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Phenotypic Expression of Oxacillin Resistance in *Staphylococcus epidermidis*: Roles of *mecA* Transcriptional Regulation and Resistant-Subpopulation Selection

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The MICs for many oxacillin-resistant (OR) *Staphylococcus epidermidis* (ORSE) strains are below the *Staphylococcus aureus* methicillin or oxacillin resistance breakpoint. The difficulty detecting the OR phenotype in *S. epidermidis* may be due to extreme heterotypy in resistance expression and/or transcriptional repression of *mecA*, the OR gene, by MecI. To determine the role of these factors in the phenotypic expression of ORSE, 17 geographically diverse *mecI*$^+$ ORSE isolates representing 14 distinct pulsed-field gel electrophoresis pulse types (>3 band differences) were investigated. Thirteen of the 14 types contained *mecI* and *mecA* promoter-operator sequences known to be associated with maximal *mecA* repression, and in all isolates, *mecA* transcription was repressed. All 17 were heterotypic in their resistance expression. Oxacillin MICs ranged from 1 to 128 μg/ml and increased for 16 of 17 isolates after β-lactam induction. Allelic replacement inactivation of *mecI* in three isolates similarly resulted in a four- to sevenfold increase in MIC. In the two of these three isolates producing β-lactamase, *mecA* transcription was regulated by both *mecI* and β-lactamase regulatory sequences. Heterotypic expression of resistance in these three isolates was unaffected by either β-lactam induction or *mecI* inactivation. However, prolonged incubation in concentrations of oxacillin just sufficient to produce a lag in growth (0.5 to 1.0 μg/ml) converted the population resistance expression from heterotypic to homotypic. Homotypic conversion could also be demonstrated in microtiter wells during MIC determinations in one isolate for which the MIC was high. We conclude that the phenotypic expression of *S. epidermidis* OR in broth can be affected both by *mecA* transcriptional regulation and by subpopulation resistance expression.

More than 70% of nosocomial *Staphylococcus epidermidis* isolates are methicillin resistant (MRSE) or oxacillin resistant (ORSE) (39), but resistance is often difficult to detect by conventional susceptibility testing methods. While *Staphylococcus aureus* clinical isolates tend to be either very susceptible or very resistant to methicillin or oxacillin (MICs of <0.5 or >8 μg/ml, respectively), *S. epidermidis* strains are not as clearly bimodal in their resistance pattern (21). The oxacillin MICs for many *S. epidermidis* strains are 0.5 to 2 μg/ml, which is below the breakpoint for oxacillin-resistant *S. aureus* (ORSA) (4 μg/ml), but these strains are found to contain *mecA*, the gene that mediates oxacillin resistance. Resistance expression in these *S. epidermidis* isolates can only be demonstrated when more laborious susceptibility testing techniques are used. When the in vitro expression of oxacillin resistance in *S. epidermidis* is examined on agar plates containing increasing concentrations of a β-lactam antibiotic, most isolates exhibit a heterotypic phenotype. This type of resistance expression is defined by a small percentage of cells (0.1%) that are able to survive on plates containing 100 μg of oxacillin per ml; the surviving colonies are of different sizes. In contrast, extreme heterotypic expression is relatively uncommon among clinical *S. aureus* isolates; resistant subpopulations comprise a greater proportion of the overall population than among *S. epidermidis* strains, and often every member of the population is highly resistant (homotypic or homogeneous resistance) (T. M. Dickinson and G. L. Archer, unpublished data).

As with *S. aureus* the production of an alternative, β-lactam-resistant penicillin-binding protein, PBP2a, encoded by *mecA*, confers OR in *S. epidermidis* (2, 4). In addition, a two-gene operon, *mecR1-mecI*, that encodes a signal transducer/inducer and repressor, respectively, is divergently transcribed from *mecA* (14, 35). MecI represses *mecA* and autorepresses *mecR1-mecI* transcription by binding to promoter-operator (P-O) sequences (32). The gene products of the *mecA* regulatory system have amino acid similarity to BlaR1 and BlaI, proteins that regulate transcription of the β-lactamase gene, *blaZ*. Previous studies have shown that the *mec* and *bla* regulatory systems are able to interact and that BlaI can regulate *mecA* transcription (10, 29, 37). Sequences hybridizing with *mecR1-mecI* probes are present in one-half to two-thirds of ORSA and ORSE clinical isolates (2), while the β-lactamase gene is found in 90% of clinical isolates (20).

It has been proposed (13) that among early ORSA strains, repression of *mecA* transcription by *mecI* yielded a β-lactam-susceptible phenotype, because repression led to little or no production of PBP2a, and *mecA* transcription was poorly inducible through MecR1. According to this hypothesis, the evolution of clinically resistant ORSA required mutations in either *mecI* or the *mecA* P-O. Additional chromosomal mutations were necessary to convert heterotypic resistance expression to homotypic (13). However, a recent analysis of clinical ORSE isolates from one hospital in Japan found that all contained *mecI* and *mecA* P-O sequences identical to those associated with maximal *mecA* repression in ORSA (17).

In the following study, we have attempted to assess the relative contributions of *mecA* transcriptional regulation and

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TABLE 1. Properties of the *S. epidermidis* strains used in this study

Strain	Pulse type	Resistance[a]	*mecI*	EOP (10^{-5}) with oxacillin		Oxacillin MIC (μg/ml)[b]	
				20 μg/ml	100 μg/ml	No induction	Induction[c]
SE12	I	Tet^r	+	1,300	300	16	64
SE20	II	Tet^r	+	3	2	8	128
SE20Δ*mecI*	II	Tet^r Min^r	−	8	6	128	512
SE22	III	Gen^r	+	36	78	64	256
SE42	IV		+	4	2	8	256
SE42Δ*mecI*	IV	Tet^r Min^r	−	7	5	128	ND[d]
SE43	V	Tet^r	+	680	2	1	8
SE53	VIa	Gen^r Chl^r	+	0.8	0.4	1	128
SE53Δ*mecI*	VIa	Gen^r Chl^r Tet^r Min^r	−	0.4	0.4	128	256
SE54	VIb	Gen^r	+	9	0.7	64	128
SE24	VIc	Gen^r Chl^r Ery^r	+	1,800	65	4	256
SE34	VII	Gen^r Chl^r Ery^r	+	2	7	4	32
SE65	VIII	Tet^r Ery^r	+	15	3	128	256
SE1	IXa	Ery^r	Ile66→Asn	1	0.6	1	32
SE2	IXb	Tet^r Ery^r	Ile66→Asn	2,700	600	2	128
SE13	X	Chl^r Ery^r	+	3,800	120	64	128
SE33	XI	Gen^r Ery^r	+	230	19	64	128
SE55	XII	Tet^r Ery^r	+	4,300	1,000	32	128
SE60	XIII	Tet^r Ery^r	+	0.1	0.1	1	1
SE407	XIV	Tet^r Ery^r	+	1,700	545	4	128

[a] Gen, gentamicin; Chl, chloramphenicol; Tet, tetracycline; Min, minocycline; Ery, erythromycin.
[b] MIC by broth dilution test.
[c] Bacteria were induced with 5 μg of CBAP per ml overnight before MIC determination.
[d] ND, not determined.

heterotypic resistance expression to the low MICs seen for many clinical ORSE isolates. We have chosen geographically diverse, genetically unrelated ORSE isolates found in a previous study to contain *mecI* by hybridization with a DNA probe. We have examined the nucleotide sequence of *mecI* and *mecA* P-O in these isolates, analyzed *mecA* transcription, and assessed phenotypic expression in both broth and on agar in the presence and absence of *mecA* regulation. Finally, we have assessed changes in the size of the OR subpopulation upon oxacillin exposure and the contribution of subpopulation selection to the MIC.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *S. epidermidis* strains used in this study are listed in Table 1. Recombinant plasmids were generated and maintained in *Escherichia coli* TB1 (38) or JM109 (9) cells. The *E. coli* cloning vector used was pUC19 (38). The *E. coli-S. aureus* shuttle vectors used were constructed by adding pE194ts (15, 33) to pUC19. The staphylococcal tetracycline and gentamicin resistance genes used for selection of colonies containing recombinant vectors in *E. coli* or *S. aureus* or *S. epidermidis* were *tetM* (34) and *aac*/*aph* (28), respectively.

Materials and media. Mueller-Hinton broth (MHB) and agar (MHA) (BBL Microbiology Systems, Cockeysville, Md.) and brain heart infusion (BHI) broth and agar (Difco Laboratories, Detroit, Mich.), with and without selective additives (Sigma, St. Louis, Mo.; United States Biochemicals, Cleveland, Ohio), were used for the subculture and maintenance of *E. coli*, *S. epidermidis*, and *S. aureus* strains. The antibiotics and concentrations used for *E. coli* strains for initial selection after transformation were as follows: ampicillin, 50 μg/ml; gentamicin, 5 μg/ml; minocycline, 1 μg/ml; and tetracycline, 5 μg/ml. The antibiotics used for *S. aureus* and *S. epidermidis* strains for determining antibiotic resistance and initial selection after electroporation or conjugative mobilization were gentamicin (5 μg/ml), chloramphenicol (10 μg/ml), erythromycin (10 μg/ml), tetracycline (5 μg/ml), minocycline (1 μg/ml), and mupirocin (20 μg/ml). The antibiotics used to select for the recipient *S. epidermidis* in conjugative mobilization were novobiocin (1 μg/ml) and rifampin (10 μg/ml). Induction experiments were performed with 2-(2′-carboxyphenol) benzoyl-6-amino penicilloic acid (CBAP) (5 μg/ml) and oxacillin (0.1, 0.3, 0.5, 0.6, or 1 μg/ml) in broth. Other selective additives, such as sodium citrate (Sigma) (8 mM, for transductions) or β-D-galactopyranoside (X-Gal) (50 μg/ml; Inalco Spa, Milano, Italy), were added to the media as required.

β-Lactamase production. β-Lactamase activity was detected by growing single colonies of bacteria for 16 h on BHI agar supplemented with 1% (wt/vol) soluble starch. Five milliliters of a 0.1 N I_2 solution in 0.4 M KI containing 20 mg of benzylpenicillin per ml was pipetted over the colonies and allowed to sit for 10 s before the excess was poured away. The presence of a rapidly spreading white halo around the colonies, as the penicilloic acid reacted with the iodine and decolorized the agar around the colony, indicated the presence of β-lactamase in the bacteria.

Quantitative analysis of β-lactamase activity to assess induction by β-lactam antibiotics was accomplished by using colorimetric detection of nitrocefin hydrolysis. Bacteria were grown in BHI at 37°C to an optical density at 600 nm (OD_{600}) of 0.6 with (induced) and without (uninduced) CBAP (5 μg/ml). Bacteria were harvested, pelleted, and resuspended in 2 ml of Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercaptoethanol, pH 7.0). One milliliter of the cell suspension and 2.5 g of 0.1-mm-diameter Zirconia beads (Biospec Products, Bartlesville, Okla.) were added to a 2-ml screw-cap tube. The samples were bead beaten (Biospec Products) for 5 min at 4°C and centrifuged for 10 min at 26,895 × *g* at 4°C. One hundred microliters of the supernatant was removed and added to 750 μl of 0.1 M sodium phosphate buffer (pH 7.0) in a visible cuvette. One hundred-fifty microliters of a 0.2-mg/ml concentration of nitrocefin (kindly provided by Bristol-Meyers Squibb) was added to the cuvette, and the sample was mixed and read at an OD_{490} at 30 min.

Cloning, transformation, and DNA manipulation. All restriction endonuclease digestions and ligation reactions were performed per the manufacturer's (New England Biolabs, Beverly, Mass.) specifications. Plasmids were electroporated (31; T. B. Luchansky, P. M. Muriana, and T. R. Klaenhammer, Bio-Rad technical bulletin no. 1350, p. 1–3. Bio-Rad Laboratories, Richmond, Calif.) into *E. coli* in the Bio-Rad Gene Pulser. Shuttle plasmids were moved from *E. coli* to *S. aureus* by electroporation into the restriction-deficient *S. aureus* strain RN4220 (18) as previously described (22). Plasmids were introduced into other *S. aureus* strains by transduction with the general transduction phage 80α (16, 26). Transductions using phage 80α and the isolation of both *E. coli* and staphylococcal plasmid and genomic DNA were performed as previously described (16, 25, 26).

Conjugative mobilization. Conjugative mobilization was used to introduce plasmid DNA from *S. aureus* into *S. epidermidis* and was performed by using a three-plasmid *S. aureus* donor strain as previously described (36). The recipient *S. epidermidis* strains were made novobiocin (1 μg/ml) and rifampin (10 μg/ml) resistant by serial passage on selective agar plates. The donor *S. aureus* strain, RN4220, contained the plasmids pGO626, pC221, and pGO400. To produce the tetracycline- and minocycline-resistant plasmid pGO626 (13.8 kb), a 700-bp nick site from pC221 (27) was added to pGO514. pGO514 is the plasmid containing *tetM*-inactivated *mecI* and a temperature-sensitive pE194 origin of replication described previously for allelic replacement of *mecI* in *S. aureus* (25). pC221 (4.6 kb) encodes chloramphenicol resistance and provides mobilization genes that act in *trans* on the nick site of pGO626 (27, 36). pGO400 (33.8 kb), a member of the pGO1 family of conjugative plasmids, encodes resistance to mupirocin and provides the conjugative apparatus (23). Following filter mating (23), colonies were sought that were resistant to novobiocin, rifampin, and minocycline, indicating mobilization of pGO626 into *S. epidermidis*, and susceptible to chloram-

phenicol and mupirocin, identifying those colonies that did not receive pC221 and pGO400 by cotransfer. Differentiation of *S. epidermidis* recipients from *S. aureus* donors was also achieved by performing matings on plates containing mannitol, resulting in white *S. epidermidis* and yellow *S. aureus* colonies.

Plasmid curing and allelic replacement. *S. epidermidis* isolates harboring plasmid constructs with the pE194ts replicon (pGO626) were cured of their plasmids in order to detect allelic replacement of chromosomal genes by homologous recombination (25). Briefly, single colonies were inoculated into 10 ml of BHI broth and grown for 16 h at 30°C with antibiotic selection. Following growth of *S. epidermidis* at the nonpermissive temperature for plasmid replication (43°C), colonies were patched to minocycline, gentamicin, and erythromycin plates. Colonies were sought that were minocycline resistant, indicating chromosomal integration into the replacement locus, and either gentamicin or erythromycin susceptible, indicating secondary recombination to remove plasmid DNA. The inclusion of erythromycin and gentamicin resistance genes in the pGO626 plasmid allowed us to include in this study *S. epidermidis* strains that were resistant to either erythromycin or gentamicin, but not both. However, all isolates used had to be minocycline susceptible, so that chromosomal insertion of the *tetM* marker could be identified.

EOP. Phenotypic expression of OR was determined by the efficiency of plating (EOP) procedure described by Hackbarth et al. (11), except that oxacillin was used instead of methicillin or nafcillin. Staphylococcal strains were inoculated into 5 ml of BHI broth and incubated for 16 h at 37°C with constant shaking. Cultures were then serially diluted and plated on MHA containing 0, 10, 20, 50, 100, 250, 500, and 800 μg of oxacillin per ml. The plates were incubated at 30°C for 96 h, after which CFU were counted and expressed as a ratio of cells growing in plates containing oxacillin to the number of cells on antibiotic-free medium. We defined heterotypic resistance as a population decrease of at least 2 $\log_{10}$ on 20-μg/ml oxacillin plates and of at least 3 $\log_{10}$ on 100-μg/ml oxacillin plates.

Southern blot analysis. Alkaline capillary transfer, fixation of DNA to positively charged Zeta-Probe nylon membrane (Bio-Rad, Hercules, Calif.), and hybridization were performed per the directions of the manufacturer (Bio-Rad, Hercules, Calif.) and as previously described (1).

Northern blot and PBP analysis. Cellular RNA isolation, formaldehyde gel separation, 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) capillary transfer, fixation of RNA to neutrally charged nylon membrane (Qiagen, Valencia, Calif.), and hybridization were performed according to established protocols (25). PBPs were analyzed by Michael Pucci at the Bristol-Myers Squibb Pharmaceutical Research Institute by methods previously described (25). Transcript and protein abundance on gels were quantified by scanning densitometry with an AlphaImager 1000 digital imaging system (Alpha Innotech Corp., San Leandro, Calif.).

PCR amplification. PCR amplification of DNA sequences was performed to generate fragments for cloning into pGO626 and to create the *mecA* probe for Northern blot analysis. The primer sequences and amplification protocols were performed as previously described (25).

Sequence analysis. DNA sequence analysis was performed to confirm the sequence of *mecI* and the *mecA-mecR1* or *-mecI* P-O in the different *S. epidermidis* isolates. Sequencing was performed by the automated laser fluorescence technique employing fluorescein-labeled oligonucleotides (Applied Biosystems and the Virginia Commonwealth University Nucleic Acid Synthesis and Analysis Core Facility).

PFGE. Genomic DNA was prepared and cut with the restriction enzyme *Sma*I for pulsed-field gel electrophoresis (PFGE) by established methods (8). The following parameters (22 h at 14°C) were used to visualize large fragments of DNA: 6 V/cm; initial pulse time, 1 s; final pulse time, 30 s.

Susceptibility testing. MICs for *S. epidermidis* isolates were determined in cation-supplemented MHB containing 2% NaCl by using a 10^5-CFU/ml inoculum, according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines (24). MIC determinations were performed in quadruplicate and read after an 18- to 24-h incubation at 37°C. The only exception to the NCCLS guidelines was incubation of the MIC plates at 37°C; the guidelines stipulate an incubation temperature of 35°C.

RESULTS

Characterization of clinical *S. epidermidis*. We chose to study 22 geographically and chronologically diverse clinical ORSE isolates previously shown to contain the *mecR1-mecI* regulatory region by DNA hybridization. This collection of multiresistant *S. epidermidis* isolates came from five areas of North America (Richmond, Boston, Toronto, Birmingham, and Portland) collected over 19 years (1970 to 1989). Southern blot analysis of genomic DNA digested with the restriction endonuclease *Bam*HI was probed with a *mecR1-mecI* DNA probe to confirm the presence of the regulatory region in each of the isolates. Pulsed-field gel (contour-clamped homogeneous electric field [CHEF]) analysis of *S. epidermidis* genomic DNA digested with *Sma*I was performed and identified 17 unique banding patterns (at least 2 band differences) representing 14 pulse types (>3 band differences) listed in Table 1. The *S. epidermidis* isolates were then screened for antibiotic resistance to gentamicin, tetracycline, minocycline, chloramphenicol, erythromycin, and oxacillin. All isolates were resistant to oxacillin, and most of the isolates were resistant to two or three of the additional antibiotics tested (Table 1). All but one isolate (SE42) produced β-lactamase.

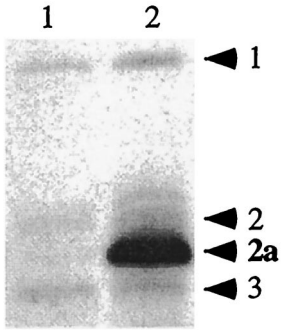

FIG. 1. PBP profiles. Determination of relative amounts of PBP2a in the cell membranes of *S. epidermidis* strains SE42 (lane 1) and SE42Δ*mecI* (lane 2). PBPs are designated by numbers to the right of the panel. This image was scanned from the original film by using an AlphaImager 1000 (Alpha Innotech) and was cropped and labeled by using Canvas 5 graphics software (Deneba, Miami, Fla.). The same systems were used to prepare Fig. 2.

Oxacillin MICs. Broth microdilution oxacillin MICs were determined for all 17 *S. epidermidis* isolates (Table 1). The range of MICs obtained was consistent with the findings of others for *S. epidermidis* clinical isolates containing the OR gene *mecA* (21). For five isolates (29%), the MIC was ≤2 μg/ml; for three isolates (18%), the MIC was 4 μg/ml (the NCCLS oxacillin resistance breakpoint for *S. aureus*); and for the remaining nine isolates (53%), the MIC was ≥8 μg/ml.

EOP. EOPs examined to determine the OR phenotypes of the 17 clinical *S. epidermidis* isolates revealed that each had a heterotypic phenotype (Table 1). All isolates had at least a 2-$\log_{10}$ drop in colony counts on plates containing ≥20 μg of oxacillin per ml, and all but one isolate (SE55) had at least a 3-$\log_{10}$ drop in colony count on plates containing ≥100 μg of oxacillin per ml. The reduction in colony count on plates containing 10 μg of oxacillin per ml was more variable.

Analysis of *mecI* and *mecR1*. PCR amplification and subsequent nucleotide sequencing of the *mecI* gene and the *mecA-mecR1* P-O revealed that 13 of the 14 *S. epidermidis* pulse types were identical to the wild-type *S. aureus mecI* and P-O sequences associated with maximal *mecA* transcriptional repression (*S. aureus* strain N315 [14]). The lone mutation observed was a single A-to-T point mutation in the *mecI* of pulse type IX, resulting in an amino acid change (isoleucine→asparagine) at position 66.

Northern blot analysis of uninduced *mecA* transcript in the clinical *S. epidermidis* isolates showed heavily repressed (~50-fold reduction) transcript in the all isolates containing *mecI*, including the isolate with the single-amino-acid change, compared to that of the *mecA* transcript in a *mecI* mutant unregulated isolate. A PBP profile of one uninduced *mecI*$^+$ *S. epidermidis* isolate, SE42, showed little PBP2a production, consistent with the *mecA* transcript data (Fig. 1). Northern blot analysis also showed that when induced for 16 h with a β-lactam antibiotic, CBAP (5 μg/ml), all but 1 (SE34) of the 17 *mecI*-positive *S. epidermidis* isolates produced *mecA* transcript equivalent to that of an unregulated isolate (~50-fold in-

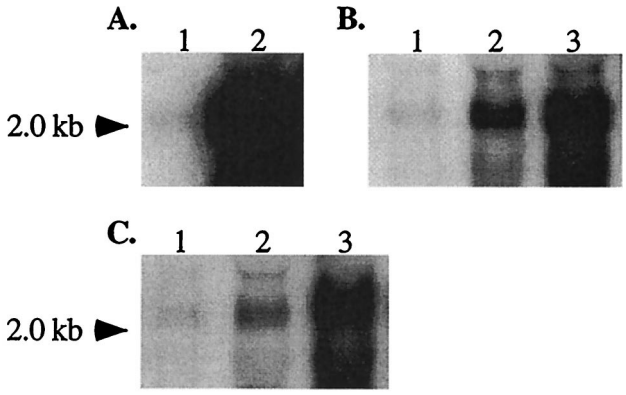

FIG. 2. Northern blot analysis of the 2-kb *mecA* transcript. The relative abundance of *mecA* transcript in SE42 (lane 1) and SE42Δ*mecI* (lane 2) (A); SE20 (lane 1), SE20Δ*mecI* (lane 2), and SE20Δ*mecI* grown in 5 μg of CBAP per ml (lane 3) (B); or SE53 (lane 1), SE53Δ*mecI* (lane 2), and SE53Δ*mecI* grown in 5 μg of CBAP per ml (lane 3) (C) is shown.

crease). *mecA* transcript in SE34 induced with 5 μg of CBAP per ml increased ~10-fold over uninduced SE34 *mecA* transcript. These data indicate that in the *S. epidermidis* clinical isolates, *mecI* and *mecR1* are largely intact and functional. MecI represses *mecA* transcription and consequently PBP2a production, while MecR1 provides the signal sensor/transducer necessary to relieve MecI repression following induction with β-lactams.

***mecI* allelic replacement mutagenesis.** *mecI* allelic replacement mutagenesis was performed with three *mecI*$^+$ *S. epidermidis* isolates (SE20, SE42, and SE53) to observe the relationship of *mecA* transcriptional repression to the OR phenotype. Deletion of *mecI* was confirmed by Southern blot hybridization by noting the loss of a 0.7-kb *Bgl*II fragment from deletion mutants.

Northern blot analysis of *mecA* transcript in the *mecI* mutant *S. epidermidis* isolates showed an increase in *mecA* transcript compared to the level of *mecA* transcript in *mecI*$^+$ parents. The SE42 *mecI* knockout resulted in an ~50-fold increase in *mecA* transcription, as determined by scanning densitometry (Fig. 2A). The PBP profile of SE42 and SE42Δ*mecI* also showed a comparable increase in PBP2a after the deletion of *mecI* (Fig. 1), correlating with the Northern blot data. SE42 produces no β-lactamase and contains no *blaZ* regulatory sequences. However, in the two *S. epidermidis* isolates (SE20 and SE53) that produced β-lactamase, there was only a three- to sixfold increase in *mecA* transcription after *mecI* inactivation (Fig. 2B and C), presumably due to the presence of the *blaZ* repressor BlaI, which has also been shown to repress *mecA* transcription. Both SE20 and SE53 were shown to contain *blaI* sequences by Southern blot hybridization. Maximal *mecA* transcription in SE20Δ*mecI* and SE53Δ*mecI* was achieved by induction with CBAP (Fig. 2B and C).

A colorimetric β-lactamase assay was performed to determine if the β-lactamase repressor BlaI was functional in isolates SE20Δ*mecI* and SE53Δ*mecI*. The *mecI* mutant isolates known to contain a β-lactamase plasmid were induced for 3 h with and without 5 μg of CBAP per ml. β-Lactamase was barely detectable in the negative control (SE42Δ*mecI*, OD_{600} of 0.042) and the β-lactamase-positive isolates (SE20Δ*mecI*, OD_{600} of 0.085; SE53Δ*mecI*, OD_{600} of 0.08) in the absence of inducer. In the presence of inducer, the β-lactamase-positive isolates (SE20Δ*mecI*, OD_{600} of 0.65; SE53Δ*mecI*, OD_{600} of 0.86) produced 8- and 11-fold more β-lactamase, respectively, while the negative control (SE42Δ*mecI*, OD_{600} of 0.043) showed no increase in activity. Thus, the β-lactamase repressor BlaI was functional in these isolates until inducer was added to the media.

Induction of *mecA* transcription. SE42 *mecA* transcription was induced by growing the isolate in various concentrations of oxacillin (0.1, 0.3, and 0.5 μg/ml) to an OD_{600} of 0.6. A progressive increase in *mecA* transcription, as determined by scanning densitometry measurement of transcripts on Northern blots, was seen as the amount of oxacillin in the medium increased (Table 2). SE42 *mecA* transcript was repressed in the absence of inducer and was 86% of the maximum seen in SE42Δ*mecI* when grown with 0.5 μg of oxacillin per ml, while 0.1 and 0.3 μg of oxacillin per ml provided relatively less induction (17 and 65% of maximal, respectively). Those strains inducibly producing β-lactamase (SE20 and SE53) displayed a stepwise pattern of *mecA* induction similar to that of SE42 at the same concentrations of oxacillin and CBAP. However, in SE20Δ*mecI* and SE53Δ*mecI* with only partially repressed *mecA* transcription, presumably due to the presence of the β-lactamase regulators, maximal induction was achieved with only 0.1 μg of oxacillin per ml. The data suggest that transcription of *mecA* is induced approximately fivefold more easily through *bla* than through *mec* regulatory sequences.

MICs and EOPs of induced isolates. Oxacillin MICs (Table 1) for the *mecI* mutant strains increased in each of the isolates from either 8 μg/ml (SE20 and SE42) or 1 μg/ml (SE53) to 128 μg/ml (SE20Δ*mecI*, SE42Δ*mecI*, and SE53Δ*mecI*). However, all three of the *mecI* knockout strains still had heterotypic EOP phenotypes that were within 1 $\log_{10}$ of the parent at all oxacillin concentrations (Fig. 3).

To determine if the increase in MICs seen in the *mecI* mutant isolates could be reproduced by induction of *mecA* transcription in the *mecI*$^+$ strains, all 17 *mecI*$^+$ *S. epidermidis* isolates were grown for 16 h with and without the inducer CBAP (5 μg/ml), and the MICs were measured the following day (Table 1). The MICs for 5 of the 17 isolates either did not increase at all following CBAP induction (1 isolate) or only increased 1 dilution (4 isolates). The MICs for the remaining 12 isolates increased by 2 dilutions (3 isolates) or >2 dilutions (9 isolates).

In contrast to the increase in MICs seen after induction, EOPs of the 17 *S. epidermidis* isolates grown with 5 μg of CBAP per ml overnight showed phenotypes that were within 1 $\log_{10}$ of the isolates grown without CBAP, with one exception. SE33 induced with CBAP overnight showed a 2-$\log_{10}$ increase in EOP at oxacillin concentrations of 20, 50, 100, and 500 μg/ml over SE33 grown without CBAP.

Oxacillin growth curves. The effect of *mecI*-mediated *mecA* repression on the growth of *S. epidermidis* exposed to β-lactam antibiotics in broth was next sought. SE42 and SE42Δ*mecI*

TABLE 2. Northern blot analysis of *mecA* induction with oxacillin

Strain	% of maximal *mecA* induction at oxacillin concn[a]:		
	0.1 μg/ml	0.3 μg/ml	0.5 μg/ml
SE42	17	65	86
SE42Δ*mecI*	100	ND[b]	ND
SE20	37	87	97
SE20Δ*mecI*	95	ND	ND
SE53	52	81	94
SE53Δ*mecI*	94	ND	ND

[a] Maximal *mecA* transcript normalized to SE42Δ*mecI*.
[b] ND, not determined.

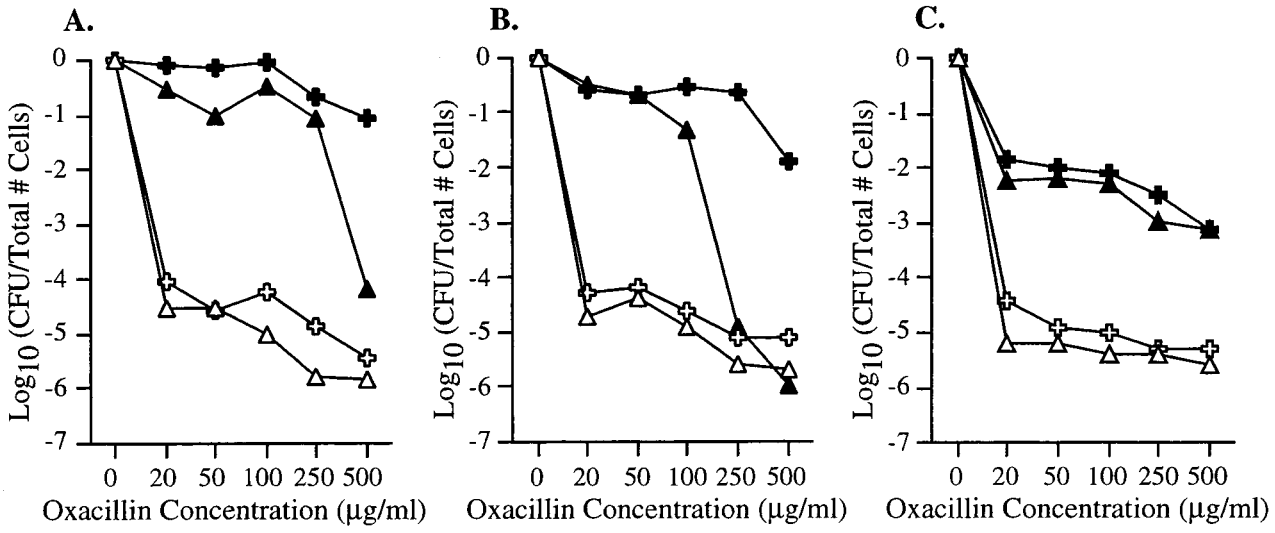

FIG. 3. EOP curves. Shown on the *y* axis are the numbers of *S. epidermidis* cells (in $\log_{10}$ CFU per milliliter on oxacillin/CFU per milliliter on MHA) remaining on the plates containing various concentrations of oxacillin (micrograms per milliliter [shown on the *x* axis]). Open triangles and crosses represent bacteria grown first in BHI broth without antibiotic, while solid triangles and crosses represent bacteria grown in BHI broth containing concentrations of oxacillin that caused a lag in growth (0.5 μg/ml for *mecI*$^+$ isolates and 1.0 μg/ml for *mecI* mutant isolates). Broth-grown bacteria were then diluted and plated on agar containing increasing concentrations of oxacillin. (A) EOP results for SE20 (△) and SE20Δ*mecI* (⌖) with no oxacillin, SE20 (▲) grown with oxacillin at 0.5 μg/ml, and SE20Δ*mecI* (✚) grown with oxacillin at 1.0 μg/ml. (B) Results for SE42 (△) and SE42Δ*mecI* (⌖) with no oxacillin, SE42 (▲) grown with oxacillin at 0.5 μg/ml, and SE42Δ*mecI* (✚) grown with oxacillin at 1.0 μg/ml. (C) Results for SE53 (△) and SE53Δ*mecI* (⌖) with no oxacillin, SE53 (▲) grown with oxacillin at 0.5 μg/ml, and SE53Δ*mecI* (✚) grown with oxacillin at 1.0 μg/ml.

were both grown for 6 h at 37°C in the presence and absence of 0.6 μg of oxacillin per ml in BHI broth. Growth of the bacteria was monitored at OD_{600}. In the presence of oxacillin, SE42 *mecI*$^+$ displayed a considerable lag in early growth, with an OD_{600} at 6 h of 0.026 (Fig. 4). In contrast, the OD_{600} of SE42Δ*mecI* at 6 h in the presence and absence of oxacillin was similar to that seen for SE42 in the absence of antibiotics. Results similar to those described above for SE42 were seen when SE20 and SE53 and their *mecI*-deleted derivatives were grown in the presence of oxacillin.

Heterotypic-to-homotypic conversion in the presence of oxacillin. When sufficient oxacillin was added to *S. epidermidis* to cause a lag in growth, the EOP phenotype increased between 3 and 5 $\log_{10}$, changing the isolates from a heterotypic to a more homotypic resistance expression (Fig. 3). Incubation of *S. epidermidis* in concentrations of oxacillin or CBAP that caused no growth lag produced no change in the EOP phenotype despite maximal induction of *mecA* transcription.

The presence or absence of *mecI* regulation of *mecA* transcription influenced the concentration of oxacillin required to produce a growth lag. For all three *mecI*$^+$ strains, growth inhibition and conversion from heterotypic to homotypic EOP expression could be achieved by incubation in 0.5 μg of oxacillin per ml. However, for each *mecI* mutant derivative, growth inhibition was only achieved by incubation in 1.0 μg of the antibiotic per ml.

Relationship between MIC and the size of the resistant population. Oxacillin MICs were determined for isolates SE42, SE20, and SE53 before and after converting expression from heterotypic to homotypic growth. In all cases, the MICs for isolates exhibiting homotypic resistance expression were 128 or 256 μg/ml. In addition, two isolates, SE33 and SE65, that exhibited extremely heterotypic resistance expression, yet for which the MICs were high (64 and 128 μg/ml, respectively [Table 1]), were examined following oxacillin MIC determination. Bacteria were removed from microtiter wells containing 1, 4, 16, and 32 (SE33) or 64 (SE65) μg of oxacillin per ml following overnight incubation, and EOP experiments were performed. SE33 from the MIC microtiter wells became progressively more homotypic in resistance expression with increasing concentrations of oxacillin. In contrast, colonies from wells of SE65 showed no change in the heterotypic subpopulation profile, even at oxacillin concentrations as high as 64 μg/ml (Fig. 5). Colonies taken from 1-μg/ml-oxacillin wells of two isolates for which MICs were low (SE53 and SE60) exhibited no change in EOP.

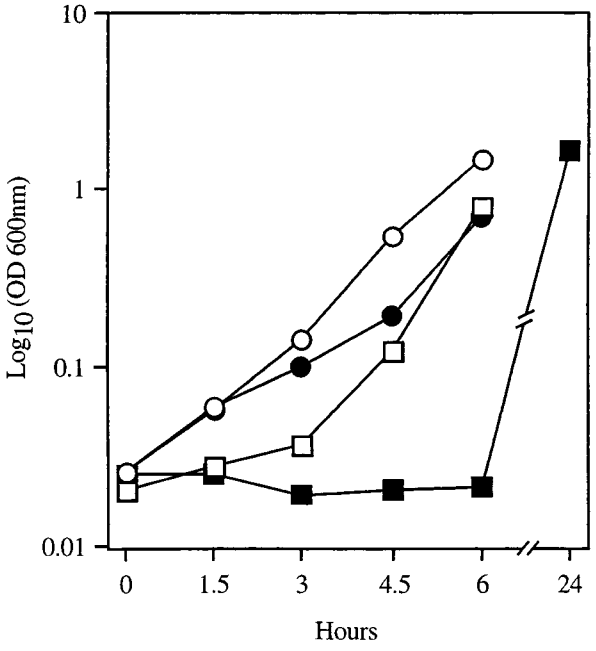

FIG. 4. Growth curve of SE42 and SE42Δ*mecI* with and without oxacillin (0.6 μg/ml). Cell density was measured by OD_{600} (*y* axis). □ and ○, SE42 (□) and SE42Δ*mecI* (○) grown in BHI without oxacillin; ■ and ●, SE42 (■) and SE42Δ*mecI* (●) grown in BHI with 0.6 μg of oxacillin per ml.

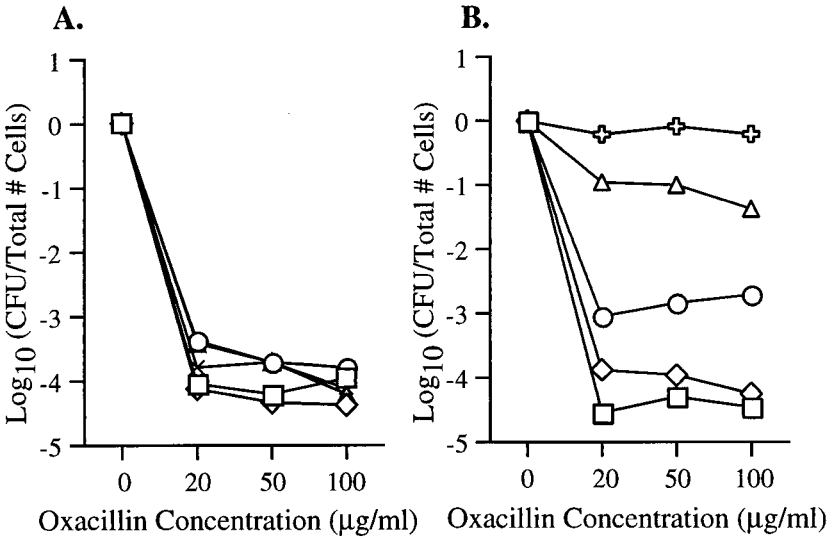

FIG. 5. EOP curves of bacteria taken from the microtiter wells of an oxacillin MIC. Shown on the *y* axis are the numbers of *S. epidermidis* cells (in $\log_{10}$ CFU per milliliter on oxacillin/CFU per milliliter on MHA) remaining on the plates containing various concentrations of oxacillin (in micrograms per milliliter [shown on the *x* axis]). (A) EOP results for SE65 taken from the 0-μg/ml oxacillin microtiter well (□), the 1-μg/ml oxacillin well (◇), the 4-μg/ml oxacillin well (○), the 16-μg/ml oxacillin well (△), and the 64-μg/ml oxacillin well (×). (B) EOP results for SE33 taken from the 0-μg/ml oxacillin microtiter well (□), the 1-μg/ml oxacillin well (◇), the 4-μg/ml oxacillin well (○), the 16-μg/ml oxacillin well (△), and the 32-μg/ml oxacillin well (✣).

DISCUSSION

The ORSE isolates chosen for this study were previously shown to contain DNA sequences that hybridized with a probe containing the *mecA* transcriptional repressor, *mecI* (2). In that study, 48% of the clinical isolates chosen hybridized with the *mecI* probe; in the other isolates, *mecI* was deleted and replaced by an IS element (IS*1272*). In the present study, we have shown that all 17 unique ORSE pulse types with *mecI*-hybridizing sequences had intact and functional *mecA* regulators: *mecI* and *mecA* P-O DNA sequences in 16 of 17 were the same as wild-type, functional ORSA sequences; baseline, uninduced *mecA* transcription was heavily repressed; transcription could be induced by β-lactam compounds; and inactivation of *mecI* led to an increase in both *mecA* transcription and MICs. The presence of intact *mecA* regulators in ORSE, in contrast to mutation of these regulators in ORSA, has been previously reported for a group of isolates from a single Japanese hospital (17). The role of these regulators in determining the phenotypic expression of β-lactam resistance in ORSE had not, however, been examined.

The relevance of assessing the relationship of *mecA* transcriptional repression to phenotype in ORSE is based on several differences between β-lactam resistance in ORSE and that in ORSA. First, as noted above, those ORSE isolates that have *mecA* regulators contain intact and functional genes in contrast to the usual presence of mutations and insertions that inactivate these genes in ORSA. Second, the oxacillin MICs for as many as 30% of clinical, *mecA*-positive *S. epidermidis* isolates, as determined by broth microdilution, are ≤2 μg/ml, below the NCCLS breakpoint that identifies more than 95% of *mecA*-positive *S. aureus* strains (21). Finally, the majority of ORSE strains exhibit extreme heterotypy when examined by EOP on oxacillin-containing agar; this expression class is less common among clinical ORSA isolates (T. M. Dickinson and G. L. Archer, unpublished data).

This study documented a direct relationship between resistance to oxacillin and *mecA* transcription when *S. epidermidis* was grown in broth. This relationship was best shown when low concentrations of the antibiotic (0.5 to 1.0 μg/ml) were used during rapid growth in broth over 6 h, but was also seen at higher antibiotic concentrations during low inoculum overnight broth MIC determinations. These data imply that, under conditions of rapid bacterial growth in broth, the presence of increasing amounts of PBP2a in membranes provides increasing resistance to β-lactam antibiotics. However, our data also showed that exposure of bacteria in broth to concentrations of oxacillin that suppressed growth selected a highly oxacillin-resistant subpopulation. This selection of a more highly β-lactam-resistant population upon β-lactam exposure has also been noted for *mecA*-positive *S. aureus* (6, 12) and has been attributed to changes in the bacterial cell wall that improve the efficiency of PBP2a in cell wall construction. The nature of the conversion of heterotypic to homotypic resistance expression among staphylococci has eluded molecular definition for some time. Data remain, therefore, largely descriptive. However, preliminary studies suggest that growth in β-lactam antibiotics selects a mutant population rather than inducing a regulatory pathway (J. E. Finan, T. M. Dickinson, A. E. Rosato, and G. L. Archer, unpublished data).

The selection of the resistant subpopulation was related to *mecA* transcription only in that strains with repressed transcription required lower concentrations of oxacillin to select high-level resistance than did strains with unregulated *mecA* transcription. The size of the highly resistant subpopulation (heterotypic expression), as determined by EOP, was unrelated to *mecA* transcription and thus to PBP2a quantity. The lack of correlation between heterotypic resistance expression and *mecA* transcription or PBP2a quantity has been noted by other investigators studying *S. aureus* (5, 25).

These observations suggest that there are at least two mechanisms that determine the survival of *mecA*-positive *S. epidermidis* strains following their exposure to penicillinase-resistant penicillins. Upon initial exposure to a β-lactam antibiotic, the growth rate of the planktonic cells that constitute the majority of the bacterial population may be directly related to their expression of PBP2a. The expression of PBP2a, regulated at

the level of *mecA* transcription, is affected by both the MecR1-MecI and BlaR1-BlaI sensor-transducers and repressors. In contrast, selection of the more highly resistant minority subpopulation occurs more slowly, is likely to require the participation of additional chromosomal factors (3, 7, 30), and may be favored by growth on solid surfaces or in biofilms.

The contribution of β-lactamase regulators to *mecA* transcriptional regulation was also observed in this study. When *mecI* was inactivated in isolates SE20 and SE53, *blaI* regulation persisted, affording partial transcriptional repression. However, *blaI* repression was easily and rapidly removed following induction with concentrations of β-lactam antibiotics that were one-fifth those required for induction of *mecA* transcription through *mecR1* and *mecI*. The difference in β-lactam induction of *mecA* through *mecR1* (slow and partial) versus that through *blaR1* (rapid and complete) has been noted previously (19, 29) in *S. epidermidis* and *S. aureus*. The inefficiency of *mecA* induction through *mecR1* by oxacillin may further explain why isolates containing these regulators have persistent phenotypic repression following relatively short incubation times in broth. In contrast, in ORSE isolates without *mecI*, most of which will contain β-lactamase regulatory sequences, *mecA* transcription is rapidly induced following β-lactam exposure, and, therefore, β-lactamase regulators contribute very little to *mecA*-related variations in phenotypic expression.

Our data showed that the ORSE oxacillin MIC, as determined in broth with the low standard inoculum, could be independently affected both by *mecA* transcriptional regulation and by a change in the size of the resistant subpopulation. Inactivation of *mecI* and induction of *mecA* transcription by CBAP were able to raise the MIC without increasing the size of the highly resistant subpopulation, as determined by EOP. However, since the MICs for only 8 of the 17 unique *mecI*-positive ORSE pulse types that we examined were ≤4 μg/ml and the MICs for some isolates with naturally occurring *mecI* deletions are low (T. M. Dickinson and G. L. Archer, unpublished data), transcriptional repression of *mecA* is not a complete explanation for low broth dilution MICs.

We also showed that even though there was no correlation between the initial size of the resistant subpopulation (baseline EOPs) and MICs, when isolates for which MICs were low were converted from heterotypic to homotypic resistance expression, MICs increased markedly. In addition, colonies removed from microtiter wells of one ORSE isolate for which the MIC was high and with a very heterotypic baseline EOP became progressively more homotypic in resistance expression as the oxacillin concentrations in the wells increased. This suggests that, with the low standard inoculum used for MIC testing, the ability of some isolates to increase the size of their highly resistant subpopulations upon β-lactam exposure can determine the MICs for them. It further suggests that the differences in MICs for some ORSE isolates may be due to genetically determined variations in their capacity to convert from heterotypic to homotypic resistance expression upon β-lactam exposure. However, the failure of SE65 to exhibit homotypic conversion in microtiter MIC wells containing high concentrations of oxacillin suggests that there are mechanisms that determine ORSE broth microtiter MICs other than those examined in this study. Furthermore, since the MIC measures rapid growth in broth and the EOP determines subpopulation distribution on agar, the two assays may be measuring different aspects of the response of some *S. epidermidis* isolates to β-lactam antibiotics.

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