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Role of *mecA* Transcriptional Regulation in the Phenotypic Expression of Methicillin Resistance in *Staphylococcus aureus*

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The gene required for methicillin resistance in staphylococci, *mecA*, encodes the low-affinity penicillin-binding protein 2a (PBP2a). Transcriptional regulation of *mecA* is accomplished in some isolates by *mecR1* and *mecI*, cotranscribed chromosomal genes that encode a putative signal transducer and a transcriptional repressor, respectively. Two *Staphylococcus aureus* strains that have identical *mecR1-mecI* nucleotide sequences, BMS1 and N315P, both exhibit low-level, heterotypic expression of methicillin resistance and contain no β-lactamase coregulatory sequences. *mecR1-mecI* was amplified from BMS1 by PCR and was shown to be functional on a high-copy-number plasmid when introduced into an *S. aureus* strain with a deleted *mecR1-mecI* locus. Cloned *mecR1-mecI* repressed phenotypic expression of methicillin resistance, *mecA* transcription and PBP2a production and mediated PBP2a induction in response to certain β-lactam antibiotics. However, *mecR1-mecI* had different regulatory activities in its native chromosomal location in N315P compared with those in BMS1. Uninduced *mecA* transcription was markedly repressed in N315P, and *mecI* inactivation increased *mecA* transcription and PBP2a production 5- and 40-fold, respectively. Furthermore, the N315P phenotype changed from low-level, heterotypic resistance with intact *mecI* to high-level, homotypic resistance in strains with disrupted *mecI*. In contrast, uninduced BMS1 produced abundant *mecA* transcript and PBP2a, while the disruption of *mecI* had no effect on phenotype and little effect on *mecA* transcription or PBP2a production. Thus, *mecI*-mediated repression of *mecA* appears to be dysfunctional in BMS1 because of the presence or absence of additional regulatory cofactors. Furthermore, heterotypic resistance expression in this strain is independent of *mecA* transcriptional regulation.

Methicillin resistance, which encompasses resistance to all beta-lactam compounds, is a common phenotype among multiresistant, nosocomial staphylococci. Methicillin-resistant isolates are not effectively treated by most antibacterial agents and are a major challenge for chemotherapy. A low-affinity penicillin-binding protein (PBP), PBP2a, encoded by the chromosomally located gene *mecA*, mediates methicillin resistance among both *Staphylococcus aureus* and coagulase-negative staphylococci (13, 30, 44). PBP2a is expressed in addition to the normal complement of resident PBPs and remains the sole unsaturated PBP in the presence of beta-lactam antibiotics, alone capable of maintaining cell wall integrity. The *mecA* gene and ≥30 kb of flanking DNA are unique to methicillin resistant staphylococci; there are no allelic equivalents in methicillin-susceptible strains (2, 8, 23).

Two methicillin resistance phenotypes are discernible by efficiency of plating (EOP) studies: homotypic and heterotypic (6, 15, 32). Homotypic (homogeneous) strains uniformly express high-level resistance, whereas heterotypic (heterogeneous) organisms exhibit a strain-unique variation in resistance expression. In heterotypic isolates, highly resistant subpopulations are present but they are a small proportion of the largely susceptible majority. However, under the selective pressure of antibiotics, the more resistant minority subpopulation predominates, providing clinical resistance. The genes that regulate or affect heterotypic resistance expression are not known, and it is not clear what role the regulation of *mecA* transcription plays in this process. Several sequences that regulate *mecA* transcription have been described. These include the beta-lactamase regulatory sequences, *blaR1-blaI*, and the analogous and partially homologous chromosomal sequences, *mecR1-mecI*. *mecR1* and *mecI* are located immediately 5′ to *mecA* and are divergently transcribed from it (23). MecR1 is predicted to be a transmembrane signal transducer protein and MecI is predicted to be a repressor, on the basis of amino acid sequence similarities of gene products that regulate beta-lactamase production in both *S. aureus* and *Bacillus licheniformis* (6, 24, 37, 42).

BMS1 is a methicillin-resistant *S. aureus* (MRSA) strain that displays a low-level, heterotypic methicillin resistance phenotype and appears clinically susceptible to methicillin (3). We have previously analyzed the BMS1 *mecR1-mecI* regulatory region and found it to have complete nucleotide sequence identity with the same region of N315, a Japanese MRSA strain also exhibiting susceptibility to methicillin (3, 24). N315 contains a beta-lactamase plasmid with *blaR1-blaI* regulatory sequences as well as *mecR1-mecI*. However, when the beta-lactamase plasmid was cured to produce N315P, the strain was still low-level resistant and slowly inducible, showing an increase in MIC and PBP2a production upon exposure to certain beta-lactam antibiotics (24). Thus, *mecR1-mecI* was proposed to be responsible for *mecA* expression regulation and the low-level-resistance phenotype. Contrary to this hypothesis, however, we noted a high basal PBP2a production level in BMS1

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype[a]	Remarks and references
E. coli strains		
TB1	*recA lacI*^{q}ZΔM15	Host for *lacZ*-containing cloning vectors (9)
JM109	*recA1 endA1 gyr96 thi-hsdR17* (s^- m^{k+}) *supE44 relA1* α-(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ZΔM15]	Recombination deficient host (18) for *lacZ*-containing cloning vectors
S. aureus strains		
RN450	Cm^s Em^s Gm^s Tc^s Bla^-	ATCC 8325-4
RN4220	Restriction-deficient mutagenized RN450	Shuttle plasmid host (39)
450M	Cm^s Em^s Gm^s Mc^r (HE) Tc^s Bla^-	450 transformed with COL *mec region DNA (3)*
COL	Cm^s Em^s Gm^s Mc^r (HO) Tc^r Bla^-	*mecR1* truncated, *mecI* deleted (3)
BMS1	Cm^s Em^r Gm^s Mc^r (HE) Tc^s Bla^-	Intact *mecR1-mecI* (3); clinical isolate from New Jersey, 1988
N315	Cm^s Em^s Gm^s Mc^r (HE) Tc^s Bla^+	Intact *mecR1-mecI* (24); clinical isolate from Japan, 1982
N315P	Cm^s Em^s Gm^s Mc^r (HO) Tc^s Bla^-	N315 cured of its beta-lactamase plasmid (23)
BMS1 *mecI* mutant	Mc^r Tc^r (HE) Bla^- *mecI* interrupted	This study
N315P *mecI* mutant	Mc^r Tc^r (HO) Bla^- *mecI* interrupted	This study
E. coli plasmids		
pUC19, pUC9	Ap^r; 2.7 kb	Cloning vectors (9, 47)
pBR322	Ap^r Tc^r; 4.3 kb	Cloning vector (10, 31)
S. aureus plasmids		
pE194ts	Em^r ts; 3.0 kb	Staphylococcal temperature-sensitive replicon (26, 38)
pRN5542	Cm^r; 3.0 kb	pSK265 with *Hin*dIII site in MCS deleted; derivative of pC194 (39)
pG0648	Ap^r; 5.1 kb	*mecR1-mecI* blunt-end ligated at pUC9 *Hin*cII site (this study)
pG01	Gm^r Tp^r Qam^r Tra^+; 52 kb	Conjugative plasmid from *S. aureus* (31)
pG0164	Ap^r; 3.8 kb	1.1-kb intragenic *mecA* fragment ligated into pUC18 (3)
pG0195	Ap^r; 5.2 kb	2.36-kb *Hin*dIII BMS1 fragment containing 641 bp of *mecR1*, all of *mecI*, and downstream DNA (3)
pSK950	Sp^r Tet^r Em^r ts; 10.4 kb	*E. coli-S. aureus* shuttle vector for integration of sequences into the *S. aureus* lipase gene with the ϕL54a *att* site (28); original pCL84 plasmid was modified by addition of pE194ts at the *Pst*I site
pG0521	Sp^r Tet^r Em^r ts; 12.9 kb	*mecR1-mecI* ligated into pSK950 (this study)
pG0514	Ap^r Em^r Gm^r Tc^r ts *mecI* mutant; 13.1 kb	Plasmid for recombinational inactivation of *mecI* (this study) (Fig. 5)
pG0600	Ap^r Cm^r; 8.1 kb	pGO648 with pRN5543 ligated at the *Eco*RI site (this study)

[a] Abbreviations: Ap, ampicillin; Bla, beta-lactamase; Cm, chloramphenicol; Em, erythromycin; Gm, gentamicin; HE, heterotypic; HO, homotypic; Mc, methicillin; MCS, multiple cloning site; Qam, quaternary ammonium compound; Sp, spectonomycin; Tc, tetracycline; tra, conjugative transfer; ts, temperature sensitive. Definitions of superscripts: −, negative; +, positive; r, resistant; s, sensitive.

without induction despite an apparently intact *mecR1-mecI* region.

In order to resolve this discrepancy, we sought to determine the effect of the *mecR1-mecI* gene products on *mecA* transcriptional regulation in several different *S. aureus* strains as well as the strain-specific role of transcriptional regulation in determining methicillin resistance expression. For this analysis, we compared phenotypic expression, *mecA* transcription, and PBP2a production in four *S. aureus* strains with both intact and disrupted *mecR1-mecI* regulatory sequences. The results of this investigation are contained in the present report.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *S. aureus* and *Escherichia coli* strains used in this study are listed in Table 1. Recombinant plasmids were generated and maintained in *E. coli* TB1 (9) or JM109 (18). The *E. coli* cloning vectors used were pUC9 or pUC19 (9, 47) and pBR322 (10). *E. coli-S. aureus* shuttle vectors were constructed by adding pRN5542 (39) or pE194ts (Table 1) (26, 39) to the *E. coli* vectors. The staphylococcal tetracycline and gentamicin resistance genes used in cloning selections in *E. coli* or *S. aureus* were *tetM* (40) or *aac*/*aph* (36), respectively. The plasmids were first introduced into the restriction-deficient strain *S. aureus* RN4220 by electroporation, were harvested, and then were used to transform other *S. aureus* strains by either electroporation (29, 38) or transduction (27, 34). N315 and N315P were kind gifts of Keichi Hiramatsu.

Materials and media. Mueller-Hinton agar (MHA) (BBL Microbiology Systems, Cockeysville, Md.) with and without selective additives (Sigma, St. Louis, Mo., and United States Biochemicals, Cleveland, Ohio) was used for the subculture and maintenance of *E. coli* and *S. aureus* strains and transformants. Mueller-Hinton broth, tryptic soy broth (BBL Microbiology Systems), or brain heart infusion broth (Difco Laboratories, Detroit, Mich.) with additives were used for the culture of *E. coli* and *S. aureus* strains. The antibiotics (and concentrations) used for *E. coli* strains for initial selection after electroporation were ampicillin (20 or 50 μg/ml), gentamicin (5 μg/ml), and tetracycline (5 μg/ml). Other antibiotics (and concentrations) used for the counterselection and maintenance of *E. coli* strains were chloramphenicol (20 or 40 μg/ml) and erythromycin (200 or 500 μg/ml). The antibiotics (and concentrations) used for *S. aureus* strains for initial selection after electroporation were chloramphenicol (10 μg/ml), erythromycin (10 μg/ml), and tetracycline (3 or 5 μg/ml). Other antibiotics (and concentrations) used for the maintenance of *S. aureus* transformants were chloramphenicol (20 and 40 μg/ml) and gentamicin (5 μg/ml). Other selective additives such as sodium citrate (8 mM) (for transductions) or β-D-galactopyranoside (X-Gal) (20 mg/ml) were added or applied to the media as required.

Cloning, transformation, and DNA manipulation. All restriction endonuclease digestions and ligation reactions were performed per the manufacturers' specifications. All *E. coli* transformations were performed by electroporation using 2-mm cuvettes and the Bio-Rad (Richmond, Calif.) Gene Pulser as previously described (31). Staphylococcal transformations were performed by electroporation with the preparation of electrocompetent cells performed as described either by Luchansky et al. (29) or by Schenk and Laddaga (38). Transduction was performed with staphylococcal phage 80α and by incorporating sodium citrate in the medium in accordance with methods described by de Lencastre and Tomasz (16), Kasatiya and Baldwin (27), and Novick (34).

The quick-check method described by Akada (1) was used for initial screening of *E. coli* plasmid DNA. The alkaline lysis mini-prep described by Hattori and Sakaki (22) was used to obtain plasmid DNA suitable for restriction digestion, ligation, or electroporation. Isolation of plasmid DNA from *S. aureus* was ac-

complished by either the hexadecyltrimethylammonium bromide extraction method of Morton et al. or Townsend et al. (31, 43) or by a modification of the quick-check method using 25 μl of lysostaphin (1 mg/ml) and 10% sodium dodecyl sulfate (SDS) to achieve bacterial cell lysis (1). All plasmid DNA prepared for restriction digestion or PCR amplification was treated with RNase A (10 mg/ml). Polyethylene glycol (20%) was used to purify all DNA samples, either plasmid or genomic, prior to PCR amplification or DNA sequencing (22). Staphylococcal genomic digests as described by Archer et al. (3) were performed to obtain DNA as a target for hybridization to confirm allelic replacement mutagenesis constructs. All chemicals and reagents used in DNA preparation and resolution procedures were obtained from either Sigma or United States Biochemicals.

DNA electroeluted from agarose gels for cloning or radiolabelling to probe Northern (RNA) blots was recovered with an analytical electroeluter (IBI, New Haven, Conn.). Electroelution was carried out for 1 h at 100 V after addition of bromphenol blue dye with 7.5 M ammonium acetate and 1× Tris acetate-EDTA buffer. The DNA sample was washed successively in 95 and then 70% ethanol, dried, and dissolved in either Tris-EDTA buffer or distilled H_2O.

Plasmid curing and allelic replacement. Staphylococcal strains harboring plasmid constructs with the temperature-sensitive replicon pE194ts, were cured of their plasmids in order to detect allelic replacement of chromosomal genes by homologous recombination. Single colonies were inoculated to 10 ml of Mueller-Hinton broth and grown overnight at 30°C with antibiotic selection (tetracycline [3 μg/ml]). A 100-μl sample of this culture was transferred to 10 ml of fresh broth and grown overnight at 42°C without antibiotic selection; the curing procedure was repeated the following day. Diluted cell suspensions were then plated to MHA and MHA containing gentamicin (5 μg/ml) or tetracycline (5 μg/ml). The efficiency of curing was determined by comparing the resistant and total cell counts (39). Cells were sought that were tetracycline resistant and gentamicin susceptible, indicating chromosomal integration into the replacement locus and secondary recombination, removing integrated plasmid sequences.

EOP. Phenotypic expression of methicillin resistance was determined by following the EOP procedure as described by Hackbarth et al. (20) with minor changes. *S. aureus* strains were inoculated onto Mueller-Hinton broth containing appropriate antibiotics and incubated overnight at 37°C with constant shaking. The strains were then quantitatively plated to MHA containing 0, 5, 10, 20, 50, 100, 250, 400, 800, or 1,000 μg of methicillin per ml. The plates were incubated at 30°C for 72 h, after which time CFU were tabulated and plotted ($\log_{10}$ CFU per milliliter) versus drug concentration as previously described (15, 20).

Southern blot analysis (DNA-DNA hybridization). Alkaline (capillary) transfer, fixation of DNA to nylon membrane (Zeta-Probe), and hybridization were performed per the directions of the manufacturer (Bio-Rad) and as previously described (4).

Northern blot analysis (DNA-RNA hybridization). Staphylococcal strains grown overnight on MHA plates containing appropriate antibiotics for plasmid maintenance were resuspended in water treated with pyronocarbonic acid diethyl ester at 7×10^9 cells per ml and pelleted by centrifugation. Pellets were resuspended in 100 μl of lysis buffer (0.020 M Tris-HCl, pH 7.6; 0.01 M Na_2-EDTA; 0.05 M NaCl; 20% sucrose; 0.1% lysostaphin) and were incubated at room temperature for 15 min. Total cellular RNA was isolated from treated cells by using the Rneasy Kit (Qiagen Inc., Chatsworth, Calif.) and by following the accompanying instructions. About 2.5 μg of RNA was separated by resolution through formaldehyde-containing 1% agarose and transferred to a nylon membrane (neutral charge; Qiabrane; Qiagen Inc.) in 20× SSC (1× SSC is 0.15 NaCl plus 0.015 M sodium citrate) at room temperature overnight. RNA was immobilized on the membrane by UV irradiation (UV Stratalinker; Stratagene, La Jolla, Calif.) and prehybridized in a solution containing 1× Denhardt's solution, 50% formamide, 5× SSC, 0.05 M $NaPO_4$ (pH 6.5), and 100 μg of denatured salmon sperm DNA per ml for 2 h at 57°C. The DNA probe (1.2-kb intragenic *Bgl*II-*Xba*I fragment of the *mecA* gene from *S. aureus* COL) was radiolabelled by incorporating [α-^{32}P]dCTP to 2×10^6 cpm and added to the prehybridization solution; incubation was continued at 57°C overnight. Following hybridization, the blots were washed and exposed to X-ray film at −70°C. Autoradiographs were subjected to scanning laser densitometry (Ultrascan XL; LKB, Bromma, Sweden) to quantitate mRNA.

PCR amplification. PCR amplification of DNA sequences was performed to generate *mec* region fragments for ligations and to assist in the verification of transformants by checking fragment sizes and nucleotide sequence. The primers were synthesized and purified either by Oligos, Etc., Inc. (Wilsonville, Oreg.), or by the Medical College of Virginia Nucleic Acid Synthesis and Analysis Core Facility (Richmond, Va.). The primers generated were complimentary to either end of the published sequences accessible in GenBank (3). $Vent_R$ polymerase (New England Biolabs, Beverly, Mass.) was used, and the recommended thermocycling conditions were followed according to the manufacturer's directions. The primers used, the nature of the amplified DNA, and the size of the resulting PCR fragment were as follows. Amplification of the 2.4-kb *mecR1-mecI* fragment from BMS1 plus 56 bp of *mecA* was performed with the *mecA* primer (5′)CCA AACCCGACAACTAC(3′) and the *mecI* downstream primer (5′)GCATATGG ATTTCACTGGTG(3′). Amplification of the 1.9-kb *mecR1-mecI* fragment for allelic replacement mutagenesis was performed with the *mecR1* primer (plus the 5′ *Eco*RI site) (5′)CGGAATTCGATGGACAATGACTGTG(3′) and the *mecI* downstream primer (plus the 5′ *Bam*HI site) (5′)CGGGATCCGCGAAATAT TATGATGG(3′). Amplification of the 421-bp *mecI* fragment as a probe for Northern blot analysis was performed with the forward *mecI* primer (5′)AATA GAATGGATAATAAAACG(3′) and the reverse *mecI* primer (5′)GACTTGA TTGTTTCCTC(3′).

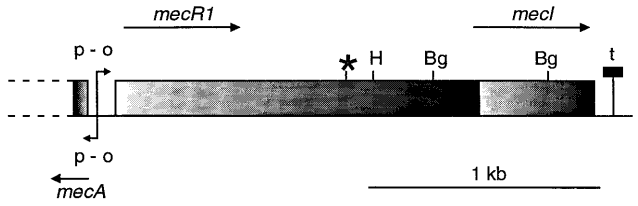

FIG. 1. A schematic diagram of the 2.3-kb *mecR1-mecI* regulator operon. The arrows labelled p-o illustrate the direction of transcription from the *mecA* and *mecR1* promoter-operator sequences. The star is the site of the natural deletion in COL and 450M, eliminating the 3′ portion of *mecR1* that contains the beta-lactam binding domain and all of *mecI*. H, *Hin*dIII; Bg, *Bgl*II; t, putative rho-independent terminator.

Sequence analysis. DNA sequencing was performed to confirm that ligated *mec* DNA contained no mutations; *mec* DNA sequence results were compared with published sequences (3). Sequencing was performed by the Sanger dideoxynucleotide chain termination method as previously described (3) or by the automated laser fluorescence technique employing fluorescein-labelled oligonucleotides (Applied Biosystems and the Medical College of Virginia Nucleic Acid Synthesis and Analysis Core Facility).

PBP analysis. *S. aureus* strains were grown to mid-logarithmic phase in brain heart infusion broth. The cells were pelleted and mechanically disrupted with glass beads (Bead-Beater; BioSpec Products, Bartlesville, Okla.). The beads were separated from the broken cells on a scintered glass filter, and the membranes were collected by ultracentrifugation at 155,000 × *g* for 30 min at 4°C and resuspended in a final 0.5-ml volume of buffer (10 mM sodium phosphate, pH 7.0). Membrane protein samples were adjusted to 100 μg of protein in a 50-μl volume of sodium phosphate buffer. [^{3}H]benzylpenicillin (27.2 Ci/mmol; DuPont NEN, Boston, Mass.) was added at 37°C and allowed to incubate for 15 min. Subsequently, 25 μl of SDS sample buffer with 2-mercaptoethanol was added and the samples were boiled for 5 min at 100°C. The entire samples were loaded into gel lanes, and the proteins were resolved by SDS-polyacrylamide gel electrophoresis (10% acrylamide, 0.08% bisacrylamide) using a long gel bed (20 cm) at a constant current of 10 mA at 4°C for 17 h to maximize resolution. Following electrophoresis, the gels were fixed, stained, and prepared for fluorography with En^{3}Hance fluor (DuPont NEN) according to the manufacturer's instructions. After drying (Bio-Rad model 583 gel dryer), the gel was exposed to prefogged X-ray film (Kodak X-Omat-AR; Kodak, Rochester, N.Y.) for 1 to 7 days at −80°C to reveal the positions of the radiolabelled PBPs. Quantitative PBP2a binding of [^{3}H]benzylpenicillin was determined by scanning laser densitometry (Ultrascan; LKB) of bands on the films to generate areas under the peak corresponding to PBP2a.

RESULTS

Analysis of cloned *mecR1-mecI*. The *mecR1-mecI* region was amplified from *S. aureus* BMS1 by PCR using a 5′ primer 56 bp into the *mecA* gene and a 3′ primer 156 bp beyond the *mecI* stop codon. This 2-kb fragment included the divergent *mecA* and *mecR1-mecI* promoters, the putative operator sequences (23, 24), and a candidate rho-independent terminator downstream of *mecI* (Fig. 1). We first tried to assess the functional status of BMS1 *mecR1-mecI* sequences by integrating them into the chromosome of *S. aureus* 450M. 450M was created by transforming *mec* region DNA from the methicillin-resistant strain COL into the methicillin-susceptible laboratory strain RN450 (3). Both COL and 450M have a natural deletion of *mecI* and the 3′ portion of *mecR1* (Fig. 1; also reference 3). *mecR1-mecI* sequences were integrated into the 450M chromosomal lipase gene by ligating them into an integration vector, pSK950, to produce pG0521. pSK950 is a modification of pCL84, described by Lee et al. (28) (Table 1), and was engineered and provided by Saleem Khan. Integration was achieved by growing pG0521 at the nonpermissive temperature for plasmid replication in the presence of integrase, provided in *trans* on a separate plasmid (28). The authenticity of the

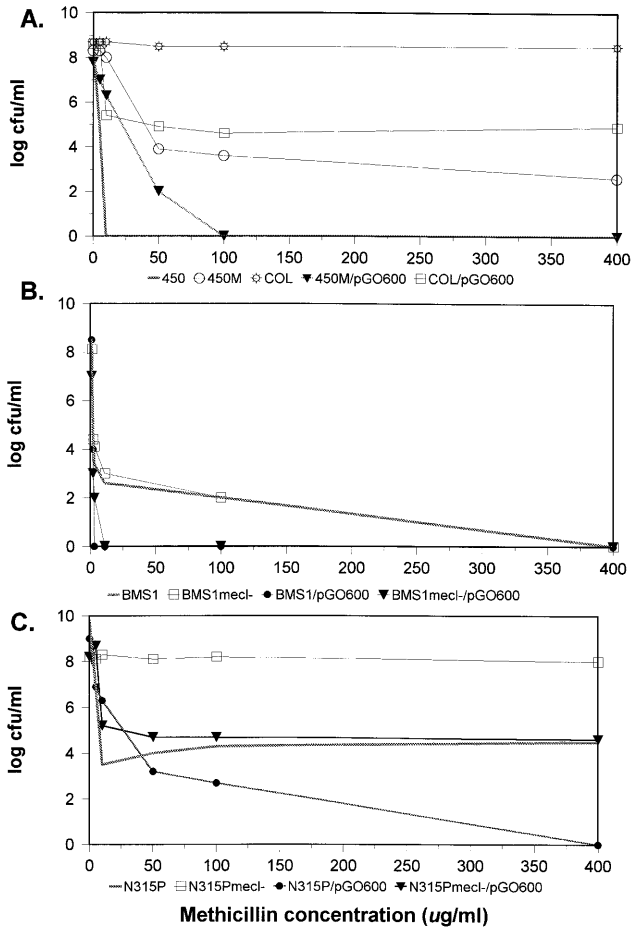

FIG. 2. EOP curves. Shown are the numbers of *S. aureus* cells (in $\log_{10}$ CFU per milliliter; shown on the *y* axis) remaining on the plates containing varied concentrations of methicillin (in micrograms per milliliter; shown on the *x* axis). (A) EOP results with 450M and COL, with or without the plasmid containing *mecR1-mecI* (pG0600); (B) results with BMS1, BMS1 with *mecI* interrupted by allelic replacement with *tetM* (BMS1mecI−), and both strains with pG0600; (C) same manipulations as for panel B, but with N315P.

integrated sequence was confirmed by DNA sequence analysis of the PCR product from 450M genomic DNA. However, there was no *trans*-active effect of these sequences in single copy, integrated at some distance from their *mecA* target, on 450M phenotype, *mecA* transcription, or PBP2a production. Therefore, we felt that the activity of *mecR1-mecI* sequences should be assessed by their inclusion on a high-copy-number plasmid. The *mecR1-mecI* fragment was blunt-end ligated into the pUC9 *Hinc*II site to create pG0648. A shuttle vector was then constructed by ligating the staphylococcal replicon pRN5542 into the pG0648 *Eco*RI site to create pG0600. Because of the high copy number of pRN5542 (Table 1; also references 35 and 39), pG0600 provided *mecR1-mecI* in high copy in *S. aureus*.

The functional status of the genes on pG0600 in *S. aureus* was examined by introducing the plasmid into 450M and COL. COL is high-level, homotypically methicillin resistant, while 450M is low-level, heterotypically methicillin resistant. The methicillin resistance phenotypes of COL and 450M were determined by EOP and compared with those of their isogenic transformants containing pG0600. As seen in Fig. 2A, *mecR1-mecI* on pG0600 converted COL from homotypic to heterotypic resistance and reduced the plating efficiency of 450M by ≥ 2 $\log_{10}$ units. In addition, as seen in Fig. 3 and 4 and Table 2, the amount of *mecA* transcript was markedly reduced and PBP2a became undetectable in membranes prepared from these isolates. These data established the functional integrity of MecI and the necessity to provide these regulatory sequences in high copy to achieve *trans*-regulatory effects.

The ability of *mecR1* on pG0600 to induce transcription of *mecI*-repressed *mecA* following exposure to beta-lactam antibiotics was tested in COL/pG0600. MecR1 has been reported to be beta-lactam selective, mediating PBP2a induction in response to some beta-lactams (e.g., cefoxitin) but not others (e.g., methicillin [23]). In contrast, BlaR1, the signal-transducing protein that mediates induction of beta-lactamase, responds to virtually all beta-lactam antibiotics (19). The repression and induction of PBP2a production in a BMS1 *mecI* mutant (see below) and COL were assessed following the exposure of cells containing pG0600 to either methicillin or cefoxitin (Fig. 4 and Table 2). In both strains, pG0600 repressed PBP2a production, confirming *mecI* activity, and mediated PBP2a induction following cefoxitin exposure, confirming the function of *mecR1*. The beta-lactam-selective response of *mecRI* was shown by the failure of PBP2a to be induced following methicillin exposure in cells containing pG0600. There also was a consistent increase in PBP1 and PBP3 in COL/pG0600 following cefoxitin exposure (Fig. 4B, lanes 5 and 6); the significance of this observation is unclear at present.

Comparison of *mecR1-mecI* in BMS1 and N315P. The contribution of *mecI*-mediated repression of *mecA* to the methicillin resistance phenotype in N315P and BMS1, two different clinical isolates with identical *mecR1-mecI* and promoter-operator nucleotide sequences, was examined. N315P is a derivative of N315 from which N315's resident penicillinase-producing plasmid was cured, leaving only the *mecR1-mecI* regulatory sequences (23). BMS1 contains no plasmids and produces no beta-lactamase. As shown in Fig. 2B and C, both strains had a heterotypic, low-level resistance phenotype although N315P grew more colonies on higher concentrations of methicillin than did BMS1. N315P had a small amount of *mecA* transcript and a barely detectable quantity of PBP2a (Fig. 3 and 4). However, uninduced BMS1 produced abundant *mecA* transcript and a large quantity of PBP2a, both nearly equal in amount to those produced by 450M, a strain containing no known transcriptional regulatory sequences.

In order to further define the role of *mecI* in these two

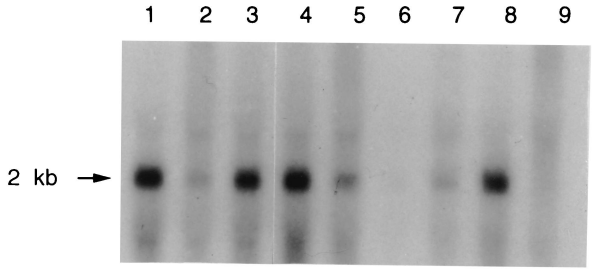

FIG. 3. Northern analysis of *mecA* transcript in *S. aureus* strains. Cells were grown overnight on MHA plates and RNA was isolated as described in Materials and Methods. The ^{32}P-labelled DNA probe was a 1.1-kb intragenic fragment of *mecA* from *S. aureus* COL. The blot was exposed on X-ray film with an intensifying screen for 2 h. The 2-kb *mecA* transcripts from the various strains are shown as follows: lane 1, 450M; lane 2, 450M/pG0600; lane 3, BMS1; lane 4, BMS1 *mecI* mutant; lane 5, BMS1 *mecI* mutant containing pG0600; lane 6, 4220 (a *mecA*-negative control); lane 7, N315P; lane 8, N315P *mecI* mutant; and lane 9, N315P *mecI* mutant containing pG0600.

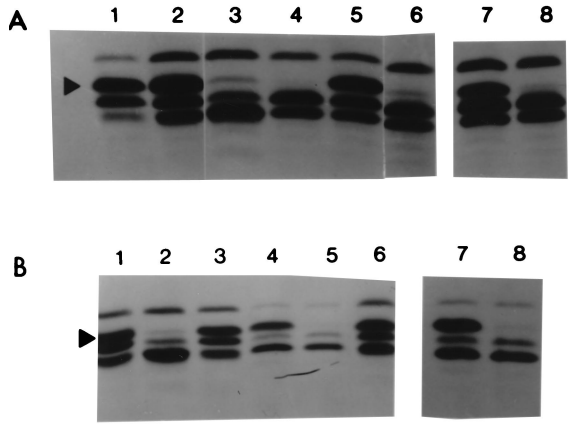

FIG. 4. PBP profiles. (A) Effect of *mecI* inactivation and/or *mecR1-mecI* present on a multicopy plasmid (pG0600) on PBP2a production. Lanes: 1, BMS1; 2, BMS1 *mecI* mutant; 3, BMS1 *mecI* mutant containing pG0600; 4, N315P; 5, N315P mecI mutant; 6, N315P *mecI* mutant containing pG0600; 7, 450M; 8, 450M/pG0600. (B) Effects of the addition of potential beta-lactam inducers to BMS1 and COL derivatives. Lanes: 1, BMS1; 2, BMS1 *mecI* mutant carrying pG0600; 3, BMS1 *mecI* mutant carrying pG0600 induced with cefoxitin (0.5 μg/ml); 4, COL; 5, COL/pG0600; 6, COL/pG0600 induced with cefoxitin (0.5 μg/ml); 7, COL; 8, COL/pG0600 induced with methicillin (0.5 μg/ml). Arrowheads indicate the position of PBP2a.

strains, the gene was inactivated by allelic replacement mutagenesis. The plasmid constructed to inactivate chromosomal *mecI* sequences, pG0514, is shown in Fig. 5. A 1.9-kb PCR fragment containing 935 bp of *mecR1*, all of *mecI*, and 548 bp of DNA 3′ to *mecI* was ligated into pUC19. A 695-bp internal *Bgl*II fragment containing 323 of the 372 bp of *mecI* was replaced with the *tetM* gene (40). A temperature-sensitive staphylococcal replicon (pE194ts) and the *aac*/*aph* gene encoding gentamicin resistance (36) were also added to the pUC19 core, and the plasmid was then introduced by electroporation into BMS1 and N315P, after passage through the restriction-deficient strain RN4220. The gentamicin resistance gene was added to the construct because both of the clinical isolates were erythromycin resistant (i.e., showed growth on plates containing 10 μg of erythromycin per ml), and gentamicin

TABLE 2. Relative amounts of *mecA* transcript and PBP2a

S. aureus strain/plasmid	*mecA* transcript (%)[a]	PBP2a (%)[a]
BMS1	100	100
BMS1 *mecI* mutant	74	149
BMS1 *mecI* mutant/pG0600	13	11
BMS1 *mecI* mutant/pG0600[b]	ND[c]	133
N315P	14	3
N315P *mecI* mutant	66	119
N315P *mecI* mutant/pG0600	<3	11
COL	ND[d]	102
COL/pG0600	ND[d]	5
COL/pG0600[b]	ND[d]	190
COL/pG0600[e]	ND[d]	5
450M	78	73
450M/pG0600	10	1
RN4220	<3	ND[c]

[a] Percent areas under the peaks of bands on autoradiographs or fluorographs after scanning laser densitometry. Values are given as the averages of at least two determinations and were normalized to BMS1 = 100%.
[b] Cefoxitin induction at 0.5 μg/ml during growth of culture.
[c] Not determined.
[d] Not determined; low levels of mRNA isolated from COL strains.
[e] Methicillin induction at 0.5 μg/ml during growth of culture.

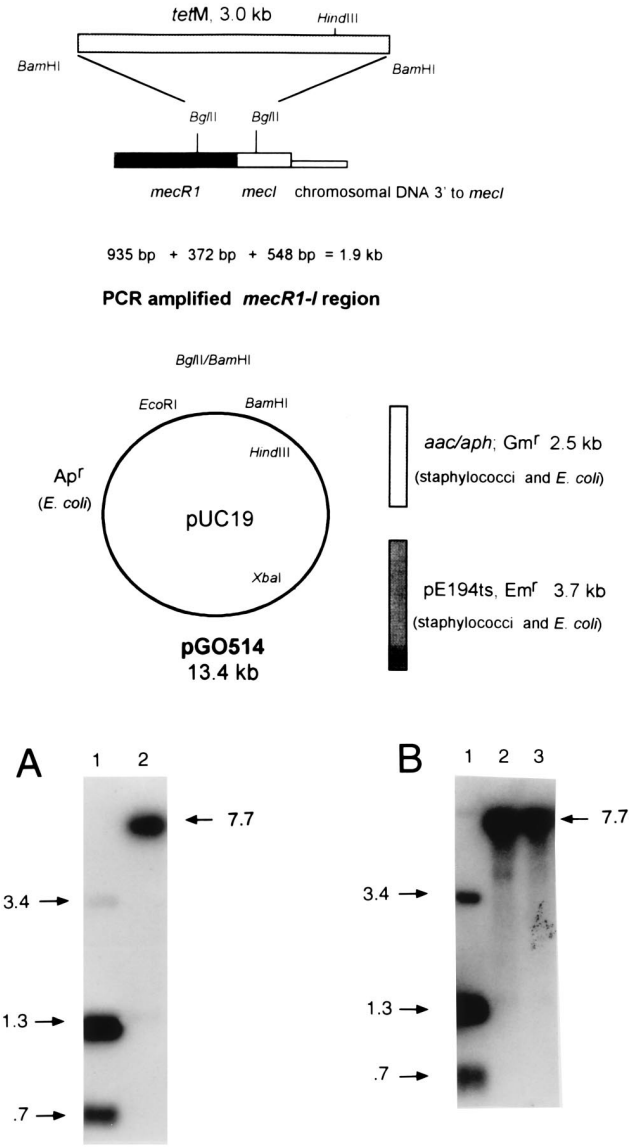

FIG. 5. Diagram showing the construction of pG0514, the plasmid introduced into *S. aureus* strains BMS1 and N315P for allelic replacement mutagenesis of *mecI*. The 2.5-kb *Hin*dIII fragment containing the *aac*/*aph* gene encoding resistance to gentamicin in staphylococci (36) was ligated into the pUC19 *Hin*dIII site. A 1.9-kb fragment that contained the *mecI* gene with both 5′ and 3′ flanking sequences was obtained from BSM1 by PCR amplification. This fragment, containing at its termini *Bam*HI and *Eco*RI sites introduced in the PCR primers, was ligated into the pUC *Eco*RI-*Bam*HI sites. The *tetM* gene, encoding resistance to tetracycline and minocycline in staphylococci (40), obtained as a 3-kb *Bam*HI fragment, was introduced into the 1.9-kb *mecR1-mecI* sequence at a *Bgl*II site that had contained a 695-bp *Bgl*II fragment encoding the terminal sequences of *mecR1* and 323 of the 372 bp of *mecI*. The summing of base pairs below the 1.9-kb fragment indicates the amount of *mecR1* (935 bp), *mecI* (372 bp), and DNA 3′ to *mecI* (548 bp) within the fragment. Finally, the temperature-sensitive staphylococcal replicon encoding erythromycin resistance (26, 39), pE194ts, was ligated into the pUC *XbaI site. Shown at the bottom of the figure are autoradiographs of the Southern hybridization of strains of BMS1 (A) and N315P (B) following integration of pG0514 into mecR1-mecI* by recombination, with the subsequent loss of integrated plasmid DNA by secondary recombination. Total genomic DNA of representative colonies was digested with *Bgl*II and probed with pG0195, which is pUC19 plus a 2.5-kb *Hin*dIII fragment. An intact *mecR1-mecI* region should contain an internal 695-bp fragment, a 3.4-kb 5′ fragment that contains most of *mecA*, and a 1.3-kb 3′ fragment. The appropriate hybridization is seen in lane 1 of both panels A (BMS1) and B (N315P). After allelic replacement, the internal 695-bp *Bgl*II fragment is replaced by the 3-kb *tetM* gene. Since the *Bgl*II sites were removed by the *Bam*HI-*Bgl*II ligation, the resultant *Bgl*II fragment should be 7.7 kb as shown in lane 2 of panel A and lanes 2 and 3 (two colonies from independent curings) of panel B.

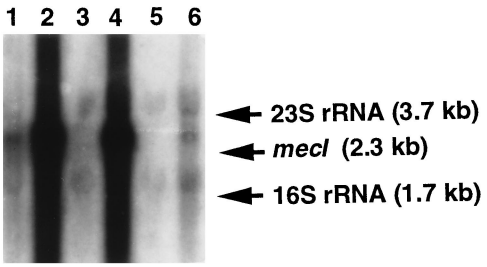

FIG. 6. Northern analysis of *mecR1-mecI* transcripts in *S. aureus* BMS1 and N315P. The ^{32}P-labelled DNA probe was the 421-bp *mecI* gene PCR amplicon. The blot was exposed for 46 h on X-ray film with an intensifying screen. The 2.3-kb transcripts from the various strains are shown as follows: Lane 1, BMS1; lane 2, BMS1/pG0600; lane 3, BMS1 *mecI* mutant; lane 4, BMS1 *mecI* mutant carrying pG0600; lane 5, RN4220; lane 6, N315P. Arrows denote rRNA bands and the *mecR1-mecI* transcript.

susceptibility was used to monitor the loss of plasmid sequences by secondary recombination. Thus, following growth of BMS1/pG0514 and N315P/pG0514 at the nonpermissive temperature for plasmid replication, colonies that were tetracycline resistant and gentamicin susceptible, indicating replacement of *mecI* with the *tetM*-interrupted construct and removal by secondary recombination of both integrated plasmid sequences and duplicated *mecR1-mecI* genes, were sought. Colonies from three independent curings for each strain were screened for the desired phenotype and analyzed by hybridization with pG0195, a plasmid containing cloned *mecR1-mecI* sequences, as previously described (3). Confirmation of *mecI* replacement in both BMS1 and N315P is shown in the autoradiograph in Fig. 5. The methicillin resistance phenotype, *mecA* transcription, and PBP2a production of the *mecI* deletion mutants were analyzed. In contrast to its heterotypic parent, the N315P *mecI* mutant phenotype was homotypic and highly methicillin resistant (Fig. 2C). Consistent with this phenotype, the amount of *mecA* transcript in the *mecI* mutant increased fivefold and the quantity of PBP2a increased 40-fold compared with those of the parent (Fig. 3 and 4; Table 2). The reintroduction of *mecR1-mecI* on pG0600 into the N315P *mecI* mutant restored the heterotypic phenotype and repression of both *mecA* transcription and PBP2a production. The data were much different for the BMS1 *mecI* mutant, however. There was no difference in phenotype between the *mecI* mutant and its parent; both remained low-level resistant and heterotypic in resistance expression. Furthermore, there were no change in *mecA* transcript and only a 50% increase in PBP2a production in the BMS1 *mecI* mutant compared with those of its parent with *mecI* intact. Introduction of pG0600 into the BMS1 *mecI* mutant repressed *mecA* transcription (Table 2 and Fig. 3) and PBP2a production (Fig. 4). However, repression had very little effect on phenotype.

If MecI is defective in repressing the transcription of *mecA* in BMS1 and if, as postulated for BlaI (5, 46), it is autoregulatory, then the *mecR1-mecI* transcript in BMS1 should be more abundant than the corresponding transcript in N315P. In order to assess this hypothesis, a Northern analysis was performed with mRNA from BMS1 and N315P as targets and the *mecI* gene as a probe. As shown in the overexposed autoradiograph in Fig. 6, there was very little *mecR1-mecI* transcript seen in either strain and the BMS1 transcript was only slightly more abundant than N315P. The low abundance of the *mecR1-mecI* transcripts can be best appreciated by comparison with the *mecA* transcripts seen in Fig. 3. The *mecA* blots were exposed for only 2 h, while *mecR1-mecI* blots received 46 h of exposure.

DISCUSSION

It has been proposed that *mecR1-mecI* sequences played a prominent role in the evolution of the staphylococcal methicillin resistance phenotype (23). In this hypothesis, the earliest MRSA isolates acquired *mecA* and *mecR1-mecI* sequences en bloc from an unknown donor strain of a different species or genus. However, these isolates would have appeared phenotypically methicillin susceptible because of the strong *mecI* repression of *mecA* transcription and PBP2a production and the failure of *mecR1* to mediate induction of resistance by derepression of *mecA* upon exposure to either penicillin G or methicillin, the only beta-lactam antibiotics available at that time. This phenotype is exemplified by the Japanese strains N315 and N315P. The MICs of methicillin for N315 have been shown to be low, similar to those of susceptible isolates (23, 25). Thus, the only way for *S. aureus* strains similar to N315 to become resistant to methicillin would be for mutations or deletions in *mecI* or its promoter-operator to occur. Inactivation of *mecI* by allelic replacement mutagenesis in N315P as we have described in this study or of N315 (N315P containing a beta-lactamase plasmid) as reported by Hiramatsu (23) and also confirmed by us (33) results in a marked increase in resistance to methicillin associated with an increase in both *mecA* transcription and PBP2a production. Furthermore, Hiramatsu and his colleagues (23, 41) have provided evidence that many clinical MRSA isolates from around the world have acquired specific deletions of *mecI*, of the type seen in the COL isolate used in our study; mutations in the presumed *mecI* operator; or point mutations in the *mecI* gene. He proposes that these mutations of regulator sequences are necessary for expression of clinically apparent methicillin resistance to occur, even though the functional significance of some of the *mecI* point mutations has not been fully determined. However, in our present work we present data supporting the argument that a fully functional *mecR1-mecI* and intact operator sequences can be present in an MRSA isolate and have little effect on *mecA* transcription, PBP2a production, or methicillin resistance phenotype.

The BMS1 strain examined in this study is a clinical *S. aureus* isolate from the United States that contains the *mecA* gene. It has a very low-level resistance to methicillin (MIC = 8 μg/ml) and sequences of *mecA*, *mecR1-mecI* and *mecA* promoter-operator and *mecR1-mecI* identical to those of N315. The BMS1 *mecR1-mecI* sequences were shown to be functional by their ability, when cloned on a high-copy-number plasmid in a heterologous strain with *mecI* deleted, to repress *mecA* transcription and PBP2a production and to be derepressed following induction with cefoxitin but not methicillin. However, in BMS1 there is abundant *mecA* transcript and PBP2a production in the uninduced strain, and interruption of *mecI* produces little change in *mecA* transcription and results in no increase in resistance to methicillin. Reintroduction of the high-copy-number *mecR1-mecI* clone into BMS1 with interrupted *mecI* produces some decrease in *mecA* transcription, showing that MecI is active in this strain when overproduced. Taken together, these data suggest that there is either an antirepressor molecule(s) produced in BMS1 or that additional factors required for *mecI* repression are underproduced.

There are older genetic data suggesting that additional chromosomal factors (*blaR2*) are required for regulation of plasmid-encoded beta-lactamase production (14). Since the plasmid-encoded genes that regulate beta-lactamase production, *blaR1* and *blaI*, bear a striking sequence, organizational, and, presumably, evolutionary relationship to *mecR1-mecI*, the requirement for additional chromosomal factors for repression

and induction of *blaZ* may indicate that the same, or similar, loci contribute to *mecR1-mecI* function. In the study reported by Cohen and Sweeney (14), chromosomal mutants ($R2^-$) each containing a functional, plasmid-encoded beta-lactamase were high-level constitutive in phenotype. This is similar to the high-level constitutive production of PBP2a in BMS1. Chromosomally encoded proteins that interact directly with MecI or the signal transduction domain of MecR1 can exist and function in a manner similar to the complex regulatory network that exists for *ampC*-encoded beta-lactamase production in *E. coli*, as hypothesized by Bennett and Chopra (5). Alternatively, DNA-binding proteins similar to integration host factor and histone-like proteins (for a review, see reference 17) might be required for optimum MecI-mediated transcriptional regulation. Finally, proteins that normally modulate the function of MecI or its interaction with target DNA can be inhibitory if they are overproduced. In fact, one hypothesis proposed to explain the varied beta-lactamase phenotypes resulting from chromosomal mutation is that BlaR2 is normally a BlaI inhibitor (5). The observation that an increased gene dosage of *mecI* provided some *mecA* transcriptional repression in BMS1 is consistent with the ability of excess MecI to overcome the activity of an inhibitor. Mutational alteration of the *mecR1-mecI* regulatory network rather than alteration of *mecI* or its target could be another mechanism by which *S. aureus* isolates containing *mecA* increase PBP2a production in response to the pressure of beta-lactam antibiotics in clinical environments.

If both the *mecA* and *mecR1-mecI* operator sequences are corepressed by MecI, as postulated for BlaI and the *blaZ* (*blaP*) and *blaR1-blaI* operators (5, 46), the BMS1 *mecR1-mecI* transcripts should be more abundant than those of N315P and similar to the highly abundant BMS1 *mecA* transcript. However, there was little *mecR1-mecI* transcript seen in either BMS1 or N315P; mRNA was seen on autoradiographs only after they were overexposed (Fig. 6). There is no obvious explanation for this paradox, but the differential binding of MecI to the two operators and the rapid degradation of these transcripts are two possibilities than can be examined in future studies.

Even though BMS1 was a high-level constitutive producer of PBP2a, the majority of cells were easily killed at clinically achievable concentrations of methicillin (Fig. 2B). Only 10^2 to 10^3 cells out of an inoculum size of 10^8 cells grew on agar containing 20 μg of antibiotic per ml, illustrating the heterotypic resistance phenotype. Other investigators have noted the lack of correlation between PBP2a amount and resistance phenotype and have speculated that genes other than *mecA*, *mecR1-mecI*, and *blaR1-blaI*, are responsible for the varied expression of the resistance phenotype in many clinical staphylococcal isolates. These include genes involved in peptidoglycan biosynthesis and cross-linking (6, 11, 21, 32, 37). However, staphylococcal isolates with heterotypic resistance expression similar to that of BMS1 have been found to be resistant to treatment with beta-lactam antibiotics in experimental animal infections, presumably because of in vivo selection of more highly resistant cells or induction of resistance through pathways other than those linked to PBP2a production (12, 45). It appears that even a small amount of PBP2a can result in the appearance of highly resistant cells within a population. For example, even though COL containing pG0600 produced little PBP2a detected by radiolabelled penicillin binding (Fig. 3), subpopulations resistant to >800 μg of methicillin per ml were still detectable (Fig. 2A). COL with *mecA* inactivated is completely susceptible to methicillin (32).

Thus, *mecA* is essential for resistance to beta-lactam antibiotics. In some isolates, like N315, *mecA* transcription and PBP2a production are tightly linked to resistance expression and, therefore, transcriptional regulators like *mecR1-mecI* and *blaR1-blaI* are important determinants of phenotype. In other isolates, like BMS1, resistance is dependent on the presence of *mecA* but the amount of transcription and PBP2a production are unlinked to resistance expression. The differences between BMS1 and N315 should provide a means for identifying pathways and factors involved in the transcriptional regulation of *mecA* and additional chromosomal loci that determine the heterotypic resistance phenotype.

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