ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1987, p. 1423–1425
0066-4804/87/091423-03$02.00/0

Vol. 31, No. 9

Increased Susceptibility to Cephamycin-Type Antibiotics of Methicillin-Resistant *Staphylococcus aureus* Defective in Penicillin-Binding Protein 2

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Received 21 January 1987/Accepted 9 June 1987

A methicillin-resistant strain of *Staphylococcus aureus* which produced low-affinity penicillin-binding protein 2′ (PBP 2′) spontaneously lost PBP 2 when the strain was cultivated at 43°C overnight. At 37°C, the mutant had increased susceptibility to cephamycin-type β-lactams, which showed high affinity for PBP 4. This result suggests that inhibition of PBP 4, in addition to that of PBP 2, is necessary to kill methicillin-resistant strains.

The primary targets of β-lactam antibiotics are penicillin-binding proteins (PBPs) which are involved in peptidoglycan biosynthesis in gram-positive and -negative bacteria (1, 15). In *Staphylococcus aureus*, only PBP 4 has been well characterized as having transpeptidase, carboxypeptidase, and penicillinase activities (8, 19). This PBP was also shown to be dispensable for bacterial growth by the isolation of a defective mutant (3). The physiological functions of the other PBPs are far from well understood, although PBP 2 and PBP 3 have been shown to be essential for bacterial growth (4, 7). In this paper, we describe a spontaneous PBP 2-deficient mutant of methicillin-resistant *S. aureus* and its increased susceptibility to cephamycin-type antibiotics.

S. aureus SR1550 was used in this study. It is a methicillin-resistant clinical isolate which produces penicillinase. Flomoxef (6315-S; 16), demethoxy-flomoxef, and demethoxy-moxalactam were synthesized in the Shionogi Research Laboratories, Osaka, Japan.

Preparation of membrane fractions, PBP assays, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and determinations of PBP 2′ and MICs were done as described elsewhere (11). Plasmids were prepared by alkaline lysis essentially as described previously (9). They were analyzed by 0.7% agarose gel electrophoresis.

When methicillin-resistant *S. aureus* SR1550 was grown in L broth at 43°C overnight (10), 80% of the bacterial cells became penicillinase negative, as assayed with nitrocefin (12). We determined the MICs of β-lactam antibiotics against 10 of these penicillinase-negative strains and PBP patterns of 2 of the 10 strains. Since these strains, one of which was designated SRM710, had similar susceptibilities to β-lactams and similar PBP patterns (as shown for strain SRM710 in Table 1 and Fig. 1), all of them were confirmed to be PBP 2 deficient, as described below. Penicillinase-negative variants were also isolated by incubating strain SR1550 with 24 μM ethidium bromide at 37°C overnight, and one of these variants was designated SRM705. A high-molecular-weight plasmid band on the agarose gel decreased in intensity by about half for strain SRM710; strain SRM705 had no band at the same position. Two kinds of plasmid bands seemed to migrate to this position. The other plasmid bands for the derivatives were the same as those for the parent plasmids.

PBP patterns and the affinity of cefazolin for PBPs (Fig. 1) showed that strain SRM705 produced typical PBPs in addition to low-affinity PBP 2′, which is associated with methicillin resistance (6, 13, 18). Strain SRM710 completely lacked PBP 2. The electrophoretogram of membrane proteins stained with Coomassie brilliant blue showed no band for strain SRM710 at the position for PBP 2 (Fig. 2). This result confirms that strain SRM710 did not produce PBP 2, as opposed to PBP 2 just losing the ability to bind to benzylpenicillin. Even if PBP 2 is essential for bacterial growth, as has been suggested previously (4, 7), the mutant was viable when it produced PBP 2′. Of the 33 strains we examined, only SR1550 lost PBP 2 when it was cultivated at 43°C.

Strain SRM710 produced an additional PBP which migrated slightly faster than PBP 3. In some other wild-type strains, this PBP was also detected by fluorography with prolonged exposure. Thus, it is more likely that more of this PBP was produced in the PBP 2-deficient strain than that the PBP was derived from PBP 2. Disturbance of peptidoglycan biosynthesis caused by the loss of PBP 2 is considered to induce production of the new PBP.

Next, we examined the effect of PBP 2 deficiency on bacterial susceptibility to β-lactam antibiotics (Table 1). *S. aureus* SR1550, SRM705, and SRM710 were similarly resistant to all drugs tested at 32°C. However, at 37°C the PBP 2-deficient strain, SRM710, was 32 times more susceptible to

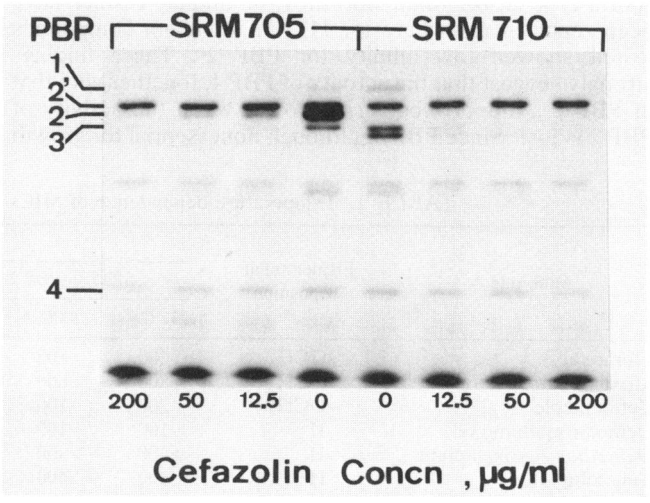

FIG. 1. PBP patterns of *S. aureus* SRM705 and SRM710 and competition of cefazolin for PBPs. Membranes were preincubated with cefazolin for 10 min at 30°C at the indicated concentrations.

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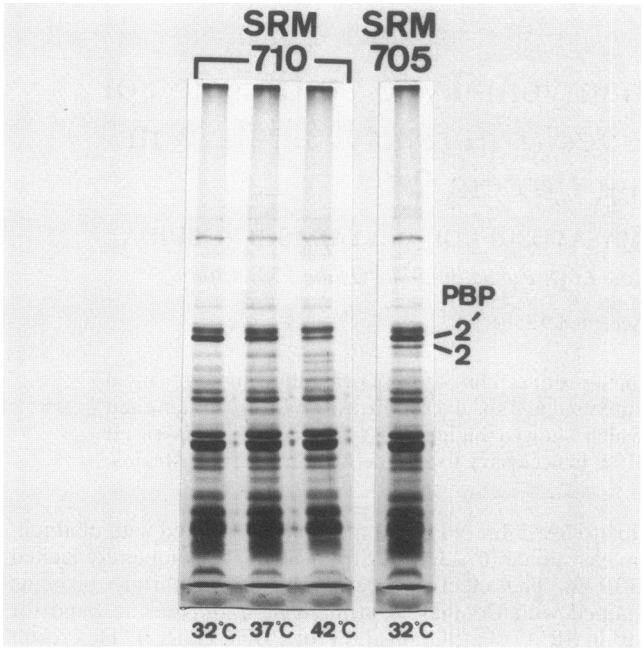

FIG. 2. Electrophoretogram of membrane proteins of *S. aureus* SRM705 and SRM710. Proteins were stained with Coomassie brilliant blue R-250. Bacteria were grown at the indicated temperatures.

cephamycin-type antibiotics than the other two strains were, whereas the susceptibility of strain SRM710 to cephalosporin-type antibiotics was similar to those of PBP 2-producing strains. All three strains became more susceptible at 42°C, and strain SRM710 was then only four times more susceptible to cephamycin-type antibiotics than the other two strains were.

The affinity of PBPs for cephamycin- and cephalosporin-type antibiotics was examined with strain SRM705 and other wild-type strains. Only PBP 4 had high affinity specifically for cephamycin-type drugs. The antibiotic concentrations required for 50% inhibition of the binding of [^{14}C]benzylpenicillin to PBP 4 were less than 0.1 μg/ml for cephamycin-type antibiotics and more than 24 μg/ml for cephalosporin-type drugs (data not shown). Similar results have been reported elsewhere (5, 11). All β-lactam compounds tested showed low affinity for PBP 2'. These findings strongly suggest that the activity of PBP 4, together with that of PBP 2', supports bacterial growth when the activity of PBP 2 is lost. Since PBP 4, although nonessential for growth (3), has transpeptidase activity (8, 19), it probably shares activity with PBP 2 to some extent.

The concentrations of flomoxef, moxalactam, and cefmetazole required to inhibit 50% of the binding of penicillin to PBP 2 are lower than 0.1 μg/ml (11; M. Murakami, M. Doi, K. Nomura, S. Nakamoto, and T. Yoshida, submitted for publication), so that at the concentrations which inhibited the growth of strain SRM710 at 37°C, most PBP 2 activity seems to be inhibited. Nevertheless, PBP 2-producing strains SR1550 and SRM705 grew at these antibiotic concentrations. This finding implies that the very small portion of the activity of PBP 2 which escapes antibiotic action is enough to support bacterial growth in the presence of PBP 2'.

The differences in MICs of cephamycin-type antibiotics against strain SRM710 at 37°C seem to reflect the differences in the affinities of these compounds for PBP 3. The concentration of flomoxef needed for 50% inhibition of penicillin binding to PBP 3 is 0.15 μg/ml, and the concentrations of cefmetazole and moxalactam needed are about 3 and 10 times higher, respectively (Murakami et al., submitted). Furthermore, these values are much smaller than the corresponding MICs. Thus, almost complete inhibition of not only PBP 2 and PBP 4 but also PBP 3 is probably necessary at 37°C to kill bacteria which produce PBP 2'.

To explain the decreased susceptibility of PBP 2-defective strains to cephamycin-type antibiotics at 32°C, we determined PBP 2' production in strain SRM710 at various temperatures (Fig. 2). The amounts produced at 37 and 42°C were 58 and 31% of the amount produced at 32°C, respectively. Induction of PBP 2' by 1 μg of flomoxef or cefazolin per ml did not occur in either parent or mutant at any temperature, a result that differs from results reported for other strains (2, 14, 17). The increased amount of PBP 2' at low temperatures probably compensates for the inhibited activities of PBP 3 and PBP 4, which raise the resistance level.

Although PBP 2 is believed to be encoded by the chromosome, it was largely lost when *S. aureus* SR1550 was cultivated at 43°C. The mechanism of the spontaneous loss is unknown but may involve an insertional inactivation of PBP 2 by some insertion sequence.

We are grateful to Takeshi Yokota, Juntendo University, Tokyo, Japan, for teaching us the PBP separation technique.

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TABLE 1. Temperature dependence of MICs of cephamycin- and cephalosporin-type antibiotics

Antibiotic	Element(s) at 7α position	MIC (μg/ml) for[a]: SR1550 (PBP 2+) 32°C	37°C	42°C	SRM705 (PBP 2+) 32°C	37°C	42°C	SRM710 (PBP 2−) 32°C	37°C	42°C
Flomoxef	-OCH_3	200	100	6.3	200	100	6.3	200	3.1	≤0.8
Moxalactam	-OCH_3	>800	>800	25	>800	>800	25	>800	50	6.3
Cefmetazole	-OCH_3	200	100	6.3	200	200	6.3	200	6.3	1.6
Demethoxy-flomoxef	-H	100	100	12.5	100	100	12.5	100	50	6.3
Demethoxy-moxalactam	-H	>400	>400	100	>400	>400	50	>400	>400	25
Cefazolin	-H	400	400	50	800	400	25	400	400	12.5
Cephalothin	-H	200	100	25	200	200	25	200	100	12.5
Cefamandole	-H	50	50	6.3	50	50	6.3	50	50	6.3

[a] PBP 2+, PBP 2 producing; PBP 2−, PBP 2 deficient.

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