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Abnormal Peptidoglycan Produced in a Methicillin-Resistant Strain of *Staphylococcus aureus* Grown in the Presence of Methicillin: Functional Role for Penicillin-Binding Protein 2A in Cell Wall Synthesis

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Upon the addition of methicillin even at relatively low concentrations (5 μg/ml or 0.3% of the MIC) to the medium, methicillin-resistant staphylococci shift to the production of a new peptidoglycan with an abnormal muropeptide composition which may be the synthetic product of penicillin-binding protein 2A.

All methicillin-resistant staphylococcal clinical isolates examined so far contain the *mec* determinant, a 1.2-kb piece of DNA that appears to be of nonstaphylococcal origin. The *mec* gene contains structural motifs characteristic of peptidoglycan transpeptidases and encodes for a 78-kDa protein that is capable of binding radioactive penicillin, although with a low affinity (8). It is generally assumed that this protein, called penicillin-binding protein (PBP) 2A, acts as a surrogate enzyme to catalyze the synthesis of peptidoglycan under conditions when normal staphylococcal PBPs are inactivated by the antibiotic in the medium.

One may expect that such a peculiar PBP has specific catalytic properties and substrate specificity, leaving its mark on the muropeptide composition of the peptidoglycan. The availability of a high-pressure liquid chromatography (HPLC) technique for analysis of the staphylococcal cell wall (2) has allowed us to test this possibility. However, the test yielded negative results: the muropeptide compositions of an isogenic pair of methicillin-resistant *Staphylococcus aureus* (MRSA), one with an active *mec* gene and the other with a transposon-inactivated *mec* gene, showed no detectable differences when the cell walls of bacteria grown in normal medium were compared (2).

It is conceivable that PBP 2A becomes functional only when the bacteria are exposed to antibiotics in the medium. In order to test this, we analyzed the peptidoglycan of the highly and uniformly resistant MRSA strain COL (4) (MIC of methicillin, 1,600 μg/ml) grown in the presence of a wide range of sub-MICs of methicillin (from 0 to 750 μg/ml). Bacterial cultures (1 liter) were grown with aeration at 37°C in tryptic soy broth containing the antibiotic. After at least 16 generations of growth, cells were harvested and extracted with boiling sodium dodecyl sulfate (4%), and peptidoglycan was prepared as described previously (2). Purified peptidoglycan was digested with a muramidase, and the muropeptides were separated by reversed-phase HPLC (2).

Figure 1 shows the elution profiles of muropeptides isolated from the muramidase hydrolysates of peptidoglycan produced by strain COL grown in the presence of 0, 2, 5, and 750 μg of methicillin per ml. The yield of peptidoglycan produced in the presence of high concentrations of methicillin was similar to the yield obtained in normal cells. It is clear from Fig. 1 that bacteria grown even at the lowest antibiotic concentration produced a completely altered muropeptide elution profile indicating major compositional changes in the peptidoglycan. Most interestingly, the compositional change reached a final, stable state at about 5 μg of methicillin per

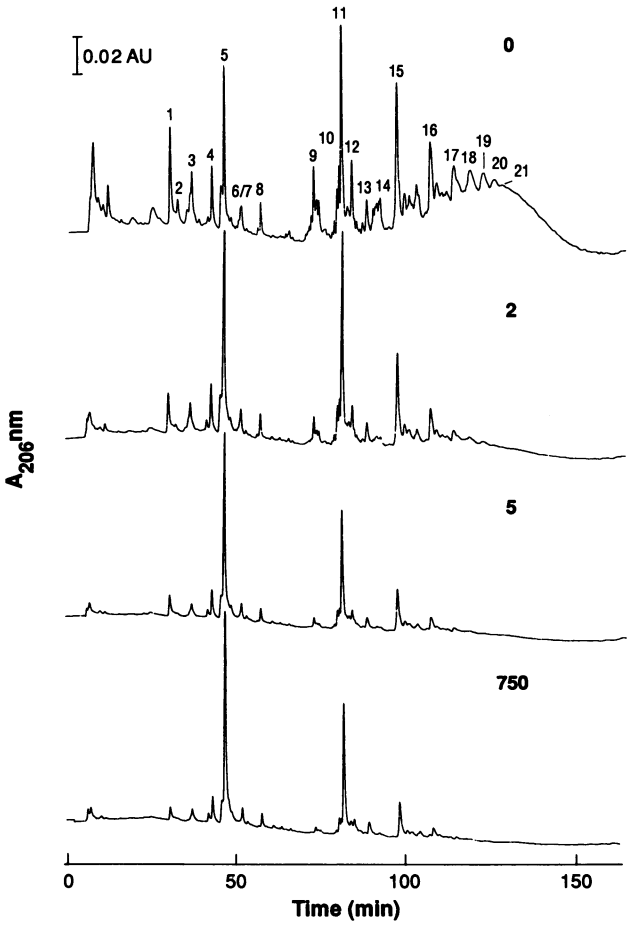

FIG. 1. Muropeptide elution profiles, analyzed by reversed-phase chromatography, of *S. aureus* COL grown in the presence of 0, 2, 5, or 750 μg of methicillin per ml. For the assignment of peaks, see reference 2. AU, absorbance units.

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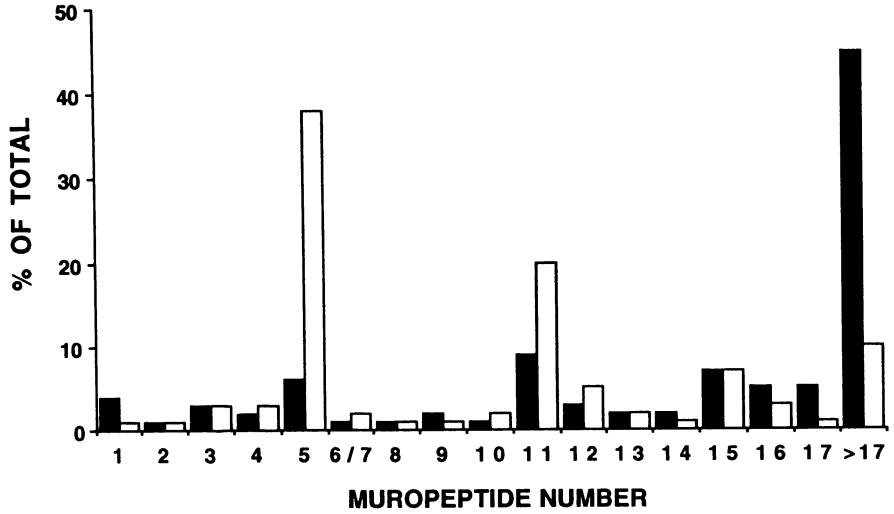

FIG. 2. Muropeptide compositions of normal (closed columns) and methicillin-grown (10 μg of methicillin per ml; open columns) staphylococcal peptidoglycans. The percentages are expressed as the fraction of the total area and were obtained from HPLC profiles, as described in the legend to Fig. 1. Muropeptide numbers refer to the peaks in Fig. 1.

ml, and the bacteria appeared to reproduce this abnormal peptidoglycan without virtually any additional change when grown in the presence of even vastly higher concentrations of the antibiotic, including 750 μg/ml, which represents about half of the MIC for this strain.

The use of the reversed-phase HPLC method (2) allowed us to identify the changes in peptidoglycan composition in terms of the particular chemically distinct muropeptides involved. Figure 2 illustrates the sharp compositional differences between the two staphylococcal peptidoglycans: the one produced in the absence of antibiotic and the other produced in the presence of methicillin within the concen-

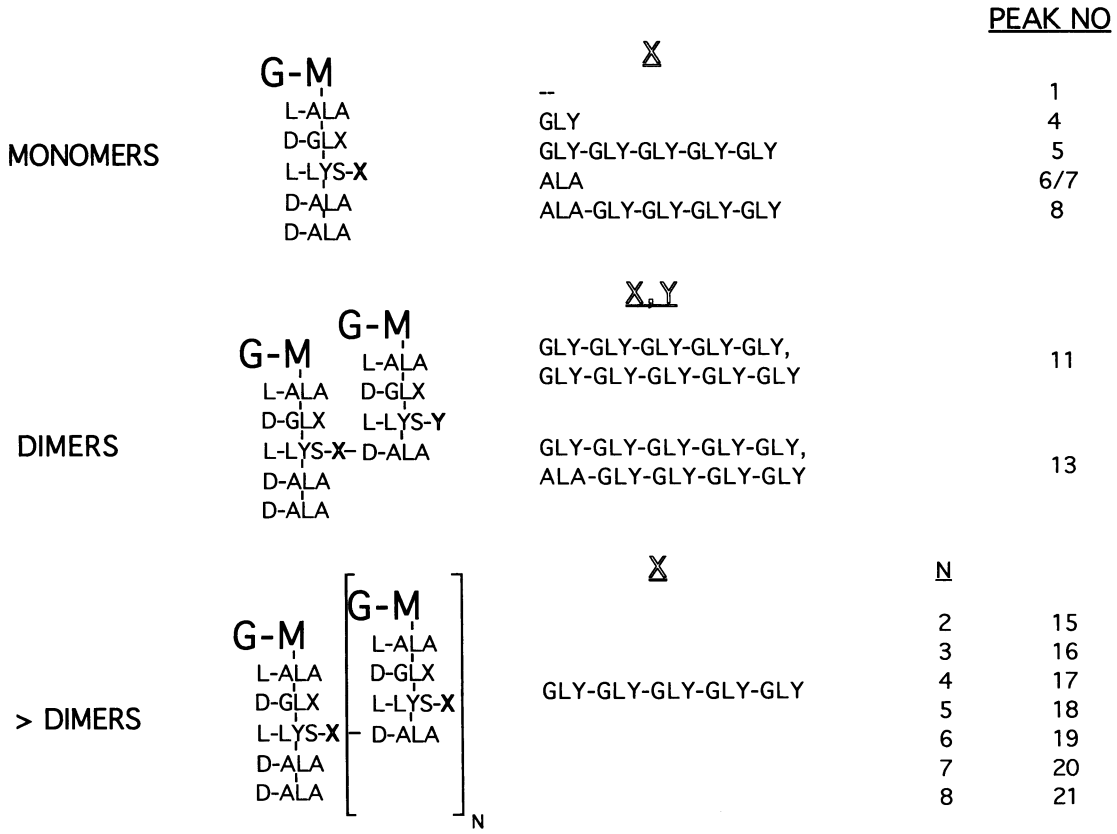

FIG. 3. Structures of some major staphylococcal cell wall muropeptides. Structures are taken from reference 2.

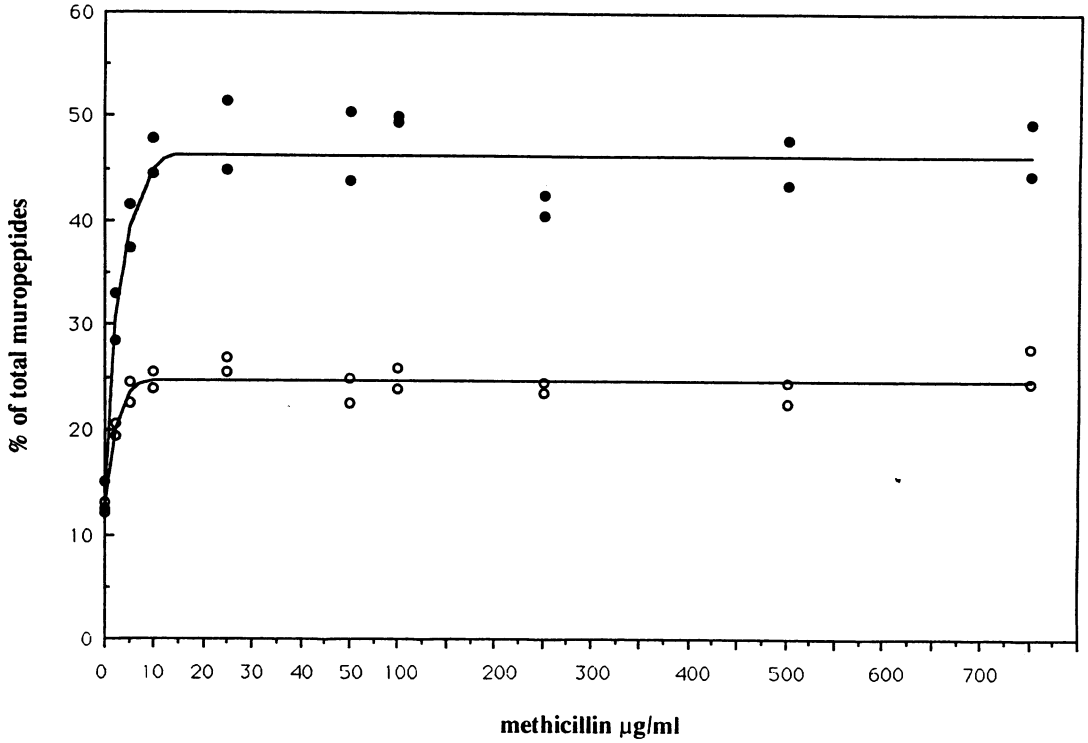

FIG. 4. Altered muropeptide composition of the peptidoglycan of *S. aureus* COL grown in the presence of various concentrations of methicillin. The area percentages of the monomers (●; peaks 1, 4, 5, 7, and 8) and dimers (○; peaks 9, 11, and 13) were expressed as the percentage of the total area and were plotted against the concentration of methicillin in the growth medium. Both here and in the experiment illustrated in Fig. 5, datum points represent two independent preparations of the cell wall for each methicillin concentration used.

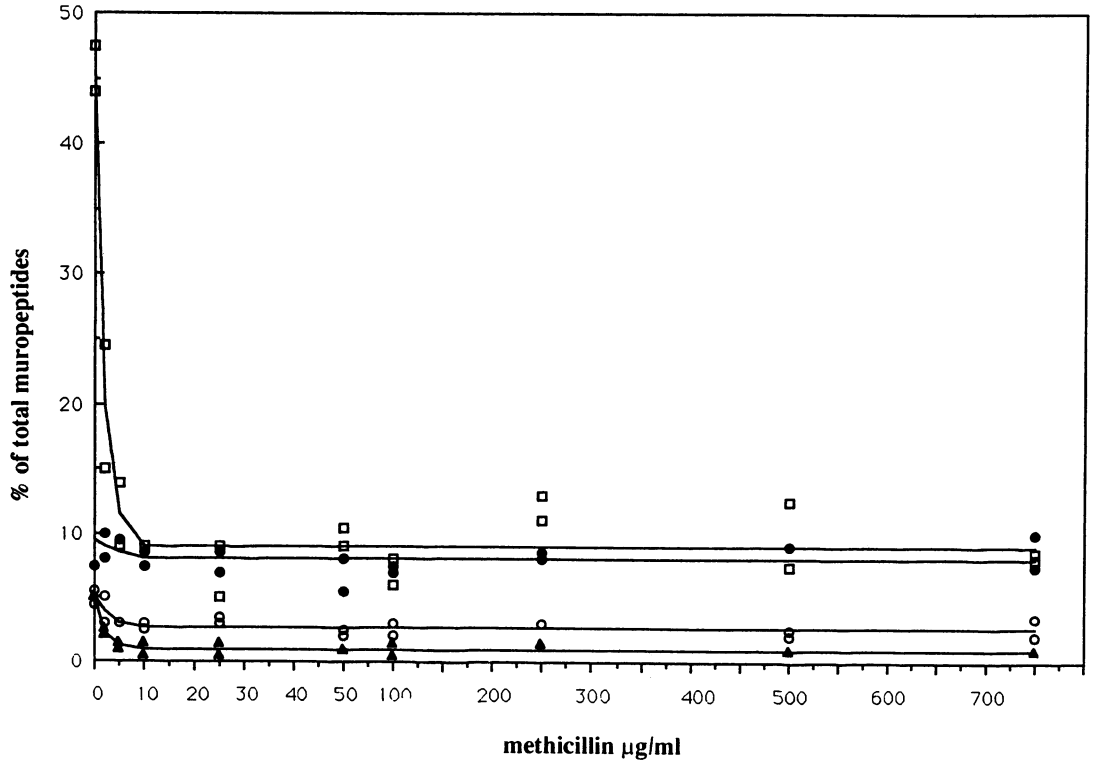

FIG. 5. Altered muropeptide composition of the peptidoglycan of *S. aureus* COL grown in the presence of various concentrations of methicillin. Area percentages of trimer (●; peak 15), tetramer (○; peak 16), pentamer (▲; peak 17), and higher oligomers (□; peak 18 and higher) were expressed as the percentage of the total area and were plotted against the concentration of methicillin in the growth medium.

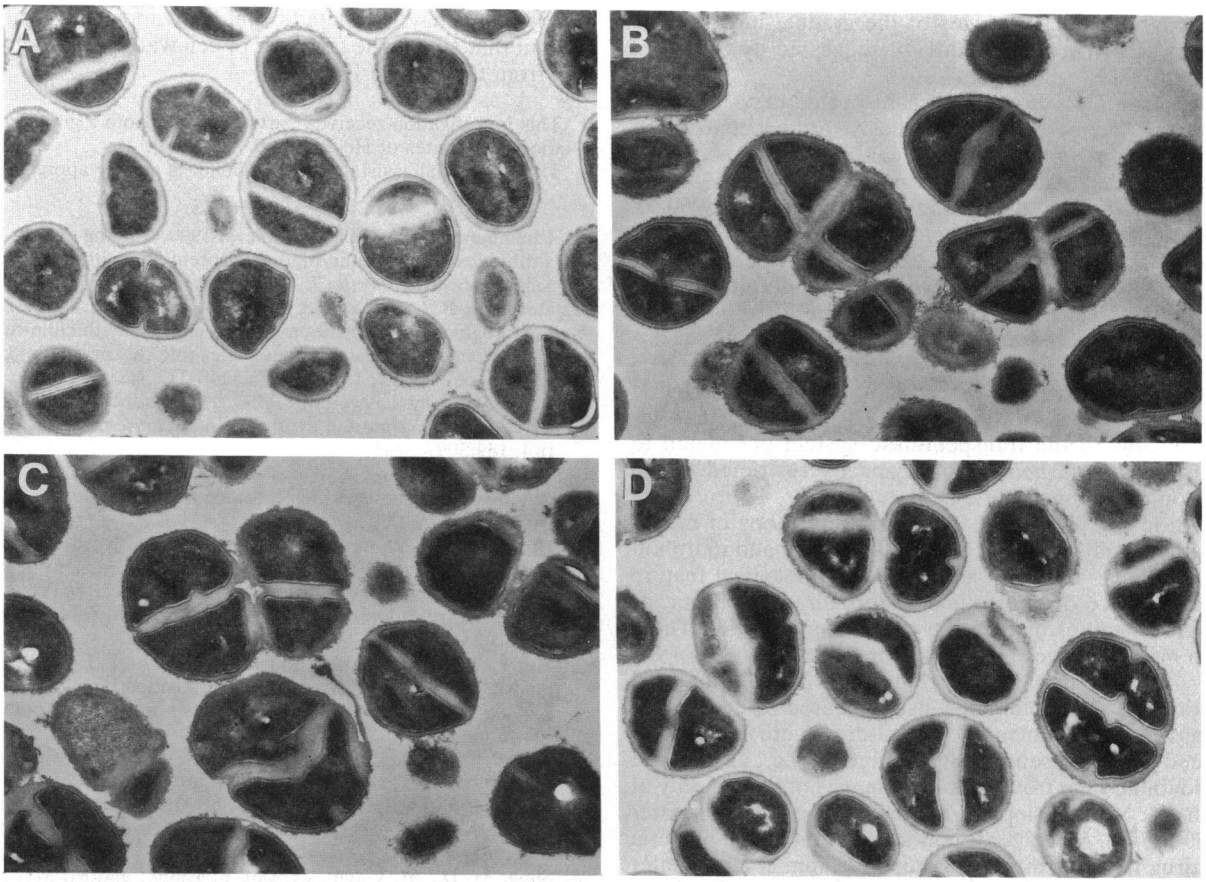

FIG. 6. Electron micrographs of cross-sections of *S. aureus* COL grown in the presence of 0 (A), 2 (B), 5 (C), and 750 (D) μg of methicillin per ml. Cells were collected by centrifugation and fixed with 2.5% glutaraldehyde at 4°C; this was followed by processing for electron microscopy (9). Magnification, ×20,000.

tration range of 5 to 750 μg/ml. In the wall of normally growing cells, over 60% of the muropeptides were made up of trimeric and higher oligomeric species (for structures, see Fig. 3). In the peptidoglycan of cells grown in the presence of methicillin, the proportions of oligomers fell to about 15% and were replaced by the monomeric and dimeric disaccharide pentapeptides carrying the pentaglycyl substituent. The fraction of other mono- and dimeric muropeptides (peaks 1, 4, 6/7, 8, 9, and 13) remained unchanged. The chemical structures of these latter compounds were identified as derivatives of the disaccharide pentapeptide: the unsubstituted monomer; the monomer with monoglycyl, alanyl, or alanyl-tetraglycine substituents; the dimers of an unsubstituted and a pentaglycyl-substituted pentapeptide; and the dimer of an alanyl-tetraglycine- and pentaglycine-substituted pentapeptide (Fig. 3). The fact that the concentration of this particular group of muropeptides remained unchanged in cells grown with or without antibiotic suggests that these compounds may have some special, essential function in the cell walls.

Figures 4 and 5 show plots of the compositional change as a function of the concentration of the antibiotic in the medium. The plots demonstrate the rapidity with which the unique muropeptide composition of cells grown in drug-free medium changes to a new (abnormal) pattern characteristic of the cells grown in the presence of methicillin.

Our findings confirm and extend the findings of earlier studies (5, 6). Most recently, Snowden and Perkins (7) used a gel filtration technique to demonstrate changes in peptidoglycan composition when methicillin-susceptible staphylococci were grown in the presence of sub-MICs of various beta-lactam antibiotics. While the context of those studies was different and the gel filtration method that the investigators used could not identify the chemical natures of the muropeptides involved, they noted that a methicillin-resistant staphylococcal strain exposed to methicillin also underwent similar changes in peptidoglycan composition, except that the changes required higher absolute concentrations of the antibiotic.

The finding that the peptidoglycan composition of COL grown with methicillin at concentrations greater than 5 μg/ml does not change with increasing concentrations of the drug, whereas the peptidoglycan composition of bacteria grown in the range of 0 to 5 μg/ml does, suggests that MRSA strains may have two distinct enzymatic machineries available for the synthesis of peptidoglycan: one that is active at less than and the other that is active at greater than 5 μg of methicillin per ml. The 5-μg/ml concentration, which seems to be the concentration at which a switch in peptidoglycan synthesis occurs, corresponds to the MIC for a Tn*551* mutant of COL with an inactivated *mec* gene (4). This mutant does not produce PBP 2A but still contains a full assembly of the normal staphylococcal PBPs. Therefore, we assume that the compositional changes observed at less than 5 μg of methi-

cillin per ml are consequences of the gradual inhibition of these normal PBPs, as was already suggested by Snowden and Perkins (7).

A likely candidate for the mechanism that takes over the catalysis of peptidoglycan synthesis at concentrations greater than 5 μg of methicillin per ml is the *mec* gene product, PBP 2A. Affinity titrations with this PBP showed that half saturation by methicillin required well over 250 μg of the drug per ml (1), so one could expect that a substantial fraction of the protein would be available in a functional form throughout the concentration range of methicillin used in our studies. The abnormal muropeptide composition of the peptidoglycan produced under these conditions suggests that PBP 2A may function as a unique transpeptidase which can cross-link efficiently to each other only monomeric muropeptide species. In this sense, PBP 2A may be considered analogous to the transpeptidase system postulated to exist in *Escherichia coli*, with a capacity restricted to the production of muropeptide dimers only (3).

Growth in the presence of high concentrations of methicillin had virtually no effect on the physiology and morphology of the cells. The only abnormal morphological feature was the presence of diffuse and abnormally wide septa, as seen in thin sections by electron microscopy (Fig. 6). There were no major changes apparent in the contour sizes of the cells or the width of the peripheral cell wall, and the orientations of the septal sites also appeared to be normal. The mass doubling times of the cultures were not greatly influenced by the presence of the antibiotic (e.g., an increase in the doubling time from 30 to 70 min was observed only at the highest concentrations of methicillin, i.e., those greater than 500 μg/ml). However, upon inoculation of the bacteria from a drug-free to a drug-containing medium at and greater than concentrations of about 10 μg of methicillin per ml, lag periods of various lengths (roughly proportional to the methicillin concentration) were observed before the resumption of growth. This lag time was about 1 and 4 h for concentrations of 10 and 25 μg of methicillin per ml, respectively. This may be related to the activation of a regulatory circuit that allows the takeover of cell wall synthesis by PBP 2A from the normal PBPs.

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