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Differential Expression of Methicillin Resistance by Different Biofilm-Negative *Staphylococcus epidermidis* Transposon Mutant Classes

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Biofilm formation mediated by polysaccharide intercellular adhesin (PIA) is the major virulence factor of *Staphylococcus epidermidis* and is often associated with methicillin resistance. Transposon Tn*917* insertions leading to a biofilm-negative phenotype in the biofilm-producing *S. epidermidis* strain 1457 (*mecA*-negative) were transferred into the methicillin-resistant, biofilm-producing *S. epidermidis* 1057 (*mecA*-positive) by transduction. According to their phenotypes and genotypes, the mutants could be separated into genetic classes I to IV (D. Mack, H. Rohde, S. Dobinsky, J. Riedewald, M. Nedelmann, J. K. M. Knobloch, H.-A. Elsner, and H. H. Feucht, Infect. Immun. 68:3799–3807, 2000). All transductants of *S. epidermidis* 1057 had phenotypes for biofilm formation similar to those of the corresponding mutants of *S. epidermidis* 1457. With a *mecA*-specific probe, identical hybridization patterns were observed for wild-type *S. epidermidis* 1057 and all the transductants. There were minor changes in oxacillin MICs for Class II and III transductants compared to those for wild-type *S. epidermidis* 1057. On population analysis, *S. epidermidis* 1057 displayed a heterogeneous expression type of resistance with an oxacillin MIC of ≥6 μg/ml for more than 90% of the cells. An almost identical profile was observed with biofilm-negative class I mutants, where the transposon insertions inactivate the *icaADBC* gene locus essential for PIA synthesis. In contrast, class III mutants were more sensitive to oxacillin with a MIC of ≤1 μg/ml for more than 90% of the cells. The class IV mutant displayed homogenous resistance with a MIC of ≥50 μg/ml for more than 90% of the cells. On oxacillin gradient plates, the class II mutant displayed decreased resistance. Apparently, different independent mutations leading to a biofilm-negative phenotype of *S. epidermidis* by influencing expression of *icaADBC* on the level of transcription significantly influence the expression of methicillin resistance. However, transcription of *mecA* was not significantly altered in the different transductants compared to the wild type, independent of *mecA* induction with oxacillin, indicating that other mechanisms influencing phenotypic expression of methicillin resistance are involved.

Today, coagulase-negative staphylococci, mostly *Staphylococcus epidermidis*, represent the most frequent causes of nosocomial sepsis and of infections of implanted medical devices (37). A major problem with these organisms is their pronounced antibiotic resistance, with up to 90% of nosocomial isolates being methicillin resistant (1, 4, 43).

Methicillin resistance is often associated with the ability of *S. epidermidis* to produce biofilms (6, 15). Biofilm formation is the preeminent virulence factor of *S. epidermidis* (23, 24, 38, 39), which is subject to phase variation leading to a biofilm-negative phenotype (5, 34, 46, 47). Pleiotropic phenotypic changes were observed with biofilm-negative phase variants of *S. epidermidis* RP62A, including decreased expression of methicillin resistance. However, the genetic mechanisms leading to these changes remain unknown (5, 34, 35).

Biofilm formation may be arbitrarily divided into two phases involving primary attachment of bacterial cells to a polymer surface, followed by accumulation of the attached bacteria in a multilayered biofilm (23, 24). The polysaccharide intercellular adhesin (PIA) is composed primarily of *N*-acetylglucosamine in β-1,6-glycosidic linkages containing deacetylated amino groups and succinate and phosphate substituents (26). PIA is functional in cell-to-cell adhesion and hemagglutination and is essential for biofilm accumulation of most clinical *S. epidermidis* strains (14, 27–29, 31, 32). The gene products of the *icaADBC* locus of *S. epidermidis* have enzymatic activity, which leads to synthesis of PIA in vivo and in vitro (17, 18, 29). Using transposon mutagenesis, we recently identified four unlinked genetic loci, whose inactivation led to a biofilm-negative phenotype (30). All class I mutants have insertions in the *icaADBC* locus, whereas apparently regulatory genetic loci in mutants of classes II, III, and IV are inactivated by the Tn*917* insertions. These genetic loci control expression of PIA synthesis and biofilm formation by directly or indirectly influencing expression of *icaADBC* on the level of transcription (30).

In the present study, we investigated the role of these different genetic loci on expression of methicillin resistance by transfer of the respective transposon insertions into the biofilm-producing, methicillin-resistant, *mecA*-positive *S. epidermidis* 1057 by transduction.

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MATERIALS AND METHODS

Bacterial strains. The biofilm-producing *S. epidermidis* 1457 and its isogenic biofilm-negative Tn*917* insertion mutants 1457-M10 and -M13 (class I), -M12 (class II), -M15 (class III), and -M17 (class IV) have been described (28, 30). Biofilm-producing *S. epidermidis* 1057 is a clinical isolate from an infected central venous catheter (36), which was methicillin resistant and *mecA* positive as determined by PCR essentially as previously described (41). *S. epidermidis* 1457 was methicillin sensitive and *mecA* negative (data not shown).

Phage transduction. Phage transduction with *S. epidermidis* phage 71, kindly provided by V. T. Rosdahl, Statens Seruminstitut, Copenhagen, Denmark, was performed as described previously (25, 36).

Adherence assay for measurement of biofilm production by *S. epidermidis*. Biofilm production by *S. epidermidis* strains grown in Trypticase soy broth (Becton Dickinson, Cockeysville, Md.) was determined by a semiquantitative adherence assay with 96-well tissue culture plates (Nunc, Roskilde, Denmark) as described previously (7, 25).

Determination of oxacillin susceptibility. The oxacillin (Sigma, Deisenhofen, Germany) MIC was determined by the broth microdilution method according to the procedure suggested by the NCCLS with Mueller-Hinton broth (Becton Dickinson) supplemented with 2% NaCl as the growth medium (19). MICs were read after 24 and 48 h of incubation at 35°C.

Phenotypic expression of methicillin resistance on oxacillin-gradient agar plates. Oxacillin gradient agar plates (oxacillin, 0 to 100 μg/ml) were prepared using Mueller-Hinton agar (Becton Dickinson) supplemented with 2% NaCl. *S. epidermidis* strains were suspended in phosphate-buffered saline to a concentration of approximately 10^8 CFU/ml, and appropriate dilutions were streaked onto the agar plate parallel to the oxacillin gradient and incubated for 48 h at 37°C.

Population analysis of phenotypic expression of methicillin resistance. *S. epidermidis* strains were subcultured overnight on blood agar and suspended in sterile phosphate-buffered saline to a concentration of 10^8 CFU/ml. Aliquots (100 μl) of these suspensions and of serial dilutions were plated onto Mueller-Hinton agar plates supplemented with 2% NaCl containing a range of concentrations of oxacillin (usually 1 to 800 μg/ml). Plates were incubated for 48 h at 37°C before the colonies were counted (42).

DNA isolation and Southern blot hybridization. Isolation of chromosomal DNA, digestion with restriction enzymes, and Southern blot analysis with [^{32}P]dCTP-labeled probes specific for Tn*917* or *mecA* were performed as described previously (25, 28, 40).

RNA extraction and Northern blot analysis. An overnight culture of the strains was diluted 1:100 in fresh, prewarmed Mueller-Hinton broth (Becton Dickinson) either unsupplemented or supplemented with oxacillin (1 μg/ml). Bacteria were cultivated under constant agitation at 160 rpm and 37°C and harvested after 6.5 h of incubation in mid-exponential growth phase (strain1457, optical density at 578 nm [OD_{578}]of ~1.0; strain1057 and 1057 mutants, OD_{578} of ~2.5). Total bacterial RNA was extracted and Northern blot analysis was performed as described (13, 30). The blots were hybridized with a ^{32}P-radiolabeled *mecA*-specific probe. This was prepared from a 1,071-bp *Xba*I/*Pst*I fragment of plasmid pBBB85 composed of a 4.2-kb *Hin*dIII fragment containing *mecA* of *Staphylococcus aureus* BB270 cloned in shuttle vector pGC-2 (40), which was kindly provided by B. Berger-Bächi, Institute of Medical Microbiology, University of Zürich, Zürich, Switzerland. Hybridization was performed at 42°C with 50% formamide, 2% sodium dodecyl sulfate, 5× SSPE (1× SSPE is 0.15 M NaCl, 10 mM NaH_2PO4, and 1 mM EDTA) (pH 7.4), 10% dextran sulfate, and 1% nonfat dry milk as described (13).

RESULTS

Transfer of Tn*917*-insertions of biofilm-negative mutants into *mecA*-positive *S. epidermidis* strain. Using *S. epidermidis* phage 71, the insertions leading to altered biofilm formation in isogenic mutants 1457-M10 and -M13 (class I), -M12 (class II), -M15 (class III), and -M17 (class IV) of *S. epidermidis* 1457 (30) were transduced into the biofilm-positive *mecA*-positive wild-type *S. epidermidis* 1057. The respective transductants 1057-M10, 1057-M13, 1057-M12, 1057-M15, and 1057-M17 had antibiotic resistance profiles identical to those of the wild type plus an additional macrolide resistance determinant introduced by Tn*917*. Similar hybridization patterns were detected with a Tn*917*-specific probe with all mutants and their respective transductants (data not shown). An identical *Eco*RI fragment of *S. epidermidis* 1057 and the respective transductants hybridized with a *mecA*-specific probe, indicating that replacement of the respective wild-type alleles by the different transposon insertions did not interfere with the *mec* locus in any transductant (data not shown). Compared to the wild-type strains *S. epidermidis* 1457 and 1057, the mutants and respective transductants displayed very similar phenotypic changes with regard to biofilm formation and colony morphology (Table 1).

TABLE 1. Phenotypic properties of biofilm-producing *S. epidermidis* wild-type strains and their corresponding biofilm-negative transductants

Mutant	Class	*S. epidermidis* 1457		*S. epidermidis* 1057	
		Biofilm (OD_{570})	Colony morphology	Biofilm (OD_{570})	Colony morphology
Wild type		2.50	White	2.50	White
M10	I	0.06	White	0.06	White
M13	I	0.02	White	0.02	White
M12	II	0.03	Grey	0.03	Grey
M15	III	0.10	Grey	0.10	Grey
M17	IV	0.30	White	0.28	White

Oxacillin MICs. MICs in broth microdilution were determined for wild-type *S. epidermidis* 1057 and the different transductants. After 24 h of incubation, a MIC of 256 μg/ml was determined for the wild type and transductants 1057-M10, 1057-M13, and 1057-M17, whereas the oxacillin MIC for transductants 1057-M12 and 1057-M15 was 128 μg/ml. After the strains underwent extended incubation for 48 h, the oxacillin MICs increased by 1 dilution, but the relative levels remained unchanged (Table 2).

Population analysis of oxacillin resistance. To investigate the resistance distribution within the bacterial cell populations of the different mutant classes, the efficiency of plating of bacterial cell populations on Mueller-Hinton agar plates supplemented with different concentrations of oxacillin was analyzed as described in Materials and Methods. The wild-type *S. epidermidis* 1057 displayed heterogeneous expression of oxacillin resistance with 90% of cells for which the MIC was ≥6 μg/ml (Fig. 1A and B). With the biofilm-negative transductants of class I 1057-M10 and 1057-M13, a very similar resistance distribution was observed (Fig. 1A and B). In contrast, the population of class III transductant 1057-M15 was shifted to a more sensitive distribution where the oxacillin MICs for more than 90% of the population were ≤1 μg/ml (Fig. 1C). Inter-

TABLE 2. Oxacillin MICs for methicillin-resistant, biofilm-producing *S. epidermidis* 1057 and its biofilm-negative transductants

Strain	Class	MIC (μg/ml)	
		After 24 h	After 48 h
1057	WT[a]	256	512
1057-M10	I	256	512
1057-M13	I	256	512
1057-M12	II	128	256
1057-M15	III	128	256
1057-M17	IV	256	512

[a] WT, wild type.

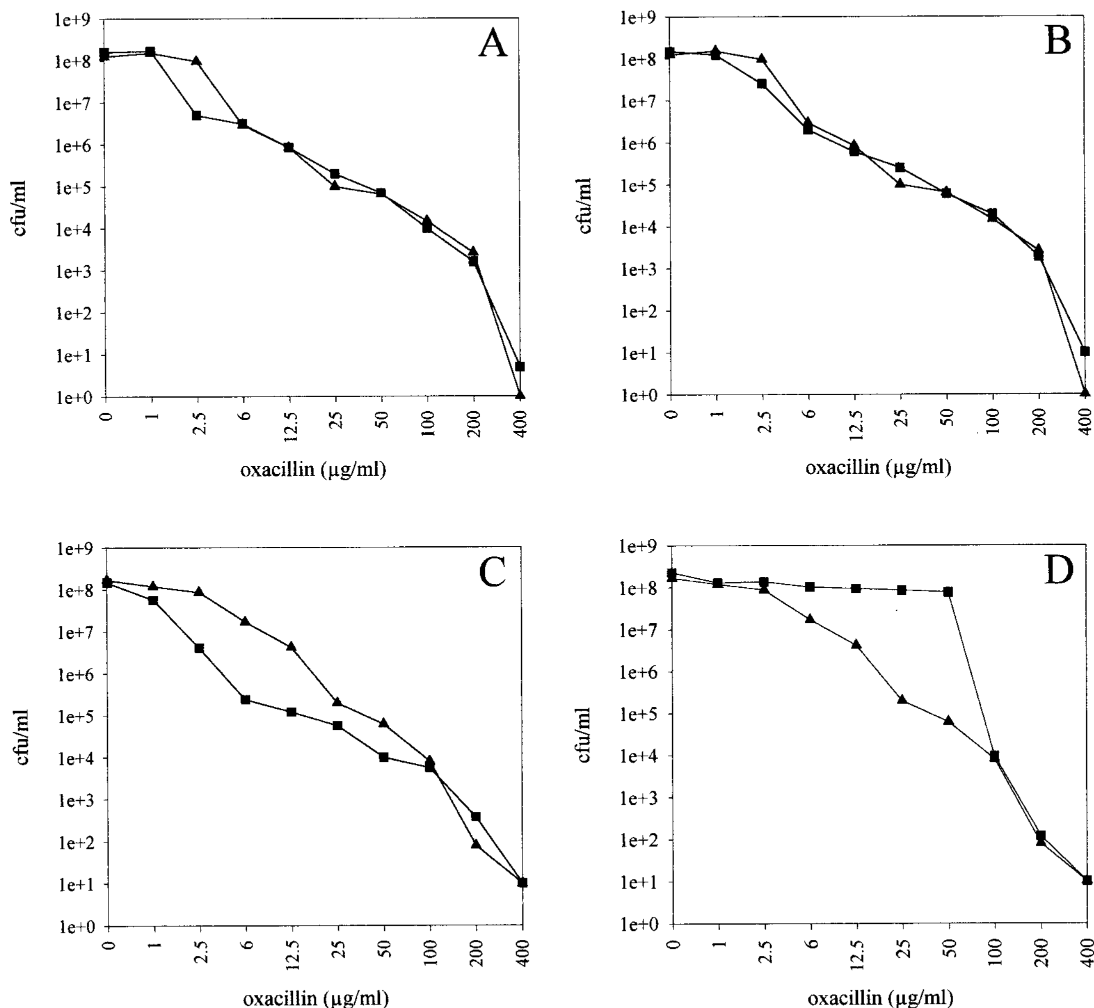

FIG. 1. Population analysis of oxacillin resistance. Suspensions of *S. epidermidis* 1057 (▲) (A to D) and biofilm-negative transductants (■) 1057-M10 (class I) (A), 1057-M13 (class I) (B), 1057-M15 (class III) (C), and 1057-M17 (class IV) (D) were spread on agar plates containing different concentrations of oxacillin as described in Materials and Methods. The CFU per milliliter are plotted against oxacillin concentration.

estingly, the resistance curve of class IV transductant 1057-M17 was shifted to a more resistant population, with an oxacillin MIC of ≥50 μg/ml for more than 90% of the cell population (Fig. 1D).

As *S. epidermidis* 1057-M12 (class II) tended to clump, the resistance distribution within the bacterial cell population of that mutant was analyzed with oxacillin gradient agar plates. A significant decrease in the size of the resistant cell population was observed for class II transductant 1057-M12 (Fig. 2). Changes in the resistance distribution of cell populations of mutants 1057-M15 (class III) and 1057-M17 (class IV) corresponding to those seen in population analysis were also observed when the oxacillin gradient plates were used (data not shown). The resistance distribution was unaltered when oxacillin gradient plates were used with mutant 1057-M10 (class I) compared to wild-type *S. epidermidis* 1057 (data not shown).

Transcriptional analysis of *mecA*. The Tn*917* insertions of the biofilm-negative mutants of classes II to IV were shown to directly or indirectly influence transcription of *icaADBC*, as no *icaADBC*-specific transcript was observed in these mutants (30). We therefore investigated if there was a similar effect on transcription of *mecA* in the respective transductants. RNA was extracted in the mid-exponential growth phase in either the presence or absence of oxacillin (1 μg/ml) and probed with a *mecA*-specific probe. A significant induction in the presence of oxacillin of a *mecA*-specific transcript was detected in wild-type *S. epidermidis* 1057 and all transductants (Fig. 3). The amount of accumulating transcripts appeared to differ only marginally between the wild type and the mutants, indicating that the observed differences in the expression of methicillin resistance was not related to alterations of transcription of *mecA*.

DISCUSSION

In the present study, we investigated the effect of four different classes of Tn*917* insertions influencing expression of biofilm formation in *S. epidermidis* 1457 on the expression of methicillin resistance in the genetic background of a *mecA*-positive *S. epidermidis* wild-type strain. After transduction of

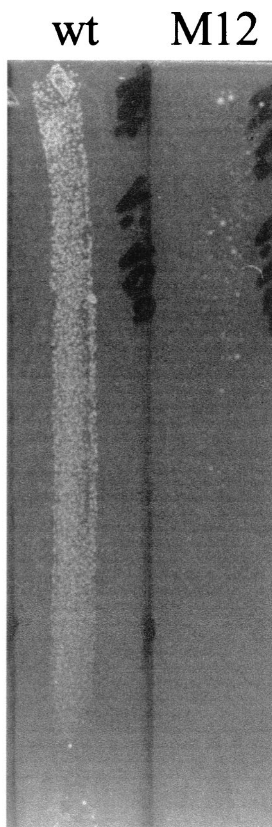

FIG. 2. Resistance expression profile on oxacillin gradient plates. Similar aliquots of suspensions of *S. epidermidis* 1057 (wt) and biofilm-negative transductant 1057-M12 (class II) (M12) were spread on a oxacillin gradient plate (0- to 100-μg/ml oxacillin) as described in Materials and Methods.

the respective insertions into *S. epidermidis* 1057, changes in expression of biofilm formation were seen, similar to those observed in *S. epidermidis* 1457, indicating that the inactivated gene loci are also relevant for biofilm formation in the differing genetic backgrounds (Table 1).

In *S. aureus*, a variety of genetic loci influencing expression of methicillin resistance known as *fem* or *aux* factors have been described (2, 3, 8–11, 21, 33, 44). However, for *S. epidermidis*, the impact of these or other similar factors on expression of methicillin resistance has not been investigated.

S. epidermidis 1057 displayed a heterotypic expression profile of methicillin resistance as is observed with most clinical *S. epidermidis* isolates (12, 42). For the different biofilm-negative transductants, only minor changes in the oxacillin MIC were observed. However, with population profile analysis of oxacillin gradient agar plates and of plates with different oxacillin concentrations, significant differences in the resistance distribution of the cell populations were observed. Inactivation of the *icaADBC* locus, encoding the enzymes for PIA synthesis, did not lead to changes in the expression profile of methicillin resistance. In contrast, transductants 1057-M15 (class III) and 1057-M12 (class II) displayed a significant increase in the susceptible cell population compared to the wild type.

Recently, we identified the molecular basis of the phenotypic change of the class III biofilm-negative mutant M15 as an insertion of Tn*917* 19 bp downstream of the translation start codon of *rsbU* of the σ^B operon of *S. epidermidis* (20). In the homotypic methicillin-resistant *S. aureus* strain COL, insertion of Tn551 into *sigB* and *rsbU* both led to a significant decrease in the methicillin MIC from 1600 to 50 μg/ml (45). The observed differences in MICs for *S. aureus* and *S. epidermidis rsbU* mutants may be related to the expression type of methicillin resistance of both strains—homotypic and heterotypic—or to differences in the regulatory pathways of the σ^B operon of *S. aureus* and *S. epidermidis*. However, inactivation of *sigB* in two other *S. aureus* strains resulted in less-pronounced alterations of expression of methicillin resistance (2; B. Berger-Bächi, personal communication). Our results demonstrate that with *S. epidermidis*, σ^B activity significantly influences the expression of methicillin resistance.

The exact mechanism of the phenotypic change of class II Tn*917* insertion of mutant 1057-M12 appears rather complex and is not yet fully elucidated. However, the respective gene locus and neighboring genes are different from known fem or aux factors of *S. aureus* described to date (2, 11; K. Bartscht, J. K.-M. Knobloch, and D. Mack, unpublished data), indicating that additional gene loci do influence expression of methicillin resistance in *S. epidermidis*.

In contrast to these other two mutant classes, the Tn*917* insertion of 1057-M17 (class IV) led to a significant shift of the cell population to higher resistance, although no difference in

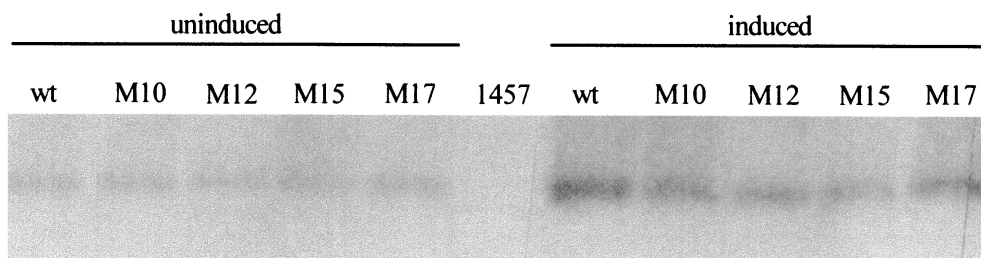

FIG. 3. Northern blot analysis of *S. epidermidis* 1057 (wt) and its isogenic biofilm-negative transposon mutants 1057-M10, 1057-M12, 1057-M15, and 1057-M17 with a *mecA*-specific probe. Bacterial cells were grown in Mueller-Hinton broth either uninduced or induced with oxacillin (1 μg/ml) to mid-exponential growth phase, and total cellular RNA was extracted. A total of 10 μg of RNA was separated on a 1% agarose-formaldehyde gel, blotted onto nylon membranes, and hybridized with a *mecA*-specific probe. The *mecA*-negative wild-type *S. epidermidis* 1457 grown in oxacillin-free medium was used as a negative control.

the overall oxacillin MIC was observed. With *S. aureus*, a chromosomal gene locus referred to as *chr** independent of *mecA* was identified as necessary for homotypic expression of methicillin resistance (40). The molecular basis of *chr** may be related to two recently identified chromosomal genes, *hmrA* and *hmrB* (22). Overexpression of these cloned genes led to a homotypic expression profile of methicillin resistance in heterotype *S. aureus* LR5 (22). However, in the original strain N315 used to isolate these genes an additional gene locus was apparently altered, as both *hmrA* and *hmrB* were unchanged in this strain. *hmrA* has homology to amidohydrolase enzymes, whereas *hmrB* has homology to acyl carrier proteins (22). Additionally, deletion of the gene of lytic protein LytH led from heterotypic to homotypic expression of methicillin resistance in *S. aureus* SR17238 (16). The insertion site of mutant 1457-M17 is unknown at present; however, a regulatory function is conferred on expression of *icaADBC* (30). As the genes involved in transition of hetero- to homo-type expression of methicillin resistance of *S. aureus* apparently do not encode regulatory proteins, no obvious relation exists to the gene locus inactivated in *S. epidermidis* 1057-M17 (16, 20).

The three classes of transposon insertions of mutants 1057-M15, 1057-M12, and 1057-M17 were identified as mutations leading to a biofilm-negative phenotype of *S. epidermidis* 1457 (30). All three mutations affected biofilm formation by directly or indirectly influencing expression of *icaADBC*, as no *icaADBC*-specific transcript was detected in biofilm-negative mutants of classes II, III, and IV (30). Similar phenotypic changes regarding biofilm formation were induced in *S. epidermidis* 1057. It is therefore of interest that these transposon insertions did not significantly alter the level of transcription of *mecA*. Apparently, as induction of the *mecA* transcript occurred similarly in all mutants in the presence of oxacillin, neither of these transposon insertions acts via the regulatory mechanisms of *mecA* induction. Obviously, the mutations induced by the transposon insertions influenced methicillin resistance of *S. epidermidis* 1057 by mechanisms independent of expression of *mecA*. These observation are in contrast to results reported for biofilm-negative phase variants of *S. epidermidis* RP62A, where a reduced oxacillin MIC was related to a complete lack of *mecA* transcription (34, 35).

Our results show an interesting link between the regulation of the major virulence factor of *S. epidermidis*, biofilm formation mediated by PIA (23, 38, 39), and methicillin resistance, which contribute to increased survival and dissemination of *S. epidermidis* as a nosocomial pathogen (1). Further studies are warranted to explore this relation in more detail.

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