MECHANISMS OF ACQUIRED PENICILLIN-RESISTANCE IN *STREPTOMYCES CACAOI*

ROLE OF PENICILLIN-BINDING PROTEINS IN PENICILLIN RESISTANT MUTANTS

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Several mutants isolated after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis of *Streptomyces cacaoi* strain KCC S-0352, were resistant to benzylpenicillin ranging in concentration from 1,000 to 5,000 μg/ml (4- to 20-fold more resistant than the parent). These mutants also acquired resistance to mecillinam, cephamycin C and methicillin. The affinity for β-lactams of penicillin-binding proteins (including PBP-2 — a possible lethal target of β-lactams in *Streptomyces cacaoi*) in the mutants decreased. Addition of Triton X-100 or ethylenediaminetetraacetic acid, but not toluene, reduced the minimum inhibitory concentration of β-lactams. *In vitro* accessibility of [^{14}C]benzylpenicillin to whole cells and membrane fractions was lower in the mutants than in the parent. The binding of β-lactams to penicillin-binding proteins in both the parent and mutants was increased by pretreatment with Triton X-100 or ethylenediaminetetraacetic acid. The results of this study of penicillin-binding suggest that penicillin-binding proteins play a major role in "acquired" resistance as well as "intrinsic" resistance.

The main mechanisms of resistance to the β-lactam antibiotics in pathogenic bacteria seem to be the following[1]: (1) change in target site (penicillin-binding proteins; PBPs), (2) action of hydrolyzing enzymes (β-lactamases) and (3) reduced access of β-lactams to the target sites (permeability barrier). However, in β-lactam producing bacteria (*Streptomyces*), it seems that PBPs are chiefly responsible for resistance. In general, PBPs of *Streptomyces* have low affinities for [^{14}C]benzylpenicillin, compared with pathogenic bacteria[2]. Furthermore, in β-lactam-producing *Streptomyces* strains there are fewer PBPs than in β-lactam non-producing strains[3]. In a previous study[4], at least five PBPs were always detected in membrane fractions of *Streptomyces cacaoi*. Of these, PBP-2 was the most likely target for killing by β-lactam antibiotics[5].

To elucidate more precisely the mechanisms of resistance to β-lactams in *Streptomyces*, we isolated mutants of *S. cacaoi* resistant to benzylpenicillin and examined their PBPs. In the present paper, we describe the properties of these PBPs and discuss the difference between "intrinsic" and "acquired" resistance to β-lactams in *S. cacaoi*.

Materials and Methods

Streptomyces Strain

Streptomyces cacaoi KCC S-0352 was a generous gift of Dr. A. SEINO of Kaken Chemicals Co.

Chemicals

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and ethylenediaminetetraacetic acid (EDTA) were purchased from Nakarai Chemicals Ltds., and Triton X-100 from Wako Pure Chemicals Industry Ltd.

Other chemicals and β-lactams used in this paper were the same as those in the preceding paper[6].

Mutant Isolation

Mutagenesis with NTG was performed as described by DERIC *et al.*[7]. The mutagenized cultures were grown at 37°C for 4 days. Colonies growing on plates containing benzylpenicillin at 1,000 μg/ml were picked, purified and characterized.

In Vitro Binding Assays

In vitro binding of [^{14}C]benzylpenicillin to whole cells and membrane fractions was assayed as described by HAKENBECK *et al.*[8] with modifications. Exponentially growing cells were harvested, washed twice with 0.01 M phosphate buffer (pH 7.0) and resuspended in the same buffer. Samples of 200 μl were added to the various concentrations of [^{14}C]benzylpenicillin and incubated for 60 minutes at 30°C. The reaction was stopped by adding a 1,000-fold excess of non-radioactive benzylpenicillin, and the mixture was cooled to 0°C. Cells were recovered by centrifugation at 15,000 rpm for 10 minutes, and washed twice. They were resuspended in the same buffer containing unlabeled benzylpenicillin in excess, and collected on a membrane filter (pore size, 0.45 μm) which was presoaked in the same buffer and washed with cold ethanol. Filters were dried and the radioactivity was counted in 5 ml of toluene-based scintillator. Non-specific binding was measured by using cells boiled for 1 minute and the value was subtracted.

Membrane fractions were prepared as previously described[4]. Experiments similar to those performed with cells were carried out with 50 μl portions of isolated membrane fractions containing 400 μg of protein. Aliquots were trapped on membrane filters and washed with 5% trichloroacetic acid. Radioactivity was counted as described above.

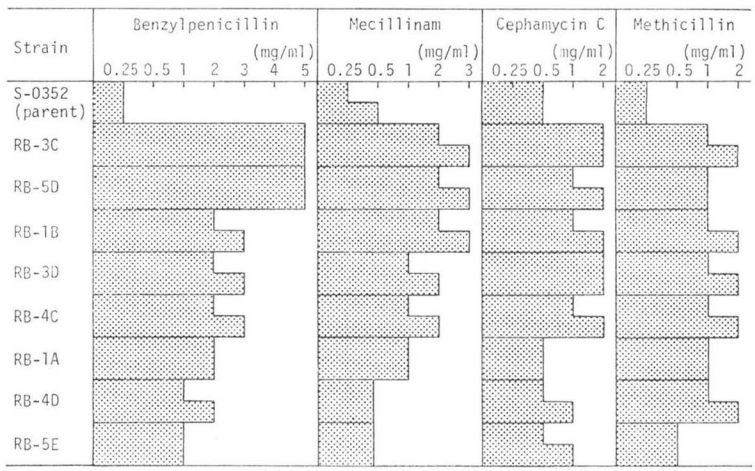
Binding of [^{14}C]benzylpenicillin to PBPs was performed as described[6].

Chemical Treatment

Minimum inhibitory concentrations (MICs) of β-lactams in the presence of Triton X-100 (3%) were estimated by a disc method[5] and Triton X-100 treatment was carried out as described by HAMILTON and LAWRENCE[9] with slight modifications. Washed logarithmic phase cells were incubated at 4°C for 30 minutes with Triton X-100 (3%) and centrifuged for 20 minutes at 10,000×*g*. Cells were washed twice with 0.01 M phosphate buffer (pH 7.0) and assayed for the binding of [^{14}C]benzylpenicillin to whole cells, membrane fractions and PBPs.

MICs of β-lactams with EDTA were measured as described by TAKATA *et al.*[10] with some modifications. EDTA-treated cells were prepared as follows. Cells were incubated for 10 minutes at 37°C with gentle shaking in 0.01 M phosphate buffer (pH 7.0) containing a sub-inhibitory concentration (1/4 of MIC) of EDTA (the MICs of EDTA for the parent and the mutants were 5~15 μg/ml).

Fig. 1. Bar graphs showing MICs of various β-lactam antibiotics for the parental and penicillin-resistant mutant strains of *S. cacaoi*.
Notched ends on bars indicate a value between those indicated.



MICs with toluene were estimated by a disc method on a nutrient agar plate containing toluene (1%). Cells were toluenized by incubation for 30 minutes at room temperature with magnetic stirring in phosphate buffer containing toluene (1%).

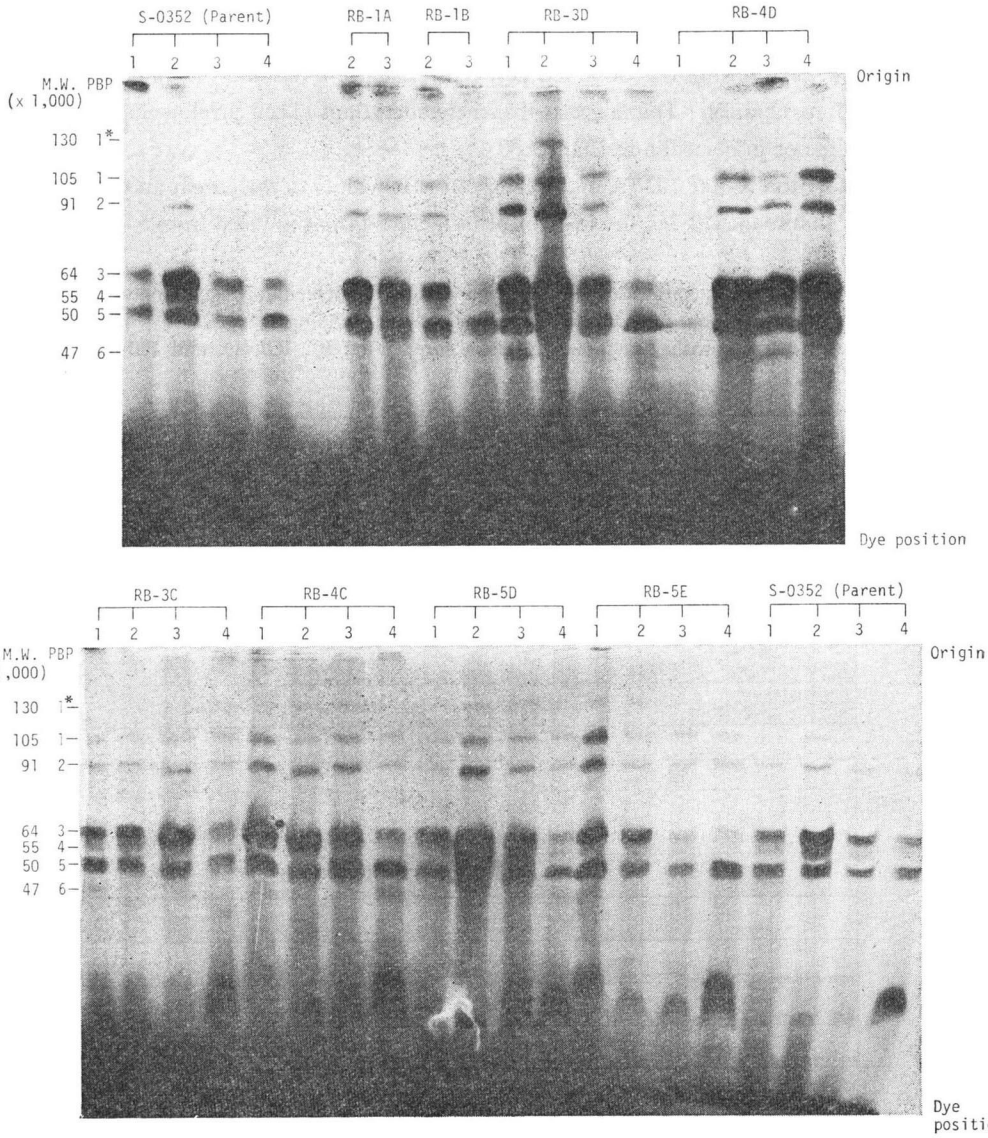
Other Method

Slab gel electrophoresis-fluorography were performed as described[6].

Results

After NTG treatment, 8 mutants were isolated on a plate containing 1,000 μg/ml of benzylpenicillin. The resistance to benzylpenicillin of the mutants was 4- to 20-times higher than that of the parent (1,000~5,000 μg/ml). Fig. 1 shows the MIC of benzylpenicillin, mecillinam, cephamycin C and me-

Fig. 2. Binding of [^{14}C]benzylpenicillin to the PBPs in membranes prepared from cells of the parent and penicillin-resistant mutants of *S. cacaoi* collected at various times.
1: Early logarithmic phase; 2: late logarithmic phase; 3: stationary phase; 4: lysis phase.



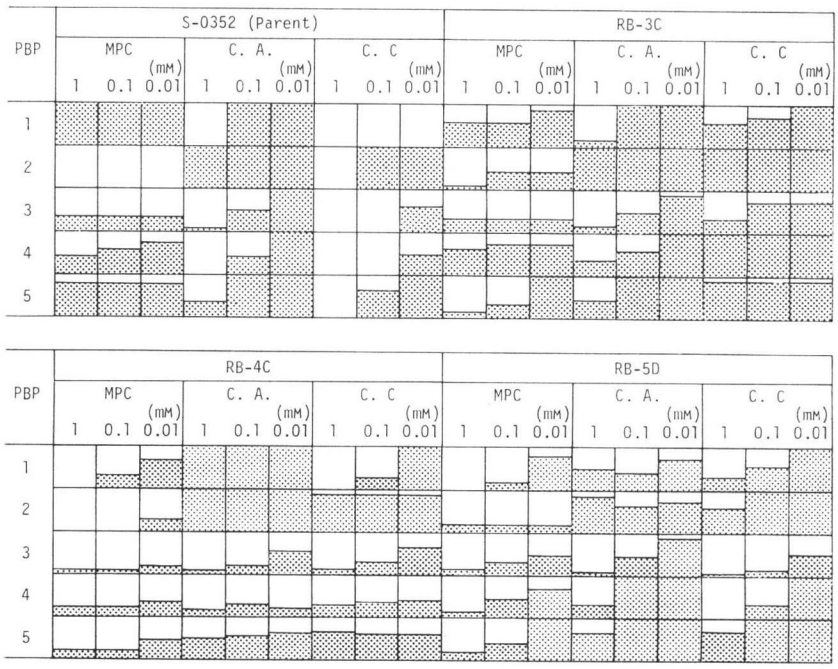
thicillin for the parental and benzylpenicillin-resistant mutant strains of *S. cacaoi*. β-Lactamase activities of the mutants were similar as those of the parent (data not shown).

In the PBPs of the mutants, the only marked change was the increased proportion of PBP-1* (Fig. 2). This PBP was sometimes detected in the parental strain but in negligible amount[4]. The relationship between appearance of this high molecular weight PBP (M.W.=130,000) in the mutants and their acquisition of resistance can not be explained at the present time.

To explore changes in the PBPs in more detail, their affinities for various β-lactams were examined by using three mutants, RB-3C, RB-4D and RB-5D. Fig. 3 illustrates the affinity patterns of β-lactams in the parent and the mutants. Mecillinam had very low affinity for PBP-1 but high affinity for PBP-2 in the parent. A slight decrease in the affinity of mecillinam for PBP-2 was observed in the mutants. However, close examination revealed quantitative alterations in the binding ability of all PBPs in the mutants, not only to mecillinam but also to clavulanic acid and cephamycin C. In order to determine how far these PBP changes contribute to penicillin-resistance, penicillin-binding to whole cells and membrane fractions was compared (Figs. 4A and 4B). Although binding in the mutants was less than in the parent, no clear-cut relationship was observed between resistance levels and the amounts of penicillin bound to cells and membranes. This suggests that these alterations in cell envelope have a role but are not the dominant factor in penicillin resistance.

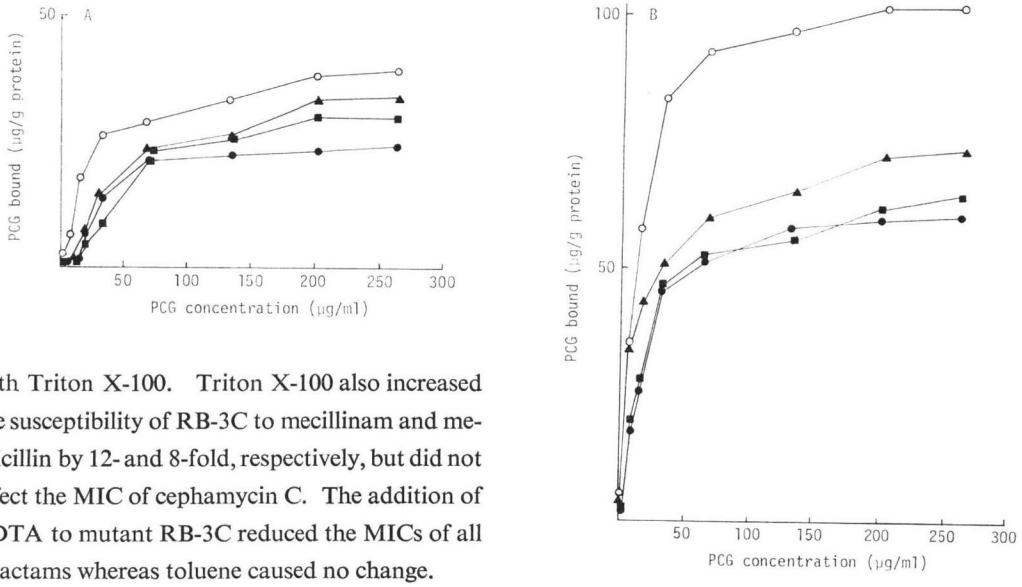
The effects of Triton X-100, EDTA and toluene on MIC values of the parent and the mutants are shown in Fig. 5. In mutant RB-3C, the MIC of benzylpenicillin was reduced from 5,000 to 500 μg/ml

Fig. 3. Affinity patterns of β-lactams for PBPs in the parent and the penicillin-resistant mutants of *S. cacaoi*.
The bars in each column indicate the percentage residual binding capacity of [^{14}C]benzylpenicillin to PBPs after treatment with β-lactams. S-0352: parent; RB-3C, RB-4D, and RB-5D: penicillin-resistant mutants.



MPC : Mecillinam, C. A. : Clavulanic acid, C. C : Cephamycin C.

Fig. 4. Binding of [^{14}C]benzylpenicillin to whole cells and isolated membrane fractions.
(A): Whole cells; (B): membrane fractions. ○: S-0352 (parent); ●: mutant RB-3C; ▲: mutant RB-4D; ■: mutant RB-5D.



with Triton X-100. Triton X-100 also increased the susceptibility of RB-3C to mecillinam and methicillin by 12- and 8-fold, respectively, but did not affect the MIC of cephamycin C. The addition of EDTA to mutant RB-3C reduced the MICs of all β-lactams whereas toluene caused no change.

To further clarify the mechanisms of this acquired resistance, we undertook a study which showed that chemical agents affecting the cell surface increased the binding of [^{14}C]benzylpenicillin to whole cells (Figs. 6A, 6B and 6C) and isolated membranes (Figs. 6D, 6E and 6F). The accessibility of [^{14}C]benzylpenicillin to the cells and membrane increased after chemical treatments but the levels and patterns varied with each strain (Figs. 6A to 6F). There was a parallel relationship between the increased incorporation of [^{14}C]benzylpenicillin into chemically treated cells (Figs. 6A to 6C) and changes in MICs caused by the chemicals (Fig. 5). However, there were some minor discrepancies in the relative incorporation into membrane fractions (Figs. 6B to 6F).

The binding patterns of [^{14}C]benzylpenicillin to the PBPs were altered by pretreatment with chemical agents. Particularly noticeable was the feature of PBP-3a to bind [^{14}C]benzylpenicillin after pretreat-

Fig. 5. Effects of chemical agents on the MICs of β-lactams for the parent and the penicillin-resistant mutants.
The concentrations of reagents are 3%, 1/4 of its MIC value, and 1% for Triton X-100, EDTA and toluene, respectively.

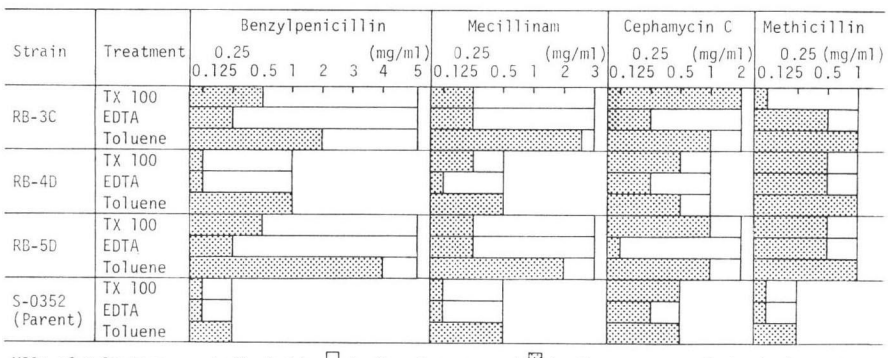


Fig. 6. Effect of chemical treatment on the binding of [^{14}C]benzylpenicillin to whole cells (A~C) and membrane fractions (D~F).

Assays were carried out as described in the text after treatment with Triton X-100 (A, D), EDTA (B, E) or toluene (C, F).

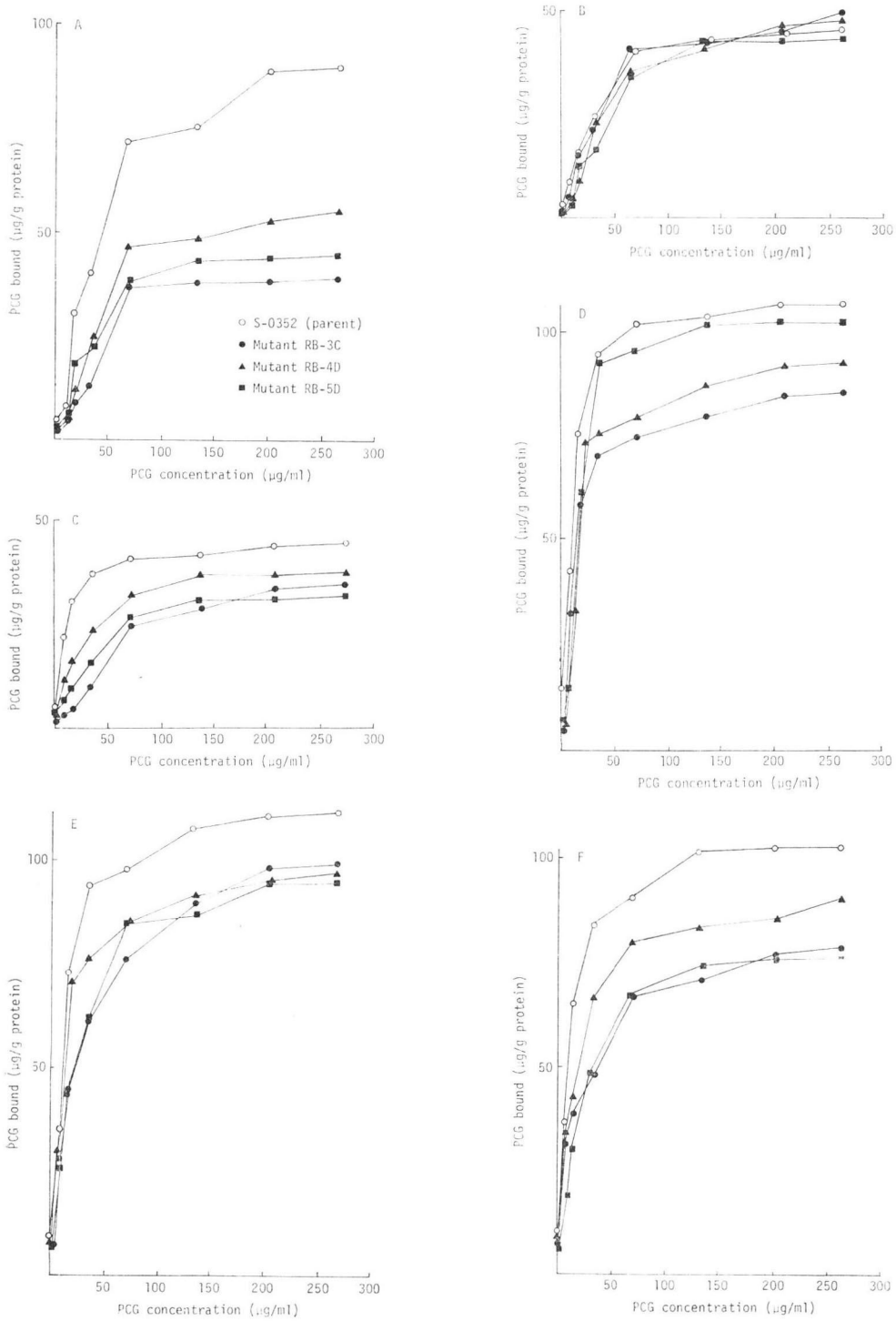
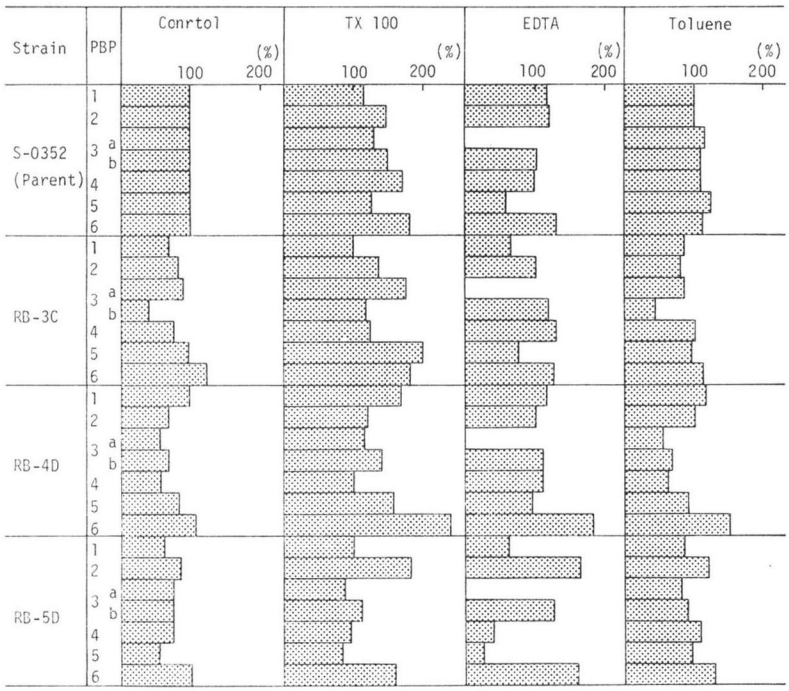


Fig. 7. The affinity patterns of [^{14}C]benzylpenicillin to PBPs after treatment with chemical agents.
The bars in each column indicate as a percentage the binding of [^{14}C]benzylpenicillin to PBPs compared to that in the parent which is not treated with any chemicals.



ment with EDTA. Such a large change in binding was not found with other treatments.

Fig. 7 illustrates the affinity patterns of [^{14}C]benzylpenicillin to PBPs pretreated with chemical agents. The bars in each column indicate the percentage binding of [^{14}C]benzylpenicillin to PBPs compared to that of the parent which was not treated with any chemical agents. Triton X-100 treatment increased the binding of [^{14}C]benzylpenicillin to all PBPs. After EDTA treatment, the most striking feature was the disappearance of binding to PBP-3a. In contrast, binding to PBP-2 and 3b was augmented. Toluene treatment caused little change.

Discussion

Recently, it became clear that "intrinsic" resistance to β-lactams in *Streptomyces* is due mainly to the low affinity of their PBPs[1]. The present work shows that the major mechanisms of "acquired" resistance in benzylpenicillin-resistant mutants of *S. cacaoi* are alterations of cell envelope and PBPs.

Compared with the parent, penicillin binding to intact cells of resistant mutants was reduced (Fig. 4A). The membrane environment may alter and prevent access of β-lactams to their site of action in the cytoplasmic membrane. The cell wall of Gram-negative bacteria is a very complex structure and is, in general, assumed to contribute to resistance to antibiotics[11]. For example, many authors suggest that "intrinsic" resistance mechanisms in *Pseudomonas* are the result of a permeability barrier to antibiotics at the outer membrane[12,13]. The cell wall of the Gram-positive bacterium, *Bacillus megaterium*, also acts as a permeability barrier to β-lactams[14]. These observations suggest that a permeability barrier may also be present on the cell surface of benzylpenicillin-resistant mutants of *S. cacaoi*. Binding to the whole cells of mutants decreased in proportion to the increased resistance (Fig. 4A). The decrease was restored, to varying extents, by chemical treatment. Triton X-100 acts on the cell surface and solu-

bilizes proteins from the cytoplasmic membrane. In accord with this, Triton X-100 increased not only the incorporation of [^{14}C]benzylpenicillin into whole cells (Fig. 6A) but also the binding of [^{14}C]benzylpenicillin to PBPs (Fig. 7). This may be because the PBPs are exposed by Triton X-100 treatment[9]. EDTA treatment also increases [^{14}C]benzylpenicillin binding to whole cells (Fig. 6B). Treatment with EDTA partially releases lipopolysaccharide and also proteins and phospholipids[15] from the cells and chelates various cations. These actions damage the permeability barrier. It is reported that intrinsic resistance in *Staphylococcus aureus* is suppressed by certain chelating agents[16]. It is particularly interesting that the PBP-3a band in treated cells disappeared (Fig. 7) while the corresponding PBP-3a band in the soluble fraction remained (data not shown). Toluene treatment affects the accessibility of [^{14}C]-benzylpenicillin to whole cells (Fig. 6C) but does not alter its binding to PBPs (Fig. 7). This may be why the toluenized cells of mutant strains show little change in MIC (Fig. 5).

Drastic alterations of PBPs are observed in β-lactam-resistant mutants of some bacteria[17~20]. Therefore, it must be considered significant that, in *Streptomyces*, the PBPs of penicillin-resistant mutants are not drastically changed (Fig. 2). That small alterations in PBPs cause a large increase in resistance to β-lactams can be best understood by assuming that, in *S. cacaoi*, alteration of PBPs is the primary mechanism of acquired β-lactam resistance in the mutants as well as the mechanism of intrinsic resistance.

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