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Enzymatic Production of Amoxicillin by β-Lactamase-deficient Mutants of *Pseudomonas melanogenum* KY 3987

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A search was undertaken for microorganisms that can catalyze the enzymatic production of amoxicillin. *Pseudomonas melanogenum* KY 3987 was selected from our stock cultures. β-Lactamase-deficient mutants were isolated from amoxicillin-sensitive mutants derived from this strain. These mutants became sensitive to other penicillins and cephalosporins as well as amoxicillin and did not produce β-lactamase even in the presence of an inducer, such as penicillin V. Amoxicillin was effectively synthesized from D-2-*p*-hydroxyphenylglycine methyl ester and 6-aminopenicillanic acid (6-APA) with these mutants as the enzyme sources. The conversion ratio from 6-APA to amoxicillin was about 90% as a mol ratio.

Many reports have appeared on penicillin acylase (penicillin amidohydrolase EC 3.5.1.11) produced in various microorganisms.[1～6] This enzyme catalyzes the synthetic and hydrolytic reactions of various β-lactam antibiotics. In the previous papers,[7,8] we reported that the enzymes from *kluyvera citrophila* KY 3641 and *Pseudomonas melanogenum* KY 3987 could effectively catalyze the synthetic reaction of ampicillin. We attempted to produce amoxicillin enzymatically, which was synthesized from D-2-*p*-hydroxyphenylglycine methyl ester and 6-APA. When cells or crude cell-extracts of these microorganisms were used as enzyme sources, β-lactamases contained in them led to the decomposition of β-lactam compounds, and the yield of this reaction was low consequently. It was previously reported that β-lactamase-deficient mutants were isolated from *Kluyvera citrophila* KY 3641 capable of carrying out enzymatic synthesis of ampicillin.[9] In this report the selection of β-lactamase-deficient mutants derived from *Pseudomonas melanogenum* KY 3987 and the production of amoxicillin by these mutants are described.

MATERIALS AND METHODS

Microorganisms used. About two hundred bacterial strains preserved in our laboratory were used for the screening experiment. *Pseudomonas melanogenum* KY 3987 and amoxicillin-sensitive mutants derived from KY 3987 by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) treatment were used in the succeeding studies.

Cultivation of microorganisms. Bacteria were inoculated from lyophile tubes on agar slant media which contained 1% peptone, 0.5% yeast extract, 0.25% NaCl and 2% agar at pH 7.0 before sterilization. For liquid cultivation of bacteria, one loopful of cells was inoculated into a medium containing 1% peptone, 0.5% meat extract, 1% yeast extract, 0.25% NaCl and 0.5% monosodium L-glutamate at pH 7.0 before sterilization. Each 50 ml of the inoculated medium in a Sakaguchi flask was shaken reciprocally at 28°C for 16 hr.

Determination of the enzyme activity. Cells were collected by centrifugation from cultured broth, washed with M/30 phosphate buffer (pH 6.0), and then freeze-dried for use in this series of experiments. For the determination of penicillin acylase, 20 mg of dry cells, 10 mg of 6-APA and 20 mg of D-2-*p*-hydroxyphenylglycine methyl ester hydrochloride were dissolved in 1 ml of M/30 phosphate buffer (pH 6.0) and incubated at 30°C for 1 hr. The reaction was terminated by boiling for 5 min. Amoxicillin formed in the supernatant of the reaction mixture was determined by high performance liquid chromatography (HPLC).

For the determination of β-lactamase activity, 10 mg of dry cells and 4 mg of amoxicillin were dissolved in 1 ml of M/30 phosphate buffer (pH 6.0) and incubated at 30°C for 30 min. The reaction was terminated by boiling for 5 min. Residual amoxicillin in the supernatant of the reaction mixture was determined by the hydroxylamine assay.[10] Especially for the determination of the β-lactamase activity of the cell-free extract, the microiodometric method[11] was applied. For this method, one unit of the enzyme activity was defined as the amount of enzyme which hydrolyzed 1 μmol of β-lactam antibiotics in 1 min at 30°C.

Preparation of cell-free enzymes. The cells were washed with 0.1 M phosphate buffer (pH 6.0) and then suspended in the same buffer at 50 mg/ml as dry cell weight. They were disrupted by ultrasonic treatment (10 kc, 100 V, 20 mA). Cell debris was removed by centrifugation at 10,000 × *g* for 30 min. The supernatant was dialyzed overnight against deionized water at 4°C. The precipitate formed during the dialysis was removed by centrifugation at 10,000 × *g* for 30 min and the supernatant was used as a cell-free enzyme.

Determination of protein. The protein concentration was determined by the method of Lowry *et al.*[12]

Determination of cephalosporins, penicillins and related compounds by HPLC. Cephalosporins, penicillins and related compounds were detected after HPLC using Microbondapak/C_{18} columns (Waters Assoc. Inc.) with an ultraviolet lamp with a solvent system of methanol and 0.2 M KH_2PO_4 (7:93) (Fig. 1a), and a solvent system of methanol and 0.05 M KH_2PO_4 (20:80) (Fig. 1b).

RESULTS AND DISCUSSION

Screening for amoxicillin synthesizing microorganisms

About two hundred strains of bacteria belonging to the families *Pseudomonadaceae* and *Enterobacteriaceae* were examined for amoxicillin synthesizing activity. As shown in Table I, several microorganisms belonging to the genera *Pseudomonas* and *Xanthomonas* were found to synthesize amoxicillin efficiently. In particular, *Pseudomonas melanogenum* KY 3987 which was previously reported as an ampicillin producing strain[7,8] showed the highest activity of amoxicillin synthesis. However, β-lactamase activity was recognized in all the microorganisms listed in Table I. The penicillin acylase activity in Table I was affected by the β-lactamase without an inhibitor.

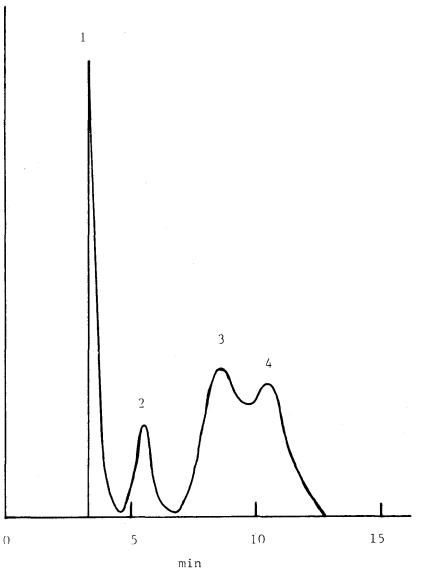

FIG. 1a. HPLC of Amoxicillin and Related Compounds.

1, D-2-*p*-hydroxyphenylglycine; 2, 6-aminopenicillanic acid; 3, amoxicillin; 4, D-2-*p*-hydroxyphenylglycine methyl ester.

The column used was μ Bondapak C_{18} (30 cm × 3.9 mm) and the solvent a mixture of 930 ml of 0.2 M KH_2PO_4 and 70 ml of methanol. The flow rate was 1.0 ml/min.

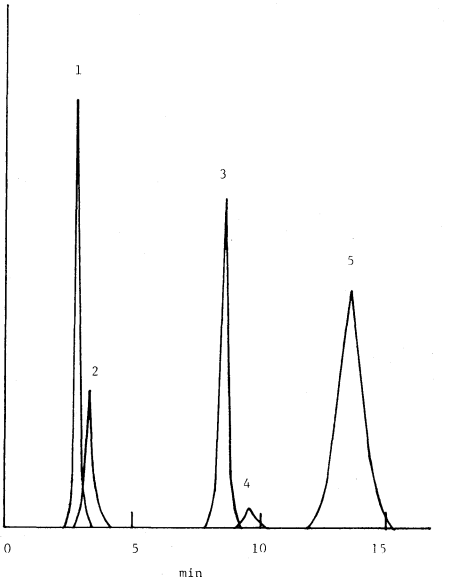

FIG. 1b. HPLC of Penicillins and Cephalosporins.

1, cephadroxil; 2, amoxicillin; 3, cephalexin; 4, ampicillin; 5, cephaloglycin.

The column used was μ Bondapak C_{18} (30 cm × 3.9 mm) and the solvent a mixture of 800 ml of 0.05 M KH_2PO_4 and 200 ml of methanol. The flow rate was 1.5 ml/min.

TABLE I. SCREENING OF AMOXICILLIN-PRODUCING MICROORGANISMS

Strains		Penicillin acylase (Synthesis of Amx. mg/ml·hr·20 mg cells)	β-Lactamase (Degradation of Amx. mg/ml·30 min·10 mg cells)
Pseudomonas melanogenum	KY 3987	2.55	3.45
Pseudomonas maltophilia	KY 4676	1.95	2.10
Pseudomonas geniculosa	KY 4678	2.45	1.50
Xanthomonas citri	KY 4215	0.43	1.05
Xanthomonas cucurbitae	KY 4217	1.17	1.85
Xanthomonas physalidicola	KY 4220	2.21	1.10

But, a proper inhibitor of the β-lactamase, the presence of which highly increased the penicillin acylase activity in this reaction system, could not be found.

Isolation of β-lactamase-deficient mutants

It has been well known that β-lactam antibiotic-sensitive mutants tend to lose β-lactamase activity.[13~16] Therefore, about a hundred amoxicillin-sensitive mutants were isolated from *Pseudomonas melanogenum* KY 3987 by NTG treatment. Table II shows both penicillin acylase and β-lactamase activities, and the sensitivities of typical mutants to amoxicillin, penicillin G, carbenicillin, cephaloridine and cephalothin. The sensitivities were represented by the minimal inhibitory concentrations (MIC) in serial two-fold dilution tests. These mutants obtained from KY 3987 were more sensitive to five β-lactam antibiotics, and showed lower β-lactamase activities and higher penicillin acylase activities than the parent strain. Of these mutants, T-417 was the most sensitive to five β-lactam antibiotics, but β-lactamase activity still remained. Although KY 8540 and KY 8541 were more resistant than T-417 to β-lactam antibiotics, these strains showed a dificiency of β-lactamase activity. Since KY 8540 and KY 8541 also had high penicillin acylase activities as shown in Table II, they were thought to be the most useful mutants to synthesize amoxicillin enzymatically. *Pseudomonas melanogenum* was more resistant to cephalosporins than to penicillins, and the resistance to these drugs was lost on NTG treatment. But, in our experiments the resistance to these drugs was not lost on acridine orange treatment which eliminated the R-factor from the cells. Moreover, the β-lactamase of *Pseudomonas melanogenum* was an inducible enzyme as shown in the next section. These results showed that the β-lactamase of *Pseudomonas melanogenum* was a chromosomally mediated enzyme according to the classification of β-lactamases from gram negative organisms.[17]

Induction of β-lactamase

Induction of β-lactamase production in KY 3987 and its mutants was investigated. The cells were cultivated in the presence or absence of 10 μg penicillin V per ml medium as an inducer. The results are shown in Table III, and the data are the mean values of six experiments (average error ± 10%). KY 3987 originally had high β-lactamase activity and its β-lactamase was enhanced in the presence of penicillin V. In this experiment, β-lactamase activity of 4.00 means that the substrate was completely hydrolyzed. Although KY 8539 had lower β-lactamase activity than KY 3987, β-lactamase of KY 8539 was induced as well as that of KY 3987 in the presence of penicillin V. In comparison with the β-lactamase of KY 8539, those of KY 8540 and KY 8541 were not induced by penicillin V at all. These mutants were highly sensitive to β-lactam antibiotics and the addition of a higher concentration of penicillin V (for example 100 μg/ml) increased the lysis of these cells. In this experiment, the same results were obtained with intact cells in place of freeze-dried cells. According to the

TABLE II. PROPERTIES OF AMOXICILLIN-SENSITIVE STRAINS

Strains	MIC (μg/ml)[a]					β-Lactamase (Degradation of Amx. mg/mg·30 min·10 mg cells)	Penicillin acylase (Synthesis of Amx. mg/ml·hr·20 mg cells)
	Amoxicillin	Penicillin G	Carbenicillin	Cephaloridine	Cephalothin		
KY 3987 (Wild)	200	100	1.6	400	200	3.45	2.55
KY 8539 (Mutant)	100	50	25	400	200	1.38	3.49
KY 8540 (Mutant)	25	25	<0.1	25	6.25	0	3.55
KY 8541 (Mutant)	12.5	12.5	<0.1	100	3.13	0	3.52
T-415 (Mutant)	12.5	0.4	0.1	3.13	3.13	2.59	3.32
T-432 (Mutant)	0.8	0.1	0.1	12.5	3.13	1.45	3.45
T-417 (Mutant)	0.2	0.1	<0.1	3.13	1.57	2.04	3.24

[a] MIC values for each strain were determined by serial two-fold dilution tests in yeast-bouillon agar medium. The cell suspension used for inocula contained 10^6 cells/ml of yeast-bouillon broth. A loopful of the cell suspensions was transferred onto 15 ml agar plates containing the drugs tested.

results in Table III, the basal β-lactamase level of these mutants was reduced, and especially, KY 8540 and KY 8541 became either completely inert to induction or at least severely impaired in their inducibility.

β-Lactamase activity in the cell-free extract

Tables II and III show the experimental results for the determination of β-lactamase, with the dry cells as the enzyme sources. On the other hand, the data of β-lactamase activity at the biological level using the cell free extract as the enzyme source are shown in Table IV. This activity means the total activity of cell-associated β-lactamase. In this experiment, ampicillin, penicillin G and cephaloridine were used as substrates and the β-lactamase activity was determined by the micro-iodometric method.[11] According to Table IV, the total cell-associated β-lactamase activities of KY 8540 and KY 8541 were found to be at a level of one-seventieth compared with that of KY 3987. These trace activities of KY 8540 and KY 8541 could not be detected in the reaction catalyzed by the β-lactamase of dry cells and they were considered to have no influence on the reaction for the enzymatic synthesis of amoxicillin.

TABLE III. INDUCTION OF β-LACTAMASE BY PENICILLIN V

The microorganisms were grown as shown in MATERIALS AND METHODS except for addition of the inducers. Induction was carried out by adding 10 μg penicillin V per ml in the early logarithmic phase. The washed cells were used as the enzyme sources. The determination conditions of the β-lactamase activity were the same as shown in MATERIALS AND METHODS.

Strains	β-Lactamase (Degradation of Amx. mg/ml · 30 min · 10 mg cells)	
	−Inducer	+Inducer
KY 3987 (Wild)	3.36	4.00
KY 8539 (Mutant)	1.52	4.00
KY 8540 (Mutant)	0	0
KY 8541 (Mutant)	0	0

TABLE IV. β-LACTAMASE ACTIVITY OF CELL-FREE EXTRACT OF *Pseudomonas melanogenum*

Strains	β-Lactamase (Units/g protein)		
	Ampicillin	Penicillin G	Cephaloridine
KY 3987 (Wild)	45.7	52.1	65.5
KY 8539 (Mutant)	21.5	27.4	43.3
KY 8540 (Mutant)	0.7	3.3	1.0
KY 8541 (Mutant)	0.7	1.1	0.9

TABLE V. ENZYMATIC SYNTHESIS OF PENICILLINS AND CEPHALOSPORINS

Washed cells of *Pseudomonas melanogenum* KY 3987 and KY 8540 were added at 20 mg of dry cell weight/ml to a solution containing 10 mg/ml of penicillin or cephalosporin nucleus, and 20 mg/ml of an acyl side-chain derivative. The reaction mixture was incubated at pH 6.5 and 30°C for 3 hr.

Synthesized compounds	Acyl-donors	Acyl-acceptors	KY 3987 Wild (mg/ml)	KY 8540 Mutant (mg/ml)
Amoxicillin	D-2-*p*-Hydroxyphenylglycine methyl ester	6-Aminopenicillanic acid	3.5	7.5
Ampicillin	Phenylglycine methyl ester	6-Aminopenicillanic acid	5.0	9.2
Cephalexin	Phenylglycine methyl ester	7-Amino-3-deacetoxycephalosporanic acid	9.0	12.7
Cephaloglycin	Phenylglycine methyl ester	7-Aminocephalosporanic acid	6.1	8.3
Cephadroxil	D-2-*p*-hydroxyphenylglycine methyl ester	7-Amino-3-deacetoxycephalosporanic acid	10.0	10.4

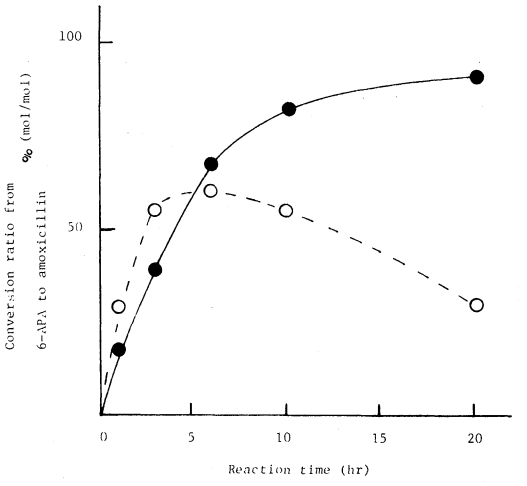

FIG. 2. Time Course of Amoxicillin Synthesis.

●—●, KY 8540; ○---○, KY 3987.

Washed cells of *Pseudomonas melanogenum* KY 3987 and KY 8540 were added at 50 mg of dry cell weight/ml to a solution containing 20 mg/ml of D-2-*p*-hydroxyphenylglycine methyl ester hydrochloride, 10 mg/ml of 6-APA and 5% (v/v) *sec*-butanol. The reaction mixture was incubated at pH 6.0 and 20°C for 20 hr.

Synthesis of amoxicillin, ampicillin, cephalexin, cephaloglycin and cephadroxil

The penicillin acylases of *Escherichia coli*[18] and *Kluyvera citrophila*[8] have relatively broad substrate spectra including various penicillins, penicillin amides, esters and cephalosporins. On the contrary, the penicillin acylase of *Pseudomonas melanogenum*[8] was specific for a limited acyl side-chain that contained α-amino acid in the case of *Xanthomonas citri*.[19,20] Table V shows the results of synthetic reaction for 3 hr, where the enzyme could catalyze the formation of amoxicillin, ampicillin, cephalexin, cephaloglycin and cephadroxil, because they had α-amino acid in the acyl side-chains. With the enzyme from KY 8540, five β-lactam antibiotics were synthesized more effectively than by that from KY3987. However, amoxicillin synthesis was not so effective in comparison with others even that by KY 8540. The synthesis rate of amoxicillin was lower than that of others, because D-2-*p*-hydroxyphenylglycine methyl ester and 6-APA were less effective substrates than phenylglycine methyl ester and 7-amino-3-deacetoxycephalosporanic acid for the penicillin acylase respectively. Therefore, β-lactamase had a remarkable influence on the synthesis of amoxicillin.

Time course of amoxicillin synthesis

The rate of amoxicillin synthesis by penicillin acylase from D-2-*p*-hydroxyphenylglycine methyl ester and 6-APA was relatively low as shown in Table V, and this reaction was improved by addition of *sec*-butanol. The reaction temperature was lowered to 20°C to reduce inactivation of the enzyme by *sec*-butanol. *sec*-Butanol was considered to inhibit the hydrolysis of D-2-*p*-hydroxyphenylglycine methyl ester to D-2-*p*-hydroxyphenylglycine in the reverse reaction. The time course of amoxicillin synthesis in the optimal conditions by β-lactamase-deficient mutant KY 8540, in comparison with the parent strain KY 3987, is shown in Fig. 2. The initial synthesis rates of amoxicillin by KY 3987 and KY 8540 were almost the same. However, the conversion ratio from 6-APA to amoxicillin by KY 3987 was about 60% as a mol ratio after 6 hr and then gradually decreased because of hydrolysis by β-lactamase. On the contrary, the conversion ratio with KY 8540 increased to 90% as a mol ratio after 20 hr.

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