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Characterization of C-P Lyase Gene Cluster by *in vivo* ^{31}P-NMR Spectroscopy

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^{31}P-NMR experiment was performed to detect phophonates (Pn) utilization and degradation in the several different C-P lyase mutants of *E. coli* and in *E. aerogenes* and the recombinants. The relative peak intensity (RPI) for the standard samples of 0.5 mM methylphosphonate (MPn) and 1.0 mM aminoethylphosphonate in glucose-MOPS medium showed 0.5 : 1.0 ratio. In the case of BW14329 (△*phnC-P*, △*phoA*), RPI did not change significantly after 24 hrs culturing, which means it nearly could not utilize Pn. *In vivo* ^{31}P-NMR spectrum of *E. aerogens* (BWKL 16627) during 3 hrs starvation showed two intense peaks at 0~2 ppm and at near −10 ppm which indicate intracellular orthophosphate (Pi) and pyrophosphate (PPi), respectively. Both of them might be released by degradation of inorganic polyphosphate pool. When MPn is supplied to the medium as an unique P source, Pi content in the cell has the constant, but PPi seems to be slightly decreased. Recombinants (BWKL 16954) grew slower than *E. aerogenes* in the glucose-MOPS media with various P sources. *In vivo* ^{31}P-NMR spectrum of recombinant did not show any intense signal in the cell. Surprisingly, under the cultivation adding with MPn, a few intense peaks in the region of Pi and phosphate monoester were detected.

Key words: ^{31}P-NMR, C-P lyase gene, phosphonate (Pn)

Phosphonates (Pn), a class of organophosphorus compounds containing a direct C-P bond are found in nature (8). The natural Pn, α-aminoethylphosphonate (AEPn) is widely distributed in the membrane of eukaryotic cells (10) and antibiotic fosfomycin is made by *Streptomyces* species (2, 8). Many synthetic Pn are used commercially as a herbicide or chemotherapeutic agents (4, 5).

Two bacterial enzymes have been described that catalyze C-P bond cleavage and release Pi. A phosphoacetaldehyde hydrolase (trivial name, phosphonatase) has been purified from extract of *Bacillus cereus*, and is active only with phosphoacetaldehyde which is produced by an AEPn transaminase (6, 7). At present, molecular genetics of phosphonatase pathway have been carried out very actively in *Enterobacter aerogenes* (11) and *Salmonella typhimurium* (9). Another enzyme, C-P lyase is found in a diverse group of bacteria (3, 4, 5, 16). This C-P lyase activity is functionally distinct from phosphoacetaldehyde hydrolase because it apparently involves direct dephosphonation of the substrate and it has broad substrate specificity.

There is a great deal of genetic and molecular information on Pn degradation by the C-P lyase. All genes encoding Pn uptake and degradation lie in the complex *phnC~P* gene cluster [*phnC,D,E*; transporter, *phnF,O*; regulator, *phnC, H, I, J, K, L, M, N, P*; C-P lyase complex] (12, 13, 14 17). Since it has been proven difficult to detect the C-P lyase activity *in vitro* (15), the biochemical mechanism of C-P bond cleavage by a C-P lyase is poorly understood.

We have conducted a biophysical investigation on the molecular genetic circumstances. ^{31}P-NMR has been used to study phosphate metabolism under a variety of metabolic conditions (1). We have run *in vivo* and *in vitro* ^{31}P-NMR experiments on several different C-P lyase mutants of *Escherichia coli, Enterobacter aerogenes* and the recombinants. This experiment was designed to elucidate degradative and transport process of Pn by comparing the efficiency of Pn transporter and substrate spectrum of C-P lyase.

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Materials and Methods

Strains and molecular biology procedures

We used the strains of *Enterobacter aerogenes* (BWKL 16627, *rpsL* of ATCC 15038), *E. coli phn*⁻ mutants (BW 14329, △*phnC-P*, △*phoA*; BWKL 16787, △*phnH-P*, △*phoA*) and recombinant (BWKL 16954, *E. aerogenes* C-P lyase positive clone with *E. aerogenes* DNA, e.g. pKL 124 in BWKL 16787).

Molecular cloning, mutagenesis, media preparation, molecular biology procedure, phenotypic tests and growth studies were done as previously described (11, 12, 14, 16).

^{31}P-NMR spectroscopy

Experiments were performed at the Korea Basic Science Institute, Taejon. The instrument used was a Bruker DMX 600 with a 10 mm broad band probe. The spectrometer was operated in the Fourier-transform mode at 242.94 MHz. Proton decoupling was not employed. Transients were averaged using either 4 K of memory over a spectral with of 23 KHz. 90° pulses were applied at 1 sec intervals. Chemical shifts were referenced relative to an external standard of 85% orthophosphoric acid. Intensities were measured by computer integration of the spectra. Spectra were run at a probe head temperature of 30℃.

^{31}P-NMR experiments were carried out in two ways. One is for the *in vitro* determination of remaining Pn in the culture medium. Cultures of strains were grown in 0.4% glucose-morpholinepropane sulfonic acid (MOPS) media with 0.01 mM Pi, chloramphenicol 25 μg/ml only for recombinant (BWKL 16954) to maintain plasmid pKL 124 plus excess methylphosphonate (MPn, 0.5 mM) as an alternative P source. Then the cells were removed by centrifugation or membrane filtration. The supernatant or filtrate was transferred to 10 mm NMR tube and the known concentration of Pn (1 mM AEPn) was added as an external standard.

The other is for the observation *in vivo* uptake and degradative process of MPn in the cell detecting the cleaved intermediates. Cultures of strains were grown in 0.4% glucose-MOPS media with 0.01 mM Pi, 0.5 mM MPn for a day. Cells were washed and incubated in 10 times of concentrated level in fresh 0.4% glucose-MOPS media with 0.5 mM MPn for 3 hrs at 30℃ before applying to ^{31}P-NMR. A part of washed cells was suspended in the fresh media without P sources for the control.

Results and Discussion

Pn utilization and growth studies

Pn utilization in *E. aerogenes* (BWKL 16627), mutants of *E. coli* (BW 14329) and in the recombinant (BWKL 16954) is described in Table 1. *E. aerogenes* has the broadest spectrum upon Pn utilization; *E. coli* BW 14322 used the same P sources except for DMPt. The recombinant BWKL 16954 (transformant of pKL 124) complemented △*phnH-P* mutant BWKL 16787, which showed broad spectrum, too. It failed to complement △*phnC-P* mutant BWKL 14329, which is due to the absence of transport genes on plasmids with genes for the *E. aerogenes* C-P lyase (11).

Growth of these strains on different P sources was shown in Table 1. and Fig. 1. *E. aerogenes*, BWKL 16627 appeared to grow well on glucose-MOPS agar with limiting or excess Pi or with limiting Pi plus excess MPn (Fig. 1-A). In contrast, *E. coli* BW 14329 grew best with excess Pi; it grew slightly slower with limiting Pi and it stopped the growth during shift on the excess MPn

Table 1. Pn utilization phenotype (P-ser, phosphoserine; AEPn, aminoethylphosphonate MPn, methylphosphonate; Pt, phosphite; DMPt, dimethylphosphite; Pi, phosphate)

Strains	Growth on glucose-MOPS agar with various P sources						
	P-Ser	AEPn	MPn	EPn	Pt	DMPt	Pi
E. aerogenes							
BWKL 16627 (*rpsL* of ATCC 15038)	+	+	+	+	+	+	+
E. coli							
BW 14332 (Pn⁺, △*phoA*)	+	−	+	+	−	−	+
BW 14329 (△*mel* △*phnC-P*, △*phoA*)	−	−	−	−	−	−	+
BWKL 16787 (△*phnH-P*, △*phoA*)	+	−	−	−	−	−	+
BWKL 16685 (△*phnE*$_{1}$::Tn5-112, △*phoA*)	−	−	−	−	−	−	+
BWKL 16686 (△*phnP*$_{3}$::Tn5-112, △*phoA*)	+	−	−	−	−	−	+
BWKL 16687 (△*phnH*$_{4}$::Tn5-112, △*phoA*)	+	−	−	−	−	−	+
BWKL 16688 (△*phnK*$_{6}$::Tn5-112, △*phoA*)	+	−	−	−	−	−	+
BWKL 16689 (△*phnJ*$_{7}$::Tn5-112, △*phoA*)	+	−	−	−	−	−	+
Recombinant							
BWKL 16954 (pKL 124 in 16787)	+	−	+	+	+	+	+

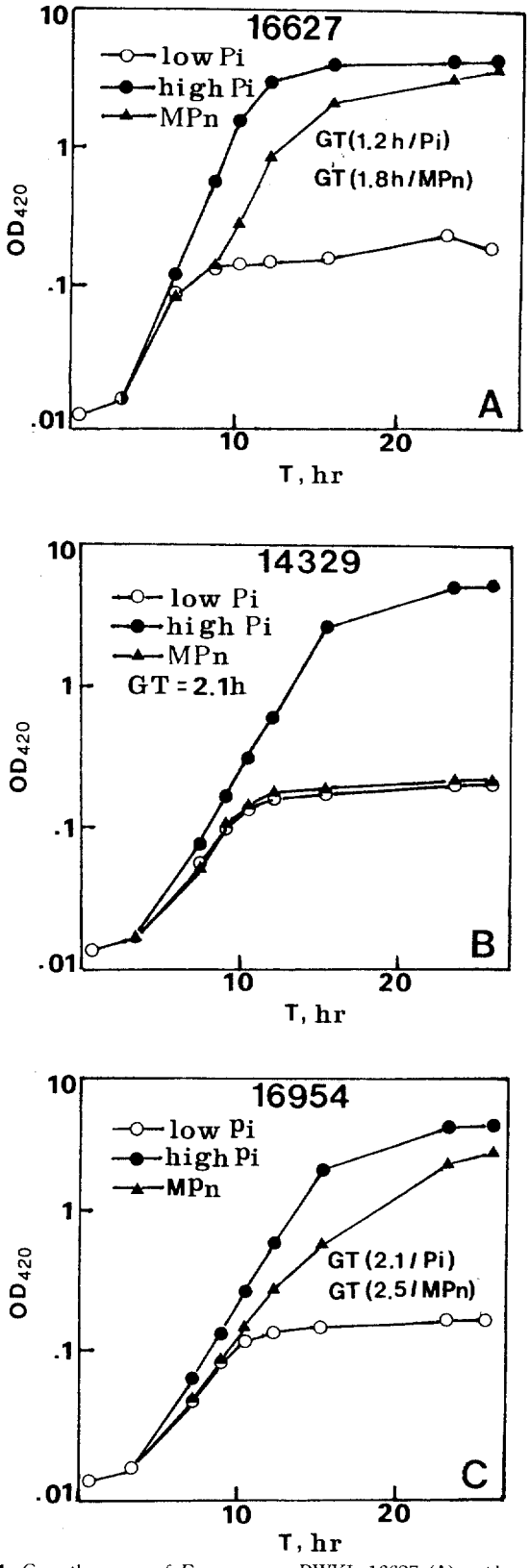

Fig. 1. Growth curve of *E. aerogenes*, BWKL 16627 (A), △*phn* mutant BW14329 (B), recombinant BWKL 16954 (C). MPn (▲) means 0.01 mM Pi plus 0.5 mM MPn; High Pi (●) means 0.51 mM Pi; Low Pi (○) means 0.01 mM Pi in glucose-MOPS media (OD=0.1, cessation of growth on 0.01 mM Pi in 10 hr culture).

(Fig. 1-B). The generation time of *E. coli* 14329 was longer than that of *E. aerogenes*. On the excess MPn, the recombinant BWKL 16954 showed the same growth yield as *E. aerogenes* BWKL 16627 except diauxic pattern for BWKL 16627, but slightly delayed generation time (Fig. 1-C). Generation time (GT) of *E. aerogenes* on the media with Pi and MPn are 1.2 hr and 1.8 hr, on the other hand those of recombinant 2.1 hr and 2.5 hr, respectively. Recombinant (BWKL 16954) grew slower than *E. aerogenes* in the glucose-MOPS media with various P sources.

Detection of remaining Pn in the medium

The concentration of Pn in the medium was estimated by comparing the relative peak intensity (RPI) with standards in ^{31}P-NMR. Intensities of standard Pn mixture (0.5 mM MPn and 1.0 mM AEPn in glucose-MOPS medium) were measured, which showed 0.5 : 1.0 ratio in accordance with concentration (Fig. 2-A). In the case of *E. coli* 14329, RPI (0.45 : 1.0=MPn : AEPn, AEPn is an external standard) didn't change significantly after 24 hrs culturing (Fig. 2-B), which is distinct from *E. aerogenes* BWKL 16627 (Fig. 2-C) and the recombinant BWKL 16954 (Fig. 2-D). These results mean *E. coli* BW 14329 nearly couldn't utilize MPn. It also implys the absence of Pn transporter system in *E. coli* 14329 or existence of another very low efficient influx system for Pn which is different from *phnCDE* transporter. Moreover, Pn utilization mode of other *E. coli phn* mutants(BWKL, 16685~16689) are similar to BW 14329.

In vivo ^{31}P-NMR

In vivo ^{31}P-NMR spectrum of *E. aerogenes* BWKL 16627 during 3 hrs starvation was shown in Fig. 3-A. An intense peak at near 0~1 ppm indicates intracellular orthophosphate (Pi) and those at near −10 ppm indicates pyrophosphate (PPi). Both of them might be released by degradation of inorganic polyphosphate pool. It seems to be correlated with the observation of late growth yield in growth curve (Fig. 1-A). If MPn is supplied to the medium as a unique P source, pyrophosphate content seems to be decreased while orthophosphate content is contant in the cell (Fig. 3-B). This phenomenon is also coincident with above results that direct the synthesis of polyphosphate rather than degradation of it for the balancing the orthophosphate content on the basis of phosphate metabolism.

In vivo ^{31}P-NMR spectrum of recombinant didn't show any intense signal in the cell (Fig. 3-C), however, under the cultivation adding with MPn, a few peaks at near 0~4 ppm were detected intensely (Fig. 3-D). The most intense one near 2.5 ppm should be an intracellular or-

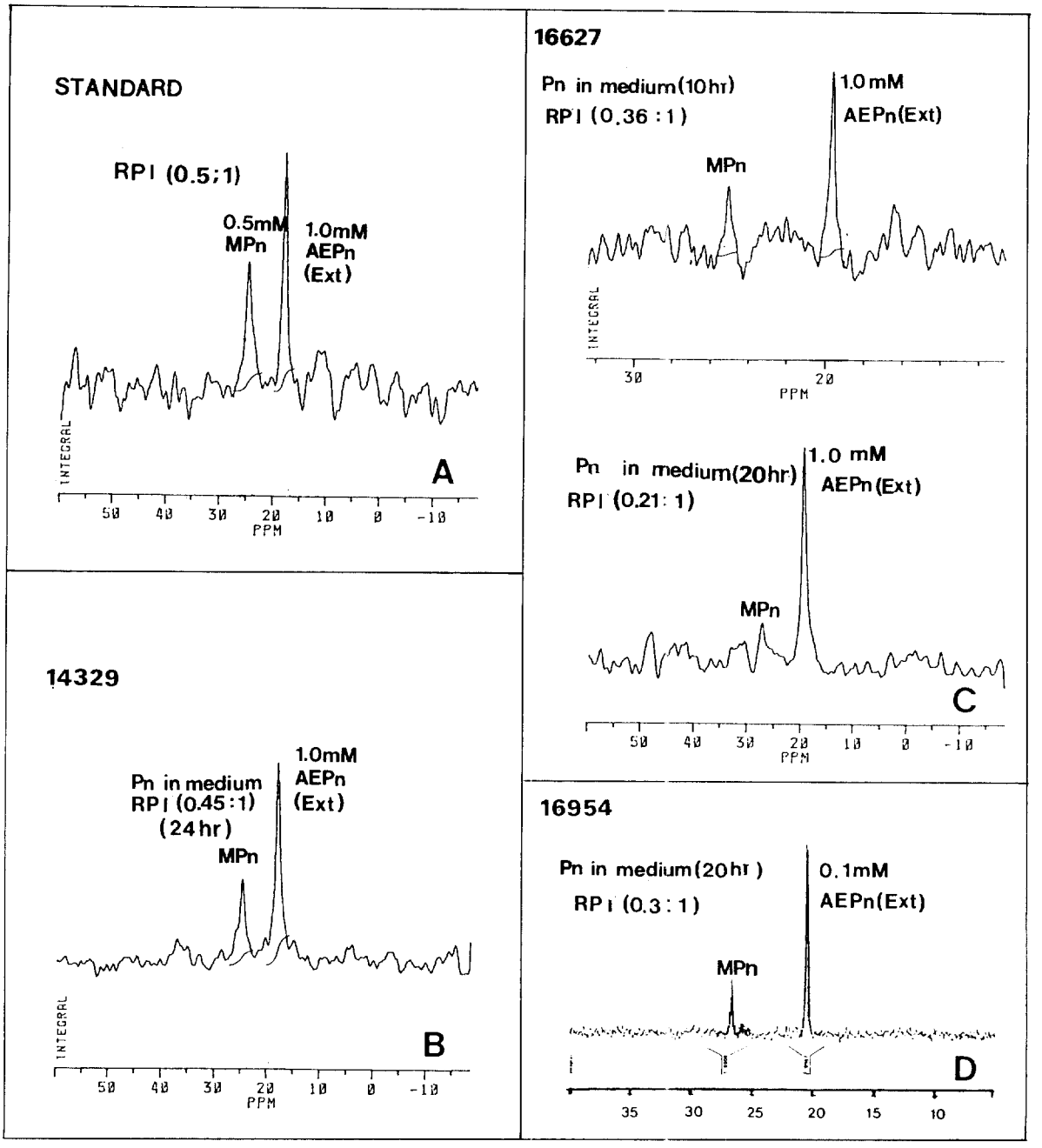

Fig. 2. The relative peak intensity (RPI) of two different concentrations of the standard in glucose-MOPS medium by *in vitro* ^{31}P-NMR, (A); RPI of remaining Pn in the medium (BW 14329), (B); RPI of remaining Pn in the medium according to the time course (BWKL 16627), (C); RPI of remaining Pn in the medium (BWKL 16954), (D).

thophosphate and the other near 3.3 ppm should be phosphate monoesters such as acetylphosphate, sugar-phosphate or tautomerized phosphonic acid, which are key intermediates by C-P lyase cleavage.

The first step process for Pn biodegradation via C-P lyase produces a phosphite (Pt), in which P is in the +3 oxidation state. In a subsequent step, C-P lyase might oxidize Pt to form Pi (+5 oxidation state). In our ^{31}P-NMR experiment, we could detect only Pi signal rather than Pt intermediate signal. This result supports a biochemical mechanism for C-P bond cleavage which involves redox chemistry at the P center (12).

In conclusion, even though *E. aerogenes* BWKL 16627 and recombinant BWKL 16954 have similar spectrum of Pn utilization, *E. aerogenes* was always faster than recombinant in the growth and the rate of Pn utilization (uptake and degradation). RPIs of remaining MPn at 20 hr culture in *E. aerogenes* and in recombinant were 0.21

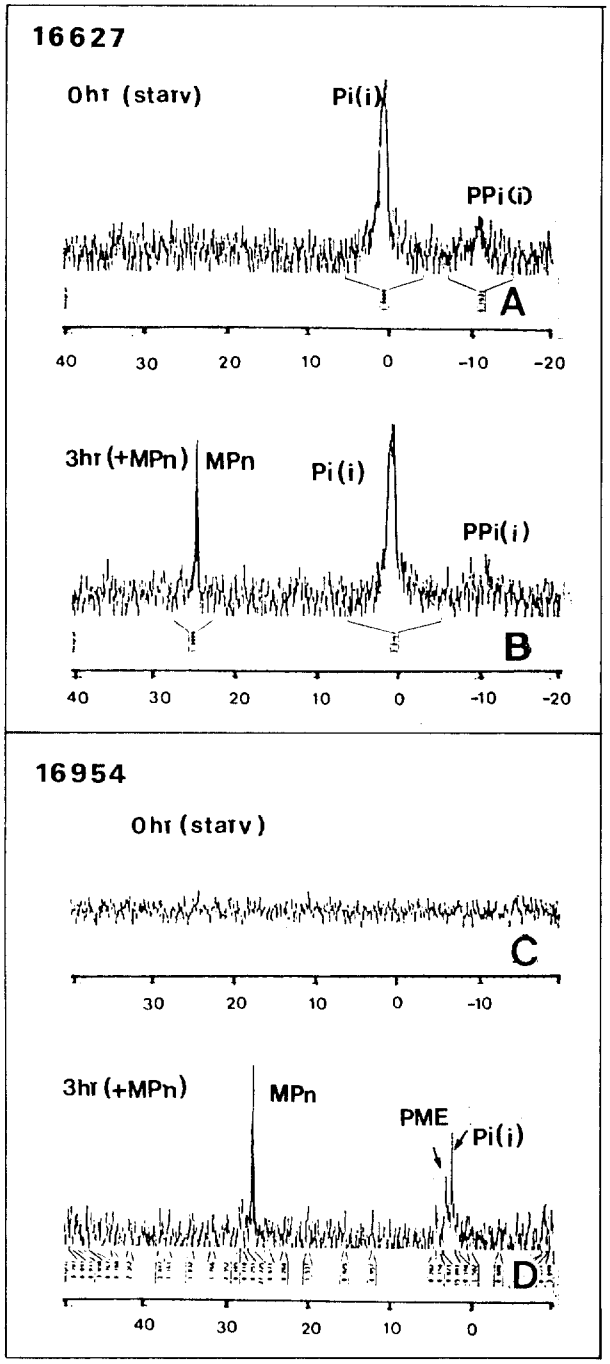

Fig. 3. *In vivo* ^{31}P-NMR of starved cell and culture fluid of *E. aerogenes*, BWKL 16627 (A, B) and recombinant, BWKL 16954 (C, D)

and 0.3, respectively. GTs on MPn in *E. aerogenes* and in recombinant were 1.8 hr and 2.5 hr, respectively. These data may allow to say that remaining Pn amount is proportional to GT. Hence, differences of C-P lyase pathway in *E. aerogenes* and *E. coli* (recombinant has the Pn transporter system of *E. coli*) could be dependent on the efficiency and affinity of Pn transporter for alternative Pn.

Considering the growth rate and RPI on the media with Pn as a sole P source, we can predict Pn transporter of *E. aerogenes* might be much more efficient in uptake and stronger in affinity for Pn utilization than those of *E. coli*.

And the recombinant can complement the DMPt$^+$ phenotype, even though the growth was poor on the DMPt$^-$ medium compared with *E. aerogenes*. C-P lyase of *E. aerogenes* may also have the broader spectrum upon Pn utilization than those of *E. coli*.

Detection of key intermediate signal (oxidized form of orthophosphate) in the C-P lyase pathway is the highlight in this study, however.

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