EFFECT OF A COMBINATION OF BENZYLPENICILLIN OR AMPICILLIN AND DICLOXACILLIN ON PEPTIDOGLYCAN SYNTHESIS IN A CELL-FREE ENZYME SYSTEM FROM A β-LACTAMASE PRODUCING STRAIN OF *CITROBACTER FREUNDII*

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The membrane fraction prepared from β-lactamase producing *Citrobacter freundii* GN346 catalyzed *in vitro* peptidoglycan synthesis from uridine-5′-diphosphate-N-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine and uridine-5′-diphosphate-N-acetylglucosamine, which was accompanied by the release of alanine from the carboxyl terminal end of the former substrate. Though this reaction was inhibited by benzylpenicillin (PCG) and ampicillin (ABPC), the reaction was relatively insensitive compared with that catalyzed by the membrane fraction from a derived β-lactamaseless mutant strain GN346/16. In contrast, the enzyme activity of the parent strain was strongly inhibited by a combination of PCG or ABPC and dicloxacillin (MDIPC). The β-lactamase present in the membrane fraction from the parent strain showed stronger activity than that from the mutant strain, and the activity was inhibited by MDIPC as in the case of the soluble enzyme localized in the periplasmic space.

The combinations of penicillins or cephalosporins and isoxazolylpenicillins have been known to exhibit synergistic antibacterial activity against β-lactamase producing Gram-negative organisms[1~6]. Isoxazolylpenicillin is an inhibitor of β-lactamase present in the periplasmic space of Gram-negative organisms[3,7~10]. In our previous paper[11], the mechanism of the synergistic activity of a combination of ampicillin (ABPC) and dicloxacillin (MDIPC) against a β-lactamase producing strain of *Citrobacter freundii* was revealed in a whole cell system: the latter antibiotic (MDIPC) inhibited the β-lactamase activity present in the periplasmic space, while the former antibiotic (ABPC) penetrated through the outer membrane[12] and periplasmic β-lactamase barriers its target sites on the cytoplasmic membrane (inner membrane).

In the present paper, the peptidoglycan synthesis of β-lactamase producing *C. freundii* GN346 was investigated in a cell-free system and the effect of a combination of benzylpenicillin (PCG) or ABPC and MDIPC on this reaction was compared with that of the same antibiotics on the reaction catalyzed by the enzyme system obtained from a derived β-lactamaseless mutant, strain GN346/16.

Materials and Methods

Organisms

The organisms empolyed were β-lactamase producing strain GN346 and its derived β-lactamase-

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less mutant strain GN346/16 of *Citrobacter freundii*[13]. Both strains were kindly provided by Drs. S. YAMAGISHI and T. SAWAI (Faculty of Pharmaceutical Sciences, Chiba University, Chiba, Japan).

Antibiotics

Benzylpenicillin (PCG), ampicillin (ABPC) and dicloxacillin (MDIPC) were supplied by Toyo Jozo Co. Ltd. (Shizuoka, Japan). [^{14}C]PCG (labeled with [1-^{14}C]phenylacetic acid) was purchased from The Radiochemical Centre (Amersham, England). It had a specific activity of 52 mCi/mmol.

Preparation of the Membrane and Soluble Fractions

Each organism was grown in Trypticase soy broth (BBL, Md., U.S.A.) at 37°C with shaking. The cells at exponential phase were harvested by centrifugation as described previously[11]. The washed cells were disrupted with a Super sonic vibrator (UR-150, Tominaga Works Ltd,. Tokyo, Japan) for 5 minutes and cell debris were removed by centrifugation at 8,000$\times g$ for 10 minutes. The membrane fraction was then sedimented at 100,000$\times g$ for 40 minutes and the supernatant was used as the soluble fraction as described previously[14].

Susceptibility Test

The minimum inhibitory concentration (MIC) of antibiotics, alone or in combination, for the strains used was estimated by the serial two-fold dilution technique in Heart infusion agar (Difco, Mich., U.S.A.) with an inoculum of about 10^6 cells/ml.

Assay for Peptidoglycan Synthesis

Synthesis of peptidoglycan in cell-free system was studied with the membrane fraction previously described[14,15]. Uridine-5′-diphosphate-N-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-[^{14}C]alanyl-D-[^{14}C]alanine (UDP-MurNAc-pentapeptide) and uridine-5′-diphosphate-N-acetylglucosamine (UDP-GlcNAc) were used as substrates.

Assay for β-Lactamase Activity

Assay for β-lactamase activity was carried out with [^{14}C]labeled PCG. The reaction mixture contained in a total volume of 20 μl, 40~50 μg of the membrane or soluble fraction proteins, or 100 μg of the whole cells (by weight), 1 μmol of phosphate buffer, pH 7.0 and 2.5 nmol of [^{14}C]PCG with or without 1 nmol of unlabeled MDIPC. After incubation at 30°C for 15 minutes the reaction was terminated in a boiling water bath for 2 minutes and the total volume of the reaction mixture was then spotted on Whatman 3MM paper. The paper was developed by ascending chromatography with *n*-butanol - *n*-propanol - water (1: 2: 1, by volume) for 3~4 hours in a thin-layer tank, and then redeveloped three times in the same direction with the same solvent. An autoradiogram was prepared as reported[16], and the area of the paper corresponding to benzylpenicilloic acid was cut out and counted in a liquid scintillation spectrometer[17].

Results

Susceptibility

Table 1 shows the MICs of PCG, ABPC, PCG plus MDIPC (2: 1, by weight) and ABPC plus MDIPC (2: 1, by weight) against the β-lactamase producing strain, *C. freundii* GN346, and its derived β-lactamaseless mutant strain, GN346/16. The former strain was extremely resistant to PCG. It was relatively resistant to ABPC and MDIPC as well. In contrast, the latter strain was relatively susceptible to PCG and highly susceptible to ABPC. The latter strain was also relatively resistant to MDIPC. The resistance mechanism in the former strain to PCG and ABPC was due to the inactivation of the antibiotics by β-lactamase localized in the periplasmic space[11].

The β-lactamase producing strain GN346 was susceptible to the combination of PCG or ABPC and MDIPC, a β-lactamase inhibitor. On the contrary, the combination did not show any synergistic activity against the β-lactamaseless mutant strain GN346/16.

Utilization of UDP-MurNAc-pentapeptide for Peptidoglycan Synthesis

The incubation of UDP-N-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-[^{14}C]-alanyl-D-[^{14}C]alanine and UDP-N-acetylglucosamine with the membrane fraction from *C. freundii* GN346 showed the incorporation of radioactivity into a lipid intermediate, followed by its incorporation into the peptidoglycan (Fig. 1). It was accompanied by the release of [^{14}C]alanine (Fig. 1). This fact indicated that the peptidoglycan synthesis in *C. freundii* GN346 was the same as in *Escherichia coli*[16] and *Pseudomonas aeruginosa*[14]. The amount of free [^{14}C]alanine liberated was in excess of the amount incorporated into the peptidoglycan, indicating that the membrane fraction contained not only transpeptidase but also D-alanine carboxypeptidase activities. The peptidoglycan synthesis catalyzed by the membrane enzyme system from the β-lactamaseless mutant strain GN346/16 was the same as in the parent strain (data not shown).

Effect of a Combination of PCG or ABPC and MDIPC on Peptidoglycan Synthesis

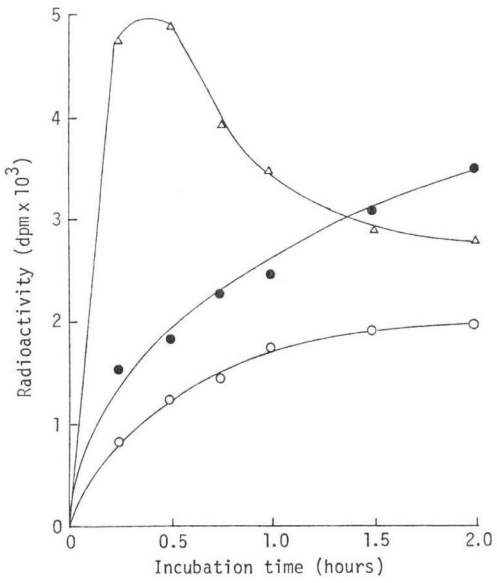
The effect of PCG or ABPC and MDIPC alone or in combination on the peptidoglycan synthesis was studied by comparing the concentration for 50% inhibition of the alanine release in the above system (Table 2). The concentrations of PCG and ABPC for 50% inhibition of the alanine release were respectively 26.5- and 131-fold higher in the β-lactamase producing *C. freundii* GN346 strain than in the β-lactamaseless mutant strain GN346/16. In contrast, the concentration of MDIPC for the same inhibition was less than two-fold in the parent strain. These results suggested that the membrane fraction from the parent strain contains β-lactamase activity which hydrolyzes PCG and ABPC as described previously[11]. The alanine release catalyzed by the membrane fraction from the parent strain was markedly inhibited by a combination of PCG and MDIPC or ABPC and MDIPC. In contrast, the synergistic action was not observed against peptidoglycan synthesis catalyzed by the membrane fraction from the mutant strain.

Table 1. MICs of benzylpenicillin, ampicillin and dicloxacillin against *C. freundii*, strains GN346 and GN346/16.

Antibiotic	MIC (μg/ml)	
	GN346	GN346/16
Benzylpenicillin	3,200	25
Ampicillin	800	1.6
Dicloxacillin	400	400
Benzylpenicillin plus dicloxacillin (2: 1, by weight)	200	12.5
Ampicillin plus dicloxacillin (2: 1, by weight)	50	1.6

Fig. 1. Time course of peptidoglycan synthesis by the membrane fraction from *C. freundii* GN346.

Assays were carried out as described in the text. Incubation was 37°C for the times shown. Data are expressed as radioactivity (dpm) incorporated into products. Symbols; peptidoglycan (○), lipid intermediate (△), released alanine (●).



Comparison of PCG-hydrolyzing Activities from *C. freundii* GN346 and GN346/16

The above results on the inhibition of alanine release by penicillins and results on the binding of

Table 2. Effect of benzylpenicillin, ampicillin and dicloxacillin alone or in combination on peptidoglycan synthesis in *C. freundii*, strains GN346 and GN346/16.

Strain	Concentration for 50% inhibition of alanine release (μg/ml)				
	PCG	ABPC	MDIPC	PCG plus MDIPC (2: 1, by weight)	ABPC plus MDIPC (2: 1, by weight)
GN346	530	170	250	31	31
GN346/16	20	1.3	170	16	1.3

Reactions were carried out as described in the text. Incubation was at 37°C for 2 hours.

Table 3. Comparison of benzylpenicillin-hydrolyzing activities of whole cells, and membrane and soluble fractions from *C. freundii*, strains GN346 and GN346/16.

Strain	Addition	Amounts of benzylpenicilloic acid		
		Whole cells (dpm/μg cells)	After cell disruption	
			Soluble fraction (dpm/μg protein)	Membrane fraction (dpm/μg protein)
GN346	None	1.5×10^4	2.1×10^5	3.8×10^3
	MDIPC*	4.1×10^2	1.4×10^3	2.4×10^2
GN346/16	None	1.7×10^2	8.8×10^2	4.4
	MDIPC*	4.2×10	9.1×10	0.3

* Final concentration; 50 nmol/ml.

PCG to the cytoplasmic membrane reported in a previous paper[11] indicated that the membrane fraction from the parent strain contained β-lactamase activity. Therefore, PCG-hydrolyzing activity in the parent strain and its derived mutant strain was compared (Table 3).

The penicillinase activity was present in the membrane fraction from the parent strain, whereas no enzyme activity was detected in that from the mutant strain. In addition, the soluble fraction mainly derived from the periplasm of the parent strain showed strong enzyme activity as compared with that of the mutant strain. The same result was obtained when whole cells were used as an enzyme source.

The addition of 50 nmol/ml of MDIPC to the above reaction mixtures markedly reduced the hydrolysis of [^{14}C]PCG.

Discussion

The β-lactamase producing *C. freundii* GN346 is resistant to PCG and ABPC, whereas the β-lactamaseless mutant strain GN346/16 is relatively susceptible to those antibiotics. The former strain, however, is susceptible to a combination of penicillin and MDIPC. In our previous paper[11] on the mechanism of the synergistic antibacterial activity of the combination, we showed that, in a whole cell system, MDIPC inhibited β-lactamase activity present in the periplasmic space and that PCG or ABPC could penetrate and bind to target sites, transpeptidase and D-alanine carboxypeptidase, which are localized on the cytoplasmic membrane (inner membrane).

The cell-free enzyme system from β-lactamase producing *C. freundii* GN346 and its derived β-lactamaseless mutant strain GN346/16 catalyzed peptidoglycan synthesis from UDP-MurNAc-pentapeptide and UDP-GlcNAc as observed in *E. coli*[16] and *P. aeruginosa*[14]; the reaction is accompanied by the release of alanine. Alanine release was inhibited by PCG and ABPC, but the inhibition in the parent strain was weaker than that in the mutant strain, suggesting the membrane fraction of the parent strain contained β-lactamase activity.

In the previous paper[11], the binding of [^{14}C]PCG to the membrane fraction from the parent strain did not follow saturation-type kinetics, whereas it did in the mutant strain. Moreover, the strong activity for the hydrolysis of [^{14}C]PCG was seen in the membrane fraction from the parent strain.

The inhibition of alanine release in peptidoglycan synthesis catalyzed by the membrane fraction from the parent strain become extremely sensitive upon addition of MDIPC. This indicated that β-lactamase activity in the membrane fraction was also inhibited by MDIPC.

In the present study, it was revealed that β-lactamase activity was present not only in the periplasmic space but also in the cytoplasmic membrane, as described in the study with *Bacillus licheniformis*.[18] A study is underway to elucidate whether the membrane-bound β-lactamase is the same as that in the periplasmic space.

The present study also revealed that the resistance mechanism of the β-lactamase producing organism to β-lactam antibiotics is not only due to the β-lactamase contained in the periplasmic space, as previously reported[19,20], but also, at least in part, to the β-lactamase bound to the cytoplasmic membrane (inner membrane). Thus, it can be concluded that a synergistic activity of the combination of PCG or ABPC and MDIPC was exhibited by the inhibition of both β-lactamase activities by MDIPC.

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