MEMBRANE-BOUND PENICILLIN-BINDING PROTEINS OF *ESCHERICHIA COLI*

COMPARISON OF A STRAIN CARRYING AN R-FACTOR AND THE PARENT STRAIN

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Membrane-bound penicillin-binding proteins of an *Escherichia coli* carrying an R factor which mediated the resistance to penicillins were examined by slab gel electrophoresis and fluorography using β-lactamase inhibitors such as methicillin, clavulanic acid and MC-696-SY2-A, and by affinity chromatography. By fluorography, it appeared that the penicillin-binding proteins of the strain carrying the R factor could not be distinguished from those of the parent strain. In both strains, methicillin had a preferential affinity for penicillin-binding proteins 2 and 3, clavulanic acid for 2 and 4, and MC-696-SY2-A for 1A at the concentration which was needed to inhibit about 75~80% of β-lactamase activity of the membrane fraction from a strain carrying an R factor. This with other facts indicates that MC-696-SY2-A has a unique character in the binding to penicillin-binding proteins. By affinity chromatography using cephalexin-CH-Sepharose 4B column, two major cephalexin-binding proteins were detected. Their molecular weights were found to be 110,000 and 32,000, respectively. These two proteins corresponded to penicillin-binding proteins 1 and 5/6. From these results it was suggested that the R factor had no influence on the penicillin-binding proteins in the *E. coli* strain examined.

The roles of β-lactamase (penicillin and cephalosporin amido-β-lactam hydrolase, EC 3. 5. 2. 6) in bacterial resistance have been extensively studied. However, little is yet known as to how the β-lactamase has been evolved in resistant bacteria. This enzyme shows tremendous variety in physico-chemical, chemical, enzymatic and immunological properties, and is a group of enzymes having only one common property, namely, catalysis of the hydrolysis of β-lactam ring of many β-lactam compounds; in other words, the β-lactamase has a common property in having a β-lactam compound-binding site. The β-lactamase which exists at the present time may have evolved in a convergent manner from many proteins with different properties, or in a divergent manner from one common precursor. In either way, the precursor should have a common property of having a β-lactam compound-binding site. Thus, one of the possible precursors is a so-called penicillin-binding protein. Recently, it has been reported that DD-carboxypeptidases from *Streptomyces* R61 and R39 degrade benzylpenicillin to phenylacetylglycine and N-formyl-D-penicillamine[1], a membrane enzyme from *Staphylococcus aureus* have transpeptidase, carboxypeptidase and penicillinase activities[2], and that the DD-carboxypeptidase from *Bacillus stearothermophilus* also degrades benzylpenicillin to phenylacetylglycine[3]. Although these degradation products are not necessarily identical with those of β-lactamase, it is speculated that DD-carboxypeptidase, one of the penicillin-binding proteins, could be an evolutional precursor of β-lactamase.

Another possible evolutional precursor could be found among the β-lactam compound-binding proteins in a broad sense, that is, so-called penicillin-binding proteins and the proteins which are obtained by affinity chromatography. If the β-lactamase is derived from such β-lactam compound-binding proteins, the production of such proteins having an ability to bind β-lactam compound could be mediated by a

plasmid, because many β-lactamases are known to be produced by the involvement of plasmid. In a previous paper[4], we reported that the soluble penicillin-binding proteins from *E. coli* K12 W3630 carrying an R factor (R^{+}_{75}) which mediated the production of a β-lactamase, could not be differentiated from those of the parent strain. This paper describes the results of comparison of the membrane-bound penicillin-binding proteins of *E. coli* K12 W3630 R^{+}_{75} and the parent strain and discusses the possible relationships between β-lactamase and penicillin-binding proteins.

Materials and Methods

Bacterial strains

Escherichia coli K12 W3630 carrying R^{+}_{75} and the parent strain were kindly provided by Prof. S. MITSUHASHI of Gunma University.

Chemicals

Sodium dodecylsulfate (SDS), acrylamide, N,N,N′,N′-tetramethylethylenediamine (TEMED) were purchased from Nakarai Chemicals Ltd., disodium ethylenediaminetetraacetate (EDTA) from Daiichi Chemicals Ltd., Triton X-100, N,N′-methylene bis(acrylamide), sodium N-lauroyl sarcosinate (Sarkosyl) and diphenyloxazole from Wako Pure Chemicals, tris (hydroxymethyl) aminomethane, soybean trypsin inhibitor (type 1-S), and egg white lysozyme from Sigma Chemicals Co. Trypsin and deoxyribonuclease I were purchased from Worthington, dithiothreitol from Boeringer Mannheim, CH-Sepharose 4B from Pharmacia Fine Chemicals, [^{14}C] benzylpenicillin (50 mCi/mmol) from Radiochemical Centre, Amersham, crystalline bovine serum albumin from Nutritional Biochemical Co. and myoglobin, chymotrypsinogen A, ovalbumin, bovine serum albumin and human gamma-globulin used for the molecular weight standards from Schwarz/Mann. MC-696-SY2-A (about 5% purity) was kindly supplied by Dr. S. TAKAHASHI of the National Institute of Health of Japan, and cephalexin by Dr. T. YOSHIDA of Shionogi Pharmaceutical Co.

Membrane preparation

Membranes were prepared by the method of SPRATT[5], but with slight simplification. *E. coli* K12 W3630 R^{+}_{75} and the parent strain were grown in 2.4 liters of nutrient broth at 37°C on a rotary shaking machine at 220 rpm. Cells were harvested in mid- to late-exponential growth phase (the absorbance at 600 nm was about 0.9~1.0). After washing with 180 ml of 0.01 M sodium phosphate buffer (pH 7.0) and re-suspending in 90 ml of the same buffer at 0°C, cells were broken by sonication (Ohtake Works, Tokyo) twice for 2 minutes at 0°C. Unbroken cells were removed by centrifugation at 8,000×*g* for 20 minutes at 4°C and cell membranes were pelletted out of the supernatant by centrifugation at 100,000×*g* for 40 minutes at 4°C. The cell membranes were resuspended in the above buffer, washed once and finally resuspended in the same buffer (1 ml buffer per 100 mg wet weight). These membrane preparations were stored at −20°C and used within 2~3 weeks.

β-Lactamase activity

This was determined as described in a previous paper[6].

Inhibitory activity against β-lactamase

The reaction mixture consisted of 1 ml of inhibitor in 0.05 M sodium phosphate buffer (pH 7.0), 200 μl of 2% starch solution, 1 ml of benzylpenicillin (0.11 mg/ml), 1 ml of starch-iodine solution (0.08M I_2 in 3.2 M KI - the above buffer - 2% starch=0.3: 180: 20) and 5 μl of the test solution. After incubation for exactly 10 minutes at 30°C, the absorbance at 600 nm was measured. As 100% inhibition reference, a mixture, which had the same composition as above except for omission of the inhibitor and which contained the membrane preparation from the parent strain instead of that from the strain carrying an R factor, was incubated under the same conditions. A zero per cent inhibition reference solution which had no inhibitor was incubated under the same conditions. With regard to the consumption of iodine by the inhibitor itself and its degradation products, a control mixture which had the same composition except for omission of the substrate was incubated under the same conditions.

Trypsin treatment

According to the method of HAKENBECK *et al.*[7], the cells which were harvested from a 400-ml cul-

ture were sonicated twice for 2 minutes at 0°C in 23 ml of 10 mM Tris-HCl buffer (pH 7.8) containing 0.75 M sucrose and 1 mM EDTA. The unbroken cells were removed by centrifugation and the cell membranes were pelletted out of the supernatant by centrifugation at 100,000×*g* for 40 minutes at 4°C. The cell membranes were resuspended in 10 ml Tris-HCl buffer (pH 7.8) containing 10 mM EDTA and the concentration was adjusted to the absorbance at 578 nm of 0.7~0.9. Then, trypsin was added (150 μg of trypsin per ml suspension). After 2 minutes at room temperature, the soybean trypsin inhibitor was added (200 μg per ml suspension). The cell membranes were recovered by centrifugation at 160,000×*g* for 60 minutes at 4°C and the membrane pellets were washed with and resuspended in 0.01 M sodium phosphate buffer (pH 7.0).

Binding of [^{14}C]benzylpenicillin to *E. coli* membrane

The membrane preparations were thawed slowly and the concentration was adjusted to 10 mg protein/ml. For the assay of penicillin-binding proteins, 200 μl of membrane preparations were treated with 20 μl of [^{14}C]benzylpenicillin (370 μg/ml; 1.0 μCi) for 10 minutes at 30°C and the reaction was stopped by the addition of 5 μl of non-radio active benzylpenicillin (120 mg/ml) and 10 μl of 20% Sarkosyl. After the inner membranes were solubilized for 20 minutes at room temperature, the Sarkosyl-insoluble outer membranes and the peptidoglycan were removed by centrifugation at 100,000×*g* for 40 minutes at 10°C. The supernatant consisting mainly of the inner membranes was used for the electrophoretic analysis. When a β-lactamase inhibitor was used, the inhibitor was added and the mixture was incubated for 10 minutes at 30°C before the addition of [^{14}C]benzylpenicillin.

Slab gel electrophoresis and fluorography

The slab gel electrophoresis was performed by the method of LAEMMLI and FAVRE[8] with a slight modification. The gel was prepared for fluorography as described by BONNER and LASKEY[9]. The diphenyloxazole-impregnated and dried gel was exposed to a Fuji X-ray film (RX-S) at −70°C in a cassette for about 85 days. The gel was stained for protein by the method of WEBER and OSBORN[10]. Slab gel electrophoresis was performed by the E-C Apparatus type EC 470 Vertical Gel Electrophoresis and the M & S GD-1 type gel drier was used.

Affinity chromatography

Cephalexin-CH-Sepharose 4B was prepared as described in a previous paper[11] and used for affinity chromatography. Membrane fractions were suspended at a concentration of 10 mg protein/ml in 0.05 M Tris-HCl buffer (pH 7.0) containing 3% Triton X-100, 1 mM EDTA and 0.2 mM dithiothreitol as described by POLLOCK *et al.*[12] and were solubilized by stirring for 1 hour at 4°C. Triton X-100-insoluble fraction was spun down by centrifugation at 100,000×*g* for 40 minutes at 4°C. The supernatant, Triton X-100-soluble fraction, was applied to a 1-ml column of cephalexin-CH-Sepharose 4B which was equilibrated with 0.05 M Tris-HCl buffer (pH 7.0) containing 3% Triton X-100, 0.2 mM dithiothreitol and 1 mM EDTA and the column was washed with 20 ml of the same buffer. Elution was performed with 10 ml each of 0.5 M and 1.0 M NaCl solution containing 0.3% Triton X-100. Finally, 3 ml of 0.05 M Tris-HCl buffer containing 1.0 M hydroxylamine and 0.3% Triton X-100 (pH 7.0) was used for releasing the proteins which bound covalently to cephalexin in the column as described by BLUMBERG and STROMINGER[13]. After the addition of SDS at a final concentration of 2% to the first part of 5 ml of each eluate and of the washing solution, and to 3 ml of hydroxylamine eluate which had been dialyzed against 0.01 M sodium phosphate buffer (pH 7.0) for 24 hours, each solution was extracted with the same volume of chloroform for the removal of Triton X-100. The aqueous phase was concentrated to 200 μl by Minicon B 15 (Amicon Corporation) and an aliquot was used directly for slab gel electrophoresis. When the binding of [^{14}C]benzylpenicillin to the components in each eluate was examined, each dialyzed eluate was concentrated as above and the [^{14}C]benzylpenicillin was added (final concentration, 34 μg/ml; 1.0 μCi). After incubation at 30°C for 10 minutes, SDS was added (final concentration, 2%) and each eluate was extracted with the same volume of chloroform to remove the Triton X-100. An aliquot was used for gel electrophoresis and fluorography.

Protein determination

Protein was determined by the method of LOWRY *et al.*[14] using crystalline bovine serum albumin as a standard. When the test solution contained Triton X-100, the protein was determined by the method of WANG and SMITH[15].

Results

β-Lactamase Activity

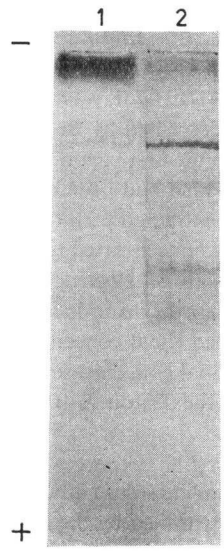
The membrane suspension from *E. coli* K12 W3630 R^{+}_{75} degraded benzylpenicillin. This was considered to be due to the β-lactamase activity, because the degradation product by the membrane suspension and that by penicillinase (Calbiochem) had the same Rf value on thin-layer chromatogram. This β-lactamase should hydrolyze [^{14}C]benzylpenicillin before its binding to penicillin-binding proteins, so we tried toprepare the β-lactamase-free membrane fraction from *E. coli* carrying an R factor. The β-lactamase of *E. coli* K12 W3630 R^{+}_{75} is located in the periplasmic space and has been purified[16]. Thus, we tried to destroy the peptidoglycan by treatment with trypsin and lysozyme in order to release the β-lactamase in the periplasmic space, since this membrane suspension was thought to be contaminated with this periplasmic β-lactamase. The assay method of β-lactamase activity by PERRET[17] was insensitive to such a low β-lactamase activity as this. Then, we determined the activity by measuring the absorbance at 620 nm.

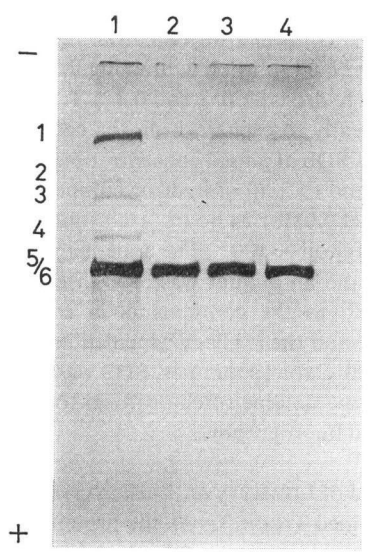
Trypsin was found to be effective in decreasing the β-lactamase activity of the membrane, but, as the fluorographic pattern in Fig. 1 shows, the penicillin-binding proteins were also destroyed by trypsin. Furthermore, the enzyme in this trypsin-treated preparation still hydrolyzed [^{14}C]benzylpenicillin, as indicated by the fluorographic patterns in an R^{+}_{75} containing strain. On the other hand, treatment with lysozyme by the method of LINDSTRÖM *et al.*[18] resulted in the degradation of peptidoglycan, as judged by rapid decrease of the absorbance at 578 nm of the membrane preparations but β-lactamase activity did not decrease. The membrane from stationary phase R^{+}_{75} cells also contained the β-lactamase

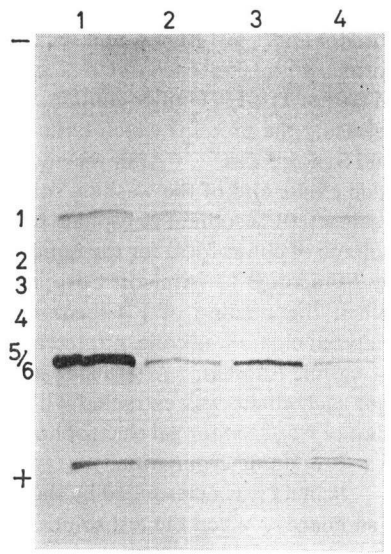
Fig. 1. The effect of trypsin on fluorographic patterns of penicillin-binding proteins.
Slot 1: the strain carrying an R factor.
Slot 2: the parent strain.

Fig. 2. Fluorographic patterns of methicillin-treated sample.
Slots 1 and 2: the parent strain.
Slots 3 and 4: R^{+}_{75} strain.
Each membrane fraction was preincubated with water (slot 1), 400 μg/ml (slot 2), 200 μg/ml (slot 3), and 400μg/ml (slot 4) of methicillin.

Fig. 3. Fluorographic patterns of clavulanic acid-treated sample.
Slots 1 and 2: the parent strain.
Slots 3 and 4: R^{+}_{75} strain.
Each membrane fraction was preincubated with water (slot 1), 40 μg/ml (slot 2), 20 μg/ml (slot 3) and 40 μg/ml (slot 4) of clavulanic acid.







activity. Accordingly, we did not use trypsin and lysozyme any further for the removal of β-lactamase from the membrane preparations.

β-Lactamase Inhibitor

For the removal of β-lactamase activity from the membrane preparations of the R factor-containing strain, many compounds related to penicillins were tested for their inhibitory activity. Among the compounds tested, methicillin, clavulanic acid[19] and MC-696-SY2-A[20,21] were effective in decreasing the β-lactamase activity of the membrane suspension from the R^{+}_{75} cells. The concentrations of these inhibitors which were used in these experiments were those which were needed to inhibit about 75~80% of β-lactamase activity of the membrane suspensions.

Penicillin-binding Proteins of *E. coli* Carrying R^{+}_{75} Factor

The fluorographic patterns using β-lactamase inhibitors are shown in Figs. 2, 3, and 4. Methicillin had higher affinity for penicillin-binding proteins 2 and 3 than [^{14}C]benzylpenicillin (Fig. 2) and clavulanic acid had higher affinity for penicillin-binding proteins 2 and 4 in both strains, as indicated by the fact that the bands 2 and 3 disappeared preferentially in the case of methicillin and bands 2 and 4 in the case of clavulanic acid. The other penicillin-binding proteins were detected in the same position with and without the R factor. Under the same conditions, MC-696-SY2-A showed strong inhibitory activity against the β-lactamase but appeared to have no affinity for any penicillin-binding proteins as indicated by Fig. 4. Fig. 4 also shows that the penicillin-binding proteins of R^{+} cells are not distinguished from those of R^{-} cells.

Fig. 4. Fluorographic patterns of MC-696-SY2-A treated sample.

Slots 1 and 2: the parent strain.

Slots 3 and 4: R^{+}_{75} strain.

Each membrane fraction was preincubated with water (slot 1), 40 μg/ml (slot 2), 20 μg/ml (slot 3) and 40 μg/ml (slot 4) of MC-696-SY2-A.

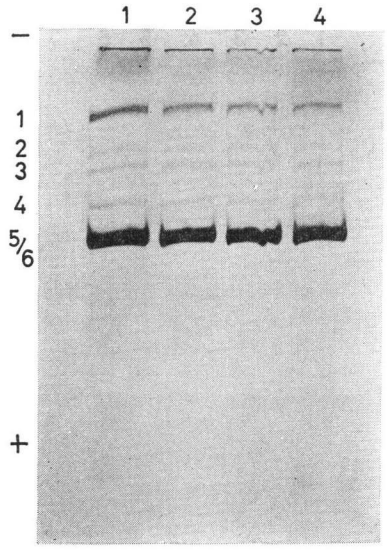
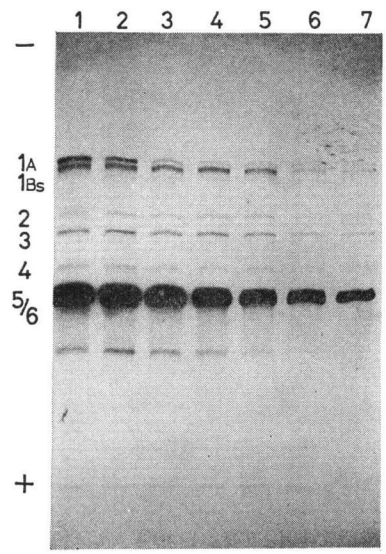


Fig. 5. Fluorographic patterns of MC-696-SY2-A treated sample.

The electrophoresis gel was composed of 7.5% acrylamide and 0.1% N, N′-methylene bis (acrylamide). The membrane fraction of the parent strain was preincubated with water (slot 1), 0.89 μg/ml (slot 2), 3.5 μg/ml (slot 3), 14 μg/ml (slot 4), 57 μg/ml (slot 5), 227 μg/ml (slot 6) and 455 μg/ml (slot 7) of MC-696-SY2-A.



Recently, penicillin-binding protein 1 has been separated into 1A and 1Bs by changing the condition of electrophoresis[22]. Using this condition, the effect of MC-696-SY2-A on the fluorographic patterns of penicillin-binding proteins was examined. Fig. 5 shows that MC-696-SY2-A had more or less a preferential affinity for penicillin-binding protein 1A. But at the same time, the ability of all the other penicillin-binding proteins to bind [^{14}C]benzylpenicillin decreased simultaneously in parallel with the concentration of this compound. This indicates that all the penicillin-binding proteins except protein 1A detected in fluorography have an almost equal affinity to MC-696-SY2-A. The same affinity patterns of MC-696-SY2-A to the penicillin-binding proteins were detected also in the R^+ strain (data not shown). From these autoradiographic and fluorographic results, it is suggested that the affinity of methicillin, clavulanic acid and MC-696-SY2-A for the penicillin-binding proteins of the strain carrying an R factor could not be distinguished from that of the parent strain and that the R factor did not affect the composition or the proportion of these binding proteins.

Affinity Chromatography

Fig. 6 shows the electrophoretic patterns of the eluates from CH-Sepharose 4B column. No protein was detected in the eluates with 0.5 M NaCl, 1.0 M NaCl and hydroxylamine. All the applied proteins were recovered in the washing. Using cephalexin-CH-Sepharose 4B column (Figs. 7 and 8), many proteins were recovered in the fraction of 0.5 M NaCl eluate but two major proteins were detected in the eluate with hydroxylamine. Their molecular weights were 110,000 and 32,000, respectively. β-Lactamase activity of the strain carrying an R factor was detected in the washing. Comparison of the electro-

Fig. 6. Electrophoretic patterns of the eluates from CH-Sepharose 4B (slot 1 to 4) and cephalexin-CH-Sepharose 4B (slot 5 to 8) columns.
Slots 1 and 5: washing.
Slots 2 and 6: 0.5 M NaCl eluates.
Slots 3 and 7: 1.0 M NaCl eluates.
Slots 4 and 8: hydroxylamine eluates.

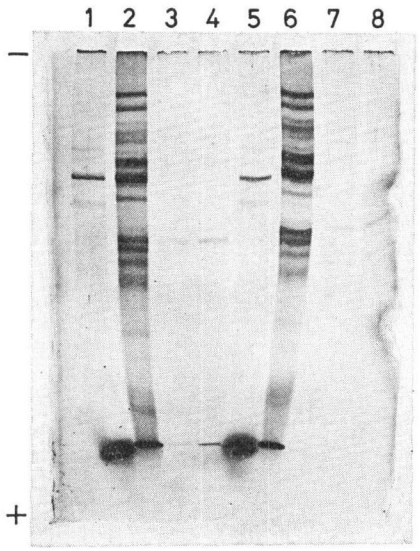


Fig. 7. Electrophoretic patterns of the eluates of affinity chromatography.
Slots 1~4: the parent strain.
Slots 5~8: R^+_{75} strain.
Slots 1 and 5: washing.
Slots 2 and 6: 0.5 M NaCl eluates.
Slots 3 and 7: 1.0 M NaCl eluates.
Slots 4 and 8: hydroxylamine eluates.
The protein was stained by Coomassie brilliant blue.

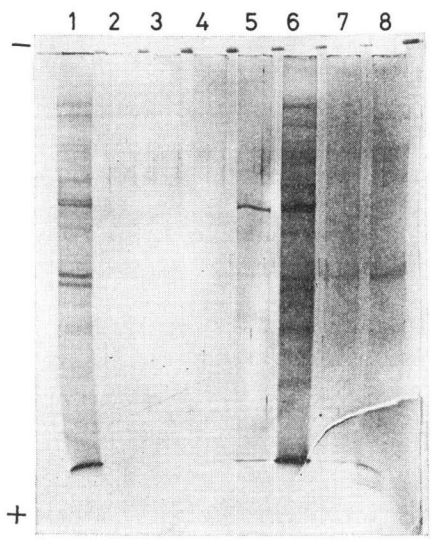
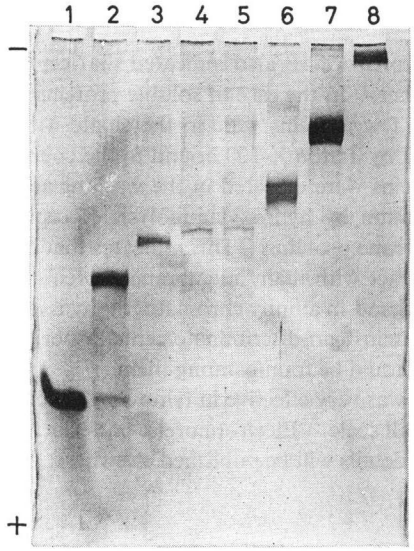


Fig. 8. Electrophoretic patterns of hydroxylamine eluate.

Slot 4: the parent strain. Slot 5: R^{+}_{75} strain. The other slots are for the molecular weight standards.

Slot 1: myoglobin (M. W., 17,800); slot 2: chymotrypsinogen A (M.W., 25,000); slot 3: deoxyribonuclease I (M. W., 31,000); slot 6: ovalbumin (M. W., 45,000); slot 7: bovine albumin (M. W., 67,000) and slot 8: gamma-globulin (M. W., 160,000).



phoretic patterns between a strain carrying an R factor and the parent strain is shown in Figs. 6 and 7. No differences were observed between these two strains.

The autoradiography of the eluates from the affinity column indicated that the one band of hydroxylamine eluate with a molecular weight of 32,000 bound [^{14}C]benzylpenicillin but another band with a molecular weight of 110,000 could not be detected in autoradiography. However, their molecular weights were in good accord with those of penicillin-binding protein 1 and 5/6. From our results, approximate molecular weights of penicillin-binding proteins 1 and 5/6 were estimated to be 110,000 and 31,000~32,000, respectively (Fig. 2).

Discussion

In a previous paper[4], we compared the penicillin-binding proteins obtained by affinity chromatography from *E. coli* K12 W3630 carrying an R factor R^{+}_{75}, with those of the parent strain. In this paper, we compared the membrane-bound penicillin-binding proteins from the same strains by autoradiography-fluorography and by affinity chromatography. Although the relationship between the soluble penicillin-binding proteins and the membrane-bound penicillin-binding proteins is not definitely clear and a few soluble proteins or their precursors may contaminate the membrane-bound penicillin-binding protein fraction, this does not matter and may even be advantageous, because they should be few in number and should show different forms in cytoplasm and in membrane and the object of the work was to look for proteins in whole cells which have a β-lactam compound-binding site. Their function is not clear at the present time, but they may be biosynthetic or evolutional precursors or they may interact with β-lactam compounds and prevent the permeability of the β-lactam compounds to some extent.

The majority of the β-lactamase in *E. coli* K12 R^{+}_{75} which was used in the present study[16] is located in periplasmic space. However, the fact that the activity could not be removed by washing and by lysozyme treatment, and the fact that trypsin that could degrade membrane proteins could decrease the β-lactamase activity of the membrane fraction, indicate that some activity remains in the membrane-bound form. The role of the bound β-lactamase is not clear at the present time.

In order to compare the penicillin-binding proteins of the strains with and without an R factor, we used the β-lactamase inhibitors with different modes of binding to the penicillin-binding proteins. Among the compounds tested, methicillin, clavulanic acid, and MC-696-SY2-A were found to have inhibitory activity against the β-lactamase. Using their inhibitory activity, we compared the electrophoretic patterns of the penicillin-binding proteins between *E. coli* strains with and without an R factor. From Figs. 2, 3, and 4, it was concluded that the penicillin-binding proteins of both strains were identical and that the R factor had no influence on the penicillin-binding proteins.

Clavulanic acid and MC-696-SY2-A were isolated from *Streptomyces clavuligerus*[19] and *Streptomyces fulvoviridis*[20,21], respectively. SPRATT has reported the binding patterns of clavulanic acid to the penicillin-binding proteins[23]. However, no reports have been published on such binding proteins

MC-696-SY2-A. Our results of the densitometric analysis of the X-ray film in Fig. 5 indicated that MC-696-SY2-A has a unique action on the penicillin-binding proteins in that this compound showed a preferential affinity to penicillin-binding protein 1A and then 1Bs, while the ability of the other penicillin-binding proteins to bind [^{14}C]benzylpenicillin decreased simultaneously in parallel with the concentration of MC-696-SY2-A. The above β-lactamase inhibitors, clavulanic acid and MC-696-SY2-A, however, had an inhibitory activity against a wide variety of β-lactamases, although these inhibitors always bound certain penicillin-binding proteins. In other words, both β-lactamase and the penicillin-binding proteins can bind not only the usual β-lactam antibiotics but also these β-lactamase inhibitors. This indicates that there are some relationships between β-lactamase and penicillin-binding proteins. Here, it is interesting that in this condition these β-lactamase inhibitors did not apparently bind penicillin-binding protein 5/6, D-alanine carboxypeptidase, which was reported to have a very weak penicillinase activity[24].

Affinity chromatography data using cephalexin-CH-Sepharose 4B also indicated that cephalexin-binding proteins of both strains were identical with each other. In the case of soluble proteins, almost all the proteins were recovered in the washing and only a few proteins were in the eluate with 0.5 M NaCl[4]. In contrast, membrane-bound proteins solubilized by Triton X-100 bound to the column and were released by elution with 0.5 M NaCl. Almost no proteins were detected in the washing and a few proteins were released from cephalexin-CH-Sepharose column by hydroxylaminolysis of cephalexin. Probably these differences are due to the character of membrane proteins. This indicates that cephalexin and probably other β-lactam compounds also can interact with many membrane proteins besides penicillin-binding proteins. It is also possible that when a ligand in affinity chromatography is changed, other membrane proteins may be eluted, because some proteins can discriminate cephalosporins from penicillins[25]. However, a possible β-lactamase precursor should be found among them.

Here, it must be mentioned that chloroform extraction was very effective in removing Triton X-100 and exchanging Triton X-100 into SDS, especially in a small scale. Electrophoretic patterns were not affected by using this method in our experiments. Further details will be published elsewhere.

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References

1) FRÈRE, J. M.; J. M. GHUYSEN, J. DEGELAEN, A. LOFFET & H. R PERKINS: Fragmentation of benzylpenicillin after interaction with the exocellular DD-carboxypeptidase-transpeptidases of *Streptomyces* R61 and R39. Nature 258: 168~170, 1975
2) KOZARICH, J. W. & J. L. STROMINGER: A membrane enzyme from *Staphylococcus aureus* which catalyzes transpeptidase, carboxypeptidase, and penicillinase activities. J. Biol. Chem. 253: 1272~1278, 1978
3) HAMMARSTRÖM, S. & J. L. STROMINGER: Degradation of penicillin G to phenylacetylglycine by D-alanine carboxypeptidase from *Bacillus stearothermophilus*. Proc. Natl. Acad. Sci. U.S.A. 72: 3463~3467, 1975
4) OGAWARA, H.: Penicillin-binding proteins of *Escherichia coli*. Comparison of a strain carrying an R-factor and the parent strain. Biochim. Biophys. Acta 491: 223~231, 1977
5) SPRATT, B. G.: Properties of the penicillin-binding proteins of *Escherichia coli* K12. Eur. J. Biochem. 72: 341~352, 1977
6) OGAWARA, H.: Production and property of β-lactamases in *Streptomyces*. Antimicr. Agents & Chemoth. 8: 402~408, 1975
7) HAKENBECK, R.; E. W. GOODELL & U. SCHWARZ: Compartmentalization of murein hydrolases in the envelope of *Escherichia coli*. FEBS Letters 40: 261~264, 1974

8) LAEMMLI, U. K. & M. FAVRE: Maturation of the head of bacteriophage T4. J. Mol. Biol. 80: 575~599, 1973
9) BONNER, W. M. & R. A. LASKEY: A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46: 83~88, 1974
10) WEBER, K. & M. OSBORN: The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4406~4412, 1969
11) OGAWARA, H. & H. UMEZAWA: *Bacillus cereus* β-lactamase. Reaction with N-bromosuccinimide and the properties of the product. Biochim. Biophys. Acta 391: 435~447, 1975
12) POLLOCK, J. J.; M. NGUGEN-DISTECHE, J. M. GHUYSEN, J. COYETTE, R. LINDER, M. R. J. SALTON, K. S. KIM, H. R. PERKINS & P. REYNOLDS: Fractionation of the DD-carboxypeptidase-transpeptidase activities solubilized from membranes of *Escherichia coli* K12, strain 44. Eur. J. Biochem. 41: 439~446, 1974
13) BLUMBERG, P. M. & J. L. STROMINGER: Isolation by covalent affinity chromatography of the penicillin-binding components from membranes of *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. 69: 3751~3755, 1972
14) LOWRY, O. H.; N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL: Protein measurement with the FOLIN phenol reagent. J. Biol. Chem. 193: 265~275, 1951
15) WANG, C. S. & R. L. SMITH: LOWRY determination of protein in the presence of Triton X-100. Anal. Biochem. 63: 414~417, 1975
16) OGAWARA, H.; K. MAEDA & H. UMEZAWA: A β-lactamase of *Escherichia coli*. Biochim. Biophys. Acta 289: 203~211, 1972
17) PERRET, C. J.: Iodometric assay of penicillinase. Nature 174: 1012~1013, 1954
18) LINDSTRÖM, E. B.; H. G. BOMAN & B. B. STEELE: Resistance of *Escherichia coli* to penicillins. VI. Purification and characterization of the chromosomally mediated penicillinase present in amp A-containing strains. J. Bacteriol. 101: 218~231, 1970
19) BROWN, A. G.; D. BUTTERWORTH, M. COLE, G. HANSCOMB, J. D. HOOD, C. READING & G. N. ROLINSON: Naturally-occurring β-lactamase inhibitors with antibacterial activity. J. Antibiotics 29: 668~669, 1976
20) UMEZAWA, H.; S. MITSUHASHI, M. HAMADA, S. IYOBE, S. TAKAHASHI, R. UTAHARA, Y. OSATO, S. YAMAZAKI, H. OGAWARA & K. MAEDA: Two β-lactamase inhibitors produced by a streptomyces. J. Antibiotics 26: 51~54, 1973
21) MAEDA, K.; S. TAKAHASHI, M. SEZAKI, K. IINUMA, H. NAGANAWA, S. KONDO, M. OHNO & H. UMEZAWA: Isolation and structure of a β-lactamase inhibitor from *Streptomyces*. J. Antibiotics 30: 770~772, 1977
22) TAMAKI, S.; S. NAKAJIMA & M. MATSUHASHI: Thermosensitive mutation in *Escherichia coli* simultaneously causing defects in penicillin-binding protein-1Bs and in enzyme activity for peptidoglycan synthesis *in vitro*. Proc. Natl. Acad. Sci. U.S.A. 74: 5472~5476, 1977
23) SPRATT, B. G.; V. JABANPUTRA & W. ZIMMERMANN: Binding of thienamycin and clavulanic acid to the penicillin-binding proteins of *Escherichia coli* K12. Antimicr. Agents & Chemoth. 12: 406~409, 1977
24) SPRATT, B. G. & J. L. STROMINGER: Identification of the major penicillin-binding proteins of *Escherichia coli* as D-alanine carboxypeptidase 1A. J. Bacteriol. 127: 660~663, 1976
25) OGAWARA, H.: A specific cephalosporin-binding protein of *Citrobacter freundii*. Biochim. Biophys. Acta 420: 155~164, 1976