., and E. Gilkerson.** 1979. Quantitation of glycosaminoglycan hexosamine using 3-methyl-2-benzothiazolone hydrazone hydrochloride. Anal. Biochem. **98:**478–480.
34. **Ludwicka, A., G. Uhlenbruck, G. Peters, P. N. Seng, E. D. Gray, J. Jaljaszewicz, and G. Pulverer.** 1984. Investigation on extracellular slime substance produced by *Staphylococcus epidermidis*. Zentralbl. Bakteriol. Mikrobiol. Hyg. 1 Abt. Orig. A **258:**256–267.
35. **Mack, D., M. Nedelmann, A. Krokotsch, and R. Laufs.** Unpublished data.
36. **Mack, D., N. Siemssen, and R. Laufs.** 1992. Parallel induction by

glucose of adherence and a polysaccharide antigen specific for plastic-adherent *Staphylococcus epidermidis*: evidence for functional relation to intercellular adherence. Infect. Immun. **60:**2048–2057.

37. **Mack, D., N. Siemssen, and R. Laufs.** Unpublished data.
38. **Marrie, T. J., and J. W. Costerton.** 1984. Scanning and transmission electron microscopy of in situ bacterial colonization of intravenous and intraarterial catheters. J. Clin. Microbiol. **19:**687–693.
39. **Martin, M. A., M. A. Pfaller, and R. P. Wenzel.** 1989. Coagulase-negative staphylococcal bacteremia. Mortality and hospital stay. Ann. Intern. Med. **110:**9–16.
40. **Muller, E., J. Hübner, N. Gutuirrez, S. Takeda, D. A. Goldmann, and G. B. Pier.** 1993. Isolation and characterization of transposon mutants of *Staphylococcus epidermidis* deficient in capsular polysaccharide/adhesin and slime. Infect. Immun. **61:**551–558.
41. **Muller, E., S. Takeda, H. Shiro, D. Goldmann, and G. B. Pier.** 1993. Occurrence of capsular polysaccharide/adhesin among clinical isolates of coagulase-negative staphylococci. J. Infect. Dis. **168:**1211–1218.
42. **Pascual, A., A. Fleer, N. A. C. Westerdaal, and J. Verhoef.** 1986. Modulation of adherence of coagulase-negative staphylococci to teflon catheters in vitro. Eur. J. Clin. Microbiol. **5:**518–522.
43. **Peters, G., R. Locci, and G. Pulverer.** 1981. Microbial colonization of prosthetic devices. II. Scanning electron microscopy of naturally infected intravenous catheters. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. B **173:**293–299.
44. **Peters, G., F. Schumacher-Perdreau, B. Jansen, M. Bey, and G. Pulverer.** 1987. Biology of *S. epidermidis* extracellular slime. Zentralbl. Bakteriol. Suppl. **16:**15–31.
45. **Pfaller, M. A., and L. A. Herwaldt.** 1988. Laboratory, clinical, and epidemiological aspects of coagulase-negative staphylococci. Clin. Microbiol. Rev. **1:**281–299.
46. **Rosdahl, V. T., B. Gahrn-Hansen, J. K. Moller, and P. Kjaeldgard.** 1990. Phage-typing of coagulase-negative staphylococci. Factors influencing typability. APMIS **98:**299–304.
47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
48. **Schmidt, D. D., D. F. Bandyk, A. J. Pequet, M. A. Malangoni, and J. B. Towne.** 1986. Mucin production by *Staphylococcus epidermidis*: a virulence factor promoting adherence to vascular grafts. Arch. Surg. **121:**89–95.
49. **Schwarzkopf, A., H. Karch, H. Schmidt, W. Lenz, and J. Heesemann.** 1993. Phenotypical and genotypical characterization of epidemic clumping factor-negative, oxacillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. **31:**2281–2285.
50. **Swanstrom, M., and M. H. Adams.** 1951. Agar layer method for production of high titer phage stocks. Proc. Soc. Exp. Biol. Med. **78:**372–375.
51. **Thomas, W. D., Jr., and G. Archer.** 1992. Mobilization of recombinant plasmids from *Staphylococcus aureus* into coagulase-negative staphylococcus species. Plasmid **27:**164–168.
52. **Timmerman, C. P., A. Fleer, J. M. Besnier, L. de Graaf, F. Cremers, and J. Verhoef.** 1991. Characterization of a proteinaceous adhesin of *Staphylococcus epidermidis* which mediates attachment to polystyrene. Infect. Immun. **59:**4187–4192.
53. **Tojo, M., N. Yamashita, D. A. Goldmann, and G. B. Pier.** 1988. Isolation and characterization of a capsular polysaccharide adhesin from *Staphylococcus epidermidis*. J. Infect. Dis. **157:**713–722.
54. **Townsend, D. E., N. Ashdown, S. Bolton, and W. B. Grubb.** 1985. Transfer of plasmid-borne aminoglycoside-resistance determinants in staphylococci. J. Med. Microbiol. **20:**169–185.
55. **Townsend, D. E., N. Ashdown, S. Bolton, and W. B. Grubb.** 1985. The use of cetyltrimethylammonium bromide for the rapid isolation from *Staphylococcus aureus* of relaxable and non-relaxable plasmid DNA suitable for in vitro manipulation. Lett. Appl. Microbiol. **1:**87–94.
56. **Udo, E. E., and W. B. Grubb.** 1990. A new class of conjugative plasmid in *Staphylococcus aureus*. J. Med. Microbiol. **31:**207–212.
57. **Udo, E. E., and W. B. Grubb.** 1990. Conjugal transfer of plasmid pWBG637 from *Staphylococcus aureus* to *Staphylococcus epidermidis* and *Streptococcus faecalis*. FEMS Microbiol. Lett. **72:**183–188.
58. **Vaudaux, P., D. Pittet, A. Haeberli, E. Huggler, U. E. Nydegger, D. P. Lew, and F. A. Waldvogel.** 1989. Host factors selectively increase staphylococcal adherence on inserted catheters: a role for fibronectin and fibrinogen or fibrin. J. Infect. Dis. **160:**865–875.
59. **Younger, J. J., G. D. Christensen, D. L. Bartley, J. C. H. Simmons, and F. F. Barret.** 1987. Coagulase-negative staphylococci isolated from cerebrospinal fluid shunts: importance of slime production, species identification, and shunt removal to clinical outcome. J. Infect. Dis. **156:**548–554.
60. **Youngman, P. J.** 1993. Transposons and their application, p. 585–596. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria. American Society for Microbiology, Washington, D.C.