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Regulation of Penicillin-Binding Protein Activity: Description of a Methicillin-Inducible Penicillin-Binding Protein in *Staphylococcus aureus*

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The penicillin-binding proteins (PBPs) of two methicillin-resistant strains of *Staphylococcus aureus* (R2 and R1) were analyzed in cells grown in the absence and in the presence of methicillin. Under the former condition, strain R2 showed the typical PBP pattern of beta-lactam-susceptible strains, while strain R1 showed a markedly increased amount of PBP-3. Under the latter condition, on the other hand, a novel PBP (PBP-2a) located between PBP-2 and -3 was detected in strain R2, while strain R1 appeared to synthesize an even greater amount of PBP-3, in respect to untreated cells. Both R2 PBP-2a and R1 PBP-3 showed a very low affinity for methicillin, which was consistent with the MICs for the respective strains.

Several data recently collected on penicillin-binding protein (PBP) functions clearly indicate that the PBPs play main and specific roles in cell wall growth and division (2, 18–20, 22). The particular nature of some of the functions in which PBPs are involved implies a control mechanism in the cell that assures the optimal functioning of the PBP(s) whose activity is required in a particular phase of the cell cycle or under particular growth conditions.

The suggestion originally derived from studies with *Streptococcus faecium* that growth conditions can influence the physiological status and the functions of PBPs and the observation, made with this same microorganism, that changes in conditions of growth are accompanied by changes in the apparent relative amount of some PBPs (8, 9) have brought about the possibility that cells could also control PBP activity by changing the amount of protein synthesized. Such a possibility is also supported by later studies showing that in *Escherichia coli* the relative amount of some PBPs depends on the state of growth (1, 5, 6) and that novel PBPs are synthesized by *Bacillus megaterium* during sporogenesis (21).

In this study, we show that in a *Staphylococcus aureus* strain, methicillin induces the synthesis of a novel PBP which has a low affinity for beta-lactams and makes cells resistant to these antibiotics.

(A preliminary description of these findings was reported at the 4th Mediterranean Congress of Chemotherapy at Rhodos, Greece, 18 to 24 October 1984.)

MATERIALS AND METHODS

Bacterial strains. *S. aureus* R2 and R1, two clinical isolates which showed high-level methicillin resistance, were used in this study. *S. aureus* A8, a susceptible control strain, was also used in some experiments. The relevant properties of these strains are described in Table 1. Bacteria were grown in tryptic soy broth (Difco Laboratories) supplemented with 5% NaCl at 37°C with shaking. Culture growth was monitored by measuring optical density with a Perkin-Elmer spectrophotometer.

Antibiotic MIC determination. The MIC was determined by a tube dilution method. A series of tubes containing suitable dilutions of antibiotic in tryptic soy broth supplemented with 5% NaCl (5 ml) were inoculated with 10^5 cells per ml. The tubes were incubated at 37°C and examined after 24 h of incubation. The MIC was determined from the first tube showing no visible turbidity.

PBP analysis of cells growing with and without methicillin. Cultures were grown to late-logarithmic phase in tryptic soy broth supplemented with 5% NaCl at 37°C with vigorous shaking. One milliliter of this culture was then diluted into 50 ml of fresh, warm tryptic soy broth supplemented with 5% NaCl containing the desired methicillin concentrations. An identical culture without antibiotic served as a untreated cell control. All the cultures were then incubated for 90 min to reach the mid-logarithmic phase of growth. The bacteria were harvested by centrifugation, washed and suspended in 5 ml of 0.05 M Tris hydrochloride buffer containing lysostaphin (10 μg/ml; Sigma Chemical Co.), DNase (5 μg/ml; Sigma), and RNase (2.5 μg/ml; Sigma). The cell suspension was incubated at 37°C until lysis was complete (10 to 15 min). Membranes were then collected by differential centrifugation in 0.01 M sodium phosphate buffer at a protein concentration of 10 mg/ml. As membranes from R2 induced cells still retained beta-lactamase activity (probably due to the presence of a membrane-bound precursor; see also reference 15), these were treated with clavulanic acid (a beta-lactamase inhibitor; see reference 17) at a concentration of 100 μg/ml, which was found to be the minimal concentration inhibiting the membrane-bound enzyme activity. Incubation with clavulanic acid was performed for 10 min at 37°C. For the best comparison of results, clavulanic acid was also added to R1 membranes, even though this strain did not produce beta-lactamase. Samples (25 μl) of all membrane preparations were then incubated with 100 μM [^{14}C]benzylpenicillin (specific activity, 59 Ci/mol; Radiochemical Centre, Amersham, England) for 10 min at 37°C. The reaction was stopped by adding nonradioactive penicillin (final concentration, 100 mM) and 25 μl of sample buffer containing sodium dodecyl sulfate (14). Membrane proteins were separated on discontinuous sodium dodecyl sulfate-polyacrylamide slab gels consisting of 10% acrylamide and 0.13% bis-acrylamide (14). PBPs were detected by fluorography as described previously (3).

Assay of PBP affinity for methicillin by competitive binding.

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TABLE 1. *S. aureus* strain properties

Strain	Beta-lactamase activity[a] in:		MIC (μg/ml) of:	
	Uninduced culture	Induced culture	Penicillin	Methicillin
A8	−	−	0.015	0.5
R2	−	+	>1280	1280
R1	−	−	10	640

[a] Beta-lactamase activity was determined by the nitrocefin method (16) in cells grown for 2 h without methicillin (uninduced) and with 0.5 μg of the antibiotic per ml (induced).

Membranes were isolated from cells grown with and without 0.5 μg of methicillin per ml as described above and suspended at a protein concentration of 10 mg/ml. Before competition experiments, membrane suspensions from cells grown in the presence of the antibiotic were incubated with 100 μg of clavulanic acid per ml for 10 min at 37°C. From all membrane preparations, 25-μl samples were taken and treated with various methicillin concentrations at 37°C for 10 min. [^{14}C]benzylpenicillin (100 μM) was then added, and incubation continued for 10 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and PBP detection were performed as described above.

RESULTS

While evaluating the growth rate of two methicillin-resistant *S. aureus* strains (R2 and R1) in the presence of a methicillin concentration far below the MIC, we observed that strain R2 showed a 10-min lag immediately after addition of the drug, whereas strain R1 continued to grow at the same rate as the untreated culture (Fig. 1).

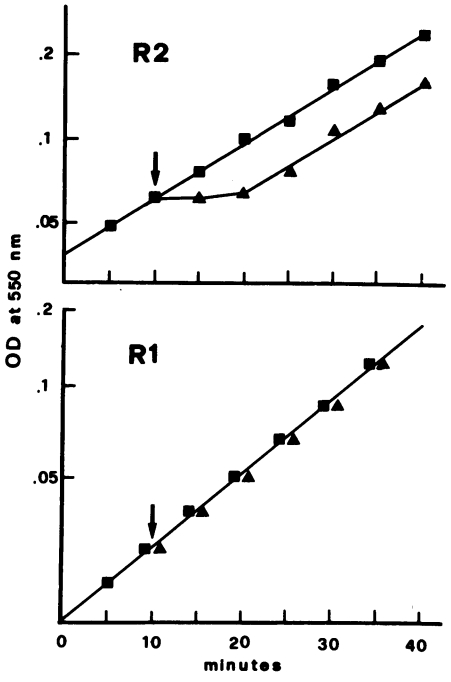

FIG. 1. Effect of methicillin on growth of *S. aureus* R2 and R1. Cultures in early exponential growth were supplemented at the time indicated by arrows with 10 μg of methicillin per ml (▲). Untreated cultures served as a control (■).

The particular response of strain R2 to growth in the presence of methicillin appeared very interesting since no inhibition of CFU was observed when cells of this strain were inoculated on solid medium containing 10 μg of methicillin per ml (data not shown).

Some properties of the two strains were then analyzed and are reported in Table 1. Both strains were much more resistant to methicillin and penicillin than the susceptible reference strain *S. aureus* A8. However, strain R2 showed methicillin and penicillin MICs that were two- and more than 128-fold higher, respectively, than those displayed by strain R1. In addition, R2 but not R1 produced a beta-lactamase whose synthesis was induced by a low methicillin concentration, as are the majority of beta-lactamase-positive *S. aureus* strains (7).

The PBP patterns of the two strains were then analyzed after growth in the absence and in the presence of low methicillin concentrations. Under the former condition, the R2 PBP pattern was similar to that of the susceptible *S. aureus* strains already described (4, 11–13), whereas the R1 PBP pattern showed a significantly greater amount of PBP-3 (Fig. 2, lane 0). R2 and R1 PBP patterns also differed from one another for the amount of PBP-4. In cells grown in the presence of various methicillin concentrations, the disappearance of ordinary PBPs was expected due to saturation by both methicillin and clavulanic acid, the latter antibiotic being used to prevent hydrolysis of radioactive penicillin by membrane-bound beta-lactamase activity. However, a single PBP that was not saturated by clavulanic acid (plus methicillin at concentrations up to 10 μg/ml) was observed in both strains. In strain R2, this PBP (PBP-2a) moved faster than PBP-2 and slightly slower than PBP-3, while in strain R1 it showed the same mobility as PBP-3 and increased in amount with respect to the control, in cells grown in the presence of 0.5 and 1 μg/ml but not 10 μg/ml (Fig. 2).

Membranes were then prepared from the two strains

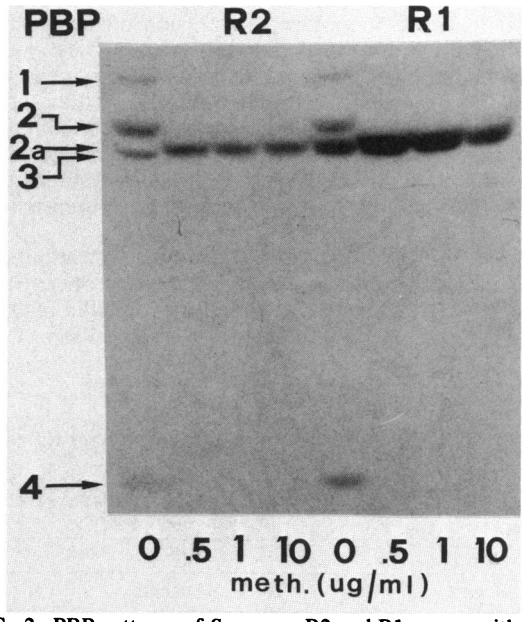

FIG. 2. PBP patterns of *S. aureus* R2 and R1 grown without and with 0.5 to 10 μg of methicillin per ml. After growth in the presence of the antibiotic, cells were harvested and exposed to 100 μM [^{14}C]benzylpenicillin as described in the text. Cells grown without methicillin served as a control (lanes 0). meth., Methicillin.

grown in the absence and in the presence of methicillin and used in competition experiments between this antibiotic and radioactive penicillin.

In membranes from strain R2 grown without the antibiotic, all PBPs were saturated by 10 μg of methicillin per ml, while in membranes from R1 grown under the same condition, PBP-3 was not saturated by this concentration but only by 1,280 μg/ml (Table 2). When the same competition experiments were performed in membranes isolated from cells grown in the presence of methicillin, R2 PBP-2a and R1 PBP-3 grown under the same conditions were saturated by 1,280 μg of the antibiotic per ml (Table 2).

Figure 3 shows the Coomassie-stained membrane protein patterns of strains R2 and R1 grown in the absence and in the presence of methicillin. A component (molecular weight, 78,000) that moved to the same position as the low-affinity PBPs increased in both strains grown in the presence of methicillin. It is also interesting that in only R2 another band (molecular weight, 24,000) besides that of the 78,000-molecular-weight component appeared to increase in membranes from cells grown in the presence of methicillin. This component did not covalently bind penicillin, and further experiments are needed to determine its function.

DISCUSSION

The data presented here clearly show that strain R2 synthesized a PBP only when methicillin was added to the medium, suggesting that this protein was most likely induced by the antibiotic itself. In fact, the finding that the cell number of strain R2 was not reduced by 10 μg of methicillin per ml ruled out the possibility that a subpopulation of resistant cells constitutively synthesizing PBP-2a was selected by the antibiotic. In addition, the 10-min lag observed in strain R2 after antibiotic addition could be more reasonably explained by a growth inhibition occurring in all the cells of the population until the gene encoding for PBP-2a was depressed and the protein was synthesized in an amount sufficient to allow phenotypic expression of methicillin resistance. The increase in the amount of the 78,000-molecular-weight component observed in membranes of cells grown in the presence of the antibiotic showed further that the appearance of the novel PBP was due more to induction of its synthesis than to activation of preexisting molecules. On the other hand, in strain R1 methicillin did not induce the de novo synthesis of a PBP but caused an increase in the amount of PBP-3.

Both R2 PBP-2a and R1 PBP-3 had very low affinities for methicillin and were saturated by antibiotic concentrations close to the MICs, suggesting that the methicillin resistance in these strains may be related to the acquisition of these PBPs. These proteins therefore shared many properties with a particular class of PBPs recently described in several gram-positive species (4, 10–13, 23). These PBPs are synthesized in large amounts by strains resistant to beta-lactams, show an unusual low affinity for these antibiotics, and can take over the functions of all the other PBPs. In *S. aureus*, the low-affinity PBPs have electrophoretic mobilities similar to those of PBP-2a and PBP-3 of strains R2 and R1, respectively (4, 11–13). In some strains, the synthesis of these proteins is constitutive (13), and in others it is dependent on the temperature and pH of growth (4, 12). Strains R2 and R1 are the first strains identified in which a beta-lactam modulates the synthesis of these PBPs, being able to both induce them (in strain R2) and increase the amount produced by cells (in strain R1). These findings are therefore totally new and offer a stimulating approach for studying regulation of activity of PBPs and their relationship with beta-lactamases, another class of proteins that bind beta-lactams and can be induced by these antibiotics.

TABLE 2. PBP saturation by methicillin in *S. aureus* R2- and R1-isolated membranes[a]

Strain	Concn of methicillin (μg/ml) resulting in 100% saturation of:				
	PBP-1	PBP-2	PBP-2a	PBP-3	PBP-4
R2, uninduced	<10	<10	Absent	<10	<10
R2, induced	<10	<10	1280	<10	<10
R1, uninduced	<10	<10	Absent	1280	<10
R1, induced	<10	<10	Absent	1280	<10

[a] Membranes were isolated from cells grown without and with 0.5 μg of methicillin per ml.

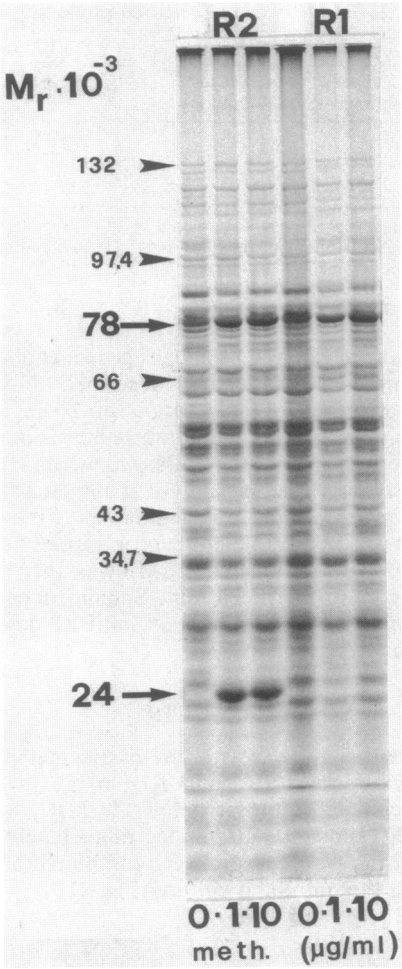

FIG. 3. Membrane protein patterns of *S. aureus* R2 and R1 grown without and with various methicillin concentrations. After growth in the presence of the antibiotic (1 to 10 μg/ml), membranes were prepared as decribed in the text. Cells grown without methicillin served as a control (lanes 0). The following molecular weight protein standards were used (arrowheads): bovine serum albumin (dimer, 132,000), phosphorylase b (97,400), bovine serum albumin (monomer, 66,000), ovalbumin (43,000), and pepsin (34,700). Arrows indicate the 78,000- and 24,000-molecular-weight components. meth., Methicillin.

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