ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1985, p. 397–403
0066-4804/85/090397-07$02.00/0

Vol. 28, No. 3

Role of an Altered Penicillin-Binding Protein in Methicillin- and Cephem-Resistant *Staphylococcus aureus*

YUKIO UTSUI* AND TAKESHI YOKOTA

Department of Bacteriology, School of Medicine, Juntendo University, 1-1, Bunkyo-ku, Tokyo 113, Japan

Received 14 January 1985/Accepted 17 June 1985

About 80% of methicillin- and cefazolin-resistant strains of *Staphylococcus aureus* isolated clinically in Japan in 1982 retained their resistance even after elimination of penicillinase-encoding plasmids. The penicillin-binding proteins (PBPs) of the penicillinase-free, methicillin- and cephem-resistant subclones of *Staphylococcus aureus* (MRSA) were compared with those of spontaneous susceptible revertants which had been obtained by the replica method after 10 subcultures in drug-free media. A new PBP fraction (PBP2′) having a molecular weight of 78,000 and low binding affinities for various β-lactam antibiotics was found in MRSA exclusively. The levels of resistance of MRSA strains were reduced markedly by culturing them at 43°C or at pH 5.2 or both. We found that the binding capacity of PBP2′ for ^{14}C-labeled penicillin G was decreased by preincubation of the membrane fractions of MRSA strains at 43°C for 60 min and that the amount of PBP2′ in MRSA strains grown at pH 5.2 was less than that the amount of PBP2′ in MRSA strains grown at pH 7.0. Temperature- and pH-dependent expression of resistance in MRSA is likely to reflect the temperature sensitivity and neutral pH-dependent production of the specific PBP fraction (PBP2′). We suggest that MRSA strains can grow in the presence of β-lactam antibiotics because of the low affinities of the specific PBP2′ fraction for various β-lactam antibiotics.

The isolation frequencies of strains of methicillin- and cephem-resistant *Staphylococcus aureus* (MRSA) from clinical specimens have been gradually increasing (3, 7, 8, 21). These strains tend to be resistant to various β-lactam antibiotics, although the levels of resistance to each drug are different. In particular, these organisms are highly resistant to many of the expanded-spectrum cephem antibiotics with few exceptions. This fact may be due to the wide clinical use of semisynthetic penicillins and third-generation cephem antibiotics which possess rather weak anti-staphylococcal activity.

Methicillin-resistant strains of *Staphylococcus aureus* were first reported in 1961 (15). These strains expressed cross resistance to other β-lactam antibiotics (22), and the resistance was intrinsic rather than due to penicillinase, as penicillinase-free mutants of the methicillin-resistant strains retained the resistance (9). Furthermore, the resistance was unusual in that the cell populations were heterogeneous (24) with respect to the levels of resistance in each cell. The levels of resistance in MRSA strains were affected markedly by growth conditions; namely, the levels of resistance of MRSA strains were higher at 30°C and pH 7.0 than at 43°C and pH 5.2, respectively (1, 18–20). The levels of resistance were also higher in media containing 5% NaCl than in salt-free media (2).

The mechanism of methicillin and cephem resistance in MRSA remained unanswered for a long time. Brown and Reynolds (4) and Hayes and colleagues (14) reported decreased affinity of penicillin-binding protein 3 (PBP3) in one strain of MRSA. In 1982 Georgopapadakou et al. (12) published a paper dealing with the decreased production of PBP3 and the low binding affinity of PBP2 in an oral cephem-resistant *Staphylococcus aureus* strain. Yokota and Sekiguchi (T. Yokota and R. Sekiguchi, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 22nd, abstr. no. 342, 1982) reported that in addition to PBP1, PBP2, PBP3, and PBP4 a new PBP fraction with molecular weight of 78,000 appeared in two penicillinase-free MRSA strains and that the new PBP fraction possessed low affinities for various β-lactam antibiotics. In this paper we describe the role of the specific PBP fraction in MRSA strains in relation to temperature- and pH-dependent expression of resistance.

MATERIALS AND METHODS

Bacterial strains. We used 60 MRSA strains isolated clinically from outpatients having primary infections (7, 8); these strains were resistant to both methicillin and cefazolin at concentrations of more than 25 μg/ml.

Elimination of penicillinase-encoding plasmids and preparation of spontaneous drug-susceptible revertants from MRSA. MRSA strains were cultured at 44°C (16) in drug-free L-broth overnight with shaking. Subcultures were streaked onto plates containing drug-free heart infusion agar (HIA; Eiken Chemical Co., Ltd., Tokyo, Japan), and penicillinase-free subclones were selected by replica plating onto HIA plates with or without 10 μg of ampicillin per ml. The clones were further confirmed to be free of penicillinase by the nitrocefin color test (17).

The penicillinase-free MRSA clones were successively subcultured in 10-ml portions of drug-free L-broth by using an inoculum size of 10^5 CFU at 37°C with shaking overnight. After 10 subcultures the cultures were diluted 10^5-fold with fresh L-broth, and 0.1-ml portions of the diluted samples were spread onto drug-free HIA plates; this was follwed by incubation at 37°C overnight. Colonies grown on drug-free HIA plates were replicated on HIA plates with and without 3.13 μg of methicillin per ml. After incubation at 37°C overnight, colonies growing on drug-free agar plates but not on methicillin-containing agar plates were selected and confirmed to be drug-susceptible spontaneous revertants.

Chemicals. Methicillin was obtained from Banyu Pharmaceutical Co., Ltd., Tokyo, Japan; ampicillin was obtained

* Corresponding author.

from Sankyo Co., Ltd., Tokyo, Japan; cefazolin was obtained from Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan; and cefotaxime was obtained from Hoechst Japan Ltd., Tokyo, Japan. ^{14}C-labeled penicillin G (potassium 6-phenyl [1-^{14}C]acetamidopenicillanate; 54 mCi/mmol) and nitrocefin were purchased from Amersham International Plc., Buckinghamshire, United Kingdom, and Glaxo Laboratories, Ltd., Greenford, United Kingdom, respectively.

Susceptibility test. Susceptibility testing of MRSA parent strains, the penicillinase-free subclones, and the drug-susceptible revertants with various β-lactam antibiotics was carried out by using the twofold plate dilution method and Mueller-Hinton agar (Becton Dickinson and Co., Madison, Wis.) on which one loopful of a 10^6-CFU/ml suspension of the test bacterium was streaked, followed by incubation at 30, 37, or 43°C for 18 h.

Preparation of membrane fractions. Cells of penicillinase-free MRSA subclones and their drug-susceptible spontaneous revertants were cultured overnight in 10 ml of L-broth with shaking at 30, 37, or 43°C. The subcultures were transferred into 200-ml portions of fresh L-broth in 500-ml flasks and incubated in a shaking water bath at 30, 37, or 43°C until late logarithmic phase. The bacterial cells were harvested by centrifugation in a refrigerated centrifuge at 6,000 × *g* for 15 min. The cells were suspended in 8 ml of 50 mM sodium phosphate buffer containing 10 mM $MgCl_2$ and disrupted by sonication 20 times at 10 kc for 30 s with 15-s intervals in ice-cold water, followed by centrifugation at 3,000 × *g* for 15 min at 4°C. The clear supernatants were centrifuged at 100,000 × *g* for 30 min at 4°C, and the membrane fractions were washed once with 50 mM sodium phosphate buffer containing 10 mM $MgCl_2$ and suspended in a small volume of the same buffer. Each membrane suspension was adjusted to a concentration of 8 mg of protein per ml.

Analysis of PBPs. The PBPs of *Staphylococcus aureus* were analyzed by the method of Spratt (23), with some modifications. Experiments to determine competitive binding of β-lactam antibiotics by the PBPs were performed by preincubation of the membrane fractions with various nonradioactive β-lactam antibiotics at 30°C for 10 min before the addition of ^{14}C-labeled penicillin G. After electrophoresis of the samples in acrylamide slab gels, 2,5-diphenyloxazole was incorporated into the gels, and the gels were dried in vacuo; this was followed by exposure to X-ray film (Eastman Kodak Co., Rochester, N.Y.) at −70°C for 14 days or more. To achieve better separation of the PBPs, a separating gel consisting of 8% (wt/vol) monoacrylamide and 0.06% (wt/vol) *N,N'*-methylenebisacrylamide (both from Seikagaku-kogyo Co., Ltd., Tokyo, Japan) in 0.25 M Tris-hydrochloride buffer (pH 8.8) (Sigma Chemical Co., St. Louis, Mo.) was used. Fluorographs of the PBPs of MRSA strains were quantitatively analyzed with a densitometer (model CS-910; Shimadzu Corp., Kyoto, Japan), when necessary.

RESULTS

Change in susceptibility to β-lactam antibiotics in cefazolin-resistant *Staphylococcus aureus* strains by elimination of penicillinase-encoding plasmid. Penicillinase-free subclones were obtained from 39 of 60 clinically isolated cefazolin-resistant *Staphylococcus aureus* strains. Tests to determine the susceptibilities of these subclones to β-lactam antibiotics were carried out at 37°C. The MICs of ampicillin, cefazolin, and methicillin for 8 of 39 subclones were 0.05 μg/ml or less, 0.78 μg/ml or less, and 6.25 μg/ml or less, respectively. These MICs are similar to the values for the standard susceptible strain of *Staphylococcus aureus* (strain FDA 209P). The restoration of susceptibility in these eight subclones by elimination of plasmids suggests that resistance in 20% of the strains was plasmid determined. In contrast, 31 subclones (80%) retained resistance, and the levels of resistance of 20 subclones were not changed at all, even after elimination of penicillinase-encoding plasmids. These results indicate that the major part of the resistance of the cefazolin-resistant *Staphylococcus aureus* strains used in this study was intrinsic, whereas a small portion of the resistance of these cefazolin-resistant *Staphylococcus aureus* strains depended on a plasmid.

Preparation of drug-susceptible spontaneous revertants from MRSA strains. A total of 31 penicillinase-free subclones retaining resistance to methicillin were subcultured 10 times in drug-free L-broth at 37°C overnight, and susceptible spontaneous revertants were selected by replica plating at a rate of 0.5 to 1.3% from the final subcultures in 6 of 31 subclones. The level of susceptibility of these revertants was comparable to that of the susceptible standard strain of *Staphylococcus aureus* (strain FDA 209P). No methicillin-resistant cells appeared from the selected susceptible revertants. Susceptible revertants were not obtained from the remaining 25 penicillinase-free, methicillin-resistant strains by this method. The proportion of susceptible cells in different MRSA strains varied.

Specific profile of PBPs in MRSA strains and competition of various β-lactam antibiotics for the PBPs. The results of fluorography of PBPs of MRSA strains and of their susceptible revertants are compared in Fig. 1. All MRSA strains tested (strains 6-1, 108-1, 123-1, and 228-1) possessed a specific PBP fraction (PBP2′) having a molecular weight of 78,000 in addition to possessing PBP2 and PBP3, which have been reported to be essential PBPs for murein synthesis by *S. aureus* and to be required for normal growth, and nonessential PBP1, PBP4, and minor components (5, 6, 11, 14, 26). The susceptible revertants (strains 6-1-4, 108-1-1, 123-1-2, and 228-1-1) were found to have lost PBP2′. The electrophoretic mobilities of PBPs other than PBP2′ in MRSA strains were identical to those of the PBPs in susceptible revertants. For a clear demonstration of the PBP2′ fraction specific for MRSA, we used a separating slab gel consisting of 8% monoacrylamide and 0.06% *N,N'*-methylenebisacrylamide. Otherwise, identification of PBP2′ became difficult because of overlapping with PBP2.

The results of fluorography to determine competition by nonradioactive methicillin, cefazolin, and cefotaxime at concentrations of 25, 100, and 400 μg/ml for ^{14}C-labeled penicillin G binding to the PBPs of MRSA strain 123-1 are shown in Fig. 2. All PBP fractions other than PBP2′ were outcompeted by relatively low concentrations (i.e., 25 μg or more of a drug per ml). In particular, PBP1 and PBP3 were fully saturated by 25 μg of methicillin per ml, 25 μg of cefazolin per ml, and 25 μg cefotaxime per ml. PBP2 showed a low binding affinity for methicillin, but it was saturated by 25 μg of cefazolin per ml and 25 μg of cefotaxime per ml. The much lower binding affinity of the PBP2′ fraction for β-lactam antibiotics compared with the other PBP fractions was confirmed, as even 400 μg of methicillin per ml, 400 μg of cefazolin per ml, and 400 μg of cefotaxime per ml did not saturate it. The MICs of methicillin, cefazolin, and cefotaxime for MRSA strain 123-1 were 400, 200, and 400 μg/ml, respectively, when the organism was tested at 37°C. The reason why MRSA is resistant to β-lactam antibiotics may be the specific low binding affinity of the PBP2′ fraction for the drugs.

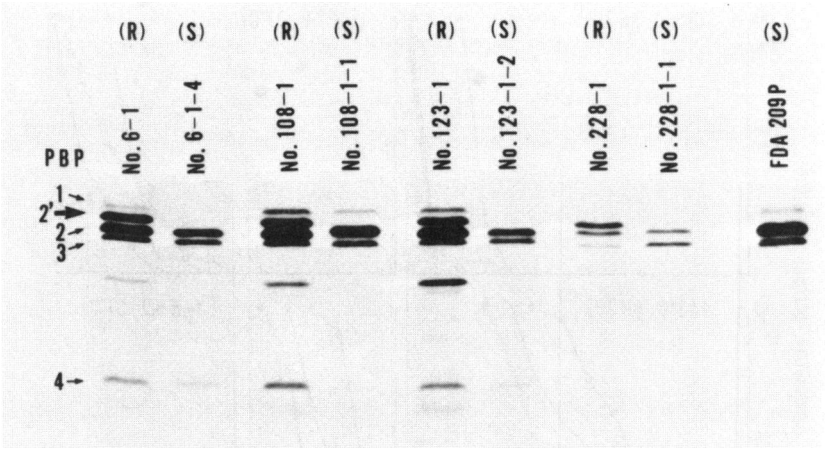

FIG. 1. PBP profiles of penicillinase-free MRSA subclones 6-1, 108-1, 123-1, and 228-1, susceptible revertant strains 6-1-4, 108-1-1, 123-1-2, and 228-1-1, and standard susceptible strain FDA 209P. A new PBP fraction (PBP2') was present only in each MRSA strain between PBP1 and PBP2. (R), Resistant strains; (S), susceptible strains.

Influence of culture temperature and pH on the levels of resistance of MRSA strains. The levels of resistance of 22 penicillinase-free MRSA subclones were examined at 30, 37, and 43°C by using Mueller-Hinton agar plates (pH 7.0). The levels of resistance of the same subclones were also examined with the same medium adjusted to pH 7.0 and 5.2 at 37°C. The MICs of methicillin, cefazolin, and cefotaxime at 30, 37, and 43°C are shown in Fig. 3. As Fig. 3 shows, all of the MRSA subclones were highly resistant at 30 and 37°C but moderately susceptible at 43°C, as reported previously by Annear (1) and Parker and Hewitt (18). The MICs of methicillin for the MRSA strains ranged from 200 to 1,600 μg/ml at 30°C, from 25 to 1,600 μg/ml at 37°C, and from 3.13 to 50 μg/ml at 43°C. The levels of resistance of the subclones to cefazolin and cefotaxime at 43°C were also lower.

The effect of pH on expression of resistance in MRSA strains was more striking than the effect of temperature. The effect of medium pH was most prominent in the MICs of cefotaxime. The levels of resistance of MRSA strains to cefotaxime ranged from 200 to 1,600 μg/ml at pH 7.0, but varied at pH 5.2 to as low as 0.78 to 3.13 μg/ml. The decreases in the MICs of cefotaxime for MRSA strains due to a lowering of the medium pH were 256-fold or more. In the same way the MICs of methicillin and cefazolin for the 22 MRSA subclones were decreased from 25 to 1,600 μg/ml (methicillin) and 50 to 400 μg/ml (cefazolin) at pH 7.0 to 1.56 to 6.25 μg/ml (methicillin) and 0.39 to 1.56 μg/ml (cefazolin) at pH 5.2. The influence of pH on the MICs of drug-susceptible strains of *Staphylococcus aureus* was essentially unchanged (20). These results indicate that recently isolated MRSA strains manifested the same behaviors as those reported previously (1, 18–20); i.e., the levels of resistance were temperature and pH dependent.

Influence of temperature on the profile of PBPs in MRSA strains. The relative amounts of every PBP fraction in MRSA strains grown for 4 h at 43°C were compared with the amounts in the same bacteria grown for 4 h at 30°C (Table 1). The amounts of each PBP fraction were measured from the fluorograms with a densitometer, and each value was expressed as the ratio of the density of PBPs prepared from the MRSA cells grown at 43°C to the density of PBPs prepared from the bacteria grown at 30°C. The penicillin-binding capacities of PBP1, PBP2', and PBP2 in four penicillinase-free MRSA subclones grown at 43°C were 70 to 80, 30 to 70, and 60 to 80%, respectively, of the capacities of the PBPs in the same strains grown at 30°C. The penicillin-binding capacities of PBP3 and PBP4 in MRSA strains grown at 43°C were almost the same as the capacities of PBP3 and PBP4 in

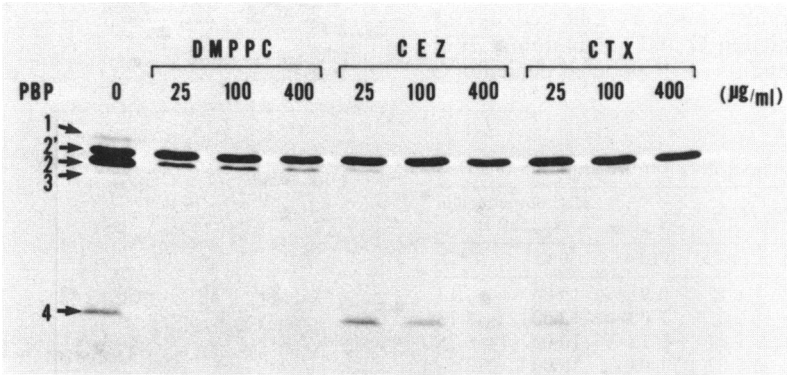

FIG. 2. Competition of methicillin (DMPPC), cefazolin (CEZ), and cefotaxime (CTX) at concentrations of 25, 100, and 400 μg/ml with ^{14}C-labeled penicillin G for binding to the PBPs of MRSA strain 123-1. Membrane fractions from cells grown at 37°C and pH 7.0 were incubated with nonradioactive β-lactams before the addition of radioactive penicillin G. The PBP2' fraction could not be saturated even by high concentrations (400 μg/ml) of the drugs.

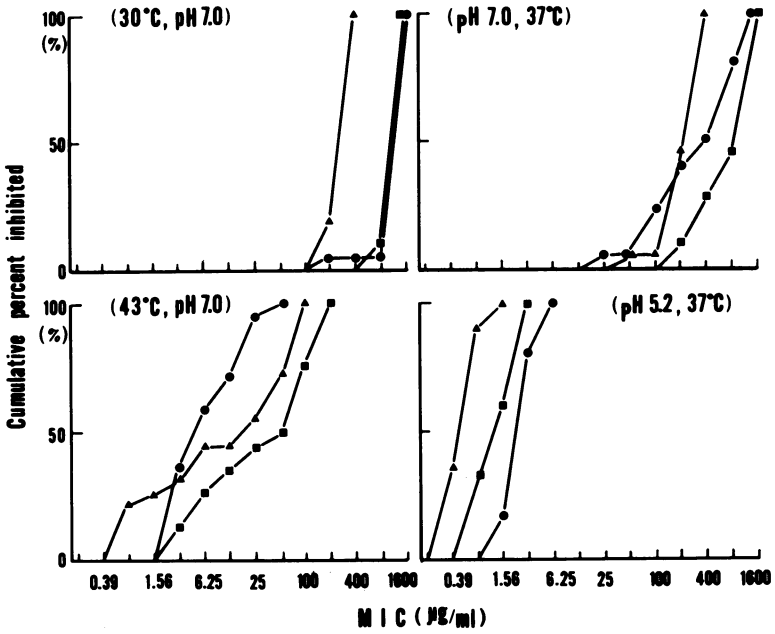

FIG. 3. Effects of temperature and pH on the MICs of methicillin, cefazolin, and cefotaxime against 22 MRSA strains. Symbols: ●, methicillin; ▲, cefazolin; ■, cefotaxime.

strains grown at 30°C. The fact that the MRSA subclones subcultured at 43°C were capable of growing on HIA plates containing 100 μg of methicillin per ml when the plates were incubated at 30°C suggested that these MRSA strains could not express resistance at 43°C, although the genetic capacity of the strains was unchanged.

To clarify the mechanism of temperature-dependent expression of resistance in MRSA, membrane fractions of MRSA strains grown for 4 h at 30°C were preincubated at 30 and 43°C for 60 min, and then ^{14}C-labeled penicillin G was added; this was followed by electrophoresis in an acrylamide slab gel. As shown in Fig. 4, the penicillin-binding capacities of PBP1, PBP2′, PBP2, and PBP4 in the membrane fractions preincubated at 43°C were much lower than the capacities of the PBPs in the membranes preincubated at 30°C, whereas the PBP3 fraction retained high penicillin-binding capacity even after preincubation at 43°C for 60 min. To study the details of the temperature sensitivity of the PBPs in MRSA strains, membrane fractions were preincubated at 30 and 43°C for 180 min, and small portions of the samples were removed at different intervals; this was followed by the addition of radioactive penicillin G and incubation at 30°C for 10 min. The relative amounts of ^{14}C-labeled penicillin G bound to the PBP2′, PBP2, and PBP3 of two MRSA subclones (strains 1-1 and 72-1) under such conditions are shown in Fig. 5. After preincubation at 43°C, the binding capacities of PBP2 and PBP2′ were decreased within 30 min to 20 to 40 and 50 to 70%, respectively, of the values before preincubation. Both fractions were further inactivated by prolonged preincubation at 43°C, and they lost their activities almost completely within 60 to 120 min. PBP1 and PBP4 were inactivated more rapidly than other PBP fractions (data not shown). However, PBP3 retained a higher penicillin-binding capacity than the other fractions even after 180 min. On the other hand, the activities of every PBP fraction in MRSA strains remained completely intact even after 180 min of preincubation at 30°C. Our results indicate that the

TABLE 1. Effect of growth temperature on the amounts of PBPs

Strain	Relative amt of PBPs (43/30°C)[a]					MIC of methicillin (μg/ml) at:	
	PBP1	PBP2′	PBP2	PBP3	PBP4	30°C[b]	43°C[b]
1-1	0.68	0.36	0.75	1.12	0.91	1,600	3.13
160-1	0.67	0.30	0.69	1.05	1.00	1,600	3.13
72-1	0.83	0.63	0.79	1.04	1.12	1,600	6.25
137-1	0.76	0.70	0.62	1.07	1.08	1,600	12.5

[a] The data are expressed as the ratio of the level of ^{14}C-labeled penicillin G bound to a PBP from bacteria grown at 43°C to the level bound to the PBP from bacteria grown at 30°C.
[b] At pH 7.0.

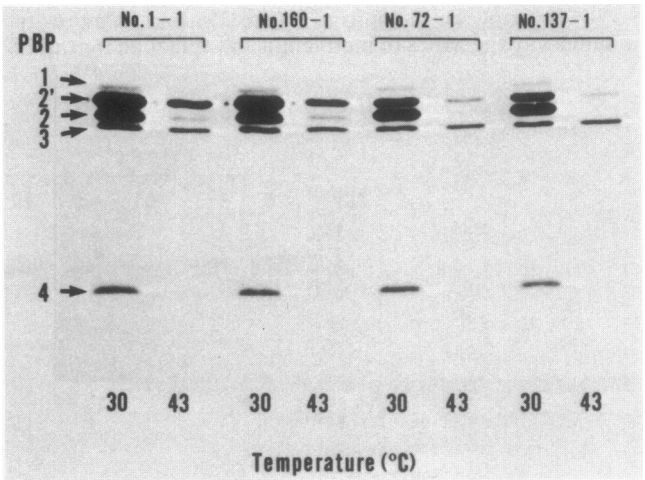

FIG. 4. Effects of preincubation temperature on the PBPs of MRSA strains 1-1, 160-1, 72-1, and 137-1. Cells were grown at 30°C and pH 7.0, and the membrane fractions were preincubated at 30 and 43°C for 60 min before addition of ^{14}C-labeled penicillin G.

decreased amounts of PBP1, PBP2', and PBP2 in MRSA strains grown at 43°C were based upon thermolability of the PBP fractions.

Effect of pH on the PBPs of MRSA strains. Membrane fractions prepared from MRSA subclones grown for 4 h at pH 7.0 in a liquid medium were suspended in phosphate buffer (pH 7.0 or 5.2) and then incubated with radioactive penicillin G at 30°C for 10 min, followed by slab gel electrophoresis and fluorography. As shown in Table 2, the relative binding capacities of each fraction of PBPs at pH 5.2 were less than those at pH 7.0. The most acid-sensitive fraction was PBP1, followed by PBP3, although the PBP2' fraction that appears to be responsible for the intrinsic resistance of MRSA was not acid sensitive. The amounts of ^{14}C-labeled penicillin G bound to PBP1, PBP2, PBP3, and PBP4 in the membrane fractions suspended at pH 5.2 were decreased to less than 10, 60 to 80, 50, and 60 to 70%, respectively, of the amounts in the membrane fractions suspended at pH 7.0. On the other hand, the amounts of radioactive penicillin G bound to PBP2' in the membrane fractions suspended at pH 5.2 were nearly the same as those in the membrane fractions suspended at pH 7.0.

To clarify the mechanism of the pH-dependent level of resistance in MRSA, the amounts of the PBP2' fraction in MRSA strains were compared in cells grown for 4 h at pH 7.0 and 5.2 at 30°C. The membrane fractions from both cell preparations were prepared with phosphate buffer (pH 7.0) and then were incubated with ^{14}C-labeled penicillin G at 30°C for 10 min; this was followed by slab gel electrophoresis and fluorography. The PBP profiles from four MRSA subclones grown at pH 7.0 and 5.2 are shown in Fig. 6. The amount of every PBP fraction in the MRSA cells grown at pH 5.2 was less than the amount in the cells grown at pH 7.0. In particular, the amounts of the PBP2' and PBP2 fractions in the cells grown at pH 5.2 were decreased to as low as 30 and 20%, respectively, of the amounts in the cells grown at pH 7.0. The amounts of PBP3 in the MRSA cells grown at pH 5.2 were 50 to 60% of the amounts in the cells grown at pH 7.0. No area corresponding to PBP1 or PBP4 was identified in the MRSA cells grown at pH 5.2.

TABLE 2. Effect of pH on the stability of PBPs

Strain	Relative stabilities of PBPs (pH 5.2/7.0)[a]					MIC of methicillin (μg/ml) at:	
	PBP1	PBP2'	PBP2	PBP3	PBP4	pH 7.0[b]	pH 5.2[b]
69-1	<0.1	0.93	0.65	0.52	0.60	800	3.13
72-1	<0.1	0.86	0.78	0.55	0.62	400	3.13
75-1	<0.1	0.88	0.62	0.51	0.68	1,600	3.13
140-1	<0.1	0.86	0.68	0.53	0.63	800	3.13

[a] The data are expressed as the ratio of the level of ^{14}C-labeled penicillin G bound to a PBP from a membrane fraction suspended in pH 5.2 buffer to the level bound to the PBP from a membrane fraction suspended in pH 7.0 buffer.
[b] At 37°C.

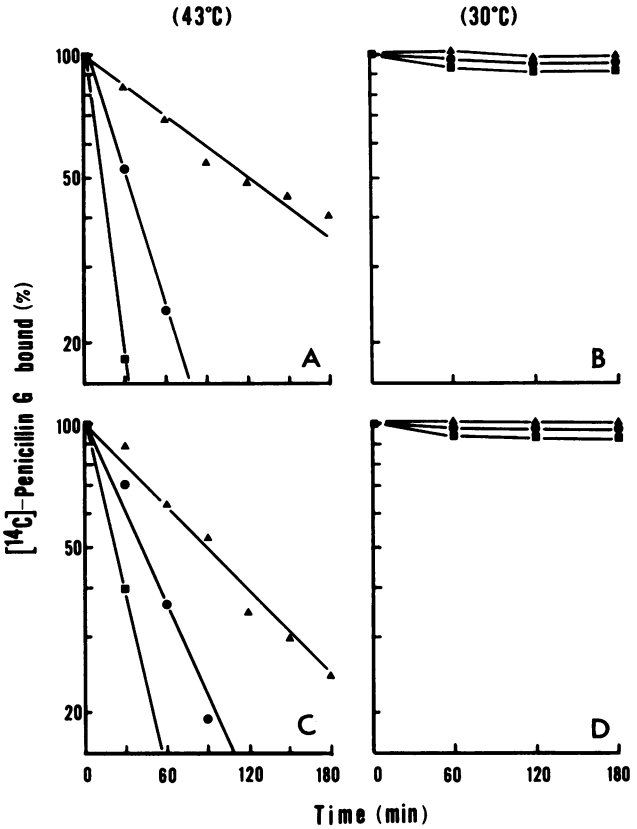

FIG. 5. Kinetic analysis of the thermostability of the PBPs in MRSA strains 1-1 (A and B) and 72-1 (C and D). Membrane fractions were preincubated at 43°C for 30, 60, 90, 120, 150, and 180 min (A and C) and at 30°C for 60, 120, and 180 min (B and D). The data are expressed as the relative amounts of ^{14}C-labeled penicillin G bound to each PBP fraction compared with the amounts bound before preincubation. Symbols: ■, PBP2; ●, PBP2'; ▲, PBP3.

DISCUSSION

A specific 78,000-dalton PBP fraction (PBP2') was identified exclusively in all of the MRSA strains, as we reported previously (Y. Utsui, M. Tajima, R. Sekiguchi, E. Suzuki, and T. Yokota, Abstr. 13th Int. Natl. Congr. Chemother., abstr. part 88, p. 2.11/3:7–10, 1983). For a clear demonstration of the PBP2' fraction, the use of slab gel containing 8% acrylamide and 0.06% bisacrylamide was essential, as otherwise the PBP2' fraction overlapped with the normal PBP2 fraction and could not be differentiated. In 1984 Hartman and Tomasz (13) reported that the presence of 78,000-dalton fraction (PBP-2a) was confirmed exclusively in two MRSA strains by using a slab gel containing 10% acrylamide and 0.13% bisacrylamide. In their case, however, the 78,000-dalton fraction had a faster mobility than PBP2. The discrepancy between our results and those of Hartman and Tomasz may be due to a difference in gel composition.

Experiments to examine competition between β-lactam antibiotics and ^{14}C-labeled penicillin G for PBPs indicated that the PBP2' fraction possessed a low binding affinity for β-lactams. The fact that even high concentrations of β-lactam antibiotics which completely inactivated other PBP fractions could not saturate the PBP2' fraction suggests that β-lactam resistance in MRSA strains is related to the presence of a low-affinity PBP2' fraction. MRSA strains may be capable of growing in the presence of certain β-lactam antibiotics because of acquisition of the specific 78,000-dalton PBP fraction which can act as murein transpeptidase for cell wall biosynthesis despite the presence of β-lactam antibiotics. This suggestion is similar to that proposed previously (10) for the possible function of a low-affinity binding protein detectable in β-lactam-resistant strains of *Streptococcus faecalis* and *Streptococcus faecium*.

The recently isolated MRSA strains also retained the

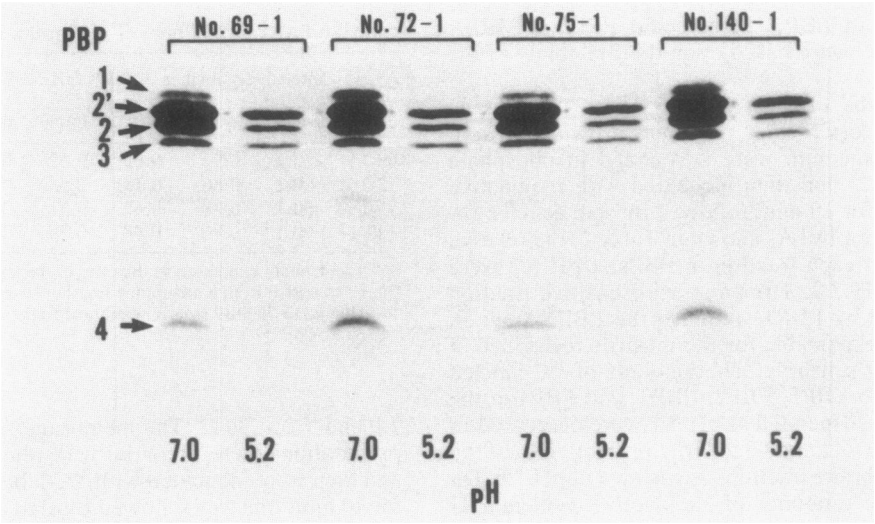

FIG. 6. Amounts of PBP fractions in MRSA strains 69-1, 72-1, 75-1, and 140-1 grown either at pH 7.0 and 30°C or at pH 5.2 and 30°C. Membrane fractions were prepared with phosphate buffer (pH 7.0), and this was followed by incubation with ^{14}C-labeled penicillin G.

temperature- and pH-dependent phenotype of β-lactam resistance, as reported previously (1, 18–20). The influence of pH on the phenotypic expression of β-lactam resistance in MRSA strains was more prominent than the influence of temperature.

The thermosensitivity of expression of a PBP has been described previously (4, 14). The ^{14}C-labeled penicillin G binding activities of the PBP fractions other than PBP3 were decreased according to the length of preincubation at 43°C and were almost lost after preincubation for more than 120 min. It is of interest to note that although the PBP1, PBP2′, and PBP2 fractions are thermosensitive, the PBP3 fraction is not. The amounts of the PBP1, PBP2′, and PBP2 fractions were decreased partially in MRSA cells grown for 4 h at 43°C, whereas these PBP fractions might be inactivated much more in MRSA cells grown overnight at 43°C. The fact that phenotypic expression of β-lactam resistance in MRSA is moderately susceptible at a high temperature may be due to the decreased amounts of the PBP1, PBP2′, and PBP2 fractions in vivo because of slow inactivation based on thermolability of these PBP fractions.

The influence of low pH on an additional PBP fraction also has been described by Hartman and Tomasz (13). These authors reported that the extra PBP fraction (PBP-2a) was not detectable in cultures grown at pH 5.2. We found that only the PBP2′ fraction was almost stable even after preincubation at pH 5.2. However, the amounts of the PBP fractions other than PBP3 in MRSA cells grown for 4 h at pH 5.2 were much less, and PBP3 might be the only fraction to be produced in MRSA cells grown overnight at pH 5.2. Our results indicate that the reason that the levels of resistance of MRSA strains are lower at pH 5.2 than at pH 7.0 is that the production of each PBP fraction is lower at an acidic pH than at neutral pH, rather than that PBP2′ is stable at pH 5.2. The reason why the influence of pH on the phenotype of β-lactam resistance in MRSA strains is more prominent than the influence of temperature is that there is no production of any PBP fraction other than PBP3 at acidic pH values.

The essential PBP fractions of *Staphylococcus aureus* have been confirmed to be PBP2 and PBP3 by Curtis and Hayes (5). The PBP2′ fraction, like PBP2, was found to be thermosensitive. Alteration of either the PBP2 fraction or the PBP3 fraction might result in the appearance of the 78,000-dalton PBP2′ fraction, but details of the origin of this specific PBP fraction are not clear yet.

Recently, Ubukata et al. (25) and Tomasz and colleagues (H. Chambers, B. Hartman, and A. Tomasz, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 24th, abstr. no. 6, 1984) reported that the specific 78,000-dalton PBP fraction (PBP2′) is inducible only when strains bear a plasmid encoding inducible penicillinase. Inducible PBP2′ changed to constitutive PBP2′ when the penicillinase-encoding plasmid was eliminated. This is a newly recognized mysterious characteristic of MRSA strains.

LITERATURE CITED

1. **Annear, D. I.** 1968. The effect of temperature on resistance of *Staphylococcus aureus* to methicillin and some antibiotics. Med. J. Aust. **1**:444–446.
2. **Barber, M.** 1964. Naturally occurring methicillin-resistant staphylococci. J. Gen. Microbiol. **35**:183–190.
3. **Boyce, J. M., O. A. Landry, T. R. Deetz, and H. L. Dupont.** 1981. Epidemiologic studies of an outbreak of nosocomial methicillin-resistant *Staphylococcus aureus* infections. Infect. Control **2**:110–116.
4. **Brown, D. F. J., and P. E. Reynolds.** 1980. Intrinsic resistance to β-lactam antibiotics in *Staphylococcus aureus*. FEBS Lett. **122**:275–278.
5. **Curtis, N. A. C., and M. V. Hayes.** 1981. A mutant of *Staphylococcus aureus* H deficient in penicillin-binding protein 1 is viable. FEMS Microbiol. Lett. **10**:227–229.
6. **Curtis, N. A. C., M. V. Hayes, A. W. Wyke, and J. B. Ward.** 1980. A mutant of *Staphylococcus aureus* H lacking penicillin-binding protein 4 and transpeptidase activity in vitro. FEMS Microbiol. Lett. **9**:263–266.
7. **Deguchi, K.** 1982. Sensitivity of *Staphylococcus aureus* isolated clinically to CEPs. Jpn. J. Antibiot. **35**:807–811.
8. **Deguchi, K.** 1982. Sensitivity of second-generation cephem agents of clinically isolated strains of bacteria in othorhinolaryngological field. Jpn. J. Antibiot. **35**:812–820.
9. **Dyke, K. G. H., M. P. Jevons, and M. T. Parker.** 1966. Penicillinase production and intrinsic resistance to penicillins in *Staphylococcus aureus*. Lancet **i**:835–838.
10. **Fontana, R., R. Cerini, P. Longoni, A. Grossato, and P. Canepari.** 1983. Identification of a streptococcal penicillin-binding protein that reacts very slowly with penicillin. J. Bacteriol. **155**:1343–1350.
11. **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.

12. **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.
13. **Hartman, B. J., and A. Tomasz.** 1984. Low-affinity penicillin-binding protein associated with β-lactam resistance in *Staphylococcus aureus*. J. Bacteriol. **158**:513–516.
14. **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.
15. **Jevons, M. P.** 1961. "Celbenin"-resistant staphylococci. Br. Med. J. **1**:124–126.
16. **May, J. W., R. H. Houghton, and C. J. Perret.** 1964. The effect of growth at elevated temperatures on some heritable properties of *Staphylococcus aureus*. J. Gen. Microbiol. **37**:157–169.
17. **O'Callaghan, C. H., A. Morris, S. M. Kirby, and A. H. Shingler.** 1972. Novel method for detection of β-lactamase by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **1**:283–288.
18. **Parker, M. T., and J. H. Hewitt.** 1970. Methicillin resistance in *Staphylococcus aureus*. Lancet **i**:800–804.
19. **Sabath, L. D.** 1977. Chemical and physical factors influencing methicillin resistance of *Staphylococcus aureus* and *Staphylococcus epidermidis*. J. Antimicrob. Chemother. **3**(Suppl. C):47–51.
20. **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.
21. **Sapico, J. F., J. Z. Montgomerie, H. N. Canawati, and G. D. Aeilts.** 1981. Methicillin-resistant *Staphylococcus aureus* bacteriuria. Am. J. Med. Sci. **281**:101–109.
22. **Seligman, S. J., and W. L. Hewitt.** 1966. Resistance to penicillins and cephalosporins, p. 387–391. Antimicrob. Agents Chemother. 1965.
23. **Spratt, B. G.** 1975. Distinct penicillin-binding proteins involved in the division, elongation, and shape of *Escherichia coli* K12. Proc. Natl. Acad. Sci. U.S.A. **72**:2999–3003.
24. **Sutherland, R., and G. N. Rolinson.** 1964. Characteristics of methicillin-resistant staphylococci. J. Bacteriol. **87**:887–899.
25. **Ubukata, K., N. Yamashita, and M. Konno.** 1985. Occurrence of a β-lactam-inducible penicillin-binding protein in methicillin-resistant staphylococci. Antimicrob. Agents Chemother. **27**:851–857.
26. **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.