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Quantitation of *mecA* Transcription in Oxacillin-Resistant *Staphylococcus aureus* Clinical Isolates

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The transcription of *mecA*, the gene required for oxacillin resistance in staphylococci, was quantified in a collection of 65 geographically and genetically diverse clinical and 8 defined laboratory *Staphylococcus aureus* isolates. *mecA* transcription was measured by real-time reverse transcription-PCR, confirmed by Northern blot analysis, and correlated with the presence and DNA sequence of the two *mecA* repressors, *mecI* and *blaI*. Isolates were first examined that contained *mecI* and/or *blaI* with wild-type sequence. BlaI provided significantly more repression of *mecA* transcription than did MecI, unrelated to *blaI* genetic location. Both together repressed *mecA* better than either one alone. In clinical isolates containing only wild-type *mecI*, *mecA* transcription repression was 10- to 25-fold less effective than that seen in previously studied constructs derived from strain N315. There was a difference in the *mecI* ribosomal binding site (RBS) between the clinical isolates (GGAA) and N315 (GGAG). The GGAA RBS was associated with 5.5- to 7.3-fold less *mecA* repression than GGAG in isogenic constructs. The values generated for wild-type repressors were compared to those in 26 isolates containing *mecI* mutations. *mecA* transcription appeared to be repressed only by BlaI in isolates with *mecI* nonsense and frameshift mutations. In contrast, *mecI* repression seemed to be partially or fully retained in many of the isolates with *mecI* and one isolate with *blaI* missense mutations, providing structure-function correlates with the site and type of mutation. We conclude that *mecA* repressor activity is highly variable in clinical *S. aureus* isolates due to *mecI* mutations, RBS polymorphisms, and unidentified genomic adaptations.

Two repressors, BlaI and/or MecI, regulate transcription of *mecA*, the gene required for oxacillin resistance in staphylococci (8, 15). *mecA* encodes a penicillin binding protein (PBP2a) that is poorly bound by β-lactam antibiotics and can perform essential functions of cell wall construction when β-lactams inactivate the cell's normal penicillin binding protein complement (3, 14). Two signal transducers, BlaR1 and MecR1, regulate repressor activity (20). MecR1 and BlaR1 are transmembrane β-lactam sensory transducers, having a surface-exposed β-lactam binding domain and a cytoplasmic zinc peptidase motif. Signal transduction is thought to occur following interaction of a β-lactam antibiotic with the binding domain, transmitting a signal through four membrane-spanning segments. This induces conformational change in the cytoplasmic metalloprotease domain causing autoproteolysis. Proteolytic cleavage of the inducer is followed by cleavage of the repressor, leading to derepression of target genes. The genes for signal transducers and repressors are contained in two-gene operons (*blaR1-blaI* and *mecR1-mecI*) that are divergently transcribed from their regulated genes (*blaZ* and *mecA*, respectively) (8, 13, 15). The repressors bind to specific palindromic sequences that overlap divergent promoters for *mecA* and *mecR1* (8, 18). A helix-turn-helix DNA binding motif has been assigned to the amino terminus of the repressor protein based on structure predictions (11), and the carboxyl terminus has been assumed to be involved in repressor dimerization by analogy to other repressors and by the location of the repressor cleavage site (19, 20). However, the structure of neither MecI nor BlaI has been solved.

In a previous study (17), we showed that at least one repressor with a wild-type nucleotide sequence was present in 91 of 95 genetically diverse clinical *Staphylococcus aureus* isolates. We hypothesized that repression was evolutionarily conserved in order to prevent overproduction of a potentially toxic, regulated gene product. However, we did not assess the effectiveness of repression by directly measuring the transcription of regulated genes. A direct assessment of transcriptional regulation is of interest for several reasons. First, in an earlier study (16), members of our group identified an isolate containing *mecI* with wild-type repressor and promoter-operator sequences in which *mecA* transcription was eightfold greater than that in another isolate containing *mecI* with the same sequence. In the present study, we sought evidence for similar variations in *mecA* transcriptional repression among clinical isolates containing *mecI* genes with wild-type sequence. Second, we sought to confirm data generated with a single, genetically manipulated strain indicating that wild-type MecI and BlaI provided additive repression of *mecA* transcription when both were present in the same strain (15). Third, we wanted to assess the extent to which mutations in *mecI* or *blaI* affected repressor function. These data might provide valuable structure-function correlates to complement studies investigating the molecular basis of MecR1-BlaR1 and MecI-BlaI signal transduction.

However, all but one of the clinical isolates with mutant *mecI* also contained the corepressor gene, *blaI*, making it difficult to assess the contribution of individual repressor mutations to *mecA* regulation. In order to assess the function of

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TABLE 1. Characterization of *mecI* mutations and quantitation of specific *mecA* mRNA

Mutation class	Strain	Mutation in *mecI* gene		Amt of specific *mecA* mRNA/16S mRNA
		Nucleotide position (change)	Amino acid change[a]	
Nonsense	SA41	202 (C→T)	Gln 68→stop codon	330
	SA35	202 (C→T)	Gln 68→stop codon	285
	SA7	202 (C→T)	Gln 68→stop codon	179, 129[b]
	SA33	202 (C→T)	Gln 68→stop codon	176
	SA18	202 (C→T)	Gln 68→stop codon	145, 83
	SA12	202 (C→T)	Gln 68→stop codon	142, 139
	SA20	202 (C→T)	Gln 68→stop codon	117
	SA9	202 (C→T)	Gln 68→stop codon	116
	SA16	202 (C→T)	Gln 68→stop codon	102
	SA19	202 (C→T)	Gln 68→stop codon	141
	SA2	202 (C→T)	Gln 68→stop codon	90
	SA11	202 (C→T)	Gln 68→stop codon	43
	SA57	343 (G→T)	Glu 115→stop codon	226, 301
Frameshift	SA10	193 (extra A)	Lys 65→12 aa to stop	306
	SA13	64 (extra A)	Lys 22→7 aa to stop	1,172
	SA42	250 (extra A)	Lys 84→5 aa to stop	834, 801
	SA56	91 (extra T)	Ile 30→8 aa to stop	330, 219
Missense	SA5	32 (C→T)	Ala 11→Val	21, 28
	SA15			35, 32
	SA24			261, 256
	SA14	347 (T→C)	Leu 116→Ser	202, 193
	SA36			1,907, 940
	SA46	22 (A→G)	Iso 8→Val	21, 35
	SA78	125 (C→T)	Pro 42→Leu	64, 75
	SA30	116 (A→G)	Asp 39→Gly	40, 52
	SA54	152 (G→T)	Arg 51→Ile	1,101, 1,227
	SA47	370 (T→A)	+18 aa to stop	66, 79
	SA27	142 (C→T)	Leu 48→Phe	66, 49

[a] aa, amino acids.
[b] For some isolates two independent RNA samples, prepared on different days, were analyzed to assess reproducibility of RT-PCR values for *mecA*-specific mRNA.

mutant repressors, we first had to establish baseline values for transcription repression in isolates with *blaI* and *mecI* that had wild-type DNA sequence. We used real-time reverse transcription PCR (RT-PCR) as our method for transcriptional quantification. We identified groups of isolates that had all possible combinations of the two repressors with wild-type sequence: both together, both absent, and each present without the other. The values for *mecA* transcription among these isolates were used as standards with which to compare isolates with *mecI* mutations. As described in a previous publication (17), we have found, among the 26 clinical isolates with *mecI* mutations, a wide variety in the type and location of mutation, making it possible to attempt structure-function predictions.

MATERIALS AND METHODS

Source of isolates. The *S. aureus* clinical isolates examined in this study came from three sources, as detailed in a previous publication (17): 30 isolates, all of which were genetically distinct, came from the Public Health Research Institute (PHRI) collection, kindly provided by Barry Kreiswirth; 26 isolates, chosen for geographical and temporal diversity, came from the Virginia Commonwealth University collection; and 9 isolates, found to have wild-type *mecI* sequence and no *blaI* coregulation, were blood culture isolates from the SCOPE collection kindly provided by Sandy Tennant and Mike Edmond (5). In addition, eight isolates were defined, genetically manipulated strains used as comparators for RT-PCR analysis.

Isolates with wild-type *mecI* and *blaI*. Isolates with both wild-type *mecI* and *blaI* together, neither repressor, or each without the other were chosen from among the clinical isolates and other well-characterized strains in our collection. The DNA sequence of the repressors was determined and described previously (17). The isolates with only wild-type *mecI* came from all three clinical collections (12 isolates); those with *blaI* only, containing the previously described IS*1272*-mediated deletion of *mecI* (2), were all from the PHRI collection, and each was of a unique *spa*-repeat type (12 isolates); those with both *mecI* and *blaI* were from the PHRI and VCU collections, and each was of a unique *spa*-repeat type (12 isolates); and those with neither repressor contained only the IS*1272*-mediated *mecI* deletion (three isolates) or laboratory-constructed *mecI* knockouts (three isolates). Well-characterized isolates (1, 12, 15, 16) were used as comparators in the appropriate groups: COL and 450 M (Δ*mecI*-Δ*blaI*), N315 (*mecI blaI*), 450 M/pI258 (Δ*mecI*-*blaI*) and N315P, BMS1, 450 M::630, and 450 M::522 (*mecI*$^+$ *blaI*).

Isolates with *mecI* and *blaI* mutations. The 26 isolates with *mecI* mutations and the single isolate with a mutation in *blaI* were described previously (17).

Media. Mueller-Hinton broth and Mueller-Hinton agar (both from BBL Microbiology Systems, Cockeysville, Md.) and brain heart infusion broth and agar (Difco Laboratories, Detroit, Mich.), with and without selective additives (Sigma, St. Louis, Mo.; United States Biochemicals, Cleveland, Ohio), were used for subculture and maintenance of *S. aureus* strains.

Southern blot analysis. The genetic location of *blaI* was determined by probing plasmid and chromosomal DNA of selected isolates with this gene. Plasmid and chromosomal DNA were extracted from bacterial isolates using Qiagen columns (Qiagen, Inc., Chatsworth, Calif.), electrophoresed through agarose, and transferred to a nylon membrane using capillary action. Probe DNA was labeled with digoxigenin and applied to membranes according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, Ind.). Signal was detected by exposing membranes to X-ray film. *blaI* was assumed to be plasmid encoded if the probe hybridized with a plasmid band of a size approximately equal to that of control plasmid pI258 and chromosomally encoded if the probe hybridized with only the chromosomal band. However, it is possible that in some isolates, plasmid DNA comigrated with chromosomal DNA and was mistakenly designated a chromosomal gene.

Northern blot analysis. *S. aureus* was grown in 30 ml of brain heart infusion broth to an optical density at 600 nm of 0.6. Cultures were centrifuged, and the bacteria were resuspended in 1,000 μl of RLT buffer (RNeasy kit; Qiagen Inc.) and added to 2-ml FastPrep Blue tubes containing ceramic matrices (Bio 101, La Jolla, Calif.). The bacteria were lysed with a Fast Prep instrument (Bio101) at setting 6 for 40 s, placed on ice for 1 min, and centrifuged at 10,000 × *g* for 5 min at 4°C. The upper aqueous phase was aspirated, and total RNA was extracted using a Qiagen RNeasy kit. About 7 μg of RNA was separated by resolution through formaldehyde-containing 1% agarose. The intensities of the 23S and 16S rRNA were visualized using a 254-nm UV short-wave lamp, and quantities were adjusted so that the same amount of RNA was loaded for each bacterium. RNA was transferred from agarose to positively charged nylon membranes (Stratagene, La Jolla, Calif.) by capillary action as previously described (15). Labeling and hybridization were done by use of the digoxigenin labeling and detection kits according to the manufacturer's instructions (Roche Molecular Biochemicals) and exposed to X-ray film.

Real-time RT-PCR. Oligonucleotide primers and probes for *mecA* and 16S rRNA were designed with Primer Express 1.0 software form ABI Prism (Perkin-Elmer Applied Biosystems, Foster City, Calif.) and purchased from Megabase Inc (Evanston, Ill.). The probes consisted of an oligonucleotide labeled at the 5′ end with the reporter dye 6-carboxyfluorescein and with the quencher dye *N*,*N*′,*N*′-tetramethyl-6 carboxytetramethylrhodamine at the 3′ end. RT-PCR was done with the TaqMan One-Step RT-PCR Master Mix Reagents kit as described by the manufacturer (PE Applied Biosystems, Foster City, Calif.). The RT-PCR mixture (25 μl contained 6.25 U of Multiscribe reverse transcriptase, 10.0 U of RNase inhibitor, 500 nM (each) gene-specific primer, 100 nM (each) probe, and 25 ng of total RNA template. Amplification and detection of specific products were performed with the ABI Prism 7700 sequence detection system (PE Applied Biosystems) with the following cycle profile: 1 cycle at 48°C for 30 min, 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min. The critical threshold cycle (C_t) is defined as the cycle at which the fluorescence becomes detectable above background levels and is inversely proportional to the logarithm of the initial number of template molecules. A standard curve was plotted for each primer-probe set with C_t values obtained from amplification of known quantities of RNA isolated from strain *S. aureus* 450 M. The standard curves were used to transform C_t values to the relative number of RNA molecules. The amount of contaminating chromosomal DNA in each sample was determined with the control reactions that did not contain reverse transcriptase. The quantity of cDNA for each experimental gene was normalized to the quantity of 16S cDNA in each sample. Each RNA sample was run in triplicate. The forward and reverse primers for *mecA* RT-PCR were GTTAGATTGGGATCATAGCGT CATT and TGCCTAATCTCATATGTGTTCCTGTAT, respectively, and for 16S RT-PCR they were TCCGGAATTATTGGGCGTAA and CCACTTTC CTCTTCTGCACTCA, respectively. The probes, labeled with carboxyfluorescein at the 5′ end and *N*,*N*′,*N*′-tetramethyl-6 carboxytetramethylrhodamine at the 3′ end, were TTCCAGGAATGCAGAAAGACCAAAGCATGA for *mecA* and AAGCCCACGGCTCAACCG for 16S RNA.

Assay for β-galactosidase activity. β-galactosidase activity was assessed in cell extracts, produced by homogenization of bacteria with glass beads for 3 min in a mini-bead-beater (BioSpec Products, Bartlesville, Okla.) using *o*-nitrophenyl-β-D-galactopyranoside as a substrate, as previously described (18).

Statistical analysis. Differences between the RT-PCR values in one group versus another were assessed by analysis of variance using SPSS for Windows (SPSS Inc., Chicago, Ill.).

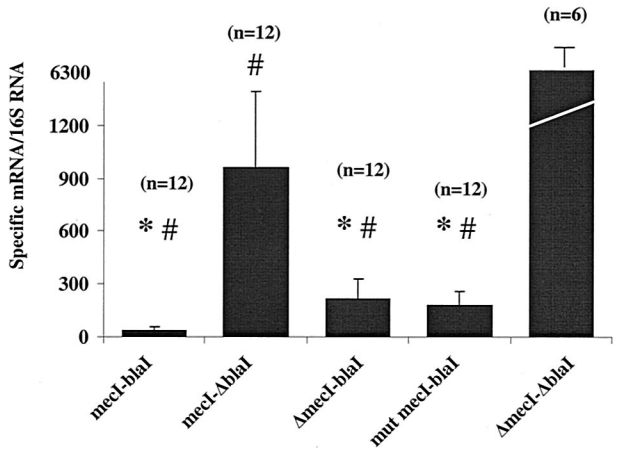

FIG. 1. Quantitation of *mecA* mRNA by Taqman Real Time RT-PCR in *S. aureus* isolates. Relative values of *mecA* mRNA over 16S mRNA are shown on the vertical axis, while groups of isolates with different repressor genotypes are shown on the horizontal axis. Each filled bar designates the mean of that group; n represents the number of strains in each group; and standard deviations are indicated by error brackets. The repressor genotype of each group is indicated below the bar. Δ indicates that the repressor is absent or deleted; *mut mecI* is the group of isolates with nonsense mutations. *, transcription repression of *mecA* significantly greater than that mediated by *mecI* alone (*mecI*-Δ*blaI*; $P < 0.05$); #, transcription repression of *mecA* significantly greater than that seen in the absence of repressor (Δ*mecI*-Δ*blaI*; $P < 0.001$).

RESULTS

Transcription of *mecA* in isolates with wild-type repressor sequence. Each RT-PCR value for the wild-type isolates and for the mutants described below is the result of the analysis of a single RNA sample for most isolates. However, the reproducibility of the RT-PCR results was supported by analysis of a second, independently prepared RNA sample on 35 isolates (10 wild types, 17 mutants, and 8 comparators). Repeat values for missense, frameshift and nonsense mutants are shown in Table 1. In addition, as described below, 36 of the 73 RNA samples analyzed by RT-PCR were also examined by Northern blotting, and the relative magnitude and difference between samples was confirmed in each case. The RT-PCR values in the groups of isolates with wild-type *mecI* and *blaI* sequence are shown in Fig. 1. The *mecA-mecR1* intergenic regions, containing the promoter-operator sequences, were wild type and identical for all isolates studied. The mean value of each group was compared to that for each other group. Transcription repression mediated by BlaI alone and that mediated by BlaI plus MecI were significantly greater than that mediated by MecI alone ($P < 0.05$). All isolates containing both repressors together also provided greater repression than BlaI alone, but that difference did not reach statistical significance due to the wide variation in values. The absence of both repressors led to a significantly greater transcription of *mecA* than was seen when either repressor was present ($P < 0.001$). The effect of genetic location on *blaI*-mediated repression was also investigated. Among the isolates with *blaI* and *mecI*, *blaI* was in the chromosome in five isolates and on a plasmid in seven, including the comparator type strain, N315. The mecA transcription values (mean ± standard deviation) were the following: chromosome, 50.7 ± 15.2; plasmid, 41.2 ± 9.2). In isolates with *blaI* alone, the repressor was in the chromosome in seven isolates and on a plasmid in six isolates, including the comparator laboratory strain, 450 M/pI258. Values were the following: chromosome, 217 ± 103; plasmid, 149 ± 102. There was no significant difference in *blaI*-mediated repression related to genetic location.

The mean ± standard deviation (960 ± 431) *mecA* transcription values for isolates containing *mecI* alone were far greater than that seen for the laboratory comparator strains, N315P and 450 M::630 (49 and 12, respectively). For 7 of 12 isolates, the *mecA* transcription values ranged from 1,077 to 1,592. The comparator strains are derived from N315, the strain providing the first published sequence of *mecR1* and *mecI* (10) and the

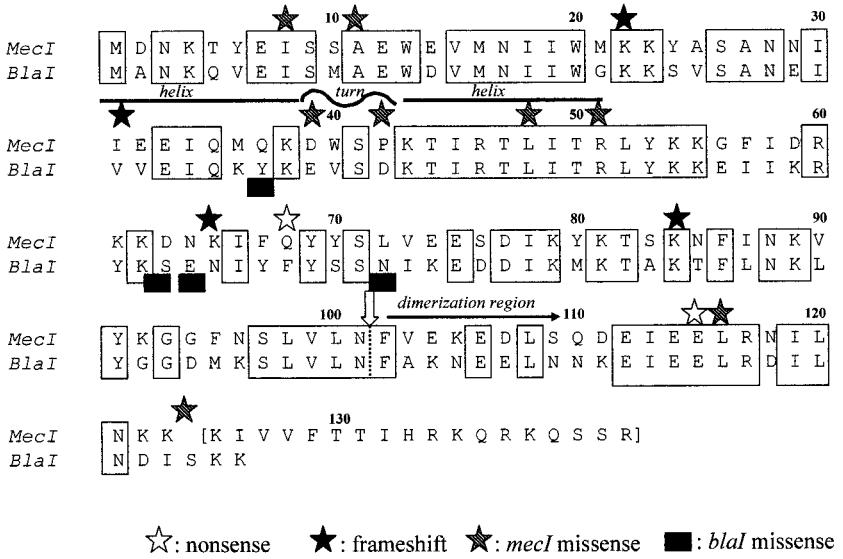

FIG. 2. Optimal alignment of the MecI and BlaI repressor protein amino acid sequences showing sites and types of mutations, indicated by stars. Identical residues are shown between boxes. Additional information about nucleotide and amino acid changes is found in Table 1. The sites of cleavage of *mecI* and *blaI* are indicated by a vertical white arrow; the putative dimerization domain is indicated by a black horizontal arrow; and the putative helix-turn-helix DNA binding motif is indicated by horizontal lines over the appropriate residues.

first published *S. aureus* genomic sequence (12). N315P is N315 cured of its penicillinase-producing plasmid. 450 M::630 contains the N315 *mecR1 mecI mecA-mecR1* promoter-operator and a portion of *mecA* introduced into the chromosomal lipase gene (*geh*) of strain 450 M (*mecR1-mecI* deletion), providing *mecA* repression in *trans*. The *lacZ* gene was fused to the first 50 bp of *mecA* in the 450 M::630 construct to provide a reporter as an additional measure of *mecA* transcription, all as previously described (18). A possible explanation for the transcription difference between N315-derived and clinical isolates was found when the ribosomal binding site (RBS) for *mecI* from N315 (GGAG) was compared to that for clinical isolates (GGAA) (see Fig. 4). All 23 isolates containing wild-type *mecI* that were examined by RT-PCR had the GGAA RBS. Among 73 isolates examined in this study, only 11 isolates contained a GGAG RBS, all of which were in isolates containing *mecI* mutations (five nonsense, three frameshift, and three missense).

In order to assess the contribution of *mecI* RBS sequence polymorphisms to differences in *mecI*-mediated *mecA* repression, we compared *mecA* transcription in isogenic constructs, 450 M::630 and 450 M::522. 450 M::522 came from isolate BMS1 (15) and differed from 450 M::630 only in that the *mecI* GGAA RBS was substituted for the N315 GGAG sequence. Repression of *mecA* transcription was 8.2-fold greater for 450 M::630 (mean, 7.0) than for 450 M::522 (mean, 57.5). These RT-PCR values were confirmed by Northern blotting (see Fig. 3) and by β-galactosidase activity (471 U of activity for 450 M::630 and 3,142 U for 450 M::522, a 7.3-fold difference). However, the RBS differences did not entirely explain the difference in *mecA* transcription between some isolates with only wild-type *mecI* and others. The mean transcription value among seven of these isolates (1,274; range, 1,097 to 1,592) was 26- and 131-fold greater than that for either N315P or 450 M::630, respectively.

Characterization of *mecI* mutations. The 28 mutant *mecI* genes could be divided into three classes (Table 1 and Fig. 2). Class one (nonsense) mutations occurred in 13 isolates; 12 of the mutations were identical. In each of the 12 isolates, a substitution at nucleotide 202 (C→T) introduced a translational stop replacing a glutamine residue. In the 13th isolate, a translational stop was introduced at nucleotide 343, eight amino acids from the carboxyl terminus of the protein.

Class two (frameshift) mutations, occurring in four isolates, introduced an A at a different site in three genes and a T in a fourth gene. The addition of adenines occurred in a group of from four to six adenine repeats; the addition of thymine created four dinucleotide TA repeats. The frameshift in these four isolates introduced a translational stop from four to eight amino acids after the mutation.

Class three (missense) mutations were found in 11 isolates. Two and three isolates had identical mutations (Leu116Ser and Ala11Val, respectively), while the final six isolates each had a unique mutation. In one of these isolates, SA47 (stop124Lys), the translational termination of the open reading frame was replaced by a lysine, extending the protein sequence by 18 amino acids. Five of the eight unique mutations in *mecI* were in amino acids that were conserved between *mecI* and *blaI*. There was a single isolate that contained mutant *blaI*; *mecI* was deleted in this isolate. There were four missense mutations in this isolate, each at a residue that was not conserved between *blaI* and *mecI*. At one position, the *blaI* mutation changed the amino acid to the one found at the comparable position in *mecI* (E64N) (Fig. 2).

Transcription of *mecA* in isolates with *mecI* and *blaI* mutations. All of the isolates with *mecI* mutations but one contained *blaI* with wild-type sequence, as shown in a previous study (17). Thus, the *mecA* transcription results for the isolates with *mecI* mutations (*mut mecI-blaI*) were compared to those for two groups of isolates (Δ*mecI-blaI*) and (*mecI-blaI*).

The nonsense mutation at amino acid 68, found in 12 isolates, was predicted to inactivate MecI, leaving only BlaI as the sole *mecA* transcriptional regulator. The data were consistent with this prediction. The values for *mecA* transcription in this group (*mut mecI blaI*, 181.6 ± 76; range, 102 to 330) were not statistically different from values for those in the group having BlaI but no MecI (Δ*mecI blaI*, 216 ± 117; $P > 0.05$). They were also not statistically different from values for the entire group having both MecI and BlaI (*mecI-blaI*). However, while the transcription values of all 11 isolates containing both repressors (*mecI blaI*) were less than 70 (mean, 34; range, 3 to 65), 11 of the 12 isolates with BlaI only (Δ*mecI blaI*) (mean, 216; range, 103 to 431) and 10 of the 12 isolates with *mecI* nonsense (*mut mecI blaI*) mutations (mean, 182; range, 102 to 330) had values greater than 100. The difference between the subset of values in the 11 isolates with both repressors (*mecI blaI*) and those of the other two groups (Δ*mecI blaI*; *mut mecI blaI*) with *mecA* transcription values greater than 100 (11 and 12 isolates, respectively) was significantly different ($P < 0.05$). This suggests that values greater than 100 resemble those for the group with BlaI but not MecI and those less than 70 resemble those for the group with two functional repressors. Therefore, we made the assumption that isolates with *mecA* transcription values of <70 or >100 were likely to have a functional or inactive *mecI* repressor, respectively.

The *mecA* transcription values of the four isolates with frameshift mutations were all greater than 200 (306, 330, 801, and 1,172), making it likely that the mutation completely inactivated *mecI*, as predicted (Table 1).

There were nine different missense mutations in 11 isolates (Table 1). Mutations in 7 of the 11 isolates had *mecA* transcription values that were less than 70 (mean, 45; range, 21 to 66), while mutations in four isolates yielded values ranging from 201 to 1,907. One of the isolates with high *mecA* transcription values (261 and 256) had the same missense mutation (A11V) as two isolates with low transcription values (22 and 35). There were no differences in *blaI* or *mec* promoter-operator sequences or *mecI* RBS sequences among these three isolates.

Northern blot analysis of *mecA* transcription. The RT-PCR results for some isolates were confirmed by Northern blot analysis. The same RNA used for RT-PCR analysis was examined by Northern blotting for the following isolates: six with the same missense mutation, all four with frameshift mutations, six with nonsense mutations, and two each with wild-type repressor combinations (both together, each without the other, and neither). The relative intensity of the *mecA* transcript by Northern blot analysis was consistent with the relative values generated using RT-PCR in each instance. Representative Northern blot results are shown in Fig. 3.

Sequence analysis of *mecR1* and induction of *mecA* transcription. In four isolates containing *mecI* without *blaI* and *mecA* transcription values >1,000, the DNA sequence of *mecR1* was determined. In all four isolates the sequence was wild- type, identical to that of N315. In addition, all of the isolates with *mecI* mutations were induced overnight by growing them in 0.3 μg of oxacillin/ml, and *mecA* transcription was determined by RT-PCR. Although there was a wide range of transcription values following induction, *mecA* transcription values increased at least twofold for each isolate. This suggests that BlaR1 was functional in these strains, because mutation would have led to constitutive BlaI-mediated repression.

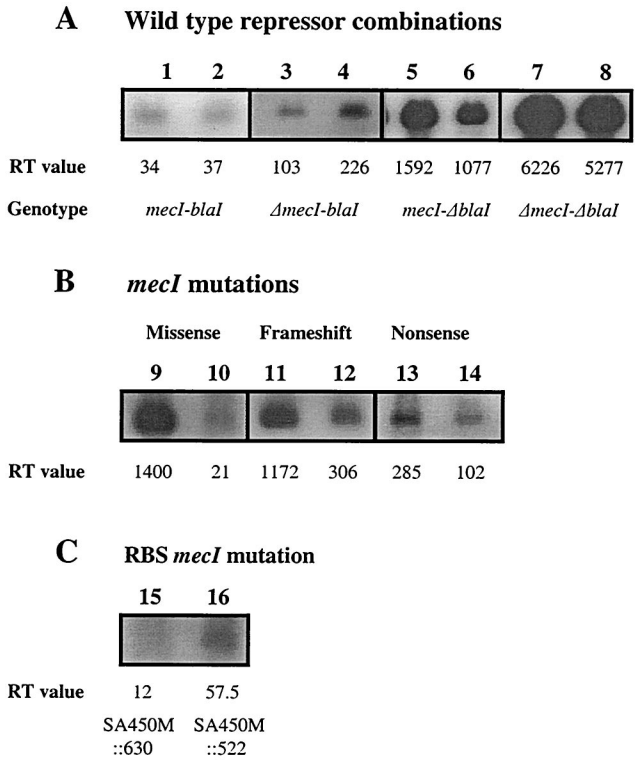

FIG. 3. Representative Northern blot of *mecA* transcription in *S. aureus* isolates. (A) Isolates with wild-type repressor combinations: both repressors together (*mecI-bla1*), SA3 (lane 1) and SA8 (lane 2); each repressor without the other (Δ*mecI-blaI*), SA79 (lane 3) and SA37 (lane 4), (*mecI-*Δ*blaI*), SA92 (lane 5) and SA85 (lane 6); both repressors absent (Δ*mecI-*Δ*blaI*), SA450M (lane 7) and SAN315PΔI (lane 8). (B) *mecI* mutations: missense mutations, SA36 (lane 9) and SA46 (lane 10); frameshift mutations, SA13 (lane 11) and SA10 (lane 12); nonsense mutations, SA35 (lane 13) and SA16 (lane 14). (C) *mecI* with ribosome binding site (RBS) sequence GGAG [SA 450M::630 (lane 15)] and GGAA [SA450M::522 (lane 16)]. All images were acquired from their original gels by using the FluorChem imaging system (Alpha Innotech) and FluorChem version 2.0 software. Labeling was added using Corel Photo-Paint software, version 8.

DISCUSSION

The present study illustrates the importance of studying clinical bacterial isolates as support for observations made using defined laboratory constructs. This is particularly true when assessing gene regulation and regulatory networks that affect antibiotic resistance. The pressure of exposure to antibiotics in hospitals may alter gene expression in ways that cannot be predicted using defined laboratory strains. Oxacillin-resistant staphylococci must be able to express high-level resistance to β-lactam antibiotics in order to survive in hospital environments where β-lactam use is high. Many staphylococci express resistance in a low-level or heterotypic manner and switch to homotypic or high-level-resistance expression following β-lactam exposure (7). This switch requires an increase in *mecA* transcription mediated by induction through MecR1 and/or BlaR1. Induction involves cleavage of the cognate repressor

1.) TTTTAAAAGAAATGGAGTTAATA**TAATG**GAT

2.) TTTTAAAAGAAATGGAATTAATA**TAATG**GAT

FIG. 4. Nucleotide sequence of the ribosomal binding site (RBS) for the *mecI* repressor genes of *S. aureus* N315 (sequence 1, top) and *S. aureus* BMS1 (sequence 2, bottom). These sequences are representative of identical sequence variants seen among clinical isolates. The RBS sequence is boxed. The ATG start codon of the *mecI* gene is in bold and italics, while the overlapping TAA translational stop codon of the preceding *mecR1* gene is in bold and underlined.

(MecI for MecR1 and BlaI for BlaR1) and is specific for each inducer's homologous repressor (15, 20). However, relief of MecI-mediated repression by induction through MecR1 is slow, taking hours, and incomplete, never achieving a level of *mecA* transcription seen when all repressors are missing or inactivated. In contrast, relief of BlaI-mediated repression by induction through BlaR1 is rapid, taking minutes, and complete (15). Thus, when MecI represses *mecA* transcription, the staphylococcal cell is at a clinical disadvantage when exposed to β-lactam antibiotics, unable to respond rapidly. As we have shown in this study, in some clinical isolates MecI is inactivated or modified by deletion or mutation, leaving *mecA* regulated only by BlaI and BlaR1. However, 37% of the clinical isolates that we examined in an earlier study (17) contained *mecI* genes and *mec* promoter-operator target sequences that were identical to those shown in laboratory constructs to provide full *mecA* repression.

In the present study, when we examined baseline, uninduced repression of *mecA* in clinical isolates that contained only *mecI* with wild-type DNA sequence, *mecA* transcription was from 6- to 131-fold greater than that seen with a well-studied comparator strain (N315P) and a laboratory construct (450 M::630). A single nucleotide difference in the *mecI* RBS (GGAA versus GGAG) (Fig. 4) between clinical isolates and laboratory strains was shown to be responsible for some of the difference in *mecA* transcription, increasing it from 7.3- to 8.2-fold in isogenic constructs. However, the magnitude of increase in *mecA* transcription due to *mecI* RBS mutations does not completely explain the 26- to >100-fold increases in transcription seen in some clinical isolates compared to laboratory constructs. It is unlikely that changes in MecR1 account for these differences, since the *mecR1* sequence was wild type in four of these isolates. It is likely, therefore, that uninduced transcription of *mecA* can be greatly increased by mechanisms that do not involve mutations in *mecI*, its RBS, or its promoter-operator target. Additional genes outside of the regulatory operon may be involved in repression and induction, as proposed initially by Cohen and Sweeney (4) and as recently documented for beta-lactamase induction in *Bacillus* sp. (6). Identification of accessory genes and gene products involved in repression and induction of *mecA* transcription will be required for a full understanding of this regulatory network.

One observation made with laboratory constructs was confirmed in this study. In a previous study, members of our group had observed that there appeared to be an additive or synergistic interaction between BlaI and MecI, and the two repressors were shown to form heterodimers in the yeast two-hybrid assay (15). In the present study, in clinical isolates containing both wild-type *mecI* and *blaI*, *mecA* transcription was at a lower level than in isolates containing either one alone. This occurred despite the apparent reduced effect of wild-type *mecI* alone. The additive activity of the two repressors may have been due to direct interactions between the proteins at the *mec* promoter-operator. We demonstrated in a previous study (15) that increasing either MecI oligomerization or *mecI* gene dosage increased *mecA* transcription repression. The addition of BlaI to MecI could effectively increase the amount of available repressor protein and the number of protein-protein interactions.

A major goal of the present study was to assess the effect that the various *mecI* mutations identified in clinical isolates had on *mecA* transcription as an aid to structure-function analysis of the repressor molecule. This goal was made difficult by the presence of corepressors, MecI and BlaI, in most of the isolates in which *mecI* was mutant and by the possible contribution, noted above, of additional unknown genes involved in repressor activity. However, evaluation of isolates with wild-type repressor sequence as comparators for isolates with mutations enabled us to make some observations about the effect of mutations on *mecI* function. First, nonsense and frameshift mutations were predicted to truncate the protein at positions that would have removed the carboxyl terminus. The cleavage site that inactivates the repressor following induction, presumably by preventing dimerization, is 22 amino acids from the carboxyl terminus in BlaI (19) and in the same relative position in MecI (G. Archer and M. Bosilevac, unpublished observations) (Fig. 2). Thus, all of the nonsense and frameshift mutations were predicted to inactivate repressor activity. The *mecA* transcription values for these isolates were similar to those for isolates in which BlaI alone regulated transcription, suggesting that MecI function was absent.

Second, we found four *blaI* missense mutations in a single isolate containing this repressor alone. The value for *mecA* transcription for this isolate (431) was slightly higher than the highest value in isolates containing *blaI* alone with wild-type sequence (346) but more than 10-fold below the value of isolates with no repressor (mean, 6,302). This suggests that these mutations did not markedly affect repressor function. The mutations (Y37N, S63Y, E64N, and N72I) were all at amino acids that were nonconserved between MecI and BlaI, two proteins that have 68% amino acid identity overall (Fig. 2). One mutation changed the amino acid from the one found in BlaI to its counterpart in MecI (E64N). It is reasonable to assume that the areas of nonidentity between the two proteins define parts of structure that are either nonessential for repressor function or that can be highly polymorphic and still not abolish activity.

Third, we examined *mecA* transcription in isolates that had eight different *mecI* missense mutations. Six of the mutations changed amino acids that were conserved between MecI and BlaI. The mutations at six nucleotides, changing five amino acids and one stop codon (A11V, I7V, D39G, P42L, L48,F and stop124K) in seven isolates, had *mecA* transcription values that were <70, not significantly different from values seen in a subset of isolates with two wild-type repressors. This suggests that these mutations left much of MecI repressor activity intact. Three mutations replaced amino acids conserved between BlaI and MecI, and although two (A11V and I7V) were in the amino terminus of the protein in an area without identified

function, the other (L48F) was in a region that is predicted to be the helix-turn-helix conformation that mediates DNA binding. Conservation of function in this case may be more related to the nature of the amino acid substitution than to its location. The other three mutations either were at amino acids that were different between MecI and BlaI (D39G and P42L) (Fig. 2) or extended the protein by 19 amino acids by mutating the translational stop. One of these mutations has been studied previously (D39G) (18). Examination of purified protein and isogenic constructs containing this mutation showed that the mutation reduced repressor activity sixfold. However, transcription was still repressed sevenfold over that seen in the absence of repressor. The clinical isolate in which this *mecI* mutation was found also contained *blaI*. The RT-PCR transcription values provide evidence that MecI-BlaI additive repression can take place even when one of the proteins has a mutation that reduces its activity.

Three final isolates had two *mecI* mutations that markedly altered transcriptional repression. Both mutations substituted amino acids that were conserved between MecI and BlaI, one (R52I) in the DNA binding domain and the other (L116S) in the carboxyl terminus, a region thought to be critical for dimerization (9). It is interesting that a nonsense mutation at the preceding amino acid (E115stop) in another clinical isolate altered *mecA* transcription to the same degree as the L116S missense mutation in one of two isolates with the same mutation (transcription values of 226 versus 201 and 194, respectively). These data support the functional importance of the carboxyl terminus of the protein and suggest that the L116S substitution has a major effect on repressor activity.

The structure-function clues gleaned from the analysis of repressor mutations in clinical isolates will have to await data from the crystal structure for confirmation of suggestions outlined above. However, the data should help the analysis of crystals and provide sites at which the molecules could be modified for more detailed structure-function analysis.

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