Acta Biochimica Polonica

Vol. 42 No. 4/1995

517–524

QUARTERLY

Text of lecture presented at the 5th International Symposium on ***Molecular Aspects of Chemotherapy***

Evolution and resistance expression of MRSA. Evaluation of β-lactam antibiotics against a set of isogenic strains with different types of phenotypic expression

Kazumi Asada, Yoko Inaba, Eiko Tateda-Suzuki, Kyoko Kuwahara-Arai, Teruyo Ito and Keiichi Hiramatsu

Department of Bacteriology, Faculty of Medicine, Juntendo University, 2-1-1 Hongo, Bunkyo-ku, Tokyo, Japan 113

Key words: MRSA, penicillin-binding protein, sulbactam, ampicillin

Methicillin-resistant *Staphylococcus aureus* (MRSA) has two mechanisms of resistance to β-lactam antibiotics; one is mediated by *mecA* gene expression, and the other by penicillinase production. It has been generally accepted in the clinical field that β-lactam antibiotics are not the drugs of choice for MRSA infection. In this report, however, ampicillin and penicillin G were shown to have relatively good activity against MRSA if combined with a β-lactamase inhibitor, sulbactam. These β-lactam antibiotics were found to have relatively high binding affinities to PBP2′, the *mecA*-encoded MRSA-specific penicillin-binding protein. The possible therapeutic application of sulbactam/ampicillin against MRSA infection in combination with arbekacin, an aminoglycoside antibiotic newly developed and introduced into clinical use in Japan, is discussed.

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first isolated in England in 1961 [1]. Since then, MRSA has been isolated throughout the world, and, in recent years, there is practically no country where MRSA is not found. Because MRSA is resistant to almost all the β-lactam antibiotics so far introduced into clinical use, and also because it easily acquires resistance to other non-β-lactam antibiotics, there is no effective chemotherapeutic agent against MRSA infection except for vancomycin, to which no resistant clinical strain has yet been isolated. However, vancomycin's efficacy is also limited because of its rather slow cytokilling activity toward MRSA, relatively high toxicity, and the future threat of emergence of resistance [2]. In this regard, MRSA has become one of the most difficult-to-manage pathogens in hospitals world-wide. Since the intrinsic resistance of MRSA to β-lactam antibiotics is due to the production of a cell-wall synthesizing enzyme, penicillin-binding protein (PBP2′), having low-binding affinities to methicillin and other β-lactam antibiotics [3], development of a new β-lactam antibiotic with high affinity to PBP2′ remains the ultimate goal of anti-MRSA chemotherapy [4]. However, we have shown that MRSA evolves with accumulation of step-wise genetic alterations to finally achieve full expression of methicillin-resistance [5]. The first step is the acquisition of *mec* region DNA by *Staphylococcus aureus* on its chromo-

Abbreviations: cfu, colony forming unit; HI-agar, heart infusion agar; MBC, minimum bactericidal concentration; MH-broth, Mueller-Hinton-broth; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; PBP2′, penicillin-binding protein 2′; PSL, photo-stimulated luminescence.

some, and the second step is the genetic destruction of the *mecI* gene function which encodes the *mecA* gene repressor [5–7]. The final step in this evolution is the conversion of phenotypic expression of methicillin resistance; i.e. the conversion from heterogeneous to homogeneous type methicillin resistance [5]. This conversion is shown to be due to the alteration of a chromosomal gene which is not associated with the *mec* region of the DNA on which the *mecA* gene coding for PBP2′ is located [5, 8]. Therefore, full expression of methicillin resistance is contributed to not only by production of PBP2′, but also by the expression of another gene product [5]. Consequently, the development of new β-lactam antibiotics against MRSA must take into consideration at least two genetic factors that contribute to the homogeneous expression of methicillin resistance. In this study, we compared anti-MRSA activity of various β-lactam antibiotics in both heterogeneous and homogeneous methicillin resistance of isogenic sets of strains, so that we can find the best β-lactam antibiotic from which to initiate development of new β-lactams.

MATERIALS AND METHODS

Bacterial strains. N315LR5 is a *mecI*-deleted heterogeneous strain derived from *mecI*-intact preMRSA strain N315 [5–7, 9]. N315LR5P-1 is a derivative of N315LR5 cured of its penicillinase plasmid, which was obtained by successive cultivation of N315LR5 for seven days at 42°C, followed by screening of colonies by nitrocefin coloration as described previously [10]. N315LR5P-1IPM8-1 is a homogeneously methicillin-resistant strain derived from N315LR5P by selection on HI (heart infusion) agar plates containing 8 μg/ml of imipenem. N315LR5CEZ64-5 is a homogeneous resistant derivative of N315LR5 selected by the presence of cefazolin at a concentration of 64 μg/ml. N315LR5IPM2-4 was a homogeneous methicillin-resistant strain derived from N315LR5 by selection with 2 μg/ml of imipenem. Frequency of emergence of homogeneous strains from N315LR5P-1, when selected with 8 μg/ml of imipenem, was 6.5×10^{-4}. Those from N315LR5 with 64 μg/ml of cefazolin and 2 μg/ml of imipenem were 4.6×10^{-4} and 6.5×10^{-4}, respectively.

Determination of minimum inhibitory concentration (MICs) and minimum bactericidal concentration (MBCs). MICs and MBCs were determined by the broth dilution technique. The 96-well microtiter plate wells containing MH (Mueller-Hinton) broth, with two-fold serial dilutions of three antibiotics (methicillin, sulbactam/ampicillin, and imipenem), were inoculated with N315LR5 or N315LR5CEZ64-5 (10^5 cfu/ml) and were incubated at 37°C for 24 h. The MIC was recorded as the lowest antibiotic concentration demonstrating no visible growth in MH broth. The MBC was determined by removing 0.1 ml of the bacterial suspension from subcultures demonstrating no visible growth and inoculating the surface of HI (heart infusion) agar. The plates were incubated at 37°C for 24 h in air. The MBC was recorded as the lowest antibiotic concentration demonstrating 99.9% killing of the bacterial inoculum.

PBP2′-binding assay. The binding affinities of β-lactam antibiotics to PBP2′ were evaluated by the method of Spratt [11] with some modifications [12]. A total of 30 μl of membrane suspension, extracted from about 10^{10} cells of N315LR5P-1, was reacted for 10 min at 30°C with 150 mCi of [^{14}C]benzylpenicillin, the specific activity of which was 50 μCi/mmol per ml. Experiments on competitive binding of the test drugs with radioactive penicillin G to PBP2′ were performed by preincubating the membrane fractions with the nonradioactive test drugs at various concentrations for 10 min at 30°C before the addition of radioactive benzylpenicillin. After polyacrylamide gel electrophoresis, and fixing the gel as described previously [12], the radioactivity of the band corresponding to the PBP2′ was quantitated with a Bioimage Analyzer (FUJIX BAS2000; Fuji Photo Film Co., Ltd. Minato-ku, Tokyo, Japan). Per cent inhibition of the photo-stimulated luminescence (PSL) was plotted against the concentration of the test drug used for the competitive binding inhibition, and the IC_{50} value was determined as the concentration of the drug which inhibited 50% of the PSL value obtained without preincubation with competitors.

Population analysis. Population analysis was performed with an AUTOPLATE MODEL 3000 (Spiral Biotech, Inc., Bethesda, MD, U.S.A.). A 50-μl portion of overnight culture and its 10-fold serial dilutants of MRSA strains

were inoculated on the HI-agar plates containing various concentrations of β-lactam antibiotics. Colonies were counted after 48 h incubation at 37°C. The number of resistant cells theoretically contained in the 50-μl portion of the original culture of bacteria was calculated and plotted on a bi-logarithmic graph.

RESULTS

Population analysis of a heterogeneous MRSA strain N315LR5 against various β-lactam antibiotics

Figure 1 represents population curves of N315LR5 for various β-lactam antibiotics. According to the pattern of the curves, the β-lactam antibiotics used in this study could be divided into three groups. Firstly, ceftizoxime had no growth inhibitory activity at drug concentrations up to 512 μg/ml. The second group comprised methicillin, ampicillin, cefotaxime, latamoxef, cloxacillin, cefotiam and cefazolin. These seven antibiotics had better activity than ceftizoxime, since, for example, at a drug concentration of 64 μg/ml, they could reduce the number of viable cells by $-3\log_{10}$ to $-4\log_{10}$ (Fig. 1). However, in the range of drug concentrations clinically attainable in the serum, the second group drugs could not reduce the cell number appreciably (e.g., see the reduction of the cell number at drug concentrations of 8 or 16 μg/ml in Fig. 1). The third group of drugs (sulbactam/ampicillin, imipenem, cefmetazole and flomoxef) was the only one which could reduce the cell number appreciably at concentrations of 4 or 8 μg/ml (e.g., in the case of imipenem, the cell number is reduced by $-4\log_{10}$ at a concentration of 4 μg/ml; Fig. 1). It should be noted that sulbactam/ampicillin, a combination of the β-lactamase inhibitor sulbactam and ampicillin, belongs to the third group, although ampicillin itself belongs to the second group. This is reasonable, because N315LR5 is a producer of penicillinase which is known to hydrolize penicillin G and ampicillin.

It was also noticed that, although the third group of drugs could reduce the cell number

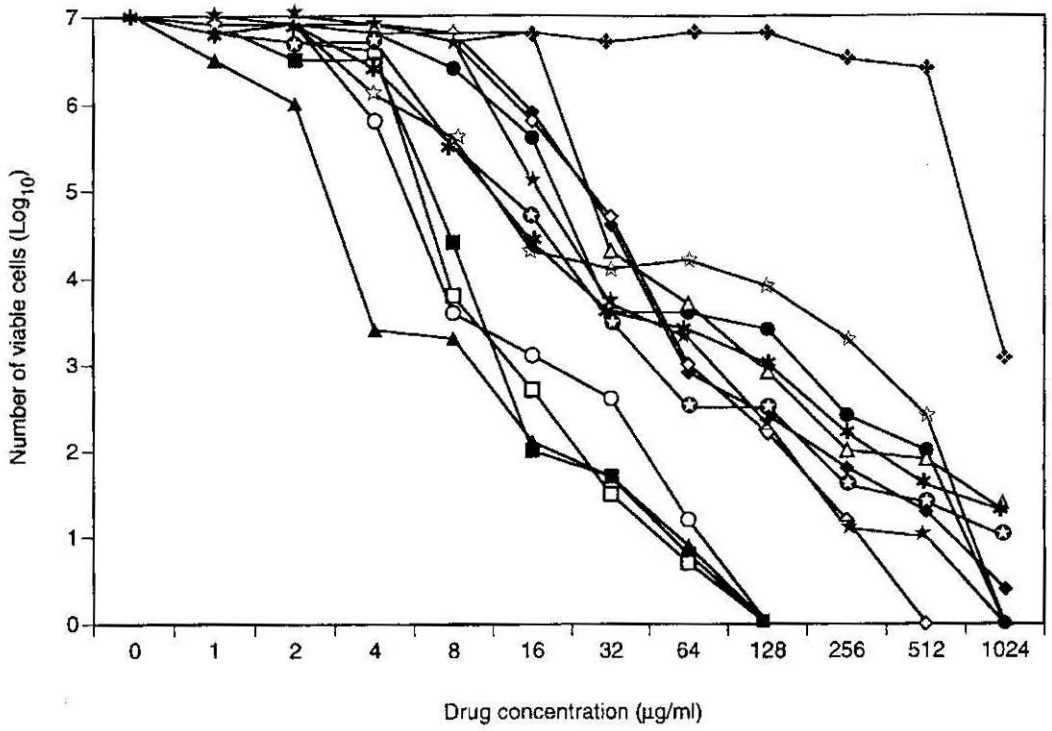

Fig. 1. Population analysis of a heterogeneous type MRSA strain N315LR5 with various β-lactam antibiotics.

The symbols are as follows: ■, sulbactam/ampicillin; ●, ampicillin; ▲, imipenem; ◆, methicillin; ❑, cefmetazole; ◊, cefazolin; ○, flomoxef; △, latamoxef; ❖, ceftizoxime; ★, cefoxitin; ✪, cloxacillin; ☆, cefdinir; ✱, cefotiam. Note that there is a significant difference in antimicrobial activity between ampicillin and sulbactam/ampicillin. Sulbactam/ampicillin, imipenem, cefmetazole, and flomoxef constitute the most potent group of β-lactam antibiotics against N315LR5 (the third group; see text for detail).

greatly at clinically feasible drug concentrations, population curves with these drugs also showed typical heterogeneous patterns; that is, small subpopulation of the strain of cells could grow even on plates containing higher concentrations of the drug, such as 32 and 64 μg/ml. The data showed that heterogeneity was not specific to methicillin but also applicable to other β-lactam antibiotics.

Change of resistance population patterns after hetero-to-homo conversion of N315LR5

Figure 2 shows the population curves of methicillin and the third group of β-lactam antibiotics against a homogeneous strain N315LR5IPM2-4 derived from N315LR5 by selection with 2 μg/ml of imipenem. The population curves became relatively upright compared to those of N315LR5 for all the drugs. It was then noted that sulbactam/ampicillin now became the most effective one among the tested antibiotics. Imipenem, cefmetazole, ampicillin and flomoxef became totally ineffective in suppressing growth even at 32 μg/ml, whereas sulbactam/ampicillin could still reduce growth by $-3\log_{10}$ at this concentration. There also was a significant difference in efficacy between ampicillin and sulbactam/ampicillin.

Population analysis of N315LR5P-1, a derivative of N315LR5 cured of penicillinase plasmid

To evaluate the activity of ampicillin against the *mecA*-mediated intrinsic resistance mechanism of MRSA, the plasmid encoding penicillinase was eliminated from the test strain N315LR5. Figure 3 shows the result of population analysis of the strain N315LR5P-1 with ampicillin, penicillin G, methicillin and the drugs of the third group. As is evident in Fig. 3, population curves of N315LR5P-1 were also heterogeneous, but their configurations were slightly shifted to include more prominent resistant subpopulations against imipenem, cefmetazole, flomoxef and methicillin compared to those with N315LR5. However, in contrast to those with other β-lactams, the population curve for ampicillin was drastically shifted to the left relative to that of N315LR3, which showed improved susceptibility of the strain to ampicillin after elimination of the penicillinase plasmid.

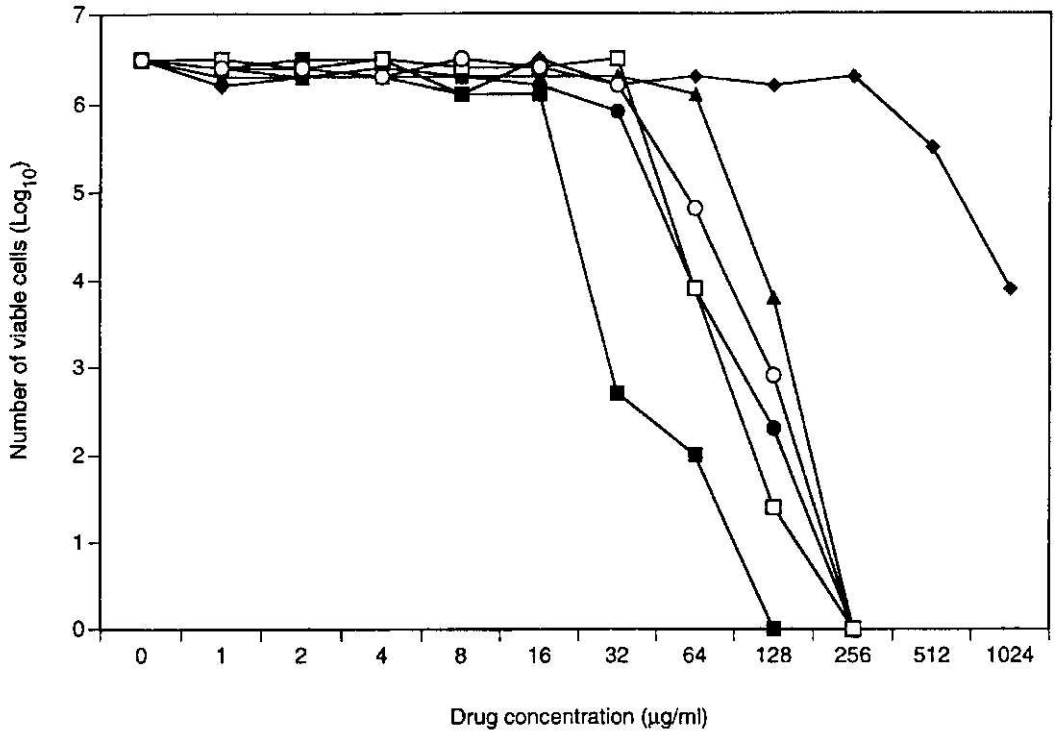

Fig. 2. Population analysis of a homogeneous type MRSA strain N315LR5IPM2-4 with the third group β-lactam antibiotics.

The symbols are as in Fig. 1. Note homogeneous patterns of population curves. Sulbactam/ampicillin was most effective among the tested β-lactam antibiotics.

The effect of hetero-to-homo conversion of N315LR5P-1 against β-lactam antibiotics

Figure 4 shows the population curves of N315L-R5P-1IPM8-1 for penicillin G, ampicillin, cefmetazole, flomoxef, imipenem, and methicillin. With its acquisition of homogeneous or highly methicillin-resistant phenotype, N315LR5P-1IPM8-1 was shown to have upright population curves for all the tested β-lactam antibiotics (Fig. 4). Imipenem was the drug whose population curve was most drastically influenced by hetero-to-homo conversion of the tested strain (compare Figs. 3 and 4); all the cells of N315LR5P-1IPM8-1 could grow in the presence of 64 μg/ml of imipenem, whereas only one out of 10^4 cells of N315LR5P-1 could grow in the presence of more than 1 μg/ml of the drug (Fig. 3). Population curves for methicillin, flomoxef, cefmetazole were also greatly influenced by the hetero-to-homo conversion of the tested strain, as clearly shown by the difference in population curves for each drug in Figs. 3 and 4. As to ampicillin and penicillin, however, relatively smaller change in the configuration of these curves were observed; for example, the concentration of ampicillin required to reduce cell growth by 99.99% was changed only four-fold from 8 μg/ml to 32 μg/ml before and after the hetero-to-homo conversion (Figs. 3 and 4). From the data shown in Fig. 4, ampicillin was found to be the most potent agent against the homogeneous strain N315LR5P-1IPM8-1, inhibiting 10^7 cells at a concentration of 32 μg/ml. This activity of ampicillin was closely followed by penicillin G (Fig. 4). On the other hand, high concentrations, 128 μg/ml, of imipenem or 285 μg/ml of cefmetazole and flomoxef were required to suppress equivalent cell number of the strain. For methicillin a concentration as high as 1024 μg/ml was still unable to suppress 10^7 cfu of the homogeneous strain (Fig. 4).

MBC and MIC of sulbactam/ampicillin against heterogeneous as well as homogeneous MRSA strains

Growth suppression activity of antibiotics is not necessarily correlated with their cytokilling effect. To test if the growth suppressive activity of sulbactam/ampicillin is mediated by cytostatic or cytokilling activity, MIC and MBC of sulbactam/ampicillin against N315LR5 and its homogeneous derivatives was determined

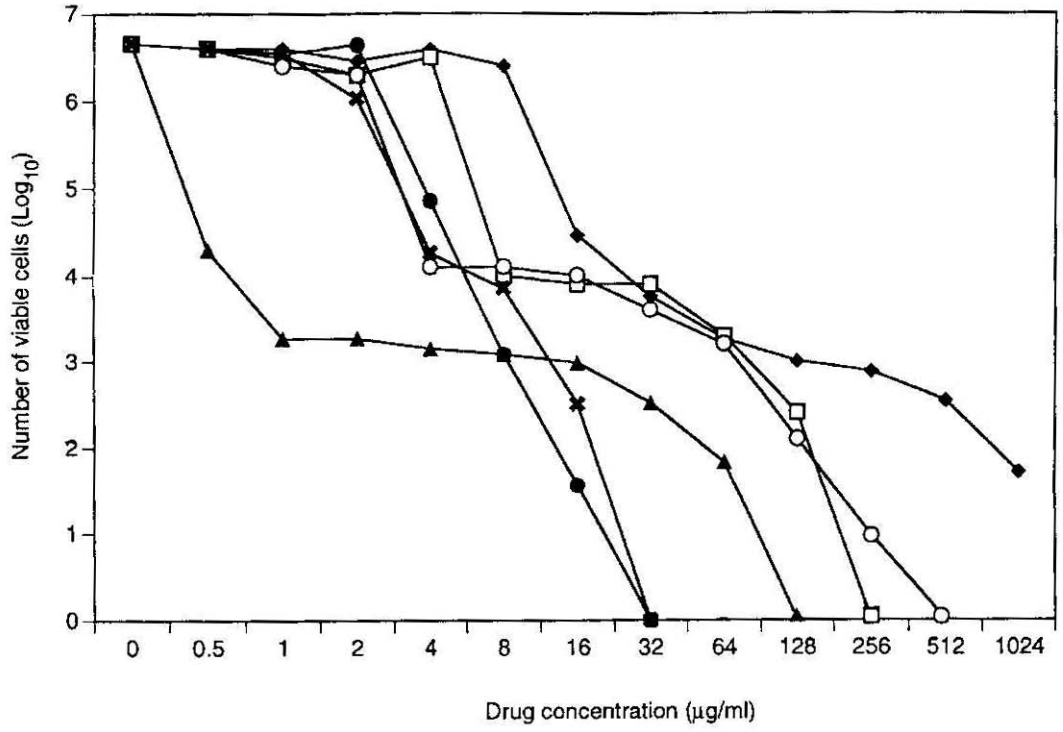

Fig. 3. Population analysis of N315LR5P-1 (penicillinase-negative derivative of N315LR5) with the third group β-lactam antibiotics.

The symbols are as in Fig. 1 except for penicillin G (✖). Imipenem was the most effective agent, but could only partially suppressed growth at lower concentrations. When compared by the ability to suppress total cfu of the bacteria, ampicillin was the most effective drug.

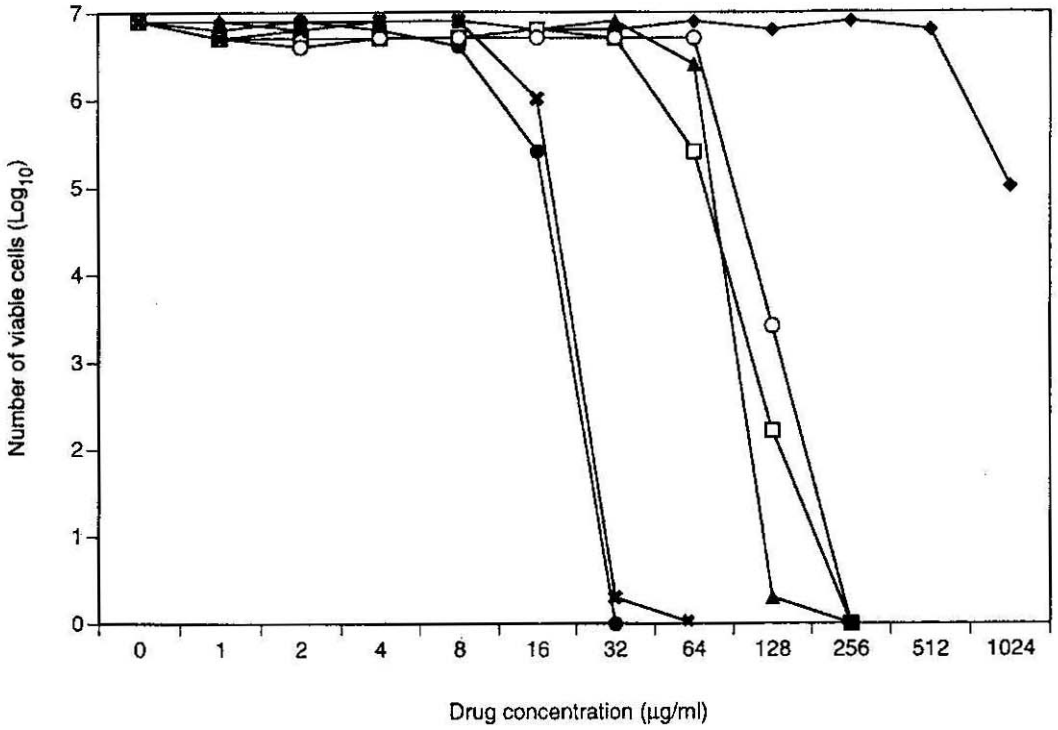

Fig. 4. Population analysis of N315LR5P-1IPM8-2 (a homogeneous strain derived from N315LR5P-1) with the third group β-lactam antibiotics.
The symbols are as in Fig. 3. Note that most effective was ampicillin whose activity was closely followed by penicillin G.

with methicillin and imipenem as controls. Table 1 shows representative results with a homogeneous strain N315LR5CEZ64-5, which gave the same MIC and MBC values of sulbactam/ampicillin for the heterogeneous strain (32 μg/ml) as well as for the homogeneous strain (64 μg/ml). On the other hand, there were discrepancies between MIC and MBC values for imipenem and methicillin. In agreement with the above result of population analyses, there was a large increase in both MIC and MBC values for imipenem and methicillin after the hetero-to-homo conversion. On the other hand, MIC and MBC values for sulbactam/ampicillin increased by only two-fold from 32 to 64 μg/ml.

Table 1
Lack of discrepancy between MIC and MBC for sulbactam/ampicillin against heterogeneous as well as homogeneous types of MRSA

Antibiotics	LR5		LR5CEZ64-5	
	MIC	MBC	MIC	MBC
Methicillin	64	256	1024	2048
Imipenem	4	32	128	512
Sulbactam/ampicillin	32	32	64	64

Evaluation of the binding affinity of penicillin G and ampicillin

Table 2 shows IC_{50} values for penicillin G and ampicillin compared with those of other β-lactam antibiotics. The IC_{50} values for ampicillin and penicillin G were estimated to be 14 and 15 μg/ml, respectively. These values were quite similar to those reported previously with other MRSA strains [13], and are the lowest among

Table 2
IC_{50} of ampicillin and other tested β-lactams in the competitive inhibition of penicillin G binding to PBP2'

	Ampicillin	Penicillin G	Imipenem	Flomoxef	Cefmetazole
IC_{50} (μg/ml)	14	15	> 200	> 200	> 200

those of the β-lactam antibiotics which have been reported previously. Therefore, ampicillin was found to have the highest binding affinity to PBP2′ among the β-lactam antibiotics hitherto tested.

DISCUSSION

An epidemiological large-scale hetero-to-homo conversion of MRSA has been experienced in Japan in the last decade [14]. Experimental *in vitro* selection of homogeneous MRSA mutants from a heterogeneous MRSA strain can be achieved with a high frequency of 10^{-3} to 10^{-5}; but the appropriate concentration of β-lactam antibiotic for effective selection of the homogeneous mutant varies from antibiotic to antibiotic. Obviously, a concentration higher than the MIC value is desirable for the selection. Therefore, it is conceivable that the outstanding nation-wide conversion of MRSA phenotype which occurred in the last decade in Japan was the result of the use of the β-lactam antibiotics possessing potent activities against heterogeneous MRSA strains, because otherwise the drug concentrations needed for selection would never be attained in the serum of the patient [14]. The β-lactams satisfying this requirement are those belonging to the third group of antibiotics described in this study. As a matter of fact, in Japan, imipenem, cefmetazole, and flomoxef were extensively used to counter MRSA infection as a single agent or in combination [14]. Sulbactam/ampicillin was not used to counter MRSA infection previously since it was not available until 1994 in Japan. Contrary to our expectation, based on sulbactam/ampicillin belonging to the third group of antibiotics against heterogeneous MRSA, it was observed that the drug has far smaller frequency in the *in vitro* selection of the hetero-to-homo converts compared to the other members of the third group antibiotics (Hiramatsu, K. & Asada, K., in preparation).

The mechanism of hetero-to-homo conversion is intriguing. There has been some evidence that genetic alterations other than the *mec* region DNA are involved in the transition [5, 8]. We have recently cloned a novel gene which can mediate homogeneous resistance when introduced and expressed in a heterogeneous MRSA strain [5]. The cloned gene was not associated with the *mec* region DNA or any other previously cloned genes which influence methicillin resistance [15]. The mechanism of expression of homogeneous resistance mediated by the cloned gene is now under investigation.

Whatever the mechanism of homogeneity is, antimicrobial efficacy of ampicillin and penicillin G was not much affected by hetero-to-homo conversion, whereas all the other β-lactam antibiotics tested in this study were greatly influenced by the conversion. Why are ampicillin and penicillin not much influenced by the hetero-to-homo conversion? Binding affinities of these agents against PBP2′, evaluated by competitive binding inhibition of radioactive penicillin G to PBP2′, were shown to be quite high compared to those of other β-lactam antibiotics whose activities were greatly influenced by the conversion. Probably the high affinity to PBP2′ possessed by ampicillin and penicillin G is the reason for the relative effectiveness of the agents against not only heterogeneous but also homogeneous or highly resistant MRSA strains. This view is a logical outcome of the observation that the function of PBP2′ is definitely required for the expression not only of heterogeneous, but also homogeneous, methicillin resistance; the inactivation of the *mecA* gene by transposon mutagenesis [16] or spontaneous loss of the *mecA* gene [10, 17] renders tested MRSA strains completely susceptible to methicillin irrespective of their original phenotypic expression.

Although sulbactam/ampicillin had the best antimicrobial activity against MRSA among the tested β-lactam antibiotics, and most of the clinical strains so far analyzed had MIC values of either 16 or 32 μg/ml, we do find some clinical MRSA strains whose MIC values are 64 μg/ml (Asada, K., unpublished observations). The value seems to be too high to be treated with sulbactam/ampicillin as a single agent. However, when the efficacy of sulbactam/ampicillin was tested against one of the strains in combination with an aminoglycoside agent, arbekacin, high synergy was observed between the two agents; in one point, MIC values decreased from 64 μg/ml of sulbactam/ampicillin and 4 μg/ml of arbekacin to 8 μg/ml and 1 μg/ml, respectively, of sulbactam/ampicillin and arbekacin in combination (Asada, K., unpublished observations). There was no synergy between sulbactam/ampicillin and vanco-

mycin. Although more extensive study with clinical MRSA isolates is required, sulbactam/ampicillin could be an effective therapeutic agent for the infection caused by both heterogeneous and homogeneous methicillin resistant *S. aureus* when combined with other antibiotics. The search for a good combination counterpart is in progress.

REFERENCES

1. Jevons, M.P. (1961) "Celbenin"-resistant staphylococci. *Br. Med. J.* **1,** 124–125.
2. Noble, W.C., Virani, Z. & Cree, R.G.A. (1992) Cotransfer of vancomycin and other resistance genes from *Enterococcus faecalis* NCTC12201 to *Staphylococcus aureus*. *FEMS Microbiol. Lett.* **93,** 195–198.
3. Utsui, Y. & Yokota, T. (1985) Role of an altered penicillin-binding protein in methicillin- and cephem-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **28,** 397–403.
4. Hanaki, H., Akagi, H., Yasui, M., Otani, T., Hyodo, A. & Hiramatsu, K. (1995) TOC-39, a novel parenteral broad-spectrum cephalosporin with excellent activity against methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **39,** 1120–1126.
5. Hiramatsu, K. (1995) Molecular evolution of MRSA. *Microbiol. Immunol.* **39,** 531–543.
6. Hiramatsu, K., Asada, K., Suzuki, E., Okonogi, K. & Yokota, T. (1991) Molecular cloning and nucleotide sequence determination of the regulator region of *mecA* gene in methicillin-resistant *Staphylococcus aureus* (MRSA). *FEBS Lett.* **298,** 133–136.
7. Suzuki, E., Kuwahara-Arai, K., Richardson, J.F. & Hiramatsu, K. (1993) Distribution of *mec* regulator genes in methicillin-resistant *Staphylococcus* clinical studies. *Antimicrob. Agents Chemother.* **37,** 1219–1226.
8. Ryffel, C., Strassle, A., Kayser, F.H. & Berger-Bachi, B. (1994) Mechanism of heteroresistance in methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **38,** 724–728.
9. Okonogi, K., Noji, Y., Kondo, M., Imada, A. & Yokota, T. (1989) Emergence of methicillin-resistant clones from cephamycin-resistant *Staphylococcus aureus*. *J. Antimicrob. Chemother.* **24,** 637–645.
10. Hiramatsu, K., Suzuki, E., Takayama, H., Katayama, Y. & Yokota, T. (1990) Role of penicillinase plasmids in the stability of the *mecA* gene in methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **34,** 600–604.
11. Spratt, B.G. (1975) Distinct penicillin-binding proteins involved in the division, elongation, and shape of *Escherichia coli* K-12. *Proc. Natl. Acad. Sci. U.S.A.* **72,** 2999–3003.
12. Suzuki, E., Hiramatsu, K. & Yokota, T. (1992) Survey of methicillin-resistant clinical strains of coagulase-negative staphylococci for *mecA* gene distribution. *Antimicrob. Agents Chemother.* **36,** 429–434.
13. Chambers, H.F. & Sachdeva, M. (1990) Binding of beta-lactam antibiotics to penicillin-binding proteins in methicillin-resistant *Staphylococcus aureus*. *J. Infec. Dis.* **161,** 1170–1176.
14. Tanaka, T., Okuzumi, K., Iwamoto, A. & Hiramatsu, K. (1995) A retrospective study on methicillin-resistant *Staphylococcus aureus* clinical strains in Tokyo University Hospital. *J. Infec. Chemother.* **1,** 40–49.
15. de Lancastre, H. & Tomasz, A. (1994) Reassessment of the number of auxiliary genes essential for expression of high-level methicillin resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **38,** 2590–2598.
16. Matthews, P. & Tomasz, A. (1990) Insertional inactivation of the *mec* gene in a transposon mutant of a methicillin-resistant clinical isolate of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **34,** 1777–1779.
17. Wada, A., Katayama, Y., Hiramatsu, K. & Yokota, T. (1991) Southern hybridization analysis of the *mecA* deletion from methicillin-resistant *Staphylococcus aureus*. *Biochem. Biophys. Res. Commun.* **176,** 1319–1325.