ORIGINAL

No relationship exists between PBP 2a amounts expressed in different MRSA strains obtained clinically and their *β*-lactam MIC values

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Abstract : After establishing a linear relationship between the amount of penicillin-binding protein (PBP) 2a and membrane proteins of methicillin-resistant *Staphylococcus aureus* (MRSA) COL by dot-blot analysis using an antibody against PBP 2a, we determined the PBP 2a quantities in membrane fractions prepared from 14 different MRSA cells. Methicillin-sensitive *S. aureus* ATCC 6538P was used as a quality control strain. The amounts of PBP 2a diverged among the strains, and no relationship to *β*-lactam MIC values were observed in the corresponding strains. J. Med. Invest. 55 : 246-253, August, 2008

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains account for >60% of *S. aureus* clinical isolates in Japan, Singapore, and Taiwan, >50% in Italy and Portugal, and 34% in the United States (1). MRSA isolates have an additional transpeptidase, a 78 Kd class B penicillin-binding protein (PBP) named PBP 2′ (PBP 2a), which has a decreased affinity to β-lactams (2-5). PBP 2a is encoded by a *mecA* gene of unknown origin (6, 7). Usually, susceptible strains do not have the gene of this PBP (3). Although it is generally believed that PBP 2a is essential for conferring broad spectrum resistance to virtually all clinically used β-lactam

antibiotics (8-11) in *S. aureus*, the β-lactam (e.g., methicillin) MIC for resistant strains varies widely, from 3.1 μg to 1600 μg/ml.

Moreover, MRSA isolates with a low level of resistance show a peculiar, so-called heterogeneous expression of resistance. Our previous population analysis study of 20 MRSA strains using oxacillin revealed that three strains (20-22) were hetero-MRSA, exhibiting relatively low methicillin MICs, ranging from 256 to 128 μg/ml, while all the others were homo-MRSA (high-level methicillin MICs, $\geq$ 1024 μg/ml) (12).

It was reported (13) that transposon mutants of MRSAs, despite the massive reductions in the mutants' methicillin MIC, retained an intact *mecA* and its product, PBP 2a. The inserts were in genes involved in staphylococcal cell wall synthesis (13). In a report with heterogeneously resistant strains, cells with increased resistance levels present at low frequency (typically, 10^{-4} to 10^{-7}) in the form of one or more subpopulations, still produced normal amounts

of PBP 2a (14).

However, there has been little study of low- and high-resistance phenotypes of the MRSA strains to β-lactams in relation to the organism-producing levels of PBP 2a. Indeed, we proposed a two-step model for the sequence of a high-level resistance mechanism of MRSA to β-lactams (12, 15). Since the ability to produce PBP 2a is essential for the methicillin resistance of *S. aureus*, it is conceivable that knowing the amount of PBP 2a produced by the strains of high- and low-resistance groups might aid in understanding PBP 2a's role in resistance to β-lactam antibiotics. In this paper, we describe a linear relationship between the protein amount of the MRSA COL (a reference strain) and the PBP 2a quantity by dot-blot analysis using an antibody against PBP 2a. Moreover, since the relationship between PBP 2a amount and β-lactam MICs has not yet been well examined in the different clinical strains, this study aims to find out whether or not any correlation exists.

MATERIALS AND METHODS

S. aureus strains

Clinical strains of MRSA (strains 1-7, 9, 20-22) were kindly supplied by the late Dr. Toru Usui (Kyoto Microbiological Institute, Kyoto, Japan). Strain N315 and vancomycin (VCM) intermediate-resistance strains (VISA) Mu3 and Mu50 were kind gifts from Dr. Keiichi Hiramatsu (Juntendo University, Tokyo, Japan), and strain COL was from Dr. John J. Iandolo (University of Oklahoma Health Sciences Center, Tulsa, OK, USA). Methicillin-sensitive *S. aureus* (MSSA) ATCC 6538P was used as a reference strain. Some properties of the clinical strains used in the present study were determined previously (12).

Drug susceptibility assay of bacterial isolates

Bacterial susceptibility to antibacterial agents was measured *in vitro* by employing a standard twofold plate-dilution method using BBL™ Mueller–Hinton II agar (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 25 μg/ml of Ca^{2+}, 50 μg/ml of Mg^{2+}, and 2% NaCl (CA–MHA) (12). Following overnight incubation of test strains at 37°C in MHB, cultures were diluted with 0.85% NaCl, and then the bacteria (about 10^6 CFU/ml) were applied with an inoculator onto the surfaces of 10 ml agar layers containing the antibiotics. The plates were incubated at 37°C for 48 h and were checked for antibacterial susceptibility. The MIC was recorded as the lowest concentration of the antibiotic that completely inhibited growth.

Dot-blotting for detection and quantification of PBP 2a followed by membrane protein preparation

For the detection of PBP 2a, an antibody against PBP 2a was prepared with a peptide of PBP 2a (16, 17) ; details of the procedure were described previously (15). The strains of MRSA were incubated for 5 h at 37°C in a BBL™ Brain Heart Infusion (BHI) broth (Becton Dickinson). The cells were subsequently harvested from the culture after washing three times with washing buffer (50 mM Tris-HCl, pH 7.5, and 145 mM NaCl) by centrifugation at $4,500 \times g$ for 15 min at 6°C. Then the pellet was resuspended in the same buffer, and 2.4 ml cell suspension was treated with 24 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 50 μg/ml lysostaphin (Sigma Chemical, St. Louis, MO, USA), 10 mg/ml DNase I (Roche Diagnostics, Indianapolis, IN, USA), 5 mM $MgCl_2$, and 100 mM phenyl-methylsulfonyl fluoride (PMSF ; Sigma Chemical) for 1 h at 37°C. After centrifugation at $45,000 \times g$ at 6°C for 1 h, the precipitant was dissolved with 10 mM phosphate buffer, pH 7.0, containing 100 mM PMSF and diluted (1 : 25) with dilution buffer containing 10 mg/ml DNase I and 200 mM $MgCl_2$. Crude debris were removed by centrifugation at $4,500 \times g$ for 1 h at 6°C, and the resultant supernatant was centrifuged at $45,000 \times g$ at 6°C for 1 h. The precipitant was suspended with 10 mM phosphate buffer, pH 7.0, containing 100 mM PMSF and purified by centrifugation at $45,000 \times g$ at 6°C for 1 h. Finally the membrane fraction was dissolved with 50 μl of 10 mM phosphate buffer, pH 7.0. Protein concentrations were determined using a colorimetric method with bovine serum albumin as a standard (15, 18). To investigate the relationship between the PBP 2a amount and the membrane protein fractions, 0.2 μg, 0.5 μg, 1.0 μg, and 2.0 μg membrane fractions from MRSA COL (a reference strain) were transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA) using the Bio-Dot-SF Microfiltration Apparatus (Bio-Rad) according to the manufacturer's instructions. PBP 2a was identified by the chemiluminescence method as described with the ECL Plus Western Blotting kit (Amersham Biosciences, Piscataway, NJ, USA). Repeated experiments were done to confirm the results.

To determine the PBP 2a quantities in membrane fractions of different MRSA clinical strains, 1.0 μg protein was used following the procedure described above with MSSA ATCC 6538P as a quality control strain.

Statistical analysis

To assess the correlation between PBP 2a levels of the strains examined and the corresponding MICs of β-lactams, the Pearson correlation coefficient was calculated by using a linear model with terms for the two variables.

RESULTS

Antibacterial activity

All of the MRSA strains examined in the present study were resistant to β-lactams (e.g., methicillin MIC, 64 - >1,024 μg/ml), but strain N315 showed a low MIC (8 μg/ml), which was close to those of β-lactam-susceptible strains. Using a PCR technique, we confirmed that all the MRSA strains (100%), including N315, carried the *mecA* gene.

Detection and quantification of PBP 2a with dot-blot

Based on the previous result that a single band (molecular mass, about 76 kD) was detected by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of a membrane fraction of MRSA (10 μg of protein) followed by Western blotting analysis with the antibody against the peptide of PBP 2a (15), we conducted the study on detection and quantification of PBP 2a with dot-blot. In the present work, PBP 2a was detected in all strains of MRSA examined, including pre-MRSA strain N315, with MSSA ATCC 6538P as a negative control strain. For the quantification study, we first examined the relationship between the PBP 2a amount and membrane proteins prepared from MRSA COL by dot-blot analysis using an antibody against PBP 2a.

As shown in Fig. 1, the relationship between PBP 2a and membrane proteins of MRSA COL was linear. Hence, using immunoblotting we determined the PBP 2a quantities from membrane fractions prepared from the different clinical MRSA cells, with MSSA ATCC 6538P as a quality control strain. The PBP 2a amounts diverged among the strains (Fig. 2). The three heteroresistant clinical strains (20-22) had higher amounts than the homoresis-

tant clinical strains, except for strains 3 and 6. However, it must be pointed out that the amounts of PBP 2a varied among the three heteroresistant strains (20-22) (Fig. 2). In the present work, VISA strain Mu3 exhibited the highest levels of PBP 2a among the examined MRSA strains (Fig. 2).

As Fig. 3 clearly demonstrates, however, the resistant phenotypes of MRSA to β-lactams were unrelated to the organism-producing level of PBP 2a. Strains N315 and 1 showed similar PBP 2a quantities, although their respective methicillin MICs were 8 μg/ml and >1024 μg/ml. Among the homoresistant strain, MRSA 6 showed the highest value of the PBP 2a although its oxacillin and cephapirin MICs were 128 μg/ml and 64 μg/ml less than MICs of MRSA isolate 2 (512 μg/ml and 128 μg/ml respectively) (Fig. 3). However, the cefotaxime MIC was found to be higher (1024 μg/ml)

(A)

(B)

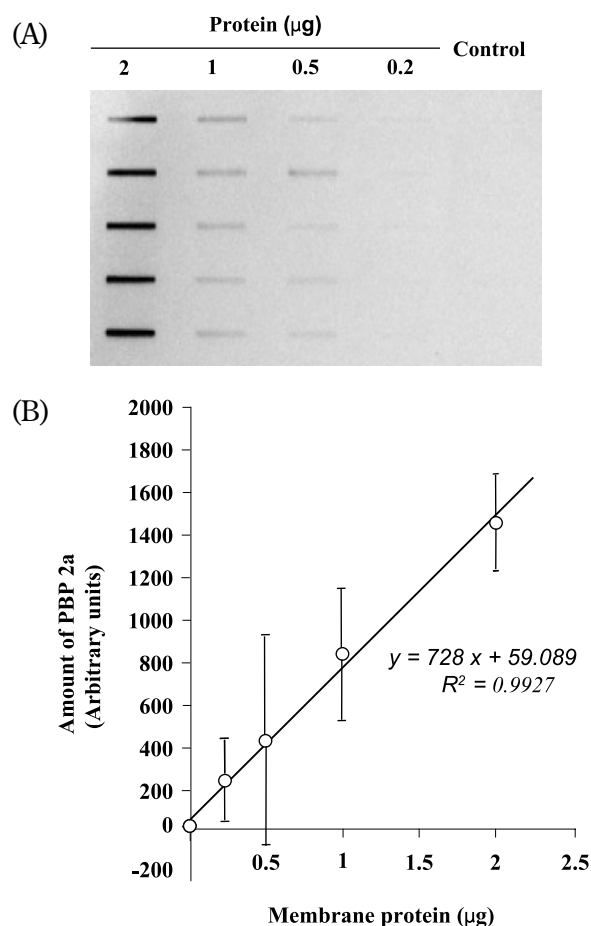$$y = 728 x + 59.089$$
$$R^2 = 0.9927$$

Fig. 1.   Immuno-blot analysis of PBP 2a in membrane fraction prepared from MRSA COL. Cell membrane proteins of MRSA COL were prepared, purified, and quantified as described in Materials and Methods. (A) Western blot analysis of different concentrations of proteins (0.2 μg, 0.5 μg, 1.0 μg, and 2.0 μg). PBP 2a was detected by the use of ECL Plus. The result shows five independent experiments. (B) A linear relationship between the amount of PBP 2a and membrane fraction of MRSA COL.

(A)

Protein (1 µg)

Strains					
MRSA 1					
MRSA 2					
MRSA 3					
MRSA 4					
Control					
MRSA 6					
MRSA 7					
MSSA					

(B)

MRSA COL

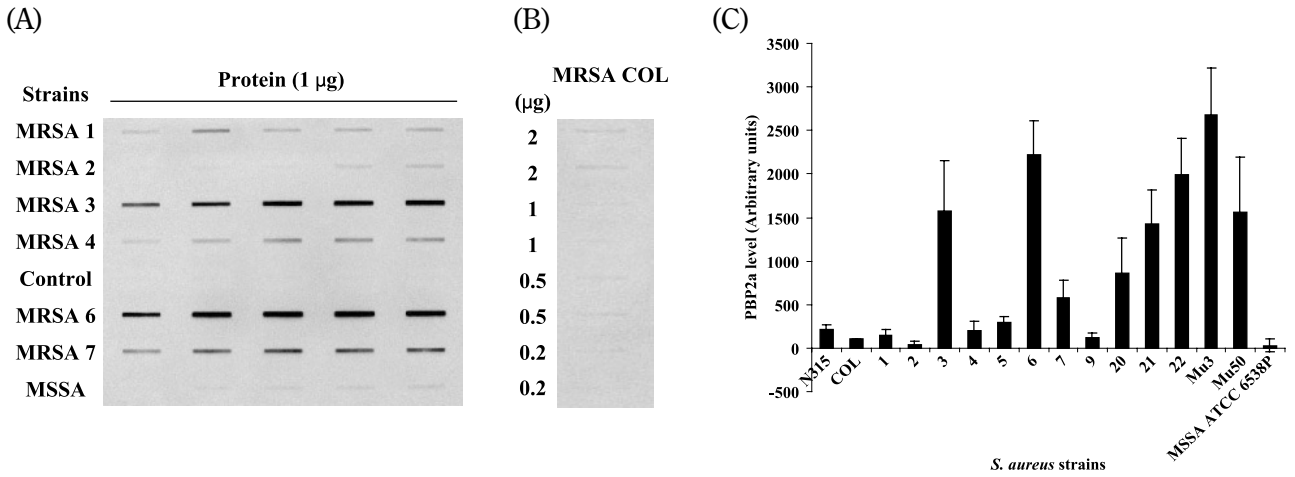(µg)

2
2
1
1
0.5
0.5
0.2
0.2

(C)

Fig. 2.   Amounts of PBP 2a in the cell membranes of different clinical strains of MRSA. (A) A typical demonstration of dot-blotting analysis of PBP 2a level. The membrane fractions of 6 strains of MRSA and MSSA (1 µg of protein) were applied. MSSA ATCC 6538P was employed as a quality control strain. The result shows five independent experiments. (B) Dot-blotting analysis of the membrane protein prepared from MRSA COL was used for the demonstration of the PBP 2a level of the other strain shown in (A). (C) Bar diagram of PBP 2a amounts of different MRSA strains obtained clinically with MSSA ATCC 6538P as a quality control strain.
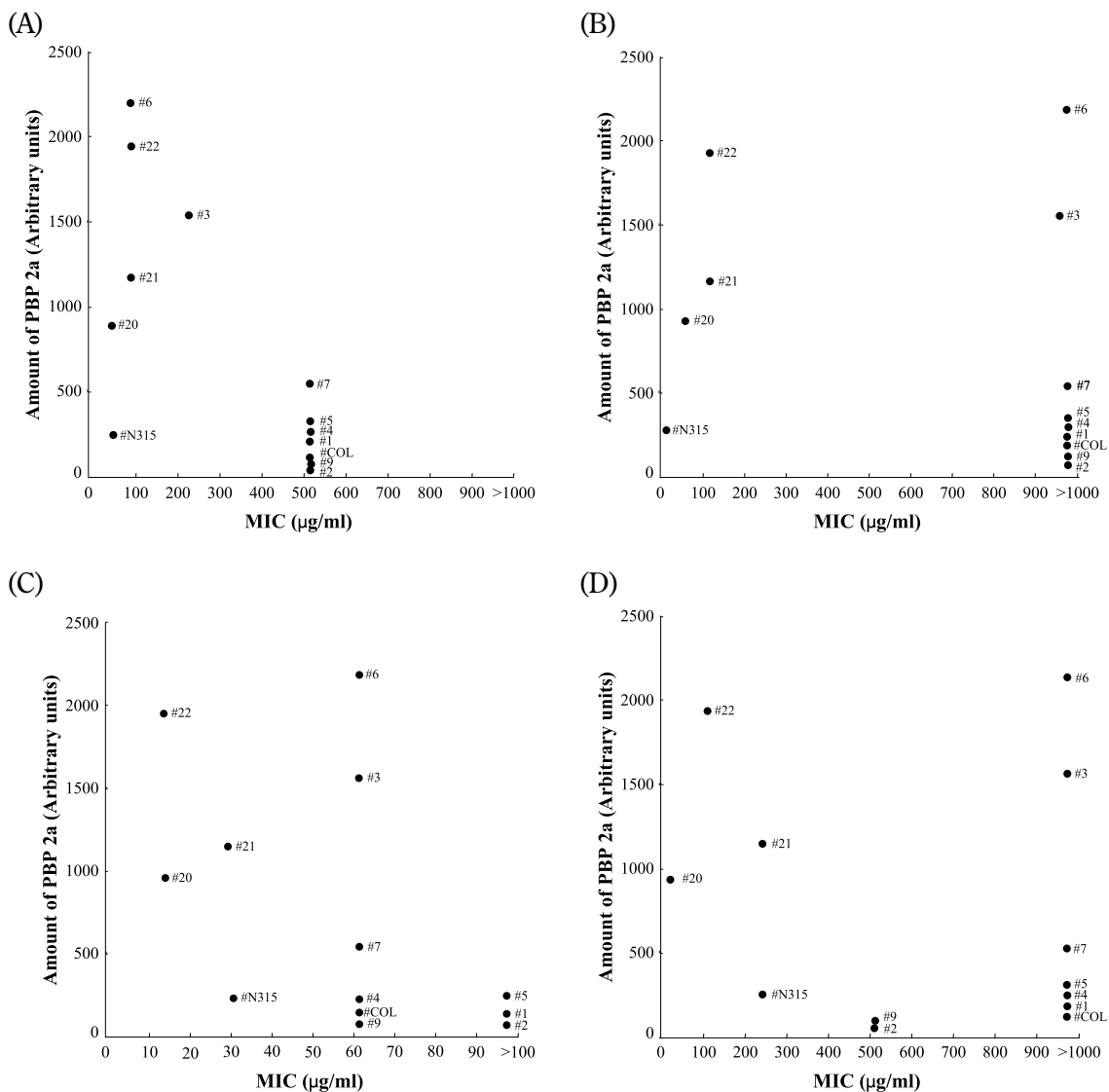
(A)

(B)

(C)

(D)

Fig. 3.   Relationship between PBP 2a levels and MIC values of oxacillin (A), methicillin (B), cephapirin (C), and or cefotaxime (D) of 13 different strains of MRSA.

in strain 6 than MRSA isolate of 2 (512 μg/ml). Discrepancy was also found with heteroresistant strain as shown in Fig. 3.

Statistical analysis

To assess the correlations between the PBP 2a levels of the strains examined and the corresponding MICs of β-lactams, we assumed a linear model with terms for the two variables and calculated the Pearson correlation coefficients for the drugs, with the exception of that for methicillin. The coefficients were -0.677 for oxacillin, -0.482 for cephapirin, and -0.153 for cefotaxime. The corresponding probability values (p-values, two-tailed) were 0.011, 0.095, and 0.618, respectively. For the latter two drugs, the results of this study suggest that there are no linear relationships between the PBP 2a levels of the MRSA strains examined and the drugs' MICs. However, in the case of oxacillin, the resulting p-value suggests that we could not exclude a linear relationship between the PBP 2a levels and the drug MICs.

DISCUSSION

A wide range of β-lactam MICs from the level of MSSA to > 4,000 μg/ml has been reported in clinical strains of MRSA, despite the fact that these MICs have an altered PBP 2a that β-lactam antibiotics do not inhibit at chemotherapeutic levels (9, 10, 14, 19). In the present study, the range of β-lactam resistance levels for MRSA also varied widely. For example, the MICs of oxacillin and cefotaxime were 64 μg/ml to 512 μg/ml and 32 μg/ml to 1024 μg/ml, respectively. On the basis of bacterial susceptibility to β-lactam antibiotics, methicillin-resistant *S. aureus* isolates could be classified into low- and high-resistance groups. Again, various phenotypic expressions of resistance (homogeneous or heterogeneous) have been reported in clinical isolates of MRSA (12, 20, 21). This leads to the question of whether or not PBP 2a production alone is related to the expression of high-level methicillin resistance in *S. aureus*. To answer this, we determined the amounts of PBP 2a in different MRSA isolates obtained clinically, following the procedure described in Materials and Methods.

Although Araj, *et al.* (22) reported that the reliable detection of MRSA cannot be based solely on the detection of the *mecA* gene in *S. aureus*, the presence of clinical isolates examined in the present study, previously classified as methicillin-resistant, were confirmed by the PCR finding that all of them carry the *mecA* gene, including strain N315 (12, 23, 24).

In the present study, we provide clear evidence with clinically isolated strains that there is no relationship between PBP 2a amount and β-lactam MIC value. First, the amount of PBP 2a varied widely among the highly and homogeneously resistant strains 1-7 and 9 (methicillin MIC ≥ 1024 μg/ml). The PBP 2a quantities were higher in strains 3, 6, and 7 than in the others. Second, strains 20-22 produced significantly high amounts of PBP 2a, yet exhibited only low-level heterogeneous resistance. Comparing strains 20 and 22, we found that, even though the cephapirin MICs were the same (16 μg/ml), the cellular amount of PBP 2a was much higher in the latter than in the former.

This is supported by the statistical analysis. For cephapirin and cefotaxime, the results of this study suggest that there are no linear relationships between PBP 2a levels and drug MICs. Unfortunately, in the case of oxacillin, we failed to exclude a certain correlation between the two variables. With respect to oxacillin, possibly as well as methicillin, the strains employed in this study were divided into two groups : those having drug MICs of 512 μg/ml (> 1,024 μg/ml for methicillin) and those having drug MICs of less than that. Overall, this appears to fit to a significant inverse correlation between the two variables. On the other hand, the PBP 2a amounts of the members of both groups ranged from low to high levels, thus demonstrating no particular relationship between the two parameters.

PBP 2a is encoded by the *mecA* gene, which is regulated by two upstream genes, *mecR1* and *mecI* (25). They can express *mecA* constitutively, or they can use the β-lactamase regulatory genes, *blaR1* and *blaI*, to optimally express *mecA*, since BlaR1 is a strong inducer of *mecA* whereas BlaI is a weak repressor (25-28). One report described that the uninduced amounts of PBP 2a from penicillinase-producing parents were lower than those for penicillinase-negative variants (29). In the present study, similar results were found with β-lactamase-nonproducing heteroresistant isolates (strains 20-22) that lack both regulatory genes *mecRI/mecI* and *blaRI/blaI* (12). In contrast with that report, we found that the β-lactamase-producing high-resistance clinical isolates exhibited relatively less PBP 2a, except for strain 1, a β-lactamase nonproducing homoresistant strain with a lower amount

of cellular PBP 2a. However, strain 1 carried *mecRI/mecI* regulatory genes, according to our previous report (12).

The apparent noncorrelation between MIC and the amount of PBP 2a suggests that some other, unknown factor contributes to the increased MIC. According to Murakami, *et al.* (29) the existence of an unknown factor was suggested previously from the observation that heterogeneously resistant strains produced significant amounts of PBP 2a under conditions in which small fractions of bacterial cells were resistant. In a study with transposon mutants of MRSAs selected for their reduced resistance, the mutants retained an intact *mecA*, and its product, PBP 2a, also remained unaffected despite the massive reduction in the methicillin MIC of the mutants (13). Biochemical analysis of these mutants indicated that the inserts were in genes involved in staphylococcal cell wall synthesis (13). Ryffel, *et al.* (19) reported that the high-level resistance expressed by a minority population of methicillin-resistant staphylococci was due to one or more chromosomal mutations (*chr**) located outside of *mecA*, the gene encoding PBP 2a. One of the most probable hypotheses to explain the mutation is that MRSA might acquire the ability to provide PBP 2a with sufficient quantities of the precursors on the nascent cross wall of the staphylococcal cell (15).

Hanaki, *et al.* (30) reported that Mu3 and Mu50 strains produced three to five times the amount of PBP 2a than VCM-susceptible *S. aureus* strains with or without methicillin resistance. In our study, VISA strain Mu3 exhibited the highest levels of PBP 2a among the 14 strains examined (Fig. 2). VCM exerts its bactericidal effect by binding to D-alanyl-D-alanine (D-Ala-D-Ala) residues of peptidoglycan and its precursor units, inhibiting cell wall peptidoglycan synthesis (31). It is commonly accepted that cell wall thickening is responsible for the VCM resistance of VCM-intermediate *S. aureus* (VISA) strains (24, 32). Cui, *et al.* (31) reported a strong positive correlation between VCM and reduced susceptibilities of VISA to daptomycin, and that this correlation is related to cell wall thickening and slower growth rates in comparison to VCM-susceptible *S. aureus*.

In MRSA, the incorporation of the peptidoglycan precursor into the growing peptidolgycan chain in the presence of a β-lactam is carried out by cooperation between the transpeptidase activity of PBP 2a (*mecA*) and the transglycosylase activity of PBP 2 (*pbp2*) (7, 33). The β-lactam ring mimics the D-Ala-D-Ala moiety of the normal peptidoglycan precursors. PBP 2a has low affinity for the β-lactam ring of β-lactams (34). Thus, PBP 2a might have low affinity for D-Ala-D-Ala in the stem peptide of the precursor, suggesting that the protein would require substantial amounts of the precursor for proper functioning as a transpeptidase. Therefore, although PBP 2a is a key protein in MRSA, not only PBP 2a production but also the acquisition of other factors, such as an increased ability to supply the precursor, would be required to mediate β-lactam resistance in MRSA.

In conclusion, there is no direct relationship between the amounts of PBP 2a expressed in MRSAs obtained clinically and MIC values of β-lactam. This finding is in good accord with the previously proposed two-step model for the development of high-level resistance to β-lactams in MRSA (12, 15, 35).

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