ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1985, p. 851–857
0066-4804/85/050851-07$02.00/0

Vol. 27, No. 5

Occurrence of a β-Lactam-Inducible Penicillin-Binding Protein in Methicillin-Resistant Staphylococci

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Received 25 October 1984/Accepted 13 February 1985

The mechanism of methicillin resistance was investigated in methicillin-resistant staphylococci (MRS) and in variants which had lost methicillin resistance. Phase-contrast microscopy showed that cells swelled at low concentrations of β-lactam antibiotics in both MRS and variants which had lost methicillin resistance. Cells of variants which had lost methicillin resistance were lysed easily when higher concentrations of antibiotic were used. In contrast, MRS cells remained swollen at even higher concentrations of antibiotics. Furthermore, bacterial growth was inhibited at antibiotic concentrations much lower than MICs for MRS. Examination of the penicillin-binding proteins (PBPs) in MRS revealed that a new PBP-2′ (molecular weight, 74,000) was induced in large quantity by exposure to β-lactams. PBP-2′ was produced constitutively in variants of MRS which had lost a penicillinase plasmid. The induction of PBP-2′ by β-lactams was not detected in variants which had lost methicillin resistance. High concentrations of β-lactam were required for saturation of PBP-2′. The optimum antibiotic concentration for the induction of PBP-2′ varied with the β-lactam used as the inducer, and PBP-2′ was produced in a larger amount at 32°C than at 37°C. From these results, we suggest that the mechanism of methicillin resistance depends on the induction of PBP-2′, which may function as a detour enzyme for PBP-2 or PBP-3 or may be a particular enzyme involved in peptidoglycan synthesis.

Methicillin-resistant strains of *Staphylococcus aureus* were first reported in 1961 (19), soon after the introduction of methicillin into clinical use. Subsequently, it has been shown that these strains also are resistant to many penicillins and cephems other than methicillin (2, 11, 15). In addition, methicillin resistance (MR) has been found to be temperature dependent (1, 7, 12) and affected by pH (27), NaCl concentration (3, 12), and inoculum size (12, 23). Although methicillin-resistant staphylococci (MRS) produce penicillinase (PCase), curing of the PCase plasmid does not reduce the level of MR (28).

Four main penicillin binding proteins (PBPs) have been identified in *S. aureus* (20), and either one or both PBP-2 and PBP-3 have been suggested to be the lethal target(s) for β-lactam action (13). In MRS, production of altered PBPs with extremely low affinity for β-lactams (14, 16, 17) or an increase of an altered PBP-3 (6) has been described. However, the double-zone phenomenon occasionally detected on a disk diffusion susceptibility test of β-lactams, such as methicillin, nafcillin (24), or imipenem (4), for MRS cannot be explained by the decreased affinities of PBPs for β-lactams. This phenomenon suggests the existence of another mechanism for MR.

Although MRS had not been a major clinical problem in Japan, after the introduction of third-generation cephems in 1982 isolates of MRS increased. We isolated two variant types from our clinical isolates of MRS. One type retained MR, whereas PCase activity became negative, and the other type lost MR along with resistance to tobramycin (MSS). From studies both of morphological changes and viable cell counts after the addition of β-lactams and of the affinities of PBPs for β-lactams in MRS and MSS, we found that a new PBP-2′ is induced by β-lactams only in MRS. In this report, we describe the newly detected PBP-2′ that may be concerned with MR.

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MATERIALS AND METHODS

Bacterial strains. Methicillin-resistant strains of *S. aureus* (four strains) and *Staphylococcus epidermidis* (34) (one strain) and methicillin-susceptible strains of *S. aureus* (two strains) were isolated from clinical materials at the Teikyo University Hospital, Tokyo, Japan, in 1983. To eliminate antibiotic resistance from these isolates, cultures were incubated at 43.5°C overnight, spread on agar plates without antibiotics, and incubated at 32°C for 24 h. All colonies on the plates were replicated on selective agar plates containing 10 μg of either benzylpenicillin or ceftizoxime per ml. The colonies which had lost resistance were picked, and the MICs of β-lactams were determined.

Antibiotics. For testing MICs and for other experiments, the following antibiotics were utilized: benzylpenicillin and cloxacillin, Meiji Seika Kaisha Co. Ltd., Tokyo, Japan; methicillin, Banyu Ph. Co. Ltd., Tokyo, Japan; cefazolin and ceftizoxime, Fujisawa Ph. Co. Ltd., Osaka, Japan; cefmetazole, Sankyo Co. Ltd., Tokyo, Japan; cephaloridine and moxalactam, Shionogi & Co. Ltd., Osaka, Japan; imipenem, Merck Banyu Japan Co. Ltd., Tokyo.

Antimicrobial susceptibility testing. The MICs of β-lactams for clinical isolates and the variants were determined by the spot method on sensitivity test agar plates (Eiken Co. Ltd., Tokyo, Japan) containing twofold dilutions of the antibiotics. Overnight broth cultures of each strain were used to inoculate these plates with a multipoint replicating apparatus, resulting in a final inoculation of 10^4 CFU per spot. The plates were incubated at 32°C for 24 h.

Analysis of plasmid DNA. Clinical isolates and the variants were grown at 37°C for 6 h in 2 ml of tryptic soy broth (Difco Laboratories, Detroit, Mich.). The cells were harvested by centrifugation at 10,000 × *g* for 5 min, washed twice in TES buffer (50 mM Tris, 9 mM EDTA, 50 mM NaCl [pH 8.0]), and suspended in 100 μl of the same buffer. Then, lysostaphin (Sigma Chemical Co., St. Louis, Mo.) was added to the cell suspension at final concentrations of 33 μg/ml for *S. aureus* and 66 μg/ml for *S. epidermidis*, together with 40 μl

TABLE 1. MICs of β-lactam antibiotics for *S. aureus* and *S. epidermidis* strains

Species and strain	β-Lactamase	R or S[a]	MIC (μg/ml)[b]							
			PCG	DMPPC	MCIPC	CER	CEZ	CMZ	CZX	IMI
S. aureus										
MS353	−	S	0.1	0.8	0.1	0.1	0.2	0.4	0.8	0.1
TK722	+	S	12.5	0.8	0.8	0.1	0.4	0.4	3.1	0.1
TK784	+	R	50	>100	12.5	12.5	100	100	>100	25
TK784E	+	S	1.6	1.6	0.4	0.1	0.2	0.8	0.8	0.1
TK803	+	R	100	>100	6.3	12.5	>100	50	>100	12.5
TK803E	+	S	1.6	1.6	0.2	0.4	0.4	1.6	6.3	0.1
TK388	+	R	50	>100	100	25	>100	100	>100	50
TK388E	−	R	6.3	100	6.3	12.5	100	25	100	25
TK731	+	R	50	>100	100	12.5	>100	50	>100	50
TK731E	−	R	3.1	100	6.3	6.3	50	25	100	6.3
S. epidermidis										
TK406	+	R	25	50	6.3	1.6	6.3	50	100	6.3
TK406E	+	S	12.5	1.6	0.2	0.2	0.4	3.1	0.8	0.1

[a] S, Methicillin susceptible; R, methicillin resistant.
[b] Agar plate dilution method with inoculum of 10^4 CFU. PCG, Benzylpenicillin; DMPPC, methicillin; MCIPC, cloxacillin; CER, cephaloridine; CEZ, cefazolin; CMZ, cefmetazole; CZX, ceftizoxime; IMI, imipenem.

of 0.5 M EDTA in TES buffer. Plasmid DNA from lysates was prepared by the method of Cohen et al. (8) and analyzed by agarose gel electrophoresis (25).

Killing curves of β-lactams. Precultured cells were inoculated at 10^6 CFU/ml into the sensitivity broth (9.5 ml) containing various concentrations of β-lactams. The samples were taken after 0, 3, 6, and 9 h of incubation at 32°C. They were put through serial 10-fold dilutions in sterile saline, and 100 μl of each dilution was spread on sensitivity disk agar plates. After incubation for 24 h, CFU were counted.

Phase-contrast microscopy. Overnight cultures (0.5 ml) were inoculated into 10 ml of sensitivity broth and grown at 32°C for 2 h. The cells were diluted 10-fold, and 5 μl of the dilution was seeded onto glass slides coated with 200 μl of sensitivity disk agar containing various concentrations of β-lactams. The glass slides were covered by cover glasses (thickness, 0.1 mm) and placed at 32°C for 6 h. Photomicrographs were made on Neopan F film (Fuji Photo Film Co. Ltd., Tokyo, Japan) under ×1,000 magnification in Nikon phase-contrast microscopy MD 1 (Nikon Co. Ltd., Tokyo, Japan).

Preparation of membrane fractions. Overnight cultured cells (20 ml) were inoculated into 300 ml of tryptic soy broth with or without 0.2 μg of ceftizoxime per ml as the inducer. The cells were routinely grown at 32°C with shaking. Late-logarithmic-growth-phase cells were harvested by centrifugation at 10,000 × *g* for 10 min and then washed once in 50 ml of ice-cold 50 mM sodium phosphate buffer (pH 7.0). The cells were suspended in 3 ml of the same buffer and incubated at 32°C for 30 min with lysostaphin at a final concentration of 50 μg/ml. The cells were disrupted with an ultrasonicator (model 200M; 9 kHz; Kubota Co. Ltd., Tokyo, Japan) at 0°C for 30 min. Unbroken cells were removed by centrifugation at 7,000 × *g* for 10 min at 4°C, and cell membranes were pelleted by ultracentrifugation at 100,000 × *g* for 60 min at 4°C (Ultracentrifuge 70P; Hitachi Koki Co. Ltd., Katsuta, Japan). The membranes were suspended in 200 μl of the same buffer and stored at a concentration of 4 mg/ml of protein at −80°C until use. The protein content was determined by the method of Bradford (5).

Binding of [^{14}C]benzylpenicillin to PBPs. The methods used for PBP assays were essentially the same as those described by Spratt (31). A 5-μl volume of 1 mM [^{14}C]benzylpenicillin (Radiochemical Centre, Amersham, England) was added to 50 μl of membranes and incubated for 10 min at 32°C. For the strains producing PCase, clavulanic acid (Beecham Ph. Co. Ltd., Brentford, England) was used at final concentrations of 0.1 to 5 μg/ml for uninduced membranes and 10 to 20 μg/ml for induced membranes and then preincubated at 32°C for 3 min before the addition of [^{14}C]benzylpenicillin.

In competition experiments, membrane fractions were preincubated with 5 μl of various concentrations of the competing nonradioactive β-lactams for 10 min (final concentration, 0 to 400 μg/ml) and postincubated with 5 μl of 1 mM [^{14}C]benzylpenicillin for another 10 min.

The procedure for sodium dodecyl sulfate-polyacrylamide gel electrophoresis was that of Laemmli and Favre (22) with slight modifications. A running gel was modified to 7.4% (wt/vol) acrylamide and 0.08% (wt/vol) bisacrylamide as described by Yokota (38).

Optimal temperature for induction of PBP-2′. To determine the optimal temperature for induction of PBP-2′, cells were cultivated until late-logarithmic-growth phase at 32, 37, or 42°C, with or without the addition of ceftizoxime at 0.2 μg/ml into 300 ml of tryptic soy broth. The preparation of membrane fractions and [^{14}C]benzylpenicillin binding experiments were by the method described above.

Optimal concentrations of β-lactams for induction of PBP-2′. The cells were incubated with 300 ml of tryptic soy broth containing various concentrations (final concentration, 0, 0.04, 0.2, 1.0, 5.0, and 25.0 μg/ml) of cloxacillin, ceftizoxime, and cefazolin at 32°C until late-logarithmic-growth phase. Preparation of membrane fractions and experiments such as binding of [^{14}C]benzylpenicillin and electrophoresis were as described above.

RESULTS

β-lactam susceptibility and plasmid DNA. Table 1 shows the β-lactam MICs for the strain used and the presence of PCase. TK784E, TK803E, and TK406E were variant strains which had lost MR from strains TK784, TK803, and TK406, respectively, by treatment at 43.5°C. The MICs for these variants were reduced to the same level as those for MSS. In contrast, strains TK731E and TK388E were PCase-negative variants that retained partial MR.

Figure 1 shows the plasmid DNA profiles of MRS and

variant strains. Strain TK731E (lane E) lost a 20-megadalton plasmid; however, the plasmid pattern of strains TK784E (lane C) and TK406E (lane G) was the same as that of the parent strains. The 2-megadalton plasmid eliminated from strain TK406E mediated tetracycline resistance.

Effect of β-lactams on viable cells. Growth of strain TK784 was clearly inhibited by 12.5 μg of cloxacillin per ml (MIC). Moreover, cell growth was affected at a concentration of 0.78 μg/ml (1/16 MIC) as compared with the control culture. The turbidity of the cultures under the same conditions increased rapidly at approximately 9 h after the addition of the antibiotic. In contrast, the amount of viable cells of strain TK784E decreased gradually with time after the addition of 0.78 μg of cloxacillin per ml (MIC). The potency of cloxacillin in the medium was not substantially reduced in both strains during the experiment. The same pattern of growth inhibition was observed with cefazolin, cefmetazole, and ceftizoxime when low concentrations, well below the MIC, were used.

Morphological changes of cells after the addition of β-lactams. Altered morphology of strains TK784 (A to F) and TK784E (G to I) when they were cultured on cloxacillin containing agar are shown in Fig. 2. With strain TK784, cells were largely swollen on agar containing 0.04 μg of cloxacillin per ml (1/312 MIC; B) as compared with the control (A). Swollen cells were observable up to 5.0 μg of cloxacillin per ml (1/2.5 MIC; E). Lysing cells were seen among swollen cells when the antibiotic concentration was increased to 25.0 μg/ml (2 MIC; F). In contrast, swollen cells were observed at a concentration as low as 0.04 μg/ml (1/10 MIC; H) in strain TK784E, and cell lysis occurred only when the antibiotic concentration was raised to 0.2 μg/ml (1/2 MIC; I). Similar findings were obtained with other β-lactams.

PBPs of uninduced and induced strains by β-lactams. Figure 3 shows the results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of membrane fractions obtained from strains induced with 0.2 μg of ceftizoxime per ml (even-numbered columns) and uninduced strains (odd-numbered columns). A markedly increased protein band was found in induced membrane fractions from strains TK784, TK731, and TK406 and in the uninduced membrane fraction from PCase-negative strain TK731E. The results of the fluorogram are shown in Fig. 4. In uninduced membrane fractions from strains TK784 and TK731, and a new PBP was evident at a slightly higher position than PBP-2 when the concentration of clavulanic acid used as a PCase inhibitor before [^{14}C]benzylpenicillin binding was greater than 0.5 μg/ml. This new protein band in induced strains of MR and in PCase-negative variants retaining MR will be termed PBP-2′ and has a molecular weight of 74,000 compared with the molecular weight of 73,000 for PBP-2.

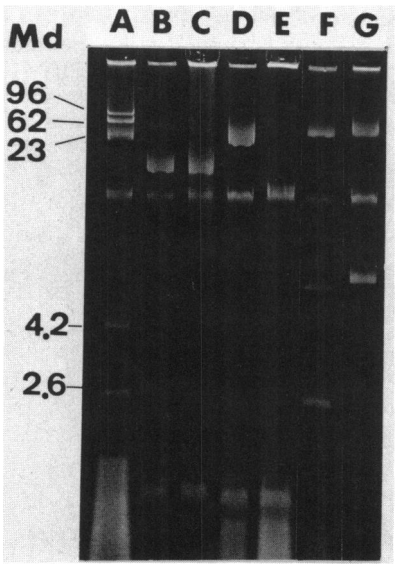

FIG. 1. Migration of *S. aureus* and *S. epidermidis* plasmid DNA in a 0.7% agarose gel. Lanes: A, plasmid size standards; B, *S. aureus* TK784; C, *S. aureus* TK784E; D, *S. aureus* TK731; E, *S. aureus* TK731E; F, *S. epidermidis* TK406; G, *S. epidermidis* TK406E. Md, Megadaltons.

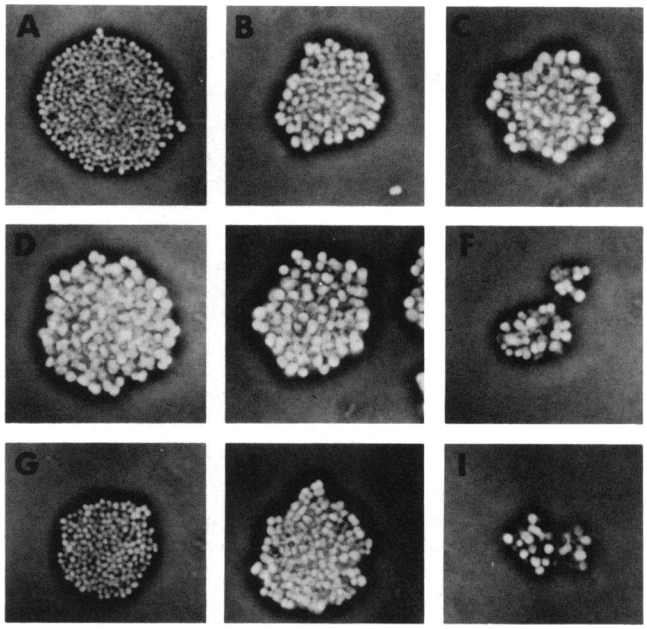

FIG. 2. Morphological changes of *S. aureus* TK784 and TK784E grown on agar containing cloxacillin for 6 h at 32°C. The observation was made with a phase-contrast microscope. Magnification, ×1,000. Lanes: A through F, strain TK784; A, no addition; B, 0.04 μg/ml (1/312 MIC); C, 0.2 μg/ml (1/62.5 MIC); D, 1.0 μg/ml (1/12.5 MIC); E, 5.0 μg/ml (1/2.5 MIC); F, 25.0 μg/ml (2 MIC); G through I, strain TK784E; G, no addition; H, 0.04 μg/ml (1/10 MIC); I, 0.2 μg/ml (1/2 MIC).

The production of PBP-2′ in all strains examined is summarized (Table 2). Induction of PBP-2′ after the addition of ceftizoxime was observed in all MRS which retained the PCase plasmid. Induction of PBP-2′ was not observed in MSS variants and in a wild-type MSS strain producing PCase. In contrast, PBP-2′ was produced constitutively in PCase-negative variants of MRS.

The same induction of PBP-2′ was observed when cefazolin, cefmetazole, and imipenem were used as inducers. Since [^{14}C]benzylpenicillin binding to PBPs decreased when the concentration of the inducer was increased, it was inferred that the inducer may have been previously bound to PBP-2′ and other PBPs, depending on the conditions of induction.

Affinity of PBP-2′ for β-lactams. Binding of cloxacillin to PBPs from strains TK784, TK784E, and TK731E without induction and from strain TK784 with induction by 0.2 μg of ceftizoxime per ml is shown in Fig. 5. Cloxacillin concentrations (Fig. 5) were those used for prebinding, and 0.1 mM [^{14}C]benzylpenicillin was used for each postbinding. In these studies, there were no detectable differences in the affinities

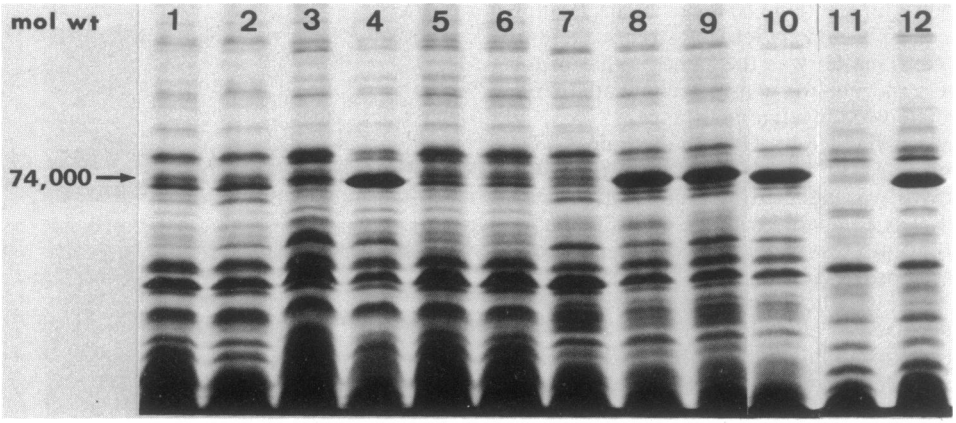

FIG. 3. Coomassie brilliant blue stain of 7% sodium dodecyl sulfate-polyacrylamide gel of membrane fractions isolated from cells induced or uninduced with β-lactam antibiotic. After the addition of 0.2 μg of ceftizoxime per ml, cells were cultivated until late-logarithmic-growth phase at 32°C. The samples of each lane are: 1, uninduced strain TK722; 2, induced strain TK722; 3, uninduced strain TK784; 4, induced strain TK784; 5, uninduced strain TK784E; 6, induced strain TK784E; 7, uninduced strain TK731; 8, induced strain TK731; 9, uninduced strain TK731E; 10, induced strain TK731E; 11, uninduced strain TK406; 12, induced strain TK406. mol wt, Molecular weight.

of PBP-1, PBP-2, and PBP-3 for cloxacillin with uninduced membrane fractions from strains TK784 and TK784E. The affinities of PBP-1 and PBP-3 for cloxacillin from induced strain TK784 and uninduced strain TK731E showed similar affinities as those of PBPs from uninduced membranes, whereas the affinity of the increased PBP-2′ was greatly different, namely, high concentrations of cloxacillin were required for saturation of PBP-2′.

Fifty percent binding levels of cefazolin, cefmetazole, and ceftizoxime for PBP-2′ were the same as those of cloxacillin.

Optimum temperature for induction of PBP-2′. Figure 6 shows the effect of the temperature of incubation on the production of PBP-2′ when 0.2 μg of ceftizoxime per ml was used as the inducer. When strains TK784 and TK731 were incubated at 32, 37, and 42°C, a large amount of PBP-2′ was produced at 32°C in both strains. Production was markedly decreased when the temperature was raised. The constitutive production of PBP-2′ by strain TK731E cultured without the inducer also was affected by the temperature of incubation, as in the parent strain. However, the productions of PBP-1 and PBP-3 were little affected by temperature (data not shown).

Optimum antibiotic concentration for induction of PBP-2′. The results of an investigation of optimum concentrations of cloxacillin and ceftizoxime for induction of PBP-2′ in strains TK784 and TK406 is shown (Fig. 7). The cloxacillin concentrations required for induction of PBP-2′ were far lower than those for ceftizoxime, demonstrating that the optimum concentration of β-lactams for induction of PBP-2′ may be different for each of the antibiotics. Furthermore, even if identical antibiotics were used as inducers, the optimum concentration for induction of PBP-2′ varied with the bacterial species.

DISCUSSION

Four main PBPs have been identified in *S. aureus* (20), but which of these PBPs is the lethal target(s) for β-lactams is not yet clear. However, PBP-1 and PBP-4 are not essential targets since a mutant lacking these PBPs is viable (37). Thus, it is highly probable that PBP-2 or PBP-3 is the lethal target. This possibility is supported by the finding that the degree of binding of β-lactams for these PBPs is correlated with the MICs of each of the antibiotics (13). Unlike the

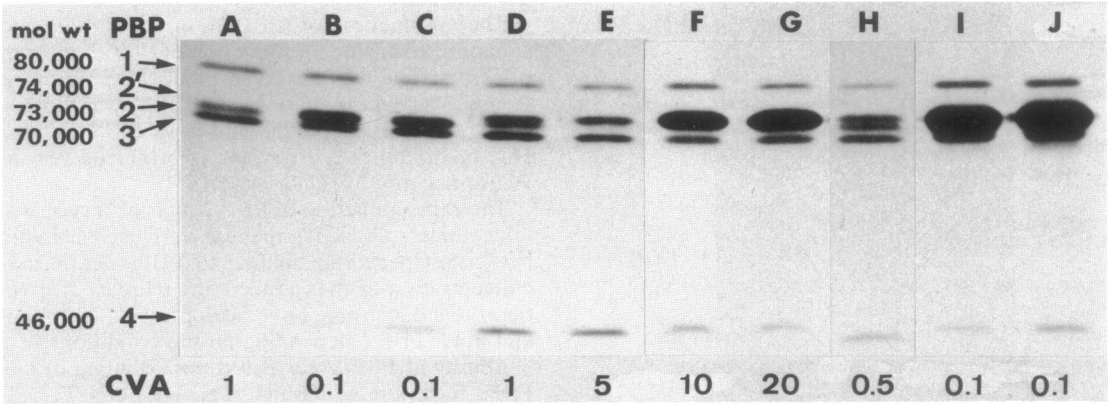

FIG. 4. Fluorogram of the PBPs of *S. aureus* MS353, TK784, TK731, and TK731E. Membrane factions were preincubated with clavulanic acid (CVA; amounts shown below each lane) to inhibit PCase activity. [^{14}C]benzylpenicillin was bound to the membrane for 10 min at 32°C, sarcosyl solubilized, and electrophoresed. The samples of each lane are: A and B, uninduced strain MS353; C through E, uninduced strain TK784; F and G, induced (0.2 μg of ceftizoxime per ml) strain TK784; H, uninduced strain TK731; I, induced (0.2 μg of ceftizoxime per ml) strain TK731; J, uninduced strain TK731E. mol wt, Molecular weight.

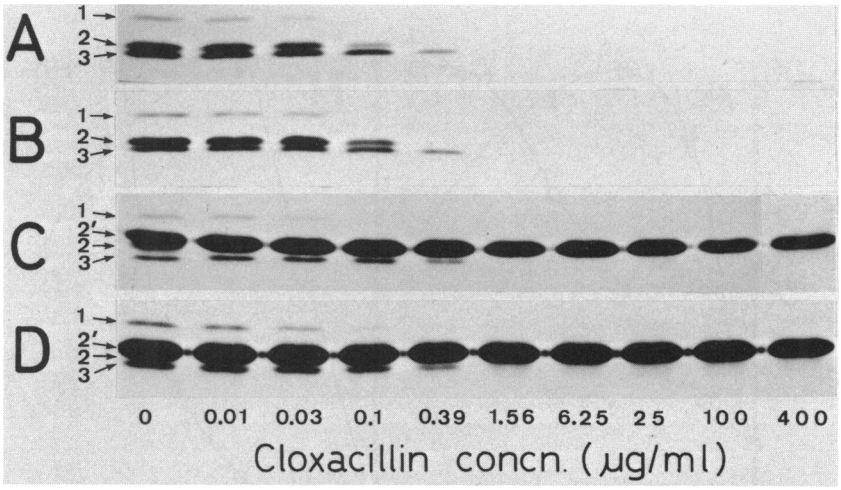

FIG. 5. Fluorogram of cloxacillin binding for the PBPs of *S. aureus* TK784, TK784E, and TK731E. Membrane fractions were solubilized after a 10-min preincubation at 32°C with cloxacillin (final concentrations, 0, 0.01, 0.02, 0.1, 0.39, 1.56, 6.25, 25, 100, and 400 μg/ml), followed by [^{14}C]benzylpenicillin binding. Uninduced strains TK784 (A), TK784E (B), and TK731E (D) were preincubated with 0.1 μg of clavulanic acid per ml, and strain TK784 (C) induced with 0.2 μg of ceftizoxime per ml was preincubated with 10.0 μg of clavulanic acid per ml for 3 min at 32°C before cloxacillin binding.

studies of PBP-1Bs (26) and PBP-1A (18) of *Escherichia coli*, these important PBPs of *S. aureus* have not yet been purified, and consequently their enzyme activity and the relationship between PBP-2 and PBP-3 are still obscure.

In contrast, it has been suggested that the mechanism of MR is due to an alteration of PBPs, since resistance is not mediated by PCase plasmids (21, 29, 33). Data demonstrating that the affinities of main PBPs (16), PBP-2 (36), or PBP-3 (17) for β-lactams are decreased or that the amount of altered PBP-3 is increased (6) already have been presented; however, there is no general agreement as yet. In studies related to a mechanism of MR other than PBPs, reports on the composition of cell walls (35) and peptidoglycan synthesis in vivo (30, 36) between MRS and MSS have been presented as well.

Even if the above results are accepted as evidence, the double-zone phenomenon with β-lactams observed in some strains of MRS recently isolated from clinical materials (4, 24) cannot be explained by the alteration of PBPs. This double-zone phenomenon and the growth curve of cells when low concentrations of β-lactams were added to the culture medium of MRS suggest that contact of the organism with β-lactams might induce a more active mechanism of resistance.

TABLE 2. Inducibility of PBP-2′ by β-lactam antibiotics in various strains of *S. aureus* and *S. epidermidis*

Strain	β-Lactamase	R or S[a]	PBP-2′
MS353	−	S	Uninducible
TK784	+	R	Inducible
TK803	+	R	Inducible
TK388	+	R	Inducible
TK731	+	R	Inducible
TK406	+	R	Inducible
TK722	+	S	Uninducible
TK784E	+	S	Uninducible
TK803E	+	S	Uninducible
TK388E	−	R	Constitutive
TK731E	−	R	Constitutive
TK406E	+	S	Uninducible

[a] S, Susceptible to methicillin; R, resistant to methicillin.

Therefore, we concentrated on MRS, which have recently emerged as an important clinical problem in Japan. Since analysis of the resistant mechanism has not been attempted with variant strains of MSS, variants eliminating MR simultaneously with tobramycin resistance were selected from

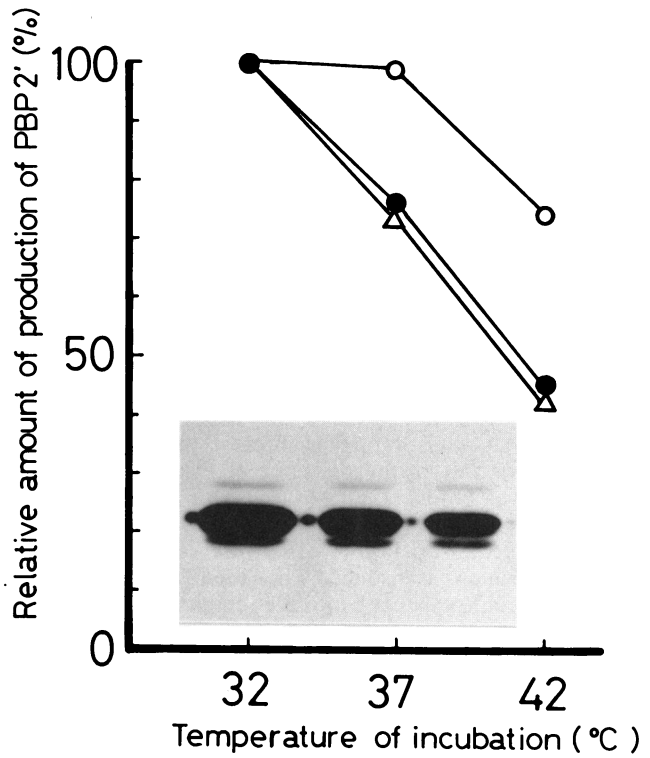

FIG. 6. Optimum temperature for induction of PBP-2′ of *S. aureus* TK784, TK731, and TK731E. Each strain was incubated at 32, 37, and 42°C, respectively. Ceftizoxime (0.2 μg/ml) as the inducer was added to the culture medium of strains TK784 and TK731 for induction of PBP-2′. For strain TK731E producing constitutive PBP-2′, the inducer was not added. Symbols: Δ, strain TK784; ○, strain TK731; ●, strain TK731E.

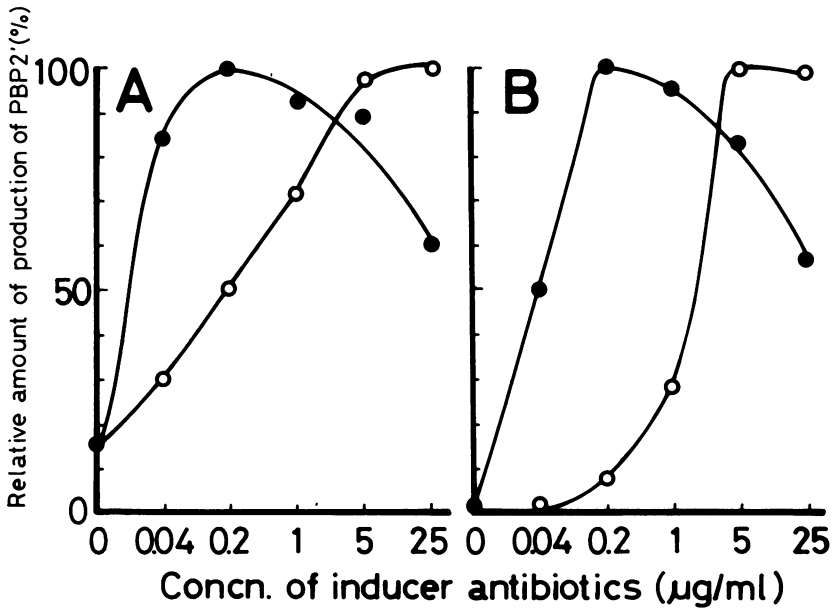

FIG. 7. Optimum antibiotic concentration for induction of PBP-2' of *S. aureus* TK784 (A) and *S. epidermidis* TK406 (B). Each antibiotic was added to the culture medium at final concentrations of 0, 0.04, 0.2, 1, 5, and 25 μg/ml. Cells were incubated until late-logarithmic-growth phase at 32°C. Symbols: ○, ceftizoxime; ●, cloxacillin.

MRS and used in our experiments. We presumed from the growth curves and morphological changes between the parent strains and the variants that some PBP related to cell lysis might be induced by β-lactams. This hypothesis has been confirmed by the finding that the PBP inducible by β-lactams is only detected in MRS, whereas it is absent in variants and in wild-type strain of MSS which produce PCase. This inducible PBP was found to be a new PBP-2' from our results with PBPs in MRS and MSS with or without induction. In addition, inducible PBP-2' appears to be identical to that first described by Yokota (38) in PCase-negative variants of MRS. Although we have reported the summary of induction of PBP-2' in MRS (K. Ubukata, N. Yamashita, R. Nonoguchi, A. Gotoh, and M. Konno, Program Abstr. 32nd Japan Soc. Chemother., abstr. no. 209, 1984), a similar nafcillin-induced PBP-2a also was recently reported by Chambers et al. (H. Chambers, B. Hartman, and A. Tomasz, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 6, 1984).

Moreover, it was of further interest that induction of PBP-2' occurs only in the presence of PCase plasmid and that PBP2' was produced constitutively in MRS which had lost the plasmid. This finding is interesting in view of the results on the transduction of MR, which showed that MR was more efficiently transduced when the PCase plasmid had previously been transduced into the recipient strains (9, 10, 32).

In brief, the results of MRS may be summarized as follows: (i) morphological changes from normal cells to swollen cells are not followed by cell lysis, (ii) PBP-2' is induced by β-lactams, (iii) a higher concentration of β-lactams is required for saturation of PBP-2', (iv) the optimum temperature for induction of PBP-2' is 32°C rather than 37°C, and (v) the optimum concentration for induction of PBP-2' varied with the various β-lactams. These findings strongly suggest that PBP-2' may be the detour enzyme of PBP-2 or PBP-3 or may have another function in the peptidoglycan synthesis. Therefore, concomitant inhibitions of PBP-2' and other PBPs may be necessary to kill the organism.

The double-zone phenomenon observed only at a low concentration range of some β-lactams in the disk diffusion susceptibility test could be explained by the fact that the concentrations of antibiotics for saturation of PBP-2 and PBP-3 are lower than those required for induction of PBP-2'. The double-zone phenomenon is more likley to occur with ceftizoxime, imipenem, and methicillin, and it is unusual with cloxacillin and cefazolin whose concentrations for induction of PBP-2' are very low (unpublished data).

Furthermore, since the β-lactam used as the inducer is assumed to be covalently bound to the induced PBP-2', measurement of the true enzyme activity of PBP-2' may be difficult unless a PCase-negative variant is used. We have not yet examined whether PBP-2' has transpeptidase activity or done any genetic analysis of the gene(s) mediating PBP-2'. Further work on these subjects will be necessary in the future.

LITERATURE CITED

1. **Annear, D. I.** 1968. The effect of temperature on resistance of *Staphylococcus aureus* to methicillin and some other antibiotics. Med. J. Aust. **1**:444–446.
2. **Barber, M.** 1962. Coagulase-positive staphylococci resistant to benzylpenicillin, methicillin, and other penicillins, p. 89–104. *In* A. V. S. de Reuck and M. P. Cameron (ed.), Resistance of bacteria to the penicillins. Little, Brown, & Co., Boston.
3. **Barber, M.** 1964. Naturally occurring methicillin-resistant staphylococci. J. Gen. Microbiol. **35**:183–190.
4. **Blumenthal, R. M., R. Raeder, C. D. Takemoto, and E. H. Freimer.** 1983. Occurrence and expression of imipemide (*N*-formimidoyl thienamycin) resistance in clinical isolates of coagulase-negative staphylococci. Antimicrob. Agents Chemother. **24**:61–69.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
6. **Brown, D. F. J., and P. E. Reynolds.** 1980. Intrinsic resistance to

β-lactam antibiotics in *Staphylococcus aureus*. FEBS Lett. **122**:275–278.
7. **Canawati, H. N., J. L. Witte, and F. L. Sapico.** 1982. Temperature effect on the susceptibility of methicillin-resistant *Staphylococcus aureus* to four different cephalosporins. Antimicrob. Agents Chemother. **21**:173–175.
8. **Cohen, M. L., E. S. Wong, and S. Falkow.** 1982. Common R-plasmids in *Staphylococcus aureus* and *Staphylococcus epidermidis* during a nosocomial *Staphylococcus aureus* outbreak. Antimicrob. Agents Chemother. **21**:210–215.
9. **Cohen, S., and H. M. Sweeney.** 1970. Transduction of methicillin resistance in *Staphylococcus aureus* dependent on an unusual specificity of the recipient strain. J. Bacteriol. **104**:1158–1167.
10. **Cohen, S., and H. M. Sweeney.** 1973. Effect of the prophage and penicillinase plasmid of the recipient strain upon the transduction and the stability of methicillin resistance in *Staphylococcus aureus*. J. Bacteriol. **116**:803–811.
11. **Collins, J. K., J. T. Mader, and M. T. Kelly.** 1983. Resistance of methicillin-resistant *Staphylococcus aureus* to third-generation cephalosporins. J. Infect. Dis. **147**:591.
12. **Dyke, K. G. H.** 1968. Penicillinase production and intrinsic resistance to penicillins in methicillin-resistant cultures of *Staphylococcus aureus*. J. Med. Microbiol. **2**:261–278.
13. **Georgopapadakou, N. H., and F. Y. Liu.** 1980. Binding of β-lactam antibiotics to penicillin-binding proteins of *Staphylococcus aureus* and *Streptococcus faecalis*: relation to antibacterial activity. Antimicrob. Agents Chemother. **18**:834–836.
14. **Georgopapadakou, N. H., S. A. Smith, and D. P. Bonner.** 1982. Penicillin-binding proteins in a *Staphylococcus aureus* strain resistant to specific β-lactam antibiotics. Antimicrob. Agents Chemother. **22**:172–175.
15. **Gravenkemper, C. F., J. L. Brodie, and W. M. M. Kirby.** 1965. Resistance of coagulase-positive staphylococci to methicillin and oxacillin. J. Bacteriol. **89**:1005–1010.
16. **Hartman, B., and A. Tomasz.** 1981. Altered penicillin-binding proteins in methicillin-resistant strains of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **19**:726–735.
17. **Hayes, M. V., N. A. C. Curtis, A. W. Wyke, and J. B. Ward.** 1981. Decreased affinity of a penicillin-binding protein for β-lactam antibiotics in a clinical isolate of *Staphylococcus aureus* resistant to methicillin. FEMS Microbiol. Lett. **10**: 119–122.
18. **Ishino, F., K. Mitsui, S. Tamaki, and M. Matsuhashi.** 1980. Dual enzyme activities of cell wall peptidoglycan synthesis, peptidoglycan transglycosylase and penicillin-sensitive transpeptidase, in purified preparations of *Escherichia coli* penicillin-binding protein 1A. Biochem. Biophys. Res. Commun. **97**:287–293.
19. **Jevons, M. P.** 1961. "Celbenin"-resistant staphylococci. Br. Med. J. **1**:124–125.
20. **Kozarich, J. W., and J. L. Strominger.** 1978. A membrane enzyme from *Staphylococcus aureus* which catalyzes transpeptidase, carboxypeptidase, and penicillinase activities. J. Biol. Chem. **253**:1272–1278.
21. **Kuhl, S. A., P. A. Pattee, and J. N. Baldwin.** 1978. Chromosomal map location of the methicillin resistance determinant in *Staphylococcus aureus*. J. Bacteriol. **135**:460–465.
22. **Laemmli, U. K., and M. Favre.** 1973. Maturation of the head of bacteriophage T4. I. DNA packaging events. J. Mol. Biol. **80**:575–599.
23. **Laverdiere, M., D. Welter, and L. D. Sabath.** 1978. Use of a heavy inoculum in the in vitro evaluation of the anti-staphylococcal activity of 19 cephalosporins. Antimicrob. Agents Chemother. **13**:669–675.
24. **McDougal, L. K., and C. Thornsberry.** 1984. New recommendations for disk diffusion antimicrobial susceptibility tests for methicillin-resistant (heteroresistant) staphylococci. J. Clin. Microbiol. **19**:482–488.
25. **Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529–1537.
26. **Nakagawa, J., S. Tamaki, and M. Matsuhashi.** 1979. Purified penicillin binding proteins 1Bs from *Escherichia coli* membrane showing activities of both peptidoglycan polymerase and peptidoglycan crosslinking enzyme. Agric. Biol. Chem. **43**:1379–1380.
27. **Sabath, L. D., S. J. Wallace, and D. A. Gerstein.** 1972. Suppression of intrinsic resistance to methicillin and other penicillins in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **2**:350–355.
28. **Seligman, S. J.** 1966. Penicillinase-negative variants of methicillin-resistant *Staphylococcus aureus*. Nature (London) **209**: 994–996.
29. **Sjöström, J. E., S. Löfdahl, and L. Philipson.** 1975. Transformation reveals a chromosomal locus of gene(s) for methicillin resistance in *Staphylococcus aureus*. J. Bacteriol. **123**:905–915.
30. **Smith, P. F., and B. J. Wilkinson.** 1981. Differential methicillin susceptibilities of peptidoglycan synthesis in methicillin-resistant *Staphylococcus aureus*. J. Bacteriol. **148**:610–617.
31. **Spratt, B. G.** 1977. Properties of the penicillin-binding proteins of *Escherichia coli* K12. Eur. J. Biochem. **72**:341–352.
32. **Stewart, G. C., and E. D. Rosenblum.** 1980. Transduction of methicillin resistance in *Staphylococcus aureus*: recipient effectiveness and beta-lactamase production. Antimicrob. Agents Chemother. **18**:424–432.
33. **Stiffler, P. W., H. M. Sweeney, and S. Cohen.** 1973. Absence of circular plasmid deoxyribonucleic acid attributable to a genetic determinant for methicillin resistance in *Staphylococcus aureus*. J. Bacteriol. **116**:771–777.
34. **Ubukata, K., N. Yamashita, A. Gotoh, and M. Konno.** 1984. Purification and characterization of aminoglycoside-modifying enzymes from *Staphylococcus aureus* and *Staphylococcus epidermidis*. Antimicrob. Agents Chemother. **25**:754–759.
35. **Wilkinson, B. J., K. J. Dorian, and L. D. Sabath.** 1978. Cell wall composition and associated properties of methicillin-resistant *Staphylococcus aureus* strains. J. Bacteriol. **136**:976–982.
36. **Wyke, A. W., J. B. Ward, and M. V. Hayes.** 1982. Synthesis of peptidoglycan *in vivo* in methicillin-resistant *Staphylococcus aureus*. Eur. J. Biochem. **127**:553–558.
37. **Wyke, A. W., J. B. Ward, M. V. Hayes, and N. A. C. Curtis.** 1981. A role *in vivo* for penicillin-binding protein-4 of *Staphylococcus aureus*. Eur. J. Biochem. **119**:389–393.
38. **Yokota, T.** 1984. Methicillin- and cephems-resistant *staphylococcus aureus*. Infect. Inflammation Immun. **14**:87–97.