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Biofilm Formation by *Staphylococcus epidermidis* Depends on Functional RsbU, an Activator of the *sigB* Operon: Differential Activation Mechanisms Due to Ethanol and Salt Stress

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Staphylococcus epidermidis* is a common pathogen in medical device-associated infections. Its major pathogenetic factor is the ability to form adherent biofilms. The polysaccharide intercellular adhesin (PIA), which is synthesized by the products of the *icaADBC* gene cluster, is essential for biofilm accumulation. In the present study, we characterized the gene locus inactivated by Tn*917* insertions of two isogenic, *icaADBC*-independent, biofilm-negative mutants, M15 and M19, of the biofilm-producing bacterium *S. epidermidis* 1457. The insertion site was the same in both of the mutants and was located in the first gene, *rsbU*, of an operon highly homologous to the *sigB* operons of *Staphylococcus aureus* and *Bacillus subtilis*. Supplementation of Trypticase soy broth with NaCl (TSB_{NaCl}) or ethanol (TSB_{EtOH}), both of which are known activators of *sigB*, led to increased biofilm formation and PIA synthesis by *S. epidermidis* 1457. Insertion of Tn*917* into *rsbU*, a positive regulator of alternative sigma factor σ^B, led to a biofilm-negative phenotype and almost undetectable PIA production. Interestingly, in TSB_{EtOH}, the mutants were enabled to form a biofilm again with phenotypes similar to those of the wild type. In TSB_{NaCl}, the mutants still displayed a biofilm-negative phenotype. No difference in primary attachment between the mutants and the wild type was observed. Similar phenotypic changes were observed after transfer of the Tn*917* insertion of mutant M15 to the independent and biofilm-producing strain *S. epidermidis* 8400. In 11 clinical *S. epidermidis* strains, a restriction fragment length polymorphism of the *sigB* operon was detected which was independent of the presence of the *icaADBC* locus and a biofilm-positive phenotype. Obviously, different mechanisms are operative in the regulation of PIA expression in stationary phase and under stress induced by salt or ethanol.

Staphylococcus epidermidis, a normal inhabitant of human skin and mucous membranes, is the predominant cause of foreign-body-associated infections (43). In addition, *S. epidermidis* is isolated with increasing frequency as the causative pathogen of nosocomial sepsis and other nosocomial infections, ranking among the five most frequent nosocomial pathogens (43, 49). The pathogenesis of *S. epidermidis* infections is correlated with the ability to form biofilms on polymer surfaces (5, 58).

Biofilm formation proceeds in two phases (23, 24). Primary attachment of bacterial cells to a polymer surface is a complex process influenced by a variety of factors, including hydrophobic interactions, presence of host proteins, and specific bacterial proteins and polysaccharides like the capsular polysaccharide adhesin, the autolysin AtlE, and other staphylococcal surface proteins (15, 17, 33, 38, 39, 50, 51). This is followed by the second phase leading to accumulation of bacteria in a multilayered biofilm embedded in an amorphous glycocalyx. Synthesis of the polysaccharide intercellular adhesin (PIA) is essential for bacterial cell accumulation because it mediates cell-to-cell adhesion of proliferating cells (26–28, 31, 32). PIA consists of two polysaccharide species which are composed of β-1,6-linked 2-deoxy-2-amino-D-glucopyranosyl residues containing non-N-acetylated amino groups, phosphate, and succinate (26) and is synthesized by the products of the *icaADBC* gene cluster (11, 16). In addition to having a function in intercellular adhesion, PIA is essential for hemagglutination mediated by *S. epidermidis* (10, 29, 42, 44). Recently, the significance of PIA as a virulence factor could be demonstrated by comparison of isogenic PIA-negative transposon mutant 1457-M10 and the corresponding wild-type strain in a central venous catheter rat infection model and a subcutaneous foreign-body mice infection model (45, 46).

By transposon mutagenesis, four unlinked gene loci were identified whose mutation leads to a biofilm-negative phenotype and abolished PIA synthesis and produces mutants that are classified, according to genotypic and phenotypic differences, as class I to IV mutants (30, 37). Class I mutants represent those in which *icaADBC* is inactivated. The other three genetic loci control expression of PIA synthesis and biofilm formation by directly or indirectly influencing expression of *icaADBC* on the level of transcription (30).

Understanding of the regulatory mechanisms regulating PIA synthesis and biofilm formation is of primary importance for the development of new preventive and therapeutic methods to combat *S. epidermidis* biomaterial-related infections. Therefore, in the present study, we characterized the genetic defect of the class III biofilm-negative mutants M15 and M19 at the molecular level.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Reference or source	Comments
S. epidermidis		
1457	31	Isolate from infected central venous catheter
M12	30	Isogenic biofilm-negative Tn*917* mutant of *S. epidermidis* 1457 (class II)
M15	30	Isogenic biofilm-negative Tn*917* mutant of *S. epidermidis* 1457 (class III)
M19	30	Isogenic biofilm-negative Tn*917* mutant of *S. epidermidis* 1457 (class III)
M16	This study	Isogenic biofilm-positive control Tn*917* mutant of *S. epidermidis* 1457
8400	31	Blood culture isolate
8400-M15	This study	Transductant of *S. epidermidis* 8400
5179	31	Blood culture isolate
RP62A	7	
SE5	42	
521	27	Isolate from infected central venous catheter
1057	27	Isolate from infected central venous catheter
939	27	Blood culture isolate
10333	31	Blood culture isolate
7837	31	Blood culture isolate
9225	31	Blood culture isolate
9896	31	Blood culture isolate
S. aureus 35005	This study	Clinical isolate
E. coli		
MC1061	12	
TOP10	Invitrogen	
JKMK186-41	This study	MC1061 containing pJKMK186-41
JKMK186-46	This study	MC1061 containing pJKMK186-46
JKMK401	This study	TOP10 containing pJKMK401
JKMK402	This study	TOP10 containing pJKMK402
Plasmids		
pBluescript II SK	Invitrogen	*E. coli* cloning vector for blue-white screening
pCRII	Invitrogen	*E. coli* cloning vector for direct cloning of PCR fragments
pJKMK186-41	This study	pBluescript II SK containing *Hin*dIII/*Sal*I fragment of 1457-M15 with chromosomal DNA flanking 5′ end (*erm*) of Tn*917*
pJKMK186-46	This study	pBluescript II SK containing *Hin*dIII/*Sal*I fragment of 1457-M19 with chromosomal DNA flanking 5′ end (*erm*) of Tn*917*
pJKMK401	This study	pCRII containing *sigB* amplification product obtained from *S. epidermidis* 1457 with oligonucleotides JKMK4 and JKMK5
pJKMK402	This study	pCRII containing amplification product obtained with oligonucleotides 3R and 5L (*sigB* operon of *S. epidermidis* 1457)

(Part of this work will appear in the Ph.D. theses of J.K.-M.K., K.B., A.S., and H.R., Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany.)

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. epidermidis* cells were grown in Trypticase soy broth (TSB_{BBL}; Becton Dickinson, Cockeysville, Md.) at 37°C. For phenotypic characterization of the *S. epidermidis* strains, TSB_{BBL} was supplemented with 4% NaCl (TSB_{NaCl}) or 4% ethanol (TSB_{EtOH}). *E. coli* cells were grown in Luria-Bertani (LB) broth or on LB agar at 37°C. Antibiotics were used at the following concentrations: erythromycin, 300 μg/ml; ampicillin, 150 μg/ml; and kanamycin, 50 μg/ml.

Phenotypic characterization. Biofilm production by *S. epidermidis* was measured by a semiquantitative adherence assay in the appropriate media in 96-well tissue culture plates (Nunclon Delta; Nunc, Roskilde, Denmark) as previously described (7, 31). For detection of PIA by immunofluorescence assay (IFA), *S. epidermidis* cells were grown in tissue culture dishes (Nunc) for 22 h in TSB_{BBL}, TSB_{NaCl}, or TSB_{EtOH}, respectively. Cells were scraped off and diluted in phosphate-buffered saline to an optical density at 578 nm (OD_{578}) of 0.3 to 0.5. The IFA procedure was then performed as previously described using a rabbit antiserum raised against purified PIA (16, 31).

For quantitation of PIA in bacterial extracts, *S. epidermidis* strains were grown in a 9-cm tissue culture dish (Nunc) for 22 h in TSB_{BBL}, TSB_{NaCl}, or TSB_{EtOH}, respectively. Cells were scraped off and centrifuged (3,000 × *g* for 15 min). The culture supernatants were cleared by an additional centrifugation step (3,000 × *g* for 15 min), and NaN_3 was added to a final concentration of 0.05%. The cell pellet was resuspended in 5 ml of phosphate-buffered saline containing 0.05% NaN_3, and bacterial extracts were prepared by sonication (two 30-s cycles with a 3/16-in. tapered Microtip at 70% of the maximal amplitude) with Digital Sonifier 250-D (Branson, Danbury, Conn.). Cells were sedimented by centrifugation (3,000 × *g* for 15 min). The cell extracts were cleared by an additional centrifugation step (12,000 × *g* for 15 min). PIA concentrations in culture supernatants and cell extracts were determined by a specific coagglutination assay with PIA-specific antiserum (31).

For quantitation of primary attachment, bacterial strains were grown in TSB_{BBL}, TSB_{NaCl}, or TSB_{EtOH} with shaking. Cells were harvested by centrifugation and resuspended and diluted in phosphate-buffered saline. Bacterial cell concentrations were determined by plating of appropriate dilutions. Bacterial dilutions (100 μl) at various concentrations were added in triplicate to the wells of 96-well cell culture plates (Nunclon Delta; Nunc) and incubated for 1 h at 37°C. After washing, attached cells were detected by enzyme-linked immunosorbent assay (ELISA) using rabbit anti-*S. epidermidis* 5179 serum and alkaline phosphatase-coupled anti-rabbit immunoglobulin G (Sigma) as previously described (25, 45).

Genetic methods. Transduction of the Tn*917* insertion of mutant M15 into the independent and biofilm-producing wild-type strain 8400 was performed essentially as described previously using *S. epidermidis* phage 71 (25, 37).

Chromosomal DNA of *S. epidermidis* was prepared as described previously

(28). DNA was cleaved with restriction enzymes as suggested by the manufacturer (Pharmacia, Freiburg, Germany), and DNA fragments were separated by electrophoresis in 0.7% agarose gels in Tris-borate buffer (47).

DNA restriction fragments of the expected size were purified from agarose gels using the Gene Clean II kit (Bio 101, Inc., Vista, Calif.) and cloned in *Escherichia coli* MC1061 using pBluescript II SK (Stratagene, La Jolla, Calif.) as a vector. Selection was for ampicillin-resistant clones on LB agar plates. Positive clones were transferred to LB agar plates containing erythromycin selecting for the erythromycin resistance gene (*erm*) of Tn*917* (12). Positive clones were further characterized.

For Southern blot analysis DNA fragments were transferred onto Zeta-Probe membranes (Bio-Rad, Munich, Germany) by alkaline capillary blotting (47). Hybridization was performed using probes labeled with [^{32}P]dCTP (Amersham, Braunschweig, Germany) by the Ready to Go labeling kit (Pharmacia) as suggested by the manufacturer. The blots were exposed to Kodak X-Omat X-ray film.

Nucleotide sequence analysis was performed on an ABI Prism 310 sequencer by capillary electrophoresis using the ABI Prism dGTP BigDye Terminator Ready Reaction Kit (PE Applied Biosystems, Foster City, Calif.). Nucleotide sequences were analyzed subsequently with HUSAR software (DKFZ, Heidelberg, Germany).

Amplification of short DNA fragments (approximately 2 kb) was performed using the DyNazyme DNA Polymerase Kit (Finzyme, Espoo, Finnland) as described by the manufacturer. Oligonucleotides specific for *rsbU* (JKMK1 [5′-GTG GAA GAA TTT AAG CAA CA-3′] and JKMK2 [5′-GGA ATA TCT GTT TTT AAG CAT-3′]) and *sigB* (JKMK4 [5′-CTG AGC AAA TTA ACC AAT GG-3′] and JKMK5 [5′-TAA CTT TGT CCC ATT TCC AT-3′]) of *S. aureus* (57) and *icaB* (*icaB*forward [5′-TGG ATC AAA CGA TTT ATG ACA-3′] and *icaB*reverse [5′-ATG GGT AAG CAA GTG CGC-3′]) of *S. epidermidis* (11, 16) and oligonucleotides JK41.rev1 (5′-AGC GAA AAT ACC AAC CCA CG-3′), JKMK11 (5′-GAG GAA ATT GGT GTG CGA GG-3′), JKMK28 (5′-TGT GAA TGT CCA TAA GCA TCC-3′), and JKMK34 (5′-TTT CTT TTA GCC TCA GTT GC-3′) were synthesized by MWG Biotech (Munich, Germany).

For long-range PCR, the Expand Long Template PCR System (Boehringer, Mannheim, Germany) was used with oligonucleotides specific for the 5′ and 3′ junctions of Tn*917* (5L [5′-CTC ACA ATA GAG AGA TGT CAC CG-3′] and 3R [5′-GGC CTT GAA ACA TTG GTT TAG TGG G-3′]) (48) as described by the manufacturer for an expected fragment length of 12 to 15 kb.

RESULTS

Cloning of DNA flanking Tn*917* insertion sites. By genetic mapping, the Tn*917* insertions of class III mutants M15 and M19 were shown to be closely linked (30). For identification of the inactivated chromosomal structures of these mutants, two identical 3.1-kb chromosomal *Sal*I/*Hin*dIII fragments containing 0.8 kb of *S. epidermidis* chromosomal DNA flanking the 5′ end of Tn*917*, including the erythromycin resistance gene (*erm*) up to the first *Hin*dIII site of Tn*917* (48), were cloned for both mutants in *E. coli* MC1061, resulting in plasmids pJKMK186-41 and pJKMK186-46. Nucleotide sequence analysis indicated that Tn*917* was inserted at the same position in both mutants M15 and M19 (data not shown). The identities of the cloned fragments with the insertion site of Tn*917* in the mutants were demonstrated by Southern blot hybridization using the cloned chromosomal fragment of mutant M15 as a probe (data not shown). The nucleotide sequence at the proximal end of the chromosomal fragment near the *Sal*I site displayed an open reading frame (ORF) encoding 120 amino acids (ORF1), while the remaining sequence appeared to be noncoding. ORF1 was highly homologous (80.9% identical bases) to an ORF, designated ORF1 or ORF136, localized proximally to the *sigB* operon of *S. aureus* (21, 57). The noncoding region near the Tn*917* insertion site of the mutants was homologous (67.6% identical bases) to the noncoding region directly preceding the *S. aureus sigB* operon.

Linkage of *sigB* of *S. epidermidis* and the Tn*917* insertion site. To ascertain the existence of a *sigB* homologue in *S. epidermidis*, we used oligonucleotides specific for the *S. aureus rsbU* (JKMK1 and JKMK3) and *sigB* (JKMK4 and JKMK6) genes for PCR amplification. Only with *sigB*-specific primers was a 696-bp fragment from *S. epidermidis* 1457 chromosomal DNA amplified which was similar in size to that amplified from a clinical *S. aureus* isolate. Nucleotide sequence analysis of the fragment obtained from *S. epidermidis* 1457(pJKMK401) revealed homology to *sigB* of *S. aureus* (21, 57). To determine the linkage of the *S. epidermidis sigB* homologue with the Tn*917* insertion site of the class III mutants M15 and M19, the PCR fragment was used as a probe in a Southern blot assay (Fig. 1). Decreased mobility of the hybridizing *Eco*RI fragment of mutants M15 and M19, consistent with insertion of 5.2-kb Tn*917*, was observed, whereas no mobility change was detected with chromosomal DNA of class II mutant M12 (used as a control). Apparently, the *sigB* homologue of *S. epidermidis* 1457 is linked to the *Eco*RI fragment containing the Tn*917* insertion of mutants M15 and M19. For chromosomal DNA of *S. aureus*, no hybridization signal was obtained when this probe was used under stringent hybridization conditions (data not shown).

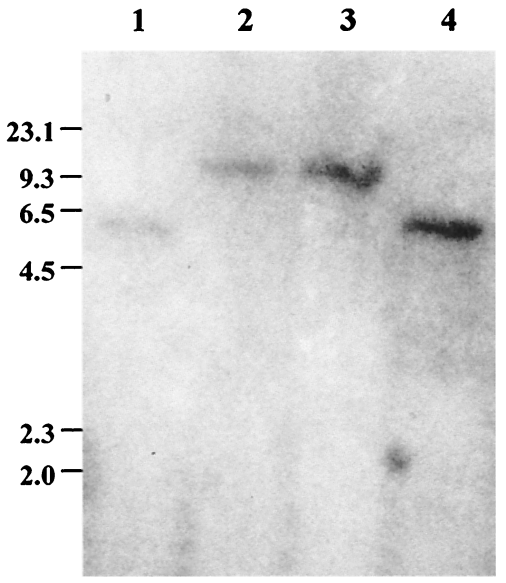

FIG. 1. Southern blot of *Eco*RI-digested chromosomal DNA using a ^{32}P-labeled *S. epidermidis sigB* PCR fragment from pJKMK401 as a probe. Lanes 1, *S. epidermidis* 1457; 2, *S. epidermidis* M15; 3, *S. epidermidis* M19; 4, *S. epidermidis* M12 (class II mutant). The values on the left are sizes in kilobases.

Analysis of the *sigB* operon of *S. epidermidis* 1457. A 12.4-kb chromosomal *Eco*RI fragment containing the Tn*917* insertion of mutant M15 was autoligated, and long-range PCR was performed using oligonucleotides 5L and 3R, which are complementary to the 5′ and 3′ junctions of Tn*917*. The resulting 7.2-kb PCR fragment was cloned, resulting in plasmid pJKMK402, which was sequenced by primer walking. Nucleotide sequences were completed by analysis of a DNA fragment generated by amplification of chromosomal DNA of wild-type *S. epidermidis* 1457 with primers JK41.rev1 and JKMK28, which overlap the transposon insertion site. Sequence analysis revealed an operon (2,700 nucleotides [nt]) consisting of four ORFs (ORF2 to ORF5) highly homologous to the *sigB* oper-

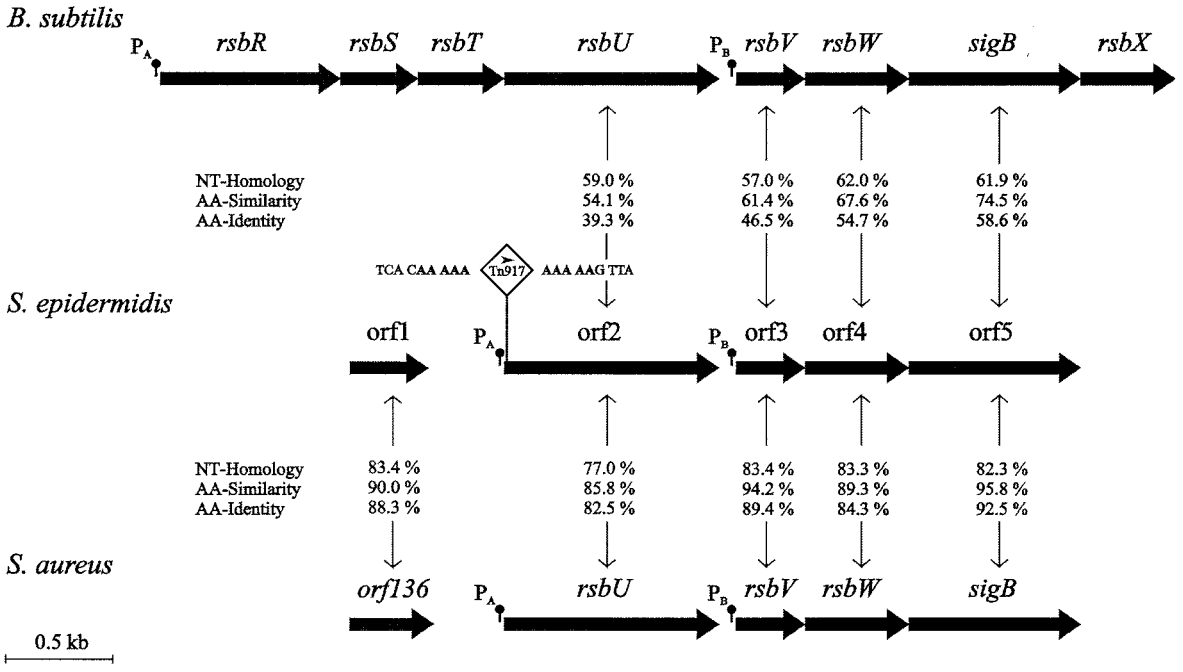

FIG. 2. Comparison of the organization of the *sigB* operon of *S. epidermidis* 1457 and that of the *sigB* operons of *S. aureus* and *B. subtilis*. In the physical maps, genes are indicated as arrows. Homology of nucleotide (NT) sequences and the identity and similarity of the deduced amino acid (AA) sequences between corresponding genes are shown. The positions of putative promoters are indicated. The Tn*917* insertion site of mutants M15 and M19 is indicated. The transcriptional direction of the *erm* gene of Tn*917* is shown by an arrowhead. The typical 5-nt duplication at the Tn*917* insertion site is in boldface.

ons of *S. aureus* (21, 57) and *Bacillus subtilis* (18, 56). The four ORFs are organized in the same conserved order as their homologous counterparts *rsbU, rsbV, rsbW*, and *sigB* (Fig. 2). For each gene, a putative Shine-Dalgarno sequence could be detected (data not shown). As described for *S. aureus* and *B. subtilis*, ORF3, which is homologous to *rsbV* of *S. epidermidis*, is preceded by a putative σ^B-dependent promoter with its −35 (TAGATTAA) and −10 (GGGTAT) promoter elements spaced by 14 nt, which conforms to the consensus sequence (13, 40). In the amino acid sequence of ORF4, which is homologous to RsbW, the residues thought to be important for ATP binding, and therefore kinase activity (19), are conserved in *S. epidermidis, S. aureus*, and *B. subtilis* (data not shown). These data permit the conclusion that these genes function similarly, and ORF2 to ORF5 were therefore named like their homologous counterparts in *S. aureus, rsbU, rsbV, rsbW*, and *sigB* (accession number AF274004).

The Tn*917* insertion sites in mutants M15 and M19 are localized 19 bp downstream of the translation start codon (GTG) of *rsbU* (Fig. 2).

Phenotypic characterization. In *S. aureus* and *B. subtilis*, σ^B is known to regulate specific genes in stationary phase and under different stress conditions like an osmotic shift and the presence of ethanol (14, 21, 22). Therefore, we compared the phenotypic properties of mutant M15 and the corresponding wild-type strain, *S. epidermidis* 1457, using different stress conditions.

Under standard biofilm assay conditions, mutant M15 exhibited a biofilm-negative phenotype in TSB_{BBL} whereas *S. epidermidis* 1457 was a strong biofilm producer (Fig. 3). In TSB_{NaCl}, mutant M15 was also biofilm negative. In this medium, the wild-type strain produced even more biofilm, which could not be quantified, however, because the respective OD values were outside the detection range of the spectrophotometer used. However, wild-type *S. epidermidis* 8400 produced less biofilm in TSB_{BBL} than did *S. epidermidis* 1457. With this strain, the increased biofilm production in TSB_{NaCl} could be quantified (Fig. 3). As with osmotic stress, both wild-type strains *S. epidermidis* 1457 and 8400 displayed increased biofilm formation in TSB_{EtOH} (Fig. 3). Interestingly, in contrast to

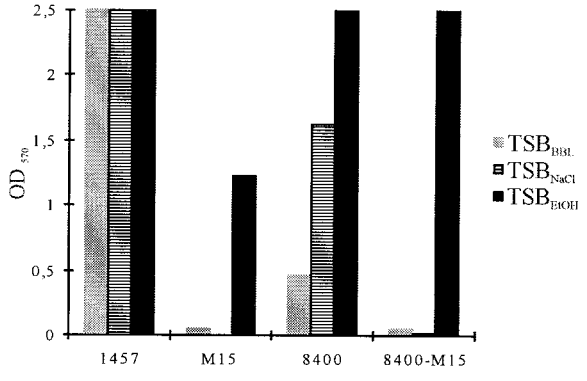

FIG. 3. Biofilm formation under different stress conditions. *S. epidermidis* 1457, isogenic mutant M15, independent wild-type *S. epidermidis* 8400, and its transductant 8400-M15 were analyzed using TSB_{BBL}, TSB_{NaCl}, and TSB_{EtOH} as the growth media. Results of a representative experiment are shown.

the response of mutant M15 to osmotic stress, this mutant was a strong biofilm producer in TSB_{EtOH} (Fig. 3). To investigate the phenotypic differences in biofilm formation resulting from insertion of Tn*917* from mutant M15 into a different genetic background, the Tn*917* insertion of this mutant was transduced into independent wild-type strain 8400, resulting in mutant 8400-M15. Insertion of Tn*917* into the transductant 8400-M15 at the expected site was demonstrated using PCR with primers JK41rev1 plus 5L and 3R plus JKMK28, respectively, followed by nucleotide sequence analysis (data not shown). With this transductant, similar phenotypic properties regarding biofilm formation in the presence of ethanol and osmotic stress were obtained (Fig. 3). Similar results were obtained for mutants M19 and 8400-M19 (data not shown).

To exclude the possibility that inactivation of *rsbU* affected the primary attachment of *S. epidermidis* to polymer surfaces under the different growth conditions analyzed, mutant M15 and *S. epidermidis* 1457 were compared for primary attachment. Staphylococcal cells attached to cell culture plates also used for the biofilm assay after 60 min of incubation were detected by ELISA with an antiserum raised against biofilm-negative *S. epidermidis* 5179 (25, 45, 45). There were no significant differences in primary attachment between mutant and wild-type cells grown in TSB_{BBL}, TSB_{NaCl}, and TSB_{EtOH} (Fig. 4). Similar results were obtained with mutant M19 (data not shown).

Wild-type *S. epidermidis* 1457 formed smaller but more compact cell clusters in TSB_{NaCl} compared to the standard medium, while in TSB_{EtOH}, significantly larger cell clusters were observed (Fig. 5A to C). In an indirect IFA using PIA-specific antiserum, expression of PIA by *S. epidermidis* 1457 was observed in all of the growth media used (Fig. 5D to F). Despite the formation of smaller cell clusters by *S. epidermidis* 1457 grown in TSB_{NaCl}, no significant difference in intensity of fluorescence was observed (Fig. 5B and D to F). In contrast, mutant M15 did not produce detectable cell clusters in TSB_{BBL} and TSB_{NaCl} (Fig. 5G and H). In parallel with reconstituted biofilm production in TSB_{EtOH}, mutant M15 was located in large cell clusters (Fig. 5I). As detected by the specific IFA, expression of PIA in TSB_{EtOH} by mutant M15 was comparable to that of wild-type cells (Fig. 5M). With mutant M15 grown in TSB_{NaCl}, only irregular, speckled fluorescence could be detected (Fig. 5L), whereas in TSB_{BBL}, PIA expression was not detected (Fig. 5K). Similar results were obtained with mutant M19 (data not shown).

As IFA allows only qualitative PIA detection, synthesis of PIA was quantified in bacterial cell extracts and culture supernatants of the cells grown as biofilms on tissue culture plates in the respective media. With wild-type *S. epidermidis* 1457, a significant eightfold increase in the PIA concentration was observed in the culture supernatant of cells grown in TSB_{NaCl} and TSB_{EtOH} compared to that of TSB_{BBL}-grown cells (Table 2). Only a minor increase in cell-associated PIA was detected with cells grown in TSB_{NaCl} or TSB_{EtOH} (Table 2). With mutant M15, consistent with its biofilm-negative phenotype in TSB_{BBL}, PIA production was hardly detectable (Table 2). In TSB_{NaCl}, a small but significant increase in the PIA concentration in the culture supernatant was detected, which could correspond to the irregular speckled fluorescence seen in these cells. In contrast, the concentrations of cell-associated PIA and

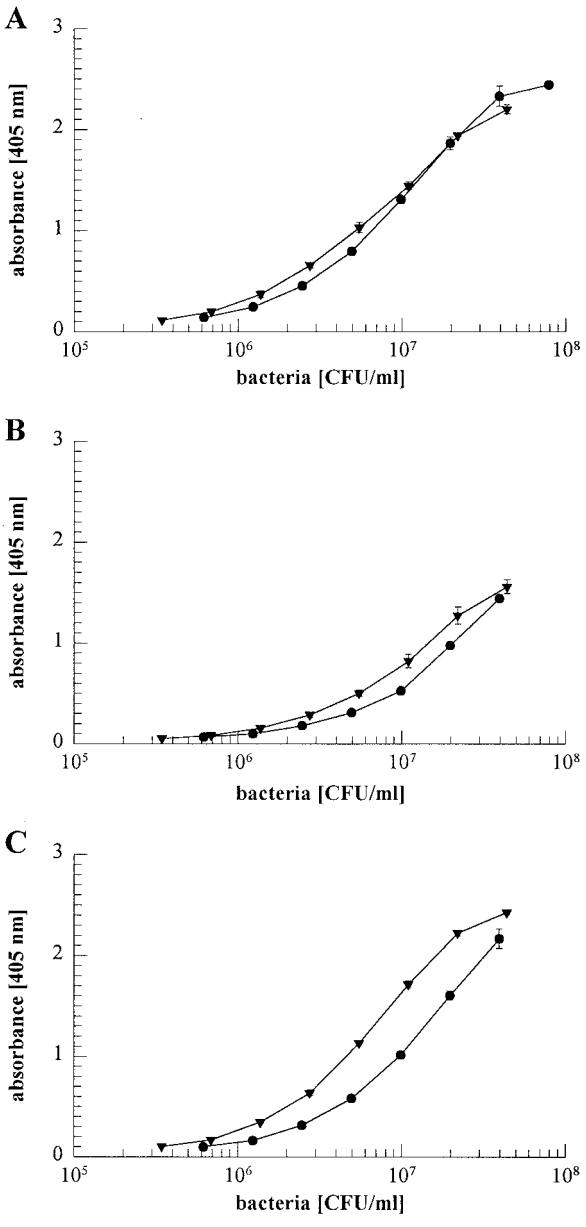

FIG. 4. Primary attachment of *S. epidermidis* 1457 (●) and isogenic mutant M15 (▼) grown in TSB_{BBL} (A), TSB_{NaCl} (B), or TSB_{EtOH} (C) to polystyrene cell culture plates (Nunclon Delta; Nunc). Bacteria were inoculated into the plates at various concentrations and incubated for 1 h at 37°C. Attached bacterial cells were detected by ELISA as described in Materials and Methods. Results of a representative experiment are shown.

PIA detected in culture supernatants of mutant M15 grown in TSB_{EtOH} were not significantly different from those of *S. epidermidis* 1457 grown in the same medium, which is consistent with reconstituted biofilm production of the mutant (Table 2). Similar results were obtained with *S. epidermidis* 8400, its transductant 8400-M15 (Table 2), and mutant M19 (data not shown).

Characterization of a *sigB* RFLP type of *S. epidermidis* wild-type strains. As the activity of *sigB* apparently influences bio-

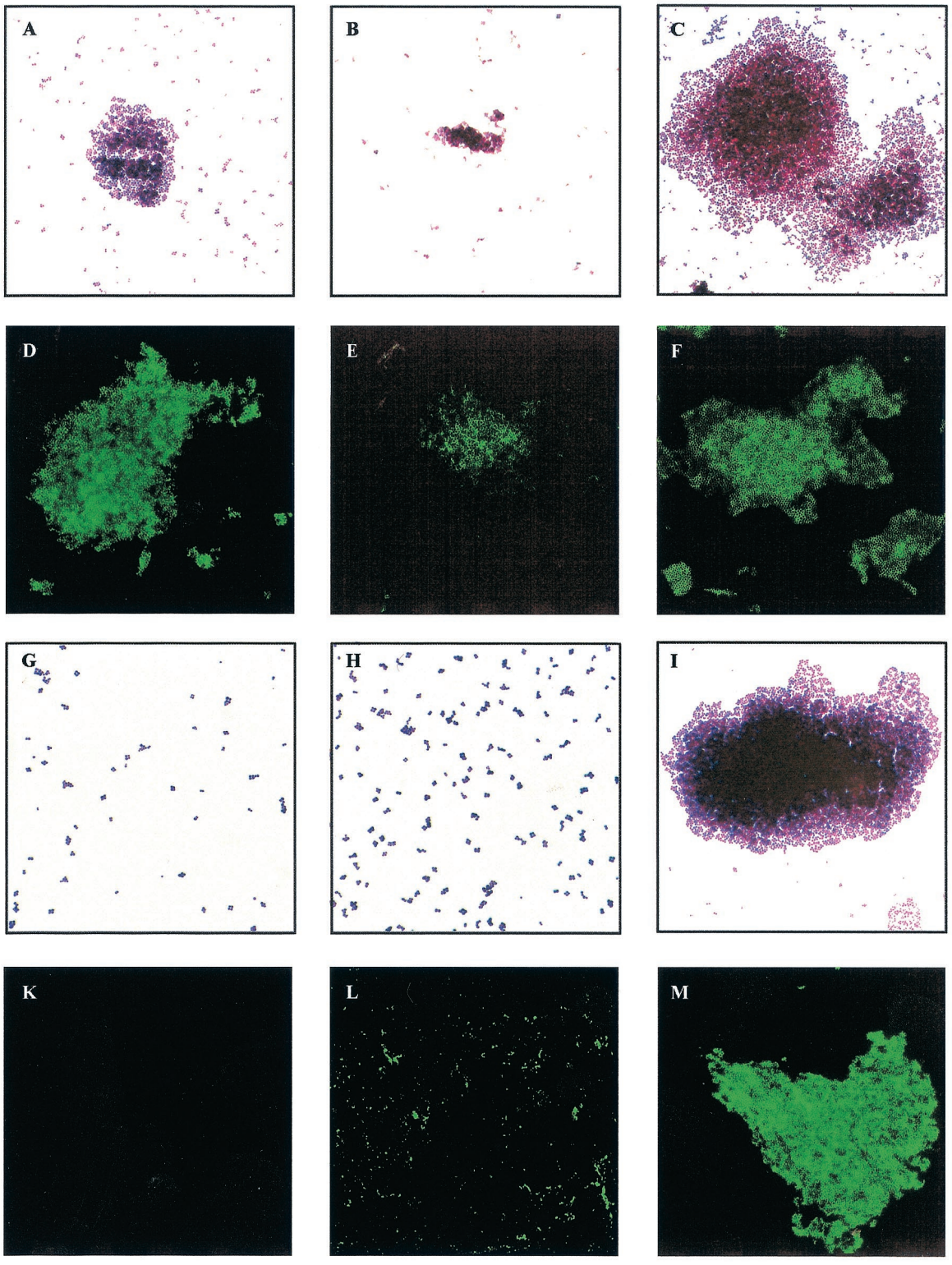

FIG. 5. Cell cluster formation and PIA expression by *S. epidermidis* 1457 and mutant M15 under different stress conditions. *S. epidermidis* 1457 (A to F) and isogenic mutant M15 (G to M) were grown in tissue culture plates as a biofilm in TSB_{BBL} (A, D, G, and K), TSB_{NaCl} (B, E, H, and L), or TSB_{EtOH} (C, F, I, and M) for 22 h at 37°C. Cells were scraped from the surface, and appropriate dilutions in phosphate-buffered saline were applied to microscope slides or immunofluorescence slides. Microphotographs of representative fields are shown after Gram staining (A to C and G to I) or after IFA using a PIA-specific antiserum as described in Materials and Methods (D to F and K to M). Results of a representative experiment are shown.

TABLE 2. Quantitation of PIA produced using osmotic and ethanol stress

Strain and PIA location	TSB_{BBL}	TSB_{NaCl}	TSB_{EtOH}
1457			
Supernatant	32[a]	256	512
Cell associated	256	512	512
M15			
Supernatant	4	64	256
Cell associated	4	16	256
8400			
Supernatant	2	128	128
Cell associated	64	256	256
8400-M15			
Supernatant	2	4	64
Cell associated	2	2	256

[a] Reciprocal titer of PIA determined by coagglutination using a PIA-specific coagglutination assay as described in Materials and Methods.

TABLE 3. Phenotypic and genotypic characterization of *S. epidermidis* wild-type strains

Strain	*icaB* PCR	*sigB* RFLP type	Biofilm formation (OD_{570})
1457	+	A	2.500
RP62A	+	A	2.446
SE5	+	B	1.800
521	+	A	1.643
1057	+	A	0.798
939	+	A	0.724
10333	+	A	0.035
5179	+	B	0.014
7837	+	B	0.009
9225	−	A	0.004
9896	−	B	0.027

film formation by *S. epidermidis*, the *sigB* operons of different *S. epidermidis* clinical isolates, including reference strains RP62A (6) and SE5 (42), were amplified by PCR using oligonucleotides JKMK11 and JKMK34, spanning the region from *rsbU* to the noncoding region downstream *sigB*. A restriction fragment length polymorphism (RFLP) of the resulting fragments (approximately 2.6 kb) was analyzed after cleavage with *Hin*fI. Two different fragment patterns, designated A and B, were detected (Fig. 6). The respective *sigB* RFLP type did not correlate with the *icaADBC* genotype characterized by PCR using *icaB*-specific oligonucleotides (*icaB*forward and *icaB*reverse) or with the expression phenotype of biofilm formation (Table 3).

DISCUSSION

In this study, we characterized the genetic defect of two isogenic PIA- and biofilm-negative Tn*917* mutants, M15 and M19 (class III), with transposon insertion sites distinct from the *icaADBC* locus (30). Sequence analysis of chromosomal DNA flanking the Tn*917* insertion sites of M15 and M19 demonstrated identical insertions at nt 19 of *rsbU*, which is the first gene of an operon of *S. epidermidis* with high homology to the *sigB* operon of *S. aureus, B. subtilis*, and *Listeria monocytogenes* (2, 21, 55, 57). In *S. epidermidis*, the same order of the genes, *rsbU, rsbV, rsbW*, and *sigB*, coding for alternative sigma factor σ^B and three regulatory proteins was detected as described for *S. aureus* (21, 57). As with *S. aureus*, no evidence of additional regulatory proteins, like RsbRST and RsbX, flanking the *sigB* operon of *S. epidermidis* was obtained (data not shown). In addition to a putative housekeeping promoter in front of *rsbU*, a σ^B-like recognition sequence was observed preceding the gene *rsbV*, as described for *S. aureus* and *B. subtilis* (21, 57). The homology of the nucleotide sequence and the organization of this operon in *S. epidermidis* suggest a general function similar to that observed for *S. aureus* or *B. subtilis*.

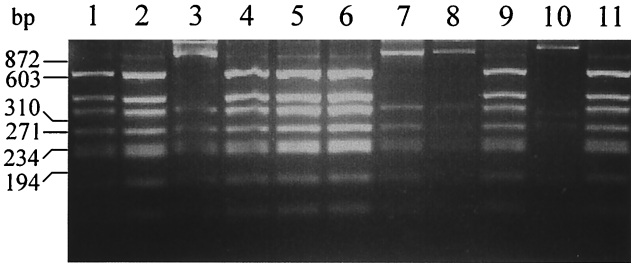

FIG. 6. *sigB* RFLP of different clinical *S. epidermidis* isolates. PCR fragments containing almost the complete *sigB* operon were cleaved with *Hin*fI. Two different *sigB* RFLP types, A and B, were detected. The *S. epidermidis* strains which display *sigB* RFLP type A were 1457 (lane 1), RP62A (lane 2), 521 (lane 4), 1057 (lane 5), 10333 (lane 6), 9225 (lane 9), and 939 (lane 11). The *S. epidermidis* strains which display *sigB* RFLP type B were SE5 (lane 3), 5179 (lane 7), 7837 (lane 8), and 9896 (lane 10).

In biofilm-producing *S. epidermidis* 1457, inactivation of *rsbU*, which is a positive regulator of σ^B (53), led to a biofilm-negative phenotype, which is caused by severely decreased PIA synthesis, indicating a defect in biofilm accumulation of *rsbU* mutant M15. This was confirmed directly, as no significant differences in primary attachment to polystyrene tissue culture plates were observed between the mutant and wild-type strains (Fig. 4). Apparently, RsbU activity is essential for expression of biofilm formation and PIA synthesis in *S. epidermidis*. As the Tn*917* insertion of mutant M15 transduced into the independent genetic background of biofilm-producing *S. epidermidis* 8400 resulted in similar phenotypic properties, it is extremely unlikely that the observed phenotypic changes are caused not by the transposon insertion into *rsbU* but by nonspontaneous mutations in association with the Tn*917* insertion, as was observed with inactivation of *xpr* in *S. aureus* KSI9051, where, in parallel with exchange of the mutated allele by transduction, independent point mutations in *agrC* occurred with high frequency (35).

Abolished PIA synthesis due to insertion of Tn*917* into *rsbU* of *S. epidermidis* suggests σ^B-dependent expression of PIA. This is corroborated by the observations that the spontaneous *rsbU* deletion mutant *S. aureus* 8325 has a phenotype similar to that of experimentally induced *sigB* deletion mutants and that its phenotype can be complemented by expression of σ^B in *trans* from an independent promoter (22). Similar to *sigB* and *rsbU* mutants of *S. aureus*, mutants M15 and M19 exhibited significant differences in colony morphology compared to wild-type *S. epidermidis* 1457 (22, 30). Recently, it was reported that

a *sigB*-null mutant of a clinical biofilm-producing *S. aureus* strain was biofilm negative (41). However, the ways in which biofilm expression by *S. epidermidis* and *S. aureus* are regulated seem to be fundamentally different, as most *icaADBC*-positive *S. epidermidis* strains produce a biofilm in vitro but almost all clinical *S. aureus* strains are biofilm negative in vitro despite the presence of *icaADBC* (8, 34; M. A. Horstkotte, J. K.-M. Knobloch, and D. Mack, unpublished results).

Mutant M15 could be complemented by expression of *icaADBC* from a xylose-dependent promoter to a biofilm-producing phenotype, indicating a defect on the level of transcription of *icaADBC* leading to the biofilm-negative phenotype of this mutant (30). Indeed, no *icaADBC*-specific transcript was observed in the mutant compared with biofilm-producing wild-type *S. epidermidis* 1457 in the mid-exponential growth phase (30). The transcriptional start site of the *icaADBC* mRNA was determined at nt 732 of the published sequence (accession number SE43366), 29 nt proximal to the start codon of the *icaA* gene (11, 16). Surprisingly, using a consensus-directed search strategy, only a putative σ^A-dependent promoter starting at nt 701 was detected. A consensus-directed search strategy for σ^B-dependent promoters (40) revealed a possible promoter (AGTGTAGT N_{18} GGAAAA) with low homology to the consensus sequence starting at nt 529 of this sequence (11, 16), which probably is not active for transcription of the *icaADBC* mRNA transcribed downstream from nt 732 (11, 13). In addition, use of the same approach to search the nucleotide sequence of *icaR*, the putative regulator of *icaADBC* (59), revealed no sequence homologous to σ^B-like recognition sequences. In the sequences of *icaR* and the *icaADBC* locus (accession number AF086783) of *S. aureus* (8), no sequence homologous to σ^B-dependent promoters preceding these genes could be detected. These observations strongly suggest that there is no direct σ^B-dependent regulation of *icaADBC* transcription. However, the possibility cannot be completely excluded that σ^B-dependent transcription of *icaADBC* starting from the putative σ^B-dependent promoter occurs under special physiological conditions.

As in *S. epidermidis*, in the closely related species *S. aureus*, the σ^B operon consists of four genes, *rsbU, rsbV, rsbW*, and *sigB*, with the same identified or predicted functions as the homologous downstream module in *B. subtilis* (21, 36, 57). The central module of σ^B regulation consists of anti-sigma factor RsbW and anti-anti-sigma factor RsbV (1, 3, 9, 20, 54). This module is additionally regulated by RsbU, an RsbV-specific phosphatase activating RsbV (53, 54, 56). For *B. subtilis*, a second RsbV-specific phosphatase, RsbP, was described (52).

Phenotypic characterization clearly shows that PIA synthesis and biofilm formation by *S. epidermidis* are significantly increased by environmental stresses like high osmolarity and the presence of ethanol. At least two different pathways of induction exist. Apparently, induction of PIA synthesis by NaCl depends on a functional *rsbU* gene, as mutants M15 and 8400-M15 were completely biofilm negative in the presence of NaCl. However, ethanol stress leads to induction of PIA synthesis and biofilm formation independent of *rsbU*. This is in contrast to observations on *B. subtilis*, where induction of σ^B by ethanol or salt stress depends on *rsbU* (53, 54). These results suggest that in *S. epidermidis*, additional pathways could exist, as suggested for *S. aureus* (4, 22), which activate σ^B by regulators substituting for RsbU or activate PIA expression by σ^B-independent pathways. Additionally, the possibility cannot be completely excluded that RsbU has two different roles in *S. epidermidis* and activates *icaADBC* transcription by a σ^B-independent mechanism.

A *Hin*fI RFLP with two different patterns (A and B) was observed in a PCR fragment containing almost the complete *sigB* operon of *S. epidermidis* in 11 clinical isolates. However, the *sigB* operon was detected in all strains and the respective *sigB* RFLP type did not correlate with the *icaADBC* genotype (Table 3). In addition, there was no correlation of the *sigB* RFLP type with the observed biofilm formation phenotype (Table 3), indicating no obvious functional genetic defects correlating with the *sigB*-RFLP types, although the possibility of point mutations or very small deletions of only a few base pairs cannot be completely excluded.

The regulatory mechanisms controlling expression of biofilm formation and PIA synthesis in *S. epidermidis* are only basically known. At least three unlinked genetic loci control expression of *icaADBC* on the level of transcription (30). One of these loci is RsbU, a positive regulator of alternative sigma factor σ^B. As is apparent from the data presented here, σ^B may act only indirectly via an additional, unknown, factor or RsbU may, by itself, be a regulator of *icaADBC* transcription. Activation of PIA expression by different stress stimuli apparently uses different pathways. It is of primary importance to further characterize the molecular mechanisms controlling expression of *icaADBC* and biofilm formation, as it is reasonable to anticipate that interference with these mechanisms will improve the therapy and prevention of biomaterial-related *S. epidermidis* infections.

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