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Altered Production of Penicillin-Binding Protein 2a Can Affect Phenotypic Expression of Methicillin Resistance in *Staphylococcus aureus*

CORINNE J. HACKBARTH,* CATHLEEN MIICK, AND HENRY F. CHAMBERS

Division of Infectious Diseases, San Francisco General Hospital, San Francisco, California 94110, and Department of Medicine, University of California, San Francisco, San Francisco, California 94143

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Altered production of penicillin-binding protein 2a (PBP 2a) may affect the phenotypic expression of resistance in methicillin-resistant *Staphylococcus aureus* (MRSA). COL, an MRSA strain that constitutively produces PBP 2a, was transformed with a recombinant plasmid containing the two β-lactamase regulatory genes, *blaI* and *blaR1*, with either the β-lactamase gene, *blaZ*, or a truncated *blaZ*. Both of the transformed MRSA strains now produced an inducible PBP 2a, and the MICs of nafcillin, methicillin, and imipenem for these strains were similar to those for the parental strain. A mutation in *blaR1* that resulted in the complete repression of PBP 2a production altered the phenotypic expression of methicillin resistance in that strain, as evidenced by efficiency-of-plating experiments. Rather than being homogeneously resistant like COL, the *blaR1* mutant strain now appeared to have a small resistant subpopulation. Gene products that regulate PBP 2a production may contribute to the organism's expression of methicillin resistance, but additional chromosomally located factors are required.

Penicillin-binding proteins (PBPs) are membrane proteins whose normal function is to catalyze the cross-linking of the bacterial cell wall (21). PBP 2a, the protein that mediates methicillin resistance, is an additional low-affinity PBP found only in methicillin-resistant staphylococci. Methicillin-resistant strains that produce PBP 2a can differ markedly in their phenotypic expression of resistance. With most clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA), the majority population is relatively susceptible to β-lactam antibiotics and a small proportion of cells (usually 1 in 10^4 to 1 in 10^7) express high levels of resistance. Although PBP 2a mediates methicillin resistance in these staphylococci, increased amounts of the protein do not correlate with an increased proportion of cells expressing resistance. This lack of correlation led to the recognition that other factors influence expression of resistance. Additional chromosomally located genes that are essential for the expression of methicillin resistance have been identified by insertional inactivation with Tn*551* (3, 12, 15). However, alterations in these loci have not, as yet, been detected in naturally occurring heterogeneously resistant strains.

Factors that control production of PBP 2a could also affect the heterogeneous expression of resistance. In most MRSA strains, PBP 2a is inducible by β-lactam antibiotics (7, 20), and it can be transcriptionally regulated by two distinct, albeit analogous, sets of regulatory genes (10, 18). The plasmid-derived *bla* regulatory genes (*blaR1* and *blaI*) are located upstream of *blaZ*, the gene that encodes β-lactamase. It is believed that *blaR1* encodes a membrane protein that binds β-lactam antibiotics and transduces the signal through the membrane into the cell. BlaI is proposed to be a repressor protein that transcriptionally regulates the production of β-lactamase as well as PBP 2a (8, 20). *mecR1* and *mecI*, two genes located upstream of the PBP 2a gene, *mecA*, are proposed to have functions similar to those of *blaR1* and *blaI*, respectively. In most methicillin-resistant isolates the *mec* regulatory genes are either deleted or altered, and these staphylococci regulate PBP 2a production with the *bla* regulatory system. However, non-β-lactamase-producing, methicillin-resistant strains that contain but strongly repress PBP 2a have recently been identified (4, 10). *mecR1* and *mecI* are present in these strains, and their gene products are believed to tightly regulate PBP 2a production.

We investigated the effect of the *bla* regulatory genes on the phenotypic expression of methicillin resistance in COL, a well-characterized strain of MRSA (15). Since the parent strain constitutively produces PBP 2a, this allowed us to determine whether induction of PBP 2a (i.e., its delayed production) makes a homogeneously resistant strain (COL) more heterogeneous in its expression of methicillin resistance. The role of PBP 2a repression in the homogeneous expression of methicillin resistance was also examined.

MATERIALS AND METHODS

Bacterial strains. By definition COL is a homogeneously resistant strain of MRSA, and 10 to 100% of a population will grow on 50 μg of methicillin per ml (15).

Plasmid constructs. DNA manipulations were as described by Ausubel et al. (2). Recombinant plasmids were constructed by using the staphylococcal cloning vector pRN5542 (17). *blaZ* and the *bla* regulatory genes, *blaR1* and *blaI*, were cloned from a 30-kb plasmid originally isolated from MRSA strain 67-O (7). pCH631, which contains intact *blaR1* and *blaI* genes and a truncated *blaZ* gene with a 150-nucleotide deletion at its 3′ end, has already been described (8). In pCH2278, the three genes are intact, whereas in pCH1988, *blaR1* contains an *Nhe*I linker insertion 250 nucleotides downstream from the promoter of the *blaR1-blaI* polycistronic message (for a map of the

* Corresponding author. Mailing address: University of California, San Francisco, Division of Infectious Diseases/San Francisco General Hospital, Box 0811, San Francisco, CA 94143. Phone: (415) 206-3349. Fax: (415) 206-6015. Electronic mail address: corinne@itsa.ucsf.edu.

TABLE 1. Phenotypic expression of methicillin resistance in COL, COL2278, COL631, and COL1988

Strain	Relevant genotype	PBP 2a	Methicillin			Nafcillin			Imipenem		
			MIC[a]		EOP[b]	MIC		EOP	MIC		EOP
			−	+		−	+		−	+	
COL	*blaZ blaI blaR1*	Constitutive	>256	>256	800	128	128	100	64	64	100
COL2278	*blaZ*$^+$ *blaI*$^+$ *blaR1*$^+$	Inducible	256	128	100	64	64	200	4	16	100
COL631	*blaZ*$_{Tr}$[c] *blaI*$^+$ *blaR1*$^+$	Inducible	>256	>256	800	64	128	100	16	64	100
COL1988	*blaZ*$^+$ *blaI*$^+$ *blaR1*	Repressed	4	>256	5	1	128	2	<0.25	128	1

[a] The MIC (micrograms per milliliter) was determined after 24 h of incubation in Mueller-Hinton broth without (−) and with (+) 2% NaCl.
[b] The efficiency of plating (EOP) (micrograms per milliliter) was the antibiotic concentration that resulted in at least a 99.9% decrease in titer compared with that of cultures grown without any drug.
[c] *blaZ*$_{Tr}$, truncated *blaZ* gene.

region, see Fig. 1 of reference 8). We hypothesized that this mutation would produce a frameshift and introduce stop codons in the *blaR1* transcript, preventing translation of a functional BlaR1 protein but not of BlaI, which has its own ribosomal binding site. By the methods of Augustin and Götz (1), first the plasmid constructs were electroporated into the restriction-deficient, modification-competent *S. aureus* strain 4220 (13), and then the stable recombinant plasmids (pCH2278 and pCH1988) were electroporated into COL. The relevant genotypes of the COL transformants are listed in Table 1.

Detection of PBP 2a and β-lactamase. Purified membranes were obtained from exponential cultures of the parent and transformed strains grown with and without 7.5 μM 2-(2′-carboxyphenyl)benzoyl-6-aminopenicillanic acid (CBAP) (11) as described previously (6, 7). Membrane aliquots were preincubated with clavulanic acid (200 μg/ml) prior to labeling of PBP 2a with [^{3}H]benzylpenicillin (20 μg/ml). At this concentration clavulanate saturates the binding sites of PBPs 1, 2, 3, and 4 but does not bind PBP 2a (5). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (14) and stained with Coomassie blue, and PBP 2a was detected by fluorography.

Susceptibility tests. MICs of methicillin, imipenem, and nafcillin were determined in Mueller-Hinton broth with and without 2% NaCl by using an inoculum of 5×10^5 CFU/ml and the broth macrodilution method (16). The MIC was determined after 24 h of incubation at 37°C. To determine the resistant subpopulation present in each of these strains, efficiency-of-plating experiments were performed as previously described (9). Each organism was grown overnight in tryptic soy broth, and the overnight culture was quantitatively cultured onto tryptic soy agar containing 0, 1, 2, 5, 10, 20, 50, 100, 200, 400, or 800 μg of methicillin, imipenem, or nafcillin per ml. Chloramphenicol (10 μg/ml) was added to cultures that contained the recombinant plasmid. The concentration that resulted in a 99.9% decrease in titer after 72 h of incubation was determined.

RESULTS AND DISCUSSION

PBP 2a was constitutively produced in COL, but it was induced in the transformed strains COL631 and COL2278 (Fig. 1). Both of these transformed strains contained the two intact *bla* regulatory genes. An inducible 31-kDa β-lactamase was produced in COL2278, whereas an inducible 28-kDa β-lactamase was produced in COL631. The size of this truncated protein corresponds to that predicted when the truncated gene was translated. Using anti-β-lactamase antiserum, we have previously shown that both of these proteins are β-lactamase (8). An additional PBP of approximately 35 kDa was also induced in COL631 and COL2278. This protein was detected only in transformants that contained a recombinant plasmid with an intact *blaR1* gene. It remains to be determined whether this inducible protein is a product of *blaR1*.

PBP 2a production was not detected by fluorography in the pCH1988 transformants grown with or without the inducer (Fig. 1, lanes 7 and 8). β-Lactamase production also was not detected in this strain by fluorography (Fig. 1), by immunoblots (data not shown), or with nitrocefin (data not shown). This linker insertion introduced stop codons in the *blaR1* message and presumably prevented the translation of BlaR1. It follows from these data that a functional BlaR1 protein was required for the induction of both PBP 2a and β-lactamase, which corresponds with its presumed function as a signal transducer located in the membrane. Because *blaI* has its own ribosomal binding site, it was predicted that BlaI was translated. Since there was complete repression of both PBP 2a and β-lactamase, this correlates with the presumed function of BlaI as a repressor protein. If the repressor protein had not been produced, there would be constitutive production of PBP 2a, just as there was in the parent strain, COL.

Susceptibility tests were performed to assess the effect of β-lactamase and the *bla* regulatory genes on the phenotypic susceptibility and resistance of these strains. MICs of nafcillin,

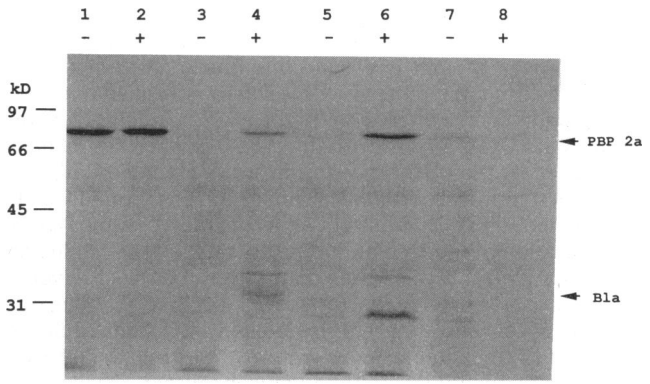

FIG. 1. Detection of PBP 2a and β-lactamase in membrane samples from COL, COL631, COL2278, and COL1988 separated by SDS-PAGE. Cultures were grown without (−) and with (+) CBAP, and aliquots were incubated with clavulanic acid (200 μg/ml) prior to being radiolabeled with [^{3}H]penicillin (20 μg/ml). Lanes 1 and 2, COL; lanes 3 and 4, COL2278; lanes 5 and 6, COL631; lanes 7 and 8, COL1988. PBP 2a and β-lactamase were induced in the COL631 and COL2278 transformed strains, and their production was repressed in the COL1988 transformed strain.

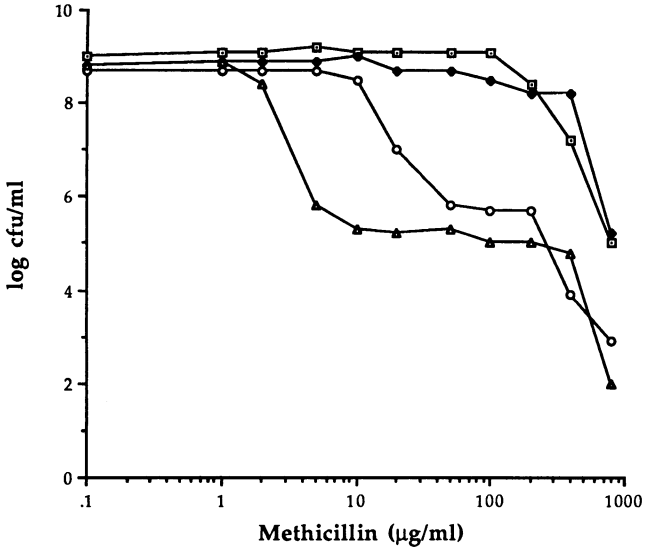

FIG. 2. Variation in response to increasing concentrations of methicillin, as determined by the efficiency-of-plating procedure. Overnight cultures of COL (⊡), COL631 (◆), COL2278 (○), and COL1988 (△) were quantitatively cultured onto tryptic soy agar containing 0, 1, 2, 5, 10, 20, 50, 100, 200, 400, or 800 μg of methicillin per ml. The growth (log_{10} CFU per milliliter) on each of those concentrations after 72 h was plotted versus concentration (log_{10}) of drug. Both COL and COL631 were homogeneous in their expression of resistance, whereas COL2278 and COL1988 displayed a heterogeneous response to the drug.

imipenem, and methicillin were obtained with and without 2% NaCl in order to determine whether these methicillin-resistant transformants now contained small resistant subpopulations or whether they were as homogeneously resistant as the parent strain. COL5542 grew on very high concentrations of all three drugs tested (data not shown), indicating that chloramphenicol, which was the plasmid selection marker for these transformants, did not affect the MICs or the efficiency-of-plating results. COL631, the transformant that contained the intact regulatory genes and a truncated *blaZ* gene, was phenotypically as resistant as the parent strain (Table 1). Interestingly, COL2278 did not appear to be as homogeneously resistant as COL631 even though it produced a functional β-lactamase. This transformant's pattern of resistance was similar to that described by Tomasz et al. (19) for a class 3 heterogeneous strain. With COL2278, as with the class 3 isolates described by Tomasz et al., the MICs and efficiencies of plating for all three antibiotics approached or were similar to those found for the homogeneously resistant strain COL (Table 1). Although the concentration of methicillin that resulted in a 99.9% reduction in viable organisms was lower than that for COL (100 versus 800 μg/ml), it was still high. However, the degree of killing over increasing concentrations of methicillin was quite different from that of the homogeneous parent strain (Fig. 2). Whereas the entire population from the homogeneously resistant strain survived until very high methicillin concentrations (≥400 μg/ml) were used, over 90% of the COL2278 population was already killed after exposure to 20 μg/ml. Similar results were found with imipenem and nafcillin. Thus, induction of PBP 2a and β-lactamase altered the phenotypic response in this transformant, and it now heterogeneously expressed methicillin resistance.

COL1988 was more heterogeneous in its expression of methicillin resistance, and its pattern of resistance was similar, although not identical, to that of the class 2 isolates previously described (19). Class 2 isolates have a small resistant subpopulation, which may not be detected by routine susceptibility testing unless 2% NaCl is added to the medium. For the other COL transformants in this study, 2% NaCl did not alter the MICs because the strains already appeared to be highly resistant. With COL1988, the MICs of methicillin, imipenem, and nafcillin approached those for susceptible strains, and the concentration of drug that killed 99.9% of the population was also low. However, unlike with susceptible strains, a subpopulation still survived at very high concentrations of methicillin (Fig. 2). Although this transformant was not producing PBP 2a or β-lactamase, the other mutations in its COL background that had made it homogeneously resistant to β-lactam antibiotics allowed this organism to survive exposure to the drugs.

Unlike the case of the naturally occurring β-lactamase plasmid in staphylococci, the β-lactamase regulatory genes are located on a high-copy-number plasmid in these transformants. Whether the same effect would occur in nature was not addressed. Regardless, this study demonstrated that other, non-*mecA*, associated factors are required for the methicillin-resistant phenotype observed in these transformants. Murakami and Tomasz (15) have previously inactivated PBP 2a production in a COL background by inserting Tn*551* into or near *mecA*. Like COL1988, their strain, RUSA4, required a slightly increased MIC of methicillin compared with susceptible strains, yet phenotypically, it was still homogeneously resistant to methicillin. Whether this additional factor is unique to COL remains to be determined.

This report indicates that inactivation of the regulatory genes can also affect the phenotypic expression of methicillin resistance. At high antibiotic concentrations, the resident PBPs are bound and the organism requires PBP 2a for cell wall metabolism. However, even when PBP 2a production was completely repressed, as evidenced with COL1988, a small subpopulation was still able to survive exposure to β-lactam antibiotics. Defining and characterizing the mutations in other loci will provide the key to understanding what determines whether these organisms are killed or are able to withstand the antibiotic pressure.

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