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Identification of Three Essential Regulatory Gene Loci Governing Expression of *Staphylococcus epidermidis* Polysaccharide Intercellular Adhesin and Biofilm Formation

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The formation of adherent multilayered biofilms embedded into a glycocalyx represents an essential factor in the pathogenesis of *Staphylococcus epidermidis* biomaterial-related infections. Using biofilm-producing *S. epidermidis* 1457 and transposon Tn*917* carried on plasmid pTV1ts, we isolated nine isogenic biofilm-negative transposon mutants. Transduction by *S. epidermidis* phage 71 was used to prove the genetic linkage of transposon insertions and altered phenotypes. Mapping of the different transposon insertions by Southern hybridization and pulsed-field gel electrophoresis indicated that these were inserted in four unlinked genetic loci. According to their phenotypes, including quantitative differences in biofilm production in different growth media, in the amount of the polysaccharide intercellular adhesin (PIA) produced, in the hemagglutination titers, and in the altered colony morphology, the mutants could be separated into four phenotypic classes corresponding with the genetic classes. Synthesis of PIA was not detectable with class I and II mutants, whereas the amount of PIA produced reflected the residual degree of biofilm production of class III and IV mutants in different growth media. Chromosomal DNA flanking the transposon insertions of five class I mutants was cloned and sequenced, and the insertions were mapped to different locations of *icaADBC*, representing the synthetic genes for PIA. Expression of *icaADBC* from a xylose-dependent promoter in the different isogenic mutant classes reconstituted biofilm production in all mutants. In a Northern blot analysis no *icaADBC*-specific transcripts were observed in RNA isolated from mutants of classes II, III, and IV. Apparently, in addition to *icaADBC*, three other gene loci have a direct or indirect regulatory influence on expression of the synthetic genes for PIA on the level of transcription.

Today, coagulase-negative staphylococci, mostly strains of *Staphylococcus epidermidis*, represent the most frequent causes of nosocomial sepsis and are the most prominent organisms responsible for infections of implanted medical devices (1, 30, 38, 40, 41). In vitro, a proportion of *S. epidermidis* strains 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 infected intravascular catheters (3, 4, 14). This phenotype is now regularly referred to as biofilm formation, whereas the somewhat ambiguous term "slime production" was used earlier (13, 15, 16).

The molecular mechanisms leading to biofilm formation of *S. epidermidis* have attracted considerable interest in recent years. Biofilm formation may be divided into two phases. First, a complex process involving multiple physicochemical, protein, and polysaccharide factors leads to primary attachment of bacterial cells to a polymer surface (9, 11, 15, 16, 26–28, 39, 44). In the second phase, the attached bacteria proliferate and accumulate in a multilayered biofilm. Using transposon mutagenesis our group identified a linear homoglycan composed primarily of *N*-acetylglucosamine in β-1,6-glycosidic linkages containing deacetylated amino groups and succinate and phosphate substituents (17, 19). This polysaccharide is referred to as polysaccharide intercellular adhesin (PIA), which is functional in cell-to-cell adhesion and is essential for biofilm accumulation of most clinical *S. epidermidis* strains (17–19, 21, 22). In addition, PIA is essential for hemagglutination of erythrocytes by *S. epidermidis* (5, 20, 29, 31). The gene products of the *icaADBC* locus of *S. epidermidis* have enzymatic activity, which leads to synthesis of PIA in vivo and in vitro (6, 10, 20). Recently, it was demonstrated using a well-characterized isogenic biofilm-negative transposon mutant 1457-M10 in two relevant animal foreign-body infection models, that a functional *icaADBC* locus and the ability to produce PIA is essential for the pathogenesis of *S. epidermidis* biomaterial-related infections (19, 25, 32, 33).

In the present study we extend our genetic analysis of the mechanisms of *S. epidermidis* biofilm formation. Our results indicate that several independent genetic loci are essential for PIA synthesis and biofilm formation by *S. epidermidis*.

(Part of this work will appear in the doctoral theses of H.R., J.R., M.N., and J.K.-M.K., Universitäts-Krankenhaus Eppendorf, Hamburg, Germany.)

MATERIALS AND METHODS

Bacterial strains. The biofilm-producing *S. epidermidis* 1457, its variant cured of an endogenous plasmid, *S. epidermidis* 1457c, and biofilm-producing *S. epidermidis* 9142, as well as the isogenic biofilm-negative mutants M10 and M11 and the biofilm-negative transductant 1457-M10, have been described (20, 21, 25). *Staphylococcus carnosus* containing the recombinant plasmids pCN27 (10) or pTX*icaADBC* (6), which contains the cloned *icaADBC* locus under control of its own or a xylose-inducible promoter, were kindly provided by Friedrich Götz (University of Tübingen, Tübingen, Germany). *S. aureus* WBG4883 carrying the conjugative plasmid pWBG636 was kindly provided by W. B. Grubb (Curtin

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TABLE 1. Strains and plasmids

Species or strain	Plasmid	Antibiotic resistance(s)	Properties	Reference(s)
1457	p1457		Biofilm positive	21, 25
1457c	pTV1ts	Ery^r Cm^r	Biofilm positive	25
9142-M10		Ery^r $Cipro^r$	Biofilm negative	20
1457-M10		Ery^r	Biofilm negative	19
M10		Ery^r $Cipro^r$ Gm^r	Biofilm negative	19
M11		Ery^r $Cipro^r$ Gm^r	Biofilm negative	19
E. coli MC1061		Sm^r	Expression of Tn*917* Ery^r	7
S. aureus WBG4881	pWBG636	Gm^r	Conjugative mobilization of plasmids	43
E. coli	pBluescript II SK	Amp^r	Cloning vector for *E. coli*	20
S. carnosus	pCN27	Cm^r	*icaADBC* cloned in pCA44	10
S. carnosus	pTX*icaADBC*	Tet^r	*icaADBC* under control of xylose-inducible promoter	6

University of Technology, Perth, Australia) (42, 43). *Escherichia coli* MC1061 (kindly provided by J. A. Gutierrez, Department of Oral Biology, University of Florida, Gainesville) was used as a host for cloning Tn*917* insertion sites in plasmid pBluescript II SK (7). The relevant plasmids and antibiotic resistance markers of these strains are listed in Table 1.

Transposon mutagenesis. Transposon mutagenesis was carried out at the nonpermissive temperature of plasmid pTV1ts using *S. epidermidis* 1457c(pTV1ts) as described previously (19, 25).

Phage transduction. Phage transduction using *S. epidermidis* phages 48 or 71, kindly provided by V. T. Rosdahl, Statens Seruminstitut, Copenhagen, Denmark, was performed as described previously (25). For transduction of chromosomal markers the phage lysates were UV irradiated as described elsewhere (19, 25).

Mobilization of pTX*icaADBC* into *S. epidermidis* by coconjugation and transduction. *S. carnosus* carrying plasmid pTX*icaADBC* and *S. aureus* WBG4883 were mated on membrane filters as described earlier (19). Donor and recipient strains were grown in brain heart infusion (BHI) broth (Oxoid, Basingstoke, England) overnight at 37°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 37°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 pTX*icaADBC* (tetracycline, 10 μg/ml) and for pWBG636 (gentamicin, 8 μg/ml) at 37°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 pTX*icaADBC* with tetracycline (10 μg/ml) and for *S. epidermidis* 9142 with 2 μg of ciprofloxacin per ml at 37°C. A transconjugant clone with the expected resistance pattern was used to move pTX*icaADBC* into different recipients using *S. epidermidis* phages 48 and 71 (25).

PFGE, DNA isolation, and Southern blot hybridization. Pulsed-field gel electrophoresis (PFGE) was performed essentially as described using *Sma*I for cleavage of DNA and a CHEF-DR II system (Bio-Rad, Munich, Germany) for the analysis of resulting fragments (36). Phage lambda DNA concatemers were used as molecular weight markers. Chromosomal and plasmid DNA was isolated as previously described (19). DNA was digested with restriction enzymes under conditions suggested by the manufacturer (Pharmacia, Freiburg, Germany). DNA fragments were separated on 0.5% agarose gels in Tris-borate buffer (34). Alkaline capillary blotting and hybridization with [^{32}P]dCTP-labeled probes were performed as described previously (19). The blots were exposed to Kodak X-Omat X-ray films.

Cloning of Tn*917* containing DNA fragments of transposon mutants. Tn*917*-containing restriction fragments were cloned directly in *E. coli* MC1061 using pBluescript II SK (Stratagene, La Jolla, Calif.) as a vector and selecting ampicillin-resistant (100 μg/ml) transformants. Positive clones were transferred using soft velvet to erythromycin-containing Luria-Bertani agar plates (300 μg/ml) directly selecting for inserts expressing the erythromycin resistance gene of Tn*917* (7). Sequences of the transposon insertion sites of the mutants were obtained using oligonucleotides 5′-GGC CTT GAA ACA TTG GTT TAG TGG G-3′ and 5′-CTC ACA ATA GAG AGA TGT CAC CG-3′ complementary to the 5′- and 3′-junctions of Tn*917* (37). DNA sequence analysis was performed using the Sequenase version 2.0 kit (U.S. Biochemicals) as described earlier (20).

Isolation of RNA and Northern blot analysis. An overnight culture of the respective strains was diluted 1:100 into fresh, prewarmed tryptic soy broth (Oxoid) TSB_{Oxoid} and then grown into the mid-exponential-growth phase (optical density at 600 nm [OD_{600}] of ~3.0). Total bacterial RNA was isolated by the FastPrep system (Bio 101, Vista, Calif.) as described earlier (2). Then, 10 μg of total RNA was analyzed on a 1% agarose-formaldehyde gel in MOPS (morpholinepropanesulfonic acid) running buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA; pH 7.0). RNA was blotted onto Zeta-Probe blotting membranes (Bio-Rad, Munich, Germany) and fixed to the membrane by baking at 80°C for 30 min. Hybridization with a ^{32}P-labeled *icaC*-specific (5′-GAA ATA GCC ATA CCA TTG TCC-3′) oligonucleotide was performed at 52°C overnight in hybridization buffer containing 7% sodium dodecyl sulfate (SDS), 20 mM sodium phosphate (pH 7.0), 10× Denhardt solution, 5× SSC (standard saline citrate), 10% dextran sulfate, and 100 μg of denatured herring sperm DNA per ml. The membranes were then washed at 52°C for 30 min in 5% SDS, 20 mM sodium phosphate (pH 7.0), 10× Denhardt solution, and 3× SSC, followed by 1 h in 1× SSC–1% SDS. The membranes were exposed to Kodak X-Omat X-ray films.

Adherence assay for measurement of biofilm production by *S. epidermidis*. Biofilm production by *S. epidermidis* strains grown in Trypticase soy broth (TSB_{BBL}; Becton Dickinson, Cockeysville, Md.) and TSB_{Oxoid} (Oxoi, Basingstoke, England), respectively, was determined with a semiquantitative adherence assay using 96-well tissue culture plates (Nunc, Roskilde, Denmark) as described previously (4, 21).

Preparation of bacterial extracts and quantitation of PIA concentration. Bacterial extracts of *S. epidermidis* strains grown in TSB_{BBL} or TSB_{Oxoid} on plastic tissue culture plates were prepared by sonication (21). The concentration of PIA in bacterial extracts was determined by a specific coagglutination assay (19–21).

Hemagglutination. Hemagglutination was assessed as described previously (20, 29). Bacteria were grown in TSB_{BBL} in plastic tissue culture dishes for 22 h at 37°C (20). The medium was aspirated, and cells were scraped from the surface into 12 ml of phosphate-buffered saline. After passage through a 23-gauge needle, the bacterial suspension was adjusted to an OD_{578} of 1.0. The hemagglutination assay was performed with 96-well (U-shaped) microtiter plates (Greiner, Nürtingen, Germany) using sheep erythrocytes (Sigma, Deisenhofen, Germany) as described earlier (20, 29).

RESULTS

Isolation of biofilm-negative transposon mutants. Using the biofilm-producing *S. epidermidis* 1457c cured of a cryptic plasmid of *S. epidermidis* 1457 and the temperature-sensitive plasmid pTV1ts, nine transposon mutants displaying altered biofilm production were isolated (25). For all mutants, genetic linkage of the observed phenotypic changes and the respective transposon insertions was demonstrated by transduction using *S. epidermidis* phage 71. Similar phenotypes and hybridization patterns using a Tn*917*-specific probe were detected with all mutants and their respective transductants (data not shown).

Genetic analysis of transposon insertion sites. (i) Characterization of isogenic biofilm-negative mutants by Southern blot hybridization and PFGE. Southern blot hybridization of *Eco*RI-cleaved chromosomal DNA of the mutants using the *icaADBC*-specific insert of plasmid pCN27 as a probe revealed single insertions of Tn*917* into the *ica*-specific 3-kb fragment of *S. epidermidis* 1457 in mutants M13 and M24, whereas in mutants M21, M22, and M23 Tn*917* inserted into the 6.4-kb *Eco*RI fragment specific for the *icaADBC* locus (Fig. 1A). In contrast, in mutants M12, M15, M17, and M19 Tn*917* insertions were apparently not related to the *icaADBC* locus, since no fragment size alterations of the two *ica*-specific *Eco*RI fragments were observed (Fig. 1A).

Hybridization with a Tn*917*-specific probe revealed that all mutants contained single insertions of Tn*917* (Fig. 1B). Two identical 14.5-kb *Eco*RI-fragments were labeled in mutants M15 and M19, suggesting that the insertion sites of these

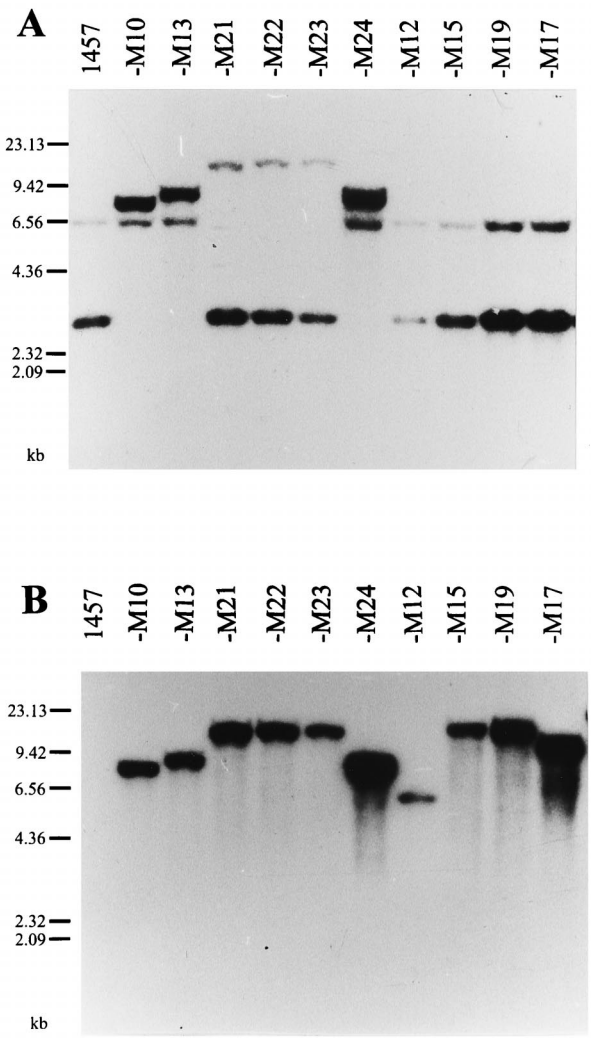

FIG. 1. Chromosomal DNA of the wild-type biofilm-producing *S. epidermidis* 1457 and the isogenic biofilm-negative Tn*917* mutants M10, M13, and M21 to M24 (class I), M12 (class II), M15 and M19 (class III), and M17 (class IV) was digested with *Eco*RI. Fragments were separated by agarose gel electrophoresis. Autoradiograms of a Southern blot hybridized with the *icaADBC*-specific *Xba*I-*Hin*dIII fragment of plasmid pCN27 (A) or a Tn*917*-specific probe (B) are shown. The discrepant intensities in panel A of the 6.4- and 3.0-kb fragments and their respective variants containing Tn*917* insertions probably result from the fact that more than two-thirds of the *icaADBC*-specific probe used covered the smaller fragment, whereas the remainder hybridized to the larger fragment.

mutants could be closely linked. Using chromosomal DNA of mutants M12 and M17, insertion of Tn*917* into *Eco*RI fragments of 6.0 and 9.8 kb was detected.

Analysis of the insertion sites of the mutants by PFGE revealed insertion of Tn*917* in a 60-kb *Sma*I fragment with mutants M13 and M22 (class I), a 40-kb *Sma*I fragment with mutant M12 (class II), a 97-kb *Sma*I fragment with mutants M15 and M19 (class III), and a 40-kb *Sma*I fragment for mutant M17 (class IV; Fig. 2). Mobility differences of fragments resulting from Tn*917* insertions can be observed by comparison with neighboring lanes with mutants of different classes in the ethidium bromide-stained gel, as indicated in the left panel of Fig. 2. In the case of mutants M12 (class II) and M17 (class IV), insertions occurred in two different fragments of approximately 40 kb, which are not separated in the wild type and mutants of classes I and III, resulting in a relatively intense band. Tn*917* insertion in mutant M12 (class II) leads to decreased mobility of that double band, probably by insertion into the smaller of the two comigrating fragments. In mutant M17 (class IV) a distinct double band is observed, probably resulting from insertion of Tn*917* into the larger of the two comigrating fragments. These studies indicate that in mutants M13 and M21 through M24, the insertion of Tn*917* occurred within or near the *icaADBC* locus, whereas the transposon insertions of the other four mutants represent gene loci, which influence biofilm formation of *S. epidermidis* differently from the *icaADBC* locus.

(ii) Mapping of the different transposon insertion sites. Using *ica*-specific probes and two *Ava*I fragments corresponding to the 5′ (*erm*) and the 3′ junctions of Tn*917*, preliminary mapping of the transposon insertions of the different mutant classes was performed using restriction enzymes *Xba*I, *Hin*dIII, and *Bgl*II, which all have cleavage sites within the Tn*917* sequence (Fig. 3). Apparently, Tn*917* inserted at different locations of the *icaADBC* locus with the identical transcriptional direction in mutants M13, M21, M22, and M23, but not with mutant M24, where Tn*917* inserted in a direction opposite to the transcriptional direction of *icaADBC*. Mapping of the mutants M12, M15, M19, and M17 confirmed the close linkage of Tn*917* insertions in mutants M15 and M19. Due to the different restriction fragment patterns observed by mapping of mutants M12 and M17, linkage of the transposon insertions of these two mutants is highly unlikely, as already indicated from the PFGE results. Apparently, in addition to Tn*917* insertions in the *icaADBC* locus, three additional classes of unlinked insertions of Tn*917* into the chromosome of *S. epidermidis* 1457 lead to a biofilm-negative phenotype.

(iii) Identification of Tn*917* insertion sites of class I biofilm-negative mutants by nucleotide sequencing. The chromosomal *Eco*RI fragments of the biofilm-negative *S. epidermidis* transposon mutants M13 and M24 containing the entire transposon Tn*917* were cloned in *E. coli* MC1061. For mutants M22 and M23 chromosomal *Eco*RI-*Sal*I fragments and for mutant M21 a *Hin*dIII-*Sal*I fragment containing the 5′ end of Tn*917*, including the *erm* gene, were cloned in *E. coli*. Sequence analysis revealed that mutants M13 and M24 had their insertions in the coding sequence of the *icaA* gene of the *icaADBC* locus, which contains synthetic genes for PIA (Table 2). In contrast, in the biofilm-negative mutants M21, M22, and M23, Tn*917* was inserted at different sites within the coding sequence of *icaC* (Table 2).

Phenotypic characterization of the transposon mutants. (i) Biofilm production. According to their differential degree of biofilm production in TSB_{Oxoid} and TSB_{BBL} and their colony morphology, the isogenic transposon mutants could be assigned to four phenotypic classes, which clearly correspond to the genetic classes defined by the different insertion sites of Tn*917*. All mutants were completely biofilm negative in TSB_{Oxoid}. In TSB_{BBL} all class I mutants were completely biofilm negative with the exception of mutant M21, in which Tn*917* inserted at the extreme end of *icaC*, apparently resulting in a low residual enzymatic activity. Mutant M12 (class II) was also completely biofilm negative in TSB_{BBL}. Mutants M15 and M19 (class III) displayed quantitatively reduced biofilm production in TSB_{BBL}, which often was near the cutoff value of the adherence assay, whereas M17 (class IV) nearly reached wild-type levels of biofilm production in TSB_{BBL} (Table 3).

(ii) PIA production. All mutants of class I did not detectably produce PIA in TSB_{Oxoid} and TSB_{BBL}, except for mutant M21, which corresponds to the residual biofilm production of M21 in TSB_{BBL} (Table 3). Mutant M12 (class II) also did not produce any PIA in either medium. Class III mutants did not produce

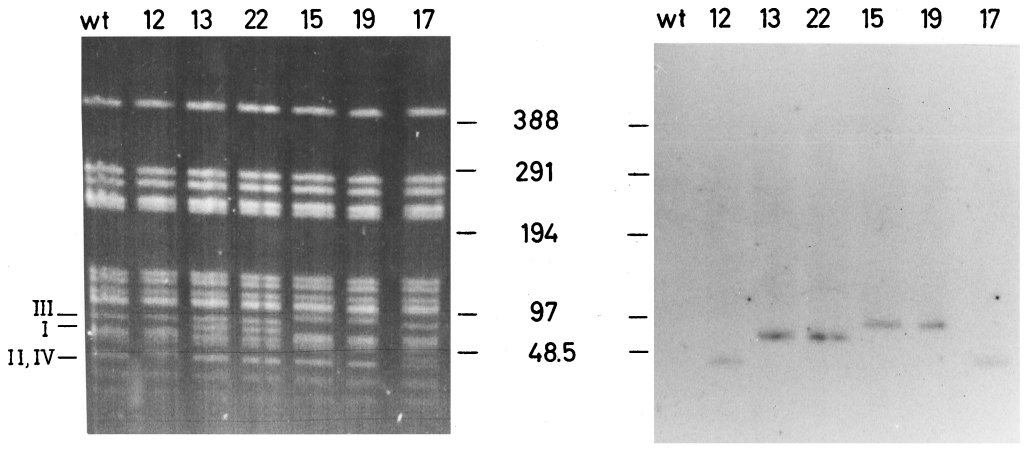

FIG. 2. Chromosomal DNA of biofilm-producing *S. epidermidis* 1457 (wt) and transposon mutants M12 (class II), M13 and M22 (class I), M15 and M19 (class III), and M17 (class IV) were cleaved by *Sma*I. Fragments were separated by PFGE. (Left panel) Ethidium bromide-stained gel. Fragments with altered mobility due to Tn*917* insertions in the different mutant classes are indicated. (Right panel) Autoradiogram after hybridization of the Southern blot with a probe specific for Tn*917*.

PIA in TSB_{Oxoid} but had 32-fold-reduced PIA titers compared to the wild type in TSB_{BBL}. The class IV mutant (M17) produced eightfold- and fourfold-reduced PIA titers in TSB_{Oxoid} and TSB_{BBL}, respectively (Table 3).

(iii) Hemagglutination. PIA was identified as the hemagglutinin of *S. epidermidis* (5, 20). In TSB_{BBL} the isogenic transposon mutants of class I were all hemagglutination negative, with the exception of mutant M21, which also displayed residual PIA synthesis (Table 3). Mutant M12 (class II) was also completely hemagglutination negative (Table 3). Mutants M15 and M19 (class III) displayed reduced hemagglutination titers proportional to their reduced synthesis of PIA (Table 3). In contrast, the hemagglutination titers displayed by mutant M17 (class IV) were significantly lower, as would have been expected from the amount of PIA synthesized by this mutant (Table 3).

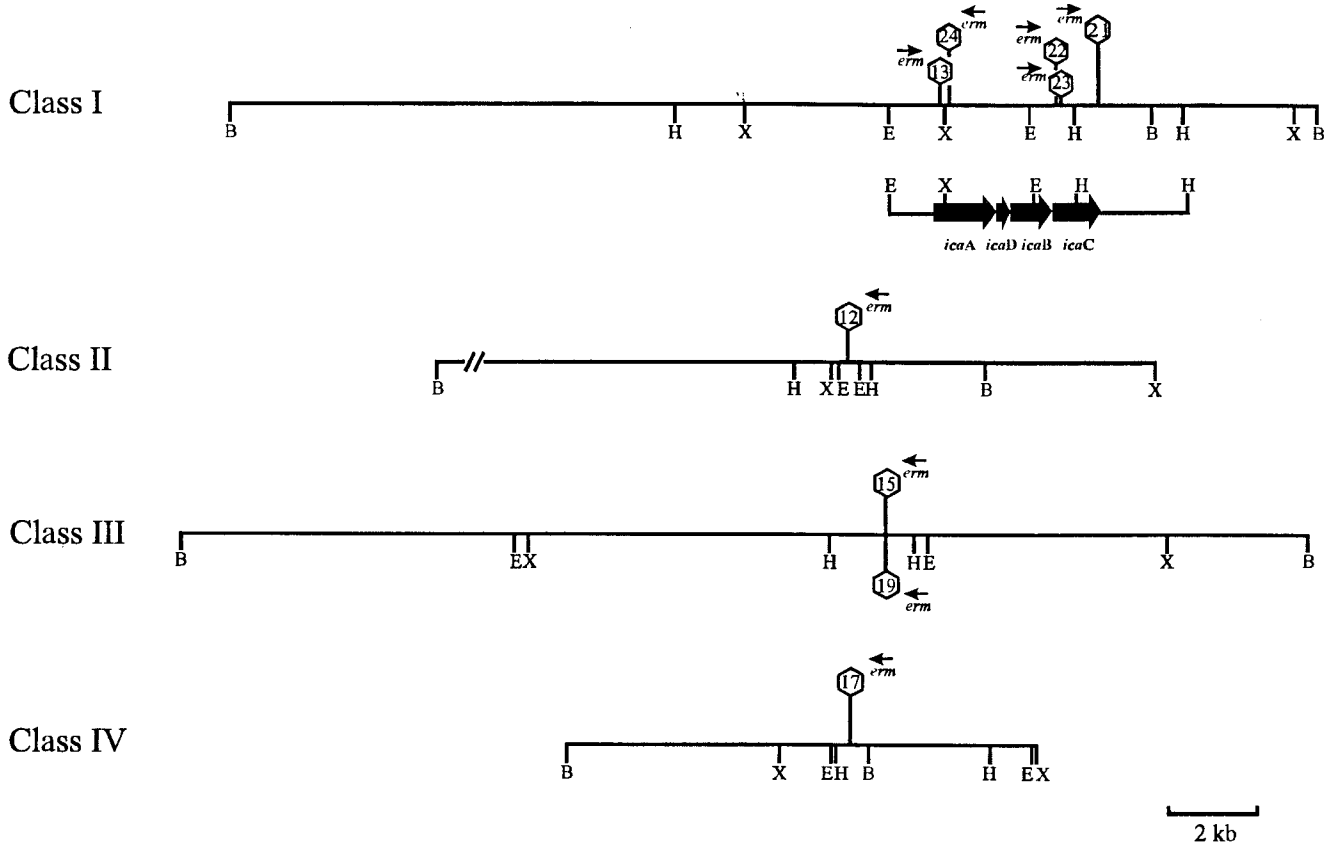

FIG. 3. Preliminary genetic map of transposon insertions of the different mutant classes. H, *Hin*dIII; X, *Xba*I; B, *Bgl*II; E, *Eco*RI. *erm* indicates the direction of transcription of the *erm* gene of Tn*917*.

TABLE 2. Nucleotide sequences flanking Tn*917* insertions of class I biofilm-negative *S. epidermidis* transposon mutants

Mutant	Host strain	Gene inactivated	Insertion site sequence (position)[a]
M13	1457	*icaA*	ATT TTT **TTA TTA** (85) Tn*917* **TAT TA**A AGA AAA
M24	1457	*icaA*	Tn*917* (331) ATA GAG GTA AAG
M22	1457	*icaC*	ATT TTT ATT TTC (154) Tn*917*
M23	1457	*icaC*	ATT GTT CAA TAG (443) Tn*917*
M21	1457	*icaC*	GTT TTA TAT ATT (1001) Tn*917*

[a] The position of the transposon insertions is indicated relative to the ATG start codon of the *icaA* gene or the *icaC* gene of the *icaADBC* gene cluster of *S. epidermidis* RP62A. The bold type indicates the 5-bp duplication typically occurring at Tn*917* insertion sites.

(iv) Changes of colony morphology. Class II and class III mutants displayed gray to translucent colonies on blood agar compared to the white colonies of the wild-type strain and the mutants of classes I and IV (Fig. 4).

(v) Expression of *icaADBC* using a xylose-inducible promoter in biofilm-negative mutants of different classes. Using the conjugative plasmid pWBG636 the recombinant plasmid pTX*icaADBC* containing the *icaADBC* locus cloned under the control of a xylose-inducible promoter (6) was mobilized from *S. carnosus* carrying pTX*icaADBC* into the biofilm-producing *S. epidermidis* 9142. Strain 9142(pTX*icaADBC*) with the expected antibiotic resistance markers was used as a donor for the production of transducing phage 48 lysates. Using this lysate the isogenic biofilm-negative transposon mutants M13 (class I), M12 (class II), M15 (class III), and M17 (class IV) were transduced with plasmid pTX*icaADBC*. All transductants had the expected antibiotic resistance profile (data not shown). Using TSB_{Oxoid} as the growth medium, mutant M13 containing a Tn*917* insertion at nucleotide 85 of the *icaA* gene was complemented to a biofilm-producing phenotype in the presence of 4% xylose, whereas in the absence of xylose this strain displayed a completely biofilm-negative phenotype (Table 4). Similarly, in TSB_{Oxoid} lacking xylose the transductants of the isogenic mutants of classes II, III, and IV were completely biofilm negative. In contrast, in the presence of 4% xylose all of these mutants expressed a strongly biofilm-producing phenotype, indicating that the genetic defect resulting from the Tn*917* insertions in these different mutant classes can be complemented, when *icaADBC* is expressed from an independent promoter (Table 4). Apparently, the mutations of the isogenic mutants of classes II, III, and IV directly or indirectly suppress expression of *icaADBC* from its own promoter.

(vi) Transcription of *icaADBC* in biofilm-negative mutants of different classes. To directly evaluate the effect of the different Tn*917* insertions of the biofilm-negative mutants of classes II, III, and IV on transcription of the *icaADBC* locus, RNA was prepared in mid-exponential-growth phase from mutants M12, M15, and M17 and from wild-type *S. epidermidis* 1457. An *icaADBC*-specific transcript (ca. 3.6 kb) was detected in RNA prepared from the biofilm-producing *S. epidermidis* 1457 grown in TSB_{Oxoid}. In contrast, an *icaADBC*-specific transcript was not detected with any of the mutants M12 (class II), M15 (class III), or M17 (class IV) (Fig. 5).

DISCUSSION

In the present study we have isolated nine isogenic biofilm-negative Tn*917* insertion mutants with Tn*917* insertions in four unlinked chromosomal loci. The resulting transposon mutants can be separated into four genetic classes according to the preliminary chromosomal mapping of their respective Tn*917* insertions. These genetic classes are reflected by phenotypic differences observed with the mutants, which include differences in the quantity of biofilm formation and PIA production in TSB_{BBL} or TSB_{Oxoid} and altered colony morphology (Table 3, Fig. 4).

The five mutants of class I have Tn*917* insertions within the coding region of the *icaADBC* locus. Mutants M13 and M24 inactivate *icaA* by insertion at nucleotides 85 and 331 of the *icaA* gene, closely resembling the isogenic mutants M10 and

TABLE 3. Phenotypic properties of biofilm-negative *S. epidermidis* transposon mutants

Strain	Class[a]	Expression on TSB_{BBL}[b]		Expression on TSB_{Oxoid}[b]		Hemagglutination[c] (reciprocal titer)
		Biofilm (OD_{570})	PIA (reciprocal titer)	Biofilm (OD_{570})	PIA (reciprocal titer)	
1457	wt	2.489	1,024	1.246	256	512
1457c	wt	1.582	1,024	1.326	128	512
M13	I	0.053	0	0.016	0	Neg
M21	I	0.155	8	0.031	0	4
M22	I	0.044	0	0.027	0	Neg
M23	I	0.047	0	0.021	0	Neg
M24	I	0.060	0	0.045	0	Neg
M12	II	0.020	0	0.013	0	Neg
M15	III	0.217	32	0.030	0	16
M19	III	0.228	32	0.033	0	16
M17	IV	1.596	256	0.039	16	16

[a] wt, wild type.

[b] For the biofilm data, strains were grown in the respective medium in 96-well tissue culture plates, the plates were washed with phosphate-buffered saline, cells were fixed with Bouin's fixative, and adherent bacterial biofilms were stained with Gentian violet. Biofilm-producing strains were arbitrarily defined to have a mean OD_{570} of >0.1. The PIA expression was determined by coagglutination of extracts of strains grown in the respective medium. A coagglutination reagent specific for PIA was prepared by sensitizing *S. aureus* Cowan I with PIA-specific absorbed antiserum as described in Materials and Methods.

[c] Hemagglutination was assessed as described in Materials and Methods. Neg, negative.

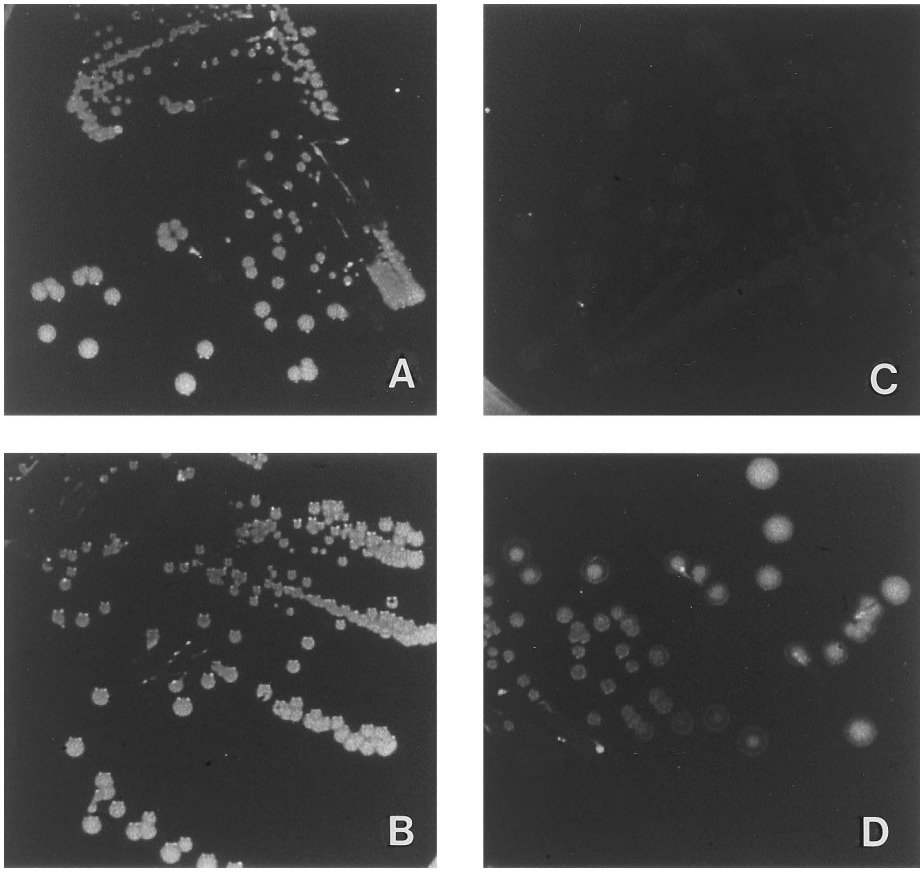

FIG. 4. Colonial morphology of biofilm-producing *S. epidermidis* 1457 (A), mutant M13 (class I) (B), M12 (class II) (C), and M15 (class III) (D) on blood agar plates.

M11 with insertions at nucleotides 87 and 1031 of the *icaA* gene (19, 20). In mutants M22 and M23 the *icaC* gene is inactivated at nucleotides 154 and 443 of the *icaC* gene. All of these insertions lead to complete inactivation of PIA synthesis. In contrast, the insertion of mutant M21 is located at nucleotide 1001 of the *icaC* gene, only 67 nucleotides from the stop codon of *icaC*, allowing residual enzymatic activity of the *icaADBC* operon leading to low-level PIA synthesis and biofilm formation in TSB_{BBL}. These observations indicate that a correct sequence of the last 21 amino acids of IcaC is essential for full functional activity for PIA synthesis.

No Tn*917* insertions in *icaB* or *icaD* were noted in our study. This could simply result from the lack of insertion sites for Tn*917* in these genes. However, this could also mean that these

TABLE 4. Complementation of biofilm formation in different biofilm-negative mutant classes by *icaADBC* expressed by a xylose-dependent promoter

Strain (class)[a]	Biofilm formation (OD_{570})[b] on TSB_{Oxoid}:	
	Without xylose	With 4% xylose
1457 (wt)	2.10	2.30
M13 (I)	0.03	0.02
M13/pTX*icaADBC*	0.04	2.20
M12 (II)	0.01	0.02
M12/pTX*icaADBC*	0.01	1.03
M15 (III)	0.02	0.02
M15/pTX*icaADBC*	0.05	1.06
M17 (IV)	0.07	0.06
M17/pTX*icaADBC*	0.09	2.50

[a] wt, wild type.
[b] Biofilm formation was assessed as described in Materials and Methods using TSB_{Oxoid} supplemented or not supplemented with 4% xylose as indicated.

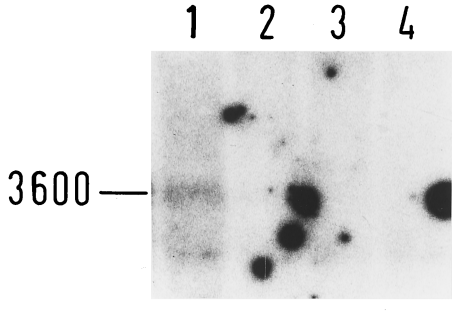

FIG. 5. Transcription of *icaADBC* in biofilm-negative class II, III, and IV mutants. Total RNA was extracted from *S. epidermidis* 1457 (lane 1) and mutants M12 (class II; lane 2), M15 (class III; lane 3), and M17 (class IV; lane 4) grown into the mid-exponential-growth phase in TSB_{Oxoid}. An *icaC*-specific oligonucleotide probe was used in Northern hybridization, and an autoradiogram is shown.

two genes are not essential for PIA synthesis and biofilm formation. This could be anticipated for *icaB* since in an in vitro-reconstituted reaction the synthesis of PIA clearly depended on the presence of IcaA, IcaD, and IcaC, but not on IcaB (6). In addition, it was reported that expression of *icaADC* without *icaB* led to cell clustering of *S. carnosus* as in *S. carnosus* expressing *icaADBC* and synthesizing PIA (6). In contrast to these observations, it was reported that the insertion of insertion sequence IS256 into *icaB* and a frame shift mutation of *icaB* led to a biofilm-negative phenotype (10, 45).

We isolated four additional isogenic biofilm-negative transposon mutants with insertions unlinked to the *icaADBC* locus. These insertions can be separated into genetic classes II, III, and IV, which correspond also with phenotypic differences of these mutants. There are several possible mechanisms which could lead to impaired PIA synthesis and biofilm formation of these mutants.

First, these gene loci could be necessary for the synthesis of an essential precursor for PIA synthesis. Since PIA is a homoglycan synthesized primarily of *N*-acetylglucosamine (17), this is unlikely as this sugar is an essential constituent of the peptidoglycan and therefore necessary for bacterial cell proliferation. However, PIA contains additional modifications, like the deacetylation of the amino groups, and the transfer of phosphate and succinate groups (17) and inactivation of the respective gene loci of mutants of class II to IV could interfere with these modifications. Second, some of these mutations could interfere with the transport of PIA outside the cell and therefore inhibit synthesis of the polysaccharide. Third, the respective gene loci could interfere with the expression of the *icaADBC* locus at the level of transcription.

To differentiate between these possibilities, we expressed *icaADBC* from a xylose-inducible promoter by transfer of the recombinant plasmid pTX*icaADBC* into mutants of classes I (M13), II (M12), III (M15), and IV (M17). All mutants containing the recombinant plasmid still had a biofilm-negative phenotype in the absence of xylose. However, in the presence of xylose the *icaA* insertion mutant M13 was complemented to a biofilm-producing phenotype as expected, indicating that the recombinant *icaADBC* locus of pTX*icaADBC* was still functional after the genetic manipulations of conjugative mobilization and transduction. Interestingly, in the presence of xylose the mutants M12, M15, and M17 containing pTX*icaADBC* produced biofilm. This clearly rules out the possibility that the respective gene loci of the mutants of classes II to IV encode functions necessary as precursors for PIA synthesis or are responsible for the transport of the polysaccharide. Since expression of *icaADBC* in *trans* from an independent promoter led to biofilm formation in these mutants, the inactivated genes apparently influence expression of *icaADBC* in a regulatory way by either directly or indirectly modulating transcription of *icaADBC*. This was confirmed directly since transcription of *icaADBC* was not detected in mutants M12, M15, and M17 compared to the wild-type *S. epidermidis* 1457 in the mid-exponential-growth phase. Apparently, functional activity of all of the inactivated genes in these mutants is essential for biofilm formation in TSB_{Oxoid} whereas in TSB_{BBL} the inactivated gene of mutant M17 seems to be dispensable for biofilm formation and PIA synthesis. In contrast to the lack of biofilm formation in TSB_{Oxoid}, it was observed over time that, although biofilm formation of mutant M17 in different lots of TSB_{BBL} varied between reaching an optical density similar to that of the wild type and being significantly reduced, a biofilm always formed. At present it is unknown which specific constituents of these complex media are relevant for the differential expression of biofilm formation by the different isogenic mutants.

Several other biofilm-negative mutants have been described previously. Tn*917* mutant Mut1 is impaired in primary attachment due to a deletion in the gene of the major autolysin *atlE* of *S. epidermidis* (9). Since this mutant still produces PIA in quantities similar to that of the wild-type strain O-47, mutant Mut1 can be easily differentiated from the mutants isolated in the present study (5, 8).

Mutant M7, obtained by chemical mutagenesis of *S. epidermidis* RP62A, is impaired in the accumulative phase of biofilm production (35). This mutant does not produce a 140-kDa accumulation associated protein (AAP) believed to be responsible for the observed defect in accumulation (12). However, since the mutant still produces PIA in quantities similar to those with the wild type (12) (D. Mack and C. de Grahl, unpublished results), it seems highly unlikely that AAP has a regulatory effect on the expression of *icaADBC* and that it is functionally related to mutants described in this study. In addition, AAP appears in the protein fraction secreted from the cells (12). It is therefore difficult to assume how AAP could directly influence expression of *icaADBC*.

Mutant M187-sn3 is a biofilm-negative transposon mutant, which is impaired in the synthesis of a polysaccharide referred to as capsular polysaccharide/adhesin (PS/A) (24). Analysis of purified PS/A revealed a composition of 54% hexoses, 20% amino sugars, and 10% uronic acids (39). As specific sugars, galactose (22%), glucosamine (15%), and galactosamine (5%) were detected (39). Recently, the composition of PS/A from *S. epidermidis* RP62A and M187 grown in a chemically defined medium was reevaluated. There was an almost identical reactivity of anti-PS/A and anti-PIA antisera with several well-characterized *S. epidermidis* strains, including the PS/A-producing *S. epidermidis* M187 and the PS/A-negative transposon mutant M187-sn3, the PIA-producing *S. epidermidis* 1457, and the isogenic PIA-negative mutant 1457-M11, as well as the recombinant *S. carnosus* containing the cloned *icaADBC* locus (23). These data suggest that PS/A and PIA are structurally related or even identical. Those authors reported that PS/A is a polysaccharide of high molecular mass composed of β-1,6-linked glucosamine residues with a high degree of substitution with succinate and acetate and that its production is dependent on the *icaADBC* locus (23). Since detailed data on the structural analysis of PS/A produced by *S. epidermidis* have not yet been reported, it remains to be determined whether PS/A is identical to polysaccharide II of PIA or whether it represents an additional variant of PIA. The genes inactivated by the transposon insertion in mutant M187-sn3 have not yet been identified; however, they are different from *icaADBC* (23) (D. Mack and H. Rohde, unpublished results). Its phenotype is very similar to class I and II mutants; however, no altered colony morphology was observed, as is typical for mutant M12, indicating that its phenotype most probably results from a different insertional gene inactivation. It is relevant to note that a similar altered colony morphology was also observed with other independent wild-type strains in which the Tn*917* insertion of mutant M12 was transferred by transduction (D. Mack and A. Sabottke, unpublished results).

Our results indicate that expression of *icaADBC* and PIA synthesis is tightly regulated in *S. epidermidis*. Cure of biomaterial-related *S. epidermidis* infections typically requires removal of the infected biomaterial and complete debridement of the infected tissue, since antibiotic therapy regularly fails when the foreign material remains in situ (1). It is believed that the resistance of an established biomaterial-related *S. epidermidis* infection to therapy is related to the biofilm mode of growth of the bacteria. Indeed, Rupp and collaborators recently demonstrated by using the isogenic *icaA* insertion mu-

tant 1457-M10 that a functional *icaADBC* locus and the ability for PIA synthesis and biofilm formation are essential virulence factors of *S. epidermidis* in a subcutaneous mouse catheter infection model and a rat central venous catheter infection model (32, 33). Further characterization of the regulatory mechanisms governing the expression of PIA and biofilm formation on a molecular level is of major importance, because it is reasonable to speculate that interference with these regulatory mechanisms may lead to improved methods of prevention and therapy of biomaterial-related *S. epidermidis* infections.

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