A New Screening Method for C-P Compound Producing Organisms by the Use of Phosphoenolpyruvate Phosphomutase

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Phosphoenolpyruvate phosphomutase (PEPPM) catalyzes the C-P bond forming reaction by intramolecular rearrangement of phosphoenolpyruvate to phosphonopyruvate, and shows stronger activity in catalyzing the reverse reaction than the forward reaction. By exploiting the activity of PEPPM to catalyze the reverse reaction, 81 strains were screened as possible C-P compound producing microorganisms from 230 soil samples. Two of these strains, B-1 and L-b, were found to produce C-P compounds by ^{31}P -NMR spectral analysis of their broth filtrates. The activity of PEPPM was detected in the cell extracts of these two strains. The strain B-1 was identified as *Pseudomonas gladioli*, and hydroxyethylphosphonic acid (HEP) was isolated from the broth filtrate of this strain as a C-P compound.

Since the first discovery of the C-P compound 2-aminoethylphosphonic acid,¹⁾ various metabolites with a C-P bond have been isolated and their physiological activities investigated. Extensive studies on their unique C-P bond formation mechanisms indicated that the first step of the biosynthetic pathway of C-P compounds is the intramolecular rearrangement of phosphoenolpyruvate (PEP) to phosphonopyruvate (PnPy)²⁾ (Fig. 1).

Recently phosphoenolpyruvate phosphomutase (PEPPM) catalyzing the C-P bond forming reaction was purified from *Tetrahymena pyriformis*³⁾ and *Streptomyces hygroscopicus*,⁴⁾ and subsequently and surprisingly, the enzyme turned out to show stronger activity in catalyzing the reverse reaction, *i.e.*, cleaving the C-P bond to give PEP, than the C-P bond

formation. Although several studies on the mechanism of this reaction using PEPPM from *Tetrahymena*⁵⁾ were reported, the detailed mechanism is still unclear. While this reaction seems to be common to all of the C-P compound producing organisms, there is little biochemical evidence for generalization. To identify the detailed mechanism of C-P bond formation and the significance of C-P compounds in living systems, other organisms producing C-P compounds need to be investigated.

To isolate such microorganisms capable of forming the C-P bond from soil samples, we used the strong activity of PEPPM to catalyze the reverse reaction. Thus, microorganisms that can produce C-P compounds are expected to be able to use PnPy as a sole phosphorus source by converting it into PEP by the reverse reaction. In addition to PEPPM, C-P cleaving enzymes such as phosphonate⁶⁾ and C-P lyase⁷⁾ are known to exist; therefore, microorganisms capable of growing in a medium containing phosphonoalanine (PnAla; 2-amino-3-phosphonopropionic acid), a biological equivalent of PnPy, as a sole phosphorus source and capable of producing PEP, could be expected to have PEPPM and produce C-P com-

Fig. 1. Proposed Biosynthetic Pathway of C-P Bond Formation.

PEP, phosphoenolpyruvate; PnPy, phosphonopyruvate; PnAA, phosphonoacetaldehyde.

pounds.

To select microorganisms truly capable of producing C-P compounds from soil isolates that were obtained as being able to use PnPy as a sole phosphorus source, we used ^{31}P -NMR spectroscopy for detecting the accumulation of C-P compounds in the fermentation broth. ^{31}P -NMR spectroscopy is an effective method for analyzing phosphorous compounds and is widely used in cell physiology and plant physiology. In the ^{31}P -NMR spectra the signals due to phosphoric acid and phosphate esters are observed at around 0 ppm, while those of the phosphorus in C-P compounds are detected at about 45–10 ppm. Therefore, C-P compounds in fermentation broth could be detected effectively with ^{31}P -NMR spectroscopy. Here we describe the screening of microorganisms that produce the C-P compounds and their PEPPM activity.

Materials and Methods

Screening of microorganisms. To facilitate isolation of C-P compound producing organisms, enrichment culture was done in a synthetic medium containing PnAla as a sole phosphorus source. Soil samples (0.5 g) were separately suspended in 5 ml of 0.9% NaCl, and samples of the supernatant (100 μl) were transferred into 50-ml culture tubes, each containing 10 ml of M(C-P) medium. M(C-P) medium contained 1% glucose, 0.05% L-asparagine, 0.01% PnAla, 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (pH 7.5). After incubation for 3 or 4 days at 27°C under aerobic condition, 100 μl of the culture was transferred into a new medium of the same composition and cultured continuously. This procedure was repeated three times to minimize the carrying over of phosphorus from soil samples. Finally samples of the cultures (200 μl) were spread on agar plates consisting of M(C-P) medium, and incubated at 27°C for several days to isolate single colonies.

Assay for ability to produce C-P compounds. Isolated strains were separately inoculated into 500-ml Erlenmeyer flasks, each containing 100 ml of M medium, and incubated at 27°C for 3 days. M medium contained 1% glucose, 0.05% L-asparagine, 0.05% K_2HPO_4 , 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. The broth filtrate was concentrated by evaporation to about 10 ml, and analyzed by ^{31}P -NMR spectroscopy under basic conditions (pH 11) adjusted by adding NaOH. ^{31}P -NMR measurements were done with a JEOL GSX500 NMR spectrometer operating at 202.35 MHz. The probehead was a JEOL 10-mm

multinuclear unit tuned for the ^{31}P nucleus. Chemical shift values are reported relative to 85% H_3PO_4 . All NMR measurements were done at 25°C.

Enzyme assay. Microorganisms were cultured in 500 ml Erlenmeyer flasks containing 100 ml of the M medium for 3 or 4 days and cell extracts were prepared by sonication of cells suspended in 50 mM Tris-HCl buffer (pH 7.5). The reverse reaction activity of PEPPM was assayed with a system consisting of 50 mM Tris-HCl (pH 7.5), 0.27 mM PnPy, 0.15 mM NADH, 0.72 mM ADP, 5 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 U/ml lactate dehydrogenase and 5 U/ml pyruvate dehydrogenase. The reaction was started by the addition of the cell extract, and then the decrease in absorbance at 340 nm was recorded at 30°C. The activity was measured by the difference of the decrease in absorbance between the presence and the absence of PnPy. One unit of PEPPM activity was defined as the amount converting 1.0 μmol of PnPy to PEP per minute. Protein was measured by the method of Lowry *et al.*⁸⁾

Isolation of C-P compounds. During isolation procedures, C-P compounds were followed by ^{31}P -NMR spectroscopy. Strain B-1 was inoculated into 500 ml Erlenmeyer flasks containing 100 ml of M medium, and incubated at 27°C for 4 days. Broth filtrate (20 liters) was treated with activated carbon and the supernatant was adsorbed on a Dowex 1 column (Cl^- form, 65 $\times$ 850 mm). After washing with water, the column was eluted with 0.1 M NaCl, and the eluate was passed through a Dowex 50 column (H^+ form, 46 $\times$ 500 mm) and the column was washed successively with water. Effluent and washing fractions containing C-P compounds were adjusted to pH 10 with conc. NH_4OH and were adsorbed on a QAE-Sephadex A-25 column (Cl^- form, 38 $\times$ 500 mm). After it was washing with water and 0.09 M NaCl, the column was eluted with 0.14 M NaCl. Fractions containing C-P compounds were combined and concentrated, and then chromatographed on a Sephadex G-25 column chromatography (20 $\times$ 1400 mm) developed with water. The combined fraction containing C-P compounds was dried by evaporation, and then the dry residue was put on a cellulose column (20 $\times$ 500 mm) equilibrated with $\text{EtOH-NH}_4\text{OH-H}_2\text{O}=8:1:2$. Development with the same solvent mixture gave 0.5 mg of the ammonium salt of the C-P compound.

Results and Discussion

C-P compound producing strains

From 230 soil samples were obtained 81 strains capable of using PnAla as a sole phosphorus source. ^{31}P -NMR spectra of broth filtrates of these isolated strains cultivated in M medium had a signal at about 18.5 ppm

Fig. 2. ^{31}P -NMR Spectrum of Broth Filtrate of Strain B-1.

The broth filtrate of B-1 was concentrated to about 1/10, adjusted to pH 11 with NaOH. A large signal at about 2–3 ppm (0 ppm at pH 7) was assigned to phosphate and a small signal at 20 ppm was phosphonate.

assignable to phosphonic acid in the broth filtrate of two strains, B-1 and L-b, suggesting accumulation of a C-P compound in the fermentation broth of these two strains (Fig. 2).

In addition to the production of a C-P compound by these strains, their abilities to use PnAla as a sole phosphorus source and to cleave the C-P bond were also examined by ^{31}P -NMR measurements. NMR analysis of the fermentation broth cultured in M(C-P) medium showed one signal at about 0 ppm in addition to the signal of PnAla, indicating the cleavage of the C-P bond of PnAla by these strains.

Production of C-P compounds by strain B-1 and L-b

Since the phosphorous chemical shifts of C-P compounds in L-b and B-1 were identical, both strains were assumed to produce the same C-P compound. Since the amount of the C-P compound produced by L-b was too small, the isolation of the C-P compound produced by strain B-1 was done as described in Materials and Methods. The structure of the isolated compound was identified as follows.

In the ^1H -NMR spectrum taken in D_2O , two methylene signals (δ_{H} 3.75 and 1.73) were coupled with each other ($J=8.2\text{ Hz}$) and phosphorus ($^2J_{\text{P-H}}=7.1\text{ Hz}$, $^3J_{\text{P-H}}=16.5\text{ Hz}$) indicating the presence of a $\text{P-CH}_2\text{-CH}_2$ unit. The downfield shift of one of the methylene signals suggests its linkage to a hydroxyl group. The ^{13}C -NMR spectrum of this compound showed a very characteristic doublet signal at δ_{C} 32.5 ($J_{\text{C-P}}=125\text{ Hz}$) due to a C-P bond.

Based on these NMR spectral analyses and comparison with a standard sample, the structure of the isolated compound was identified as hydroxyethylphosphonic acid (HEP). This compound had been obtained from the broth filtrate of a blocked mutant of a bialaphos-producing organism⁹⁾ and was known as a biosynthetic intermediate of fosfomycin.¹⁰⁾ The ability of C-P bond formation of strain B-1 was thus confirmed by HEP production.

The identification of this strain was done from Bergey's Manual of Systematic Bacteriology (Vol. 1). The strain B-1 was isolated from a soil sample collected in Hongo, Bunkyo-ku, Tokyo Prefecture, Japan. This strain is a Gram-negative and strictly aerobic rod, motile

Table I. MORPHOLOGICAL AND PHYSIOLOGICAL PROPERTIES OF STRAIN B-1

Morphology	Rod
Length	1.1–3.5 μm
Width	0.4–0.6 μm
Colony morphology	Circular, smooth surface
Pigmentation	—
Number of flagella	>1
Mobility	+
Gram stain	Negative
Endospore formation	—
Oxidase	+
Catalase	+
Urease	—
Lipase	+
OF-test	Oxidative
β -Galactosidase	+
Denitrification	—
Arginine dihydrolase	—
Nitrate reduction	+
Slime production from sucrose	+
Poly- β -hydroxybutyrate accumulation	+
Gelatin liquefaction	+
Protocatechuate, ortho cleavage	+
Utilization of ammonium	+
Nitrate	+
Adonitol	+
L-Rhamnose	—
D-Ribose	+
D-Xylose	+
D-Glucose	+
2,3-Butyleneglycol	—
D-(–)-Tartrate	+
Mesaconate	+
Levulinate	—
<i>m</i> -Hydroxybenzoate	—
Tryptamine	—
α -Aminylamine	—
Citraconate	+
Citrate	+

by polar flagella, metabolizes glucose oxidatively, and is positive for catalase and oxidase. This strain accumulated poly- β -hydroxybutyrate as a carbon reserve material, and is negative for dihydrolase and denitrification (Table I). From these characteristics and the carbon utilization, this strain was identified as *Pseudomonas gladioli* and designated *P. gladioli* B-1.

PEPPM activity of *Pseudomonas gladioli* B-1

Since *P. gladioli* B-1 produced HEP, this strain was reasonably assumed to have PEPPM

Table II. PEPPM ACTIVITY IN CELL EXTRACT OF *P. gladioli* B-1

Cell extract	Consumption of NADH (A_{340}/min)		Specific activity (10^{-3} Unit/mg protein)
	+ PnPy	– PnPy	
1	–PK	0.0345	0.0320
	+PK	0.0420	0.0323
2		0.1070	0.0488
			346.5

The cell extract (1, cultivated in flasks, 4.30 mg protein/ml, 50 μl ; 2, cultivated by jar fermentor, 1.35 mg protein/ml, 50 μl) was added to 950 μl of the assay mixture.

Fig. 3. Course of Cultivation and PEPPM Activity of *P. gladioli* B-1.

—●—, growth; ---▲---, pH; —○—, specific activity of PEPPM (10^{-3} Unit/mg protein).

activity. This enzymatic activity in the extract of *P. gladioli* B-1 cells grown in M medium was evaluated by measuring the amount of PEP spectrophotometrically, which was formed from PnPy by the reverse reaction, by ADP/pyruvate kinase-NADH/lactate dehydrogenase coupled system.¹¹⁾ The absorbance at 340 nm of the reaction mixture containing PnPy decreased faster than that without the substrate PnPy (Table II). Since the PEPPM activity of *P. gladioli* B-1 was very weak, the relationship between the enzyme activity and the cell growth was examined by cultivating strain B-1 in M medium by a jar fermentor. Under these growing conditions, about 15 hr after the start of the cultivation the enzyme activity suddenly appeared and gradu-

ally decreased to an undetectable level in about an hour (Fig. 3). Attempts to detect the forward reaction catalyzed by PEPPM were unsuccessful based on the method using NADH/malate dehydrogenase.⁴⁾ This result supports the observation that the forward reaction is weaker than the reverse reaction, in agreement with the nature of PEPPM from *S. hygroscopicus*. Although PEPPM was purified from *Tetrahymena pyriformis* and *S. hygroscopicus*, this is the first time of the detection of PEPPM activity in Gram-negative bacteria.

The PEPPM activity of strain L-b was undetectable when culture was done in Erlenmeyer flasks, but a little was observed when cultivated in a jar fermentor in the same way as strain B-1. Therefore, strain L-b is also believed to be a C-P compound producing microorganism. Thus we have succeeded in isolating two microorganisms producing C-P compounds from soil samples.

If other phosphonic acids such as 2-aminoethylphosphonic acid (AEP) or phosphonoacetic acid (PnAc) were used as a sole phosphorus source, most of the isolated microorganisms would have shown only either phosphonatease or C-P lyase activity, and could only cleave C-P bonds but not form C-P bonds. Therefore, the screening method exploiting the nature of PEPPM, using PnAla as a sole source of phosphorus turns out to be very effective in obtaining new microorganisms capable of producing C-P compounds. By application of this method, not only the promotion of the study of PEPPM and mechanism of C-P bond formation but also the discovery of new natural C-P compounds with biological activities, will become possible.

In spite of the formation of C-P compounds, the activity of the forward reaction of PEPPM is weaker than that of the opposite reaction. It may be due to the energetic stability of each

compound related to this reaction in the living system.⁵⁾ So in the living system there is a mechanism of stabilizing C-P bonds to produce C-P compounds. We propose that in the course of production of C-P compounds, the next reaction to C-P bond formation by PEPPM, perhaps PnPy to phosphonoacetaldehyde (PnAA)¹²⁾ (Fig. 1), occurred successively remove the reaction product (PnPy) to drive the reaction equilibrium irreversibly in favor of the formation of the C-P bond. Although this reaction has not been detected, the organisms which were obtained by our screening in this report will be useful in studying those mechanisms.

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