

Structural and Functional Analysis of the Phosphonoacetate Hydrolase (*phnA*) Gene Region in *Pseudomonas fluorescens* 23F

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The *Pseudomonas fluorescens* 23F phosphonoacetate hydrolase gene (*phnA*) encodes a novel carbon-phosphorus bond cleavage enzyme whose expression is independent of the phosphate status of the cell. Analysis of the regions adjacent to the phosphonoacetate hydrolase structural gene (*phnA*) indicated the presence of five open reading frames (ORFs). These include one (*phnR*) whose putative product shows high levels of homology to the LysR family of positive transcriptional regulators. Its presence was shown to be necessary for induction of the hydrolase activity. 2-Phosphonopropionate was found to be an inducer (and poor substrate) for phosphonoacetate hydrolase. Unlike phosphonoacetate, which is also an inducer of phosphonoacetate hydrolase, entry of 2-phosphonopropionate into cells appeared to be dependent on the presence of a gene (*phnB*) that lies immediately downstream of *phnA* and whose putative product shows homology to the glycerol-3-phosphate transporter. RNA analysis revealed transcripts for the *phnAB* and *phnR* operons, which are transcribed divergently; the resulting mRNAs overlapped by 29 nucleotide bases at their 5' ends. Transcripts of *phnAB* were detected only in cells grown in the presence of phosphonoacetate, whereas transcripts of *phnR* were observed in cells grown under both induced and uninduced conditions. The expression of three additional genes found in the *phnA* region did not appear necessary for the degradation of phosphonoacetate and 2-phosphonopropionate by either *Pseudomonas putida* or *Escherichia coli* cells.

Organophosphonates are a group of compounds of biogenic and xenobiotic origins that are characterized by possession of a direct carbon-phosphorus bond. Two different routes of C-P bond cleavage, each inducible only under conditions of phosphorus limitation, have been demonstrated when such molecules serve as the sole phosphorus source for microbial growth. "C-P lyase" is the trivial name given to an enzyme complex which catalyzes the cleavage of the C-P bond of both substituted and unsubstituted phosphonates by a mechanism which may involve redox or radical chemistry (27). By contrast, phosphonoacetaldehyde hydrolase ("phosphonatase" [EC 3.11.1.1]) is active in vitro and hydrolytically cleaves only the substituted C-P bond of phosphonoacetaldehyde to yield acetaldehyde and P_i (10). Since bacterial cleavage of the C-P bond by both C-P lyase(s) and phosphonatase is under control of the *pho* regulon (10) and hence occurs only under conditions of P_i limitation, organophosphonates generally fail to serve as sources of carbon for microbial growth because the excess of P_i released during the catabolism of their carbon skeletons serves to repress and/or inhibit their further mineralization. Genetic analysis of C-P bond cleavage by the C-P lyase and phosphonatase pathways has been reported (10, 18, 26), and the phosphonatase gene of *Pseudomonas aeruginosa* has been characterized (5).

We have recently reported the purification (15) and molecular cloning (14) of phosphonoacetate (PA) hydrolase (EC 3.11.1.2), a novel C-P cleavage enzyme from *Pseudomonas fluorescens* 23F which is able to mineralize PA with essentially quantitative P_i release. Expression of the enzyme is inducible in the presence of its apparently xenobiotic substrate PA and is independent of the P_i status of the cell. No mode of regulation had previously been described in which a phosphorus-containing compound is needed for induction of a gene required for its own utilization (27). To obtain a better understanding of this unique biodegradation system we now report the structural and functional analysis of those regions of the genome of *P. fluorescens* 23F adjacent to the *phnA* gene and address the function and regulation of the genes comprising the *phn* operon.

MATERIALS AND METHODS

Bacterial strains and plasmids. The PA-degrading strain *P. fluorescens* 23F has been described (17). *Escherichia coli* DH5 α and *Pseudomonas putida* AC577 *trp leu* (from A. M. Chakrabarty, University of Illinois-Chicago, Chicago) were used for the analysis of cloned PA degradation genes. Preliminary experiments showed that PA and 2-phosphonopropionate could serve as only poor sources of phosphorus for *P. putida* AC577 without P_i release. *E. coli* DH5 α could use PA but not 2-phosphonopropionate as a source of phosphorus, also without P_i release. Plasmid pRK2013 (7) was used as a helper plasmid. Cosmid pLAFR5 (12) was used for analysis of the expression of *phn* genes in *E. coli* and *P. putida*, and plasmid vectors pUC129 (12) and pUC18 were used for the analysis of the same genes in *E. coli* only. Promoter probe vector pKK232-8 (Pharmacia) was used for the analysis of *phnA* gene expression.

Media and growth conditions. *Pseudomonas* and *E. coli* strains were propagated in a rich (2YT) medium (19) solidified when required by addition of Difco agar (1.8% [wt/vol]). For the growth and detection of recombinant plasmids,

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ampicillin (100 $\mu\text{g ml}^{-1}$) or tetracycline (15 $\mu\text{g ml}^{-1}$), IPTG (isopropyl- β -D-thiogalactopyranoside) (50 $\mu\text{g ml}^{-1}$), and X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) (50 $\mu\text{g ml}^{-1}$) were used. Mineral salts medium was free of phosphorus and contained (per liter): KCl, 0.24 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; NH_4Cl , 1.0 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.0 mg; ferric ammonium citrate, 1.0 mg; HEPES buffer, 25 mM (pH 7.2); and 1 ml of trace element solution (13). Purified agar (1%) (Oxoid, Basingstoke, United Kingdom) was added when appropriate. As a source of carbon, sodium gluconate (2.0 g l^{-1}) or sodium pyruvate (2.0 g l^{-1}) was used. As a source of phosphorus, PA (2 mM), 2-phosphonopropionate (2PP) (2 mM), or P_i (2 mM) was used. A number of phosphonates (2 mM) were also screened for their ability to serve as sources of phosphorus. Medium for auxotrophic strains was supplemented with appropriate amino acids (50 mg l^{-1}) and vitamins (5 mg l^{-1}). *Pseudomonas* strains were routinely grown at 29°C, and *E. coli* was grown at 37°C.

Bacterial crosses. Recombinant plasmids constructed using pLAFR5 were transferred into the *P. putida* AC577 recipient strain using a three-factor mating procedure (4) with pRK2013 as a helper plasmid.

Analysis of PA hydrolase activity. C-P bond cleavage activity in cell extracts and resting cells was assayed at 30°C by measuring the amount of phosphate liberated from phosphonates supplied as substrates (17). In both cases cultures were grown in a mineral salts medium with P_i (2 mM) as phosphorus source to an optical density at 600 nm (OD_{600}) of 1.0. Cells were then harvested, washed with NaCl (0.85%), and transferred into fresh mineral salts medium without a carbon and phosphorus source; at this point various phosphonates (2 mM) were added as potential inducers, and incubation was carried out for another 8 to 10 h. For assay of resting cells, this culture (adjusted to an OD_{600} of 0.5) was washed and divided into aliquots, and each potential substrate phosphonate (5 mM) was added. After 16 h of incubation at 30°C with aeration, phosphate release into culture supernatants was measured by the method of Fiske and Subbarow (8). Cell extracts were prepared as described earlier (17). As a control, cells incubated in medium lacking phosphonate supplementation were used. Lack of P_i release in phosphonate-containing sterile growth medium was confirmed. PA hydrolase activity was expressed as nanomoles of P_i liberated per minute per milligram of protein. Protein concentration was determined by the method of Bradford (2).

In all cases PA hydrolase activity was measured using cell extracts. However, to investigate the role of PhnB protein as a possible transporter, activities for both cell extracts and resting cells induced with PA were measured.

DNA techniques. Recombinant DNA manipulations were carried out using standard protocols as described by Sambrook et al. (23). Restriction analysis of recombinant plasmids was performed using enzymes obtained from Pharmacia, according to the manufacturer's instructions. A SephaGlas BandPrep kit (Pharmacia) was used for the recovery of restriction fragments from agarose gels.

Construction of clones. Several recombinant plasmids (pLA82, pLA45, pUA45, pCA452, and pCA455) containing the *phn* genes were described earlier (14) and are further characterized in this paper. Plasmids pUA6, pKK2-26, and pKK6-4 were constructed in this work. To clone the defined *phn* region sequences, the following oligonucleotide primers were used: JQ92, 5'-CTGAAGCTTGTGGCGCTACGATC-3' (a reverse primer; position in the sequence 6688 to 6671 nt); JQ93, 5'-CATAAGCTTCGAACCGGTAACCTG-3' (2911 to 2929 nt); and JQ96, 5'-CGGAAGCTTCAAAAATGCGCAGCGC-3' (3863 to 3881 nt). PCRs were performed on *P. fluorescens* 23F genomic DNA using two pairs of primers: JQ92-JQ93 and JQ92-JQ96. *PfuI* polymerase was used to minimize the number of possible mistakes introduced during amplification. Reactions were carried out in volumes of 50 μl with concentrations of deoxynucleoside triphosphates at 200 μM and primers at 0.15 μM each. The following temperature profile was used: denaturing at 95°C for 3 min and then 95°C for 30 s, 60°C for 30 s, and 72°C for 7 min, for 30 cycles. The *Hind*III sites introduced into all above listed primers (underlined) were used for the cloning of the resulting PCR fragments into pKK232-8. Primers JQ63 (5'-ACTGGCGTGCTGCTGGCA-3' [2472 to 2489 nt]) and JQ76 (5'-CTTGATTGCAGGGTATCAG-3' [5404 to 5385 nt]) were used for construction of pKK38.

DNA sequencing. Plasmids pUC129 and pUC18 containing insertions from the *phnA* gene region were used as templates for the *Taq* Dye-Deoxy Terminator Cycle sequencing kit (Applied Biosystems). DNA sequences were obtained using an automatic DNA sequencer (model 373A; Applied Biosystems). The nucleotide sequences of both strands were determined. Primer synthesis was performed by C. Stevenson (School of Biology and Biochemistry, The Queen's University of Belfast, Ireland). Initial computer analysis of the sequences was performed using the DNASIS (Hitachi) software package. The alignment of sequences was performed by using CLUSTALW software (25) with parameters set at default values.

Searches for nucleotide and amino acid sequence similarities were carried

out on the National Center for Biotechnology Information web server (<http://www.ncbi.nlm.nih.gov>) in the nonredundant nucleotide (nr-nt) or amino acid (nr-aa) databases using the FASTA and BLAST programs (22).

RNA analysis. *P. fluorescens* 23F cells were grown in mineral salts medium to an OD_{600} of 0.7. In the case of induction with PA, cells were harvested by centrifugation, washed, and resuspended in the same medium without a source of carbon and phosphorus. PA was added to a final concentration of 10 mM, and cells were incubated for 24 h. Cells were lysed with a solution containing 4 M guanidine thiocyanate, 0.5% (wt/vol) laurylsarcosine, and 100 mM β -mercaptoethanol. An equal volume of phenol saturated with 0.3 M sodium acetate (pH 5.5) was added, and the preparation was mixed thoroughly and heated at 80°C for 4 min. It was then cooled, and 0.1 volume of chloroform was added. Following centrifugation the aqueous phase was collected. This procedure was repeated 3 to 4 times. The RNA preparation was ethanol precipitated and dissolved in diethyl pyrocarbonate-treated water. Northern blot analysis was carried out essentially according to the method of Sambrook et al. (23). Aliquots of total RNA (20 μg) were fractionated in 0.8% or 1.2% agarose-formaldehyde gels electroblotted onto Hybond-N⁺ membranes (Amersham) in formaldehyde gel-running buffer for 3 h at a constant current of 150 mA using the Mini Trans-Blot cell (Bio-Rad) and were UV cross-linked to the filter. Either 5'-³²P-labeled oligonucleotides or uniformly ³²P-labeled single-stranded DNA fragments were used in hybridization. Primer JQ86 (5'-CGCCGTGAGTCAGGTTCCCGGCGCCG-3') complementary to mRNA for *phnR* (positions 3803 through 3832 in the sequence) was 5'-³²P-labeled with T4 polynucleotide kinase (Promega) by the recommendations of the suppliers. The probe used for *phnA* mRNA analysis was obtained as follows. First a DNA fragment (positions 3948 through 4682), corresponding to the 5' end of the *phnA* gene, was amplified by PCR using primers JQ87 (5'-TTGTGTGAGAAAGATGCTCAGAAATACGGTG-3' [positions 3948 through 3977]) and JQ84 (5'-TGCCTGGCGCATGTTTGTGTTG-3' [positions 4682 through 4661]). Residual primers, any extraneous single-stranded DNA produced by PCR, and the remaining deoxynucleoside triphosphates were removed from the PCR mixture with exonuclease I (USB) and shrimp alkaline phosphatase (USB), using the supplier's recommendations. Second, a uniformly ³²P-labeled single-stranded DNA fragment was synthesized by asymmetric PCR using JQ84 as the primer and the product of the previous PCR as the template. RNA immobilized on filters was hybridized overnight with 5'-³²P-labeled JQ86 or the uniformly ³²P-labeled, single-stranded DNA fragment, in 6 \times SSC (1 \times SSC is 0.15 M NaCl, 0.015 M sodium citrate) at 52°C or 68°C, respectively. Following hybridization, membranes were washed with hybridization buffer under the same conditions for 30 min and subsequently washed at room temperature in 2 \times SSC, 0.5 \times SSC, and 0.1 \times SSC.

Primer extension experiments were carried out according to the method of Sambrook et al. (23). Primers JQ86 (see above) and JQ88 (5'-CAGGCGGATGGAGCGGAGTTCACGCTGAT-3' [positions 4090 through 4060]), which are complementary to mRNA for *phnR* and *phnA*, respectively, were employed for these purposes. 5'-³²P labeling was carried out as described above. Labeled primers (500 to 1,000 \times 10³ cpm) were annealed with 10 to 30 μg of RNA. Hybridization was performed at 30°C in a solution containing 40 mM piperazine-N,N'-bis(2 ethanesulfonic acid) (PIPES), pH 6.4, 1 mM EDTA, 0.4 M NaCl, and 80% formamide. Primer extension was carried out with RNase H minus Moloney murine leukemia virus reverse transcriptase (Promega). The products synthesized were analyzed by electrophoresis in 6% polyacrylamide 8 M urea gels.

GC-MS determination of propionate. Aliquots from enzyme assays containing 2PP were derivatized as the phenylphenacyl derivative of the target compound and analyzed by gas chromatography-mass spectrometry (GC-MS) with the mass spectrometer (MS) operating in the selected ion-monitoring mode. Fluoroacetate was used as an internal standard for quantitation purposes. Fluoroacetate (0.25 ml, 1 mM) was added to cell extract (1 ml), and the mixture was frozen. Following sample lyophilization, the dried product was suspended in toluene-acetonitrile (1:1 [vol/vol], 1 ml); 1,4,7,10,13,16-hexaoxacyclooctadecane (10 mg) and 4-(bromoacetyl) biphenyl (10 mg) were added; and the mixture was heated at 75°C for 12 h. The derivatized samples were cooled and analyzed by GC-MS on a Hewlett Packard 5890 series II gas chromatograph linked to a Hewlett Packard 5971 mass selective detector controlled by a HP vectra computer using ChemStation software. The gas chromatograph was fitted with an HP Ultra 2 fused-silica wall-coated open tubular capillary column (12 m by 0.22 mm internal diameter) with 5% biphenyl-95% dimethyl polysiloxane as the bonded phase. Helium was used as the carrier gas at a flow rate of 0.8 ml min⁻¹, and samples (1 μl) were injected at a split ratio of 80:1. After injection of samples, the oven temperature was held at 130°C for 1 min, then programmed upwards at 10°C min⁻¹ to 300°C. The mass selective detector was operated in selected ion monitoring mode monitoring ion currents at *m/z* 181 and 268 atomic mass units for propionate and *m/z* 181 and 272 atomic mass units for fluoroacetate. To quantify

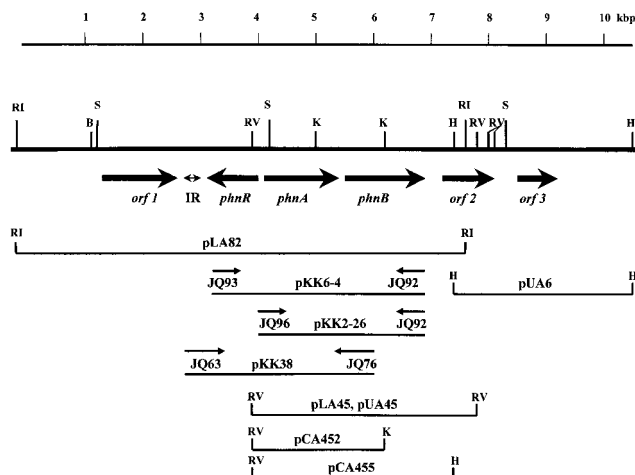


FIG. 1. Physical and genetic map of the *phnA* gene region. Restriction sites are indicated as follows: E, *EcoRI*; RV, *EcoRV*; K, *KpnI*; S, *SalI*; H, *HindIII*; IR, region of imperfect inverted repeats; *phnA*, PA-hydrolase gene locus; *phnR*, putative transcriptional regulator of the PA-hydrolase gene cluster; *phnB*, putative transporter gene for 2PP; putative genes with unknown functions found in the region are designated as *orf1*, *orf2*, and *orf3*. The direction of transcription of each gene is shown by the arrows (\rightarrow). The most important clones analyzed in this work are shown; the fragments containing the *phnA* gene were subcloned into pLAFR5 (designated as pLA plasmids), pUC129 (designated as pUA plasmids), or pUC18 (designated as pCA plasmids). Plasmid pKK2-26 was constructed by cloning a 2.8-kb PCR fragment (primers JQ96 and JQ92) into plasmid pKK232-8, and plasmid pKK6-4 was constructed by cloning a 3.78-kb PCR fragment (primers JQ93 and JQ92) into the same vector.

propionate, the peak area for ion m/z 181 at the expected retention time of propionate was compared to a standard response curve after correcting for the response of the internal standard.

Nucleotide sequence accession number. The nucleotide sequence reported in this work was deposited in GenBank under accession number L49465.

RESULTS

Nucleotide sequence analysis of the *phnA* gene region. The construction of the *P. fluorescens* 23F genomic library, and cloning and sequencing of the PA-hydrolase structural gene (*phnA*) were reported earlier (14). Here we describe the sequencing analysis of the regions (9.5 kbp) adjacent to the *phnA* gene (Fig. 1 and Table 1). The nucleotide sequences of *orf1*, *phnR*, *phnB*, and part of *orf2* were obtained using subclones produced from the pLA82 insert (Fig. 1). For sequencing of the distal part of *orf2* and the whole of *orf3*, plasmids pUA45 and pUA6 were used (Fig. 1). The latter plasmid was produced by the cloning from the *P. fluorescens* 23F genomic library of the *HindIII* fragment overlapping with the pLA82 insert.

Five open reading frames (ORFs), apart from the *phnA* gene, were found; they showed various degrees of similarity to existing database entries (Table 1):

(i) ORF1 is preceded by a putative ribosome-binding site (RBS) and encodes a putative protein with a molecular mass of 41,681 Da. This protein shows similarity to the hydroxyphenylpropionic acid transporter (6) and to several hypothetical metabolite transport proteins.

(ii) The ORF found immediately upstream of *phnA* (Fig. 1) was designated as the *phnR* gene. This gene is encoded by a

complementary chain (the direction of transcription is opposite to that of the *phnA* gene) and begins 140 bp upstream of the *phnA* gene. This ORF is also preceded by a putative RBS and encodes a putative protein of 32,827 Da. PhnR showed significant similarity to several transcriptional regulators belonging to the LysR family. The alignment of the PhnR amino acid sequence with the amino acid sequences of three proteins belonging to the LysR family is presented in Fig. 2. Analysis of this alignment clearly demonstrates that the PhnR gene product had a high degree of similarity (identical aa residues) to other LysR proteins in its N-terminal part, the first 66 amino acid residues, and much lower similarity in the C-terminal half. This mode of similarity distribution has been shown to be typical for the LysR family of transcriptional regulators (24). Evidence for the regulation of PA-hydrolase activity by the *phnR* gene is presented later in this paper.

(iii) An ORF found 96 bp downstream of *phnA* was designated as the *phnB* gene. This ORF (Fig. 1) was preceded by a putative RBS. It encoded a putative protein of 428 amino acids. The amino acid sequence of this protein was shown to be similar to several transporter proteins; for example, it showed 25% of aa identity with the glycerol-3-phosphate transporter (20). The possible role of PhnB in the biodegradation of 2PP is analyzed later in this paper.

(iv) ORF2 encoded a putative protein of 377 amino acids. This protein was found to be very similar (55% aa identity) to the spermidine/putrescine-binding protein precursor from *E. coli* (21) and to several other similar proteins.

(v) ORF3 encoded a putative protein of 209 amino acids. Significant similarity between this protein (44% aa identity) and the peptide methionine sulfoxide reductase from *Bacillus subtilis* and several other sulfoxide reductases was found.

Identification of *phnA* and *phnR* transcripts and mapping of their 5' termini. To identify the *phnA* and *phnR* transcripts, total RNA isolated from PA-induced and uninduced *P. fluorescens* 23F cells was fractionated by electrophoresis, transferred to nylon membranes, and hybridized with 32 P-labeled probes. Transcripts for *phnA* were detected only in those RNA preparations obtained from induced cells (Fig. 3A). In contrast, mRNA for *phnR* was found in cells grown in either the presence or the absence of the inducer; it migrated as a single

TABLE 1. Sequence analysis of the *phnA* gene region

Gene or feature	Coding strand ^a	Coding region (nt)	Protein (aa/Da)	Relatedness to other proteins ^b		
				Protein	Identity (%)	Accession no.
<i>orf1</i>	+	1368–2585	405/41681	MhpT	65	P77589
IR	NA	2610–2828	NA	NA	NA	NA
<i>phnR</i>	–	3009–3908	299/32827	GcvA	29	P32064
				AmpR	33	P14145
				TrpI	29	P34818
<i>phnA</i>	+	4049–5272	407/44237			
<i>phnB</i>	+	5369–6655	428/47304	GlpT	25	P37948
<i>orf2</i>	+	7040–8173	377/41291	PotF	55	P31133
<i>orf3</i>	+	8489–9118	209/23775	PmsR	44	P54154

^a In the nucleotide sequence, the strand coding for the PA-hydrolase gene (*phnA*) is designated the plus (+) strand; *phnR* is the minus (–) strand. NA, not applicable.

^b Accession numbers relate to the SWISS-PROT database; identity relates to percentage of identical amino acids in alignments.

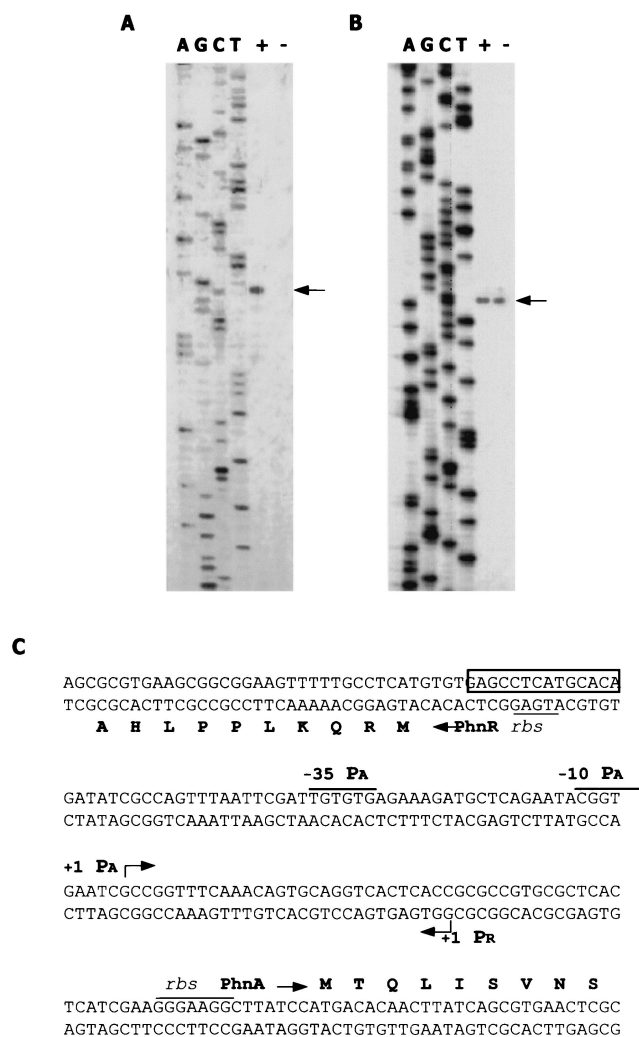


FIG. 4. Identification of *phnA* and *phnR* transcript initiation sites and putative regulatory sequences. Total RNA (30 μ g) isolated from *P. fluorescens* 23F cells grown in the presence (+) or in the absence (-) of PA was annealed with 32 P-labeled primers JQ88 (A), and 10 μ g of the same RNA was annealed to JQ86 (B) as described in Materials and Methods. Aliquots from the primer extension reactions (lanes + and -) were separated by electrophoresis in denaturing 6% polyacrylamide gels along with the sequence ladders generated with the same oligonucleotides (lanes A, G, C, and T). The corresponding primer extension products are indicated by arrows. The nucleotide sequence of the intergenic region and the 5' ends of *phnA* and *phnR* genes are presented (C). N-terminal amino acids translated from ORFs for both genes are shown in one-letter codes. Transcription start sites for P_A and P_R promoters are indicated by bent arrows. Putative -10 and -35 sequences of PA and RBS are shown. The putative recognition site for PhnR is boxed.

was mediated by the PhnA gene product (PA hydrolase) rather than by a classical C-P lyase since the latter enzyme is subject to repression by P_i and no net P_i release should occur as a result of its activity.

Cleavage of the C-P bond of 2PP was detected only in cell extracts of *P. putida* and *E. coli* recombinant strains with hybrid plasmids containing the *phnA* gene. No activity was detected when the *phnA* gene was deleted. C-P cleavage activities towards 2PP were rather low, at most some 2% of the hydrolase's

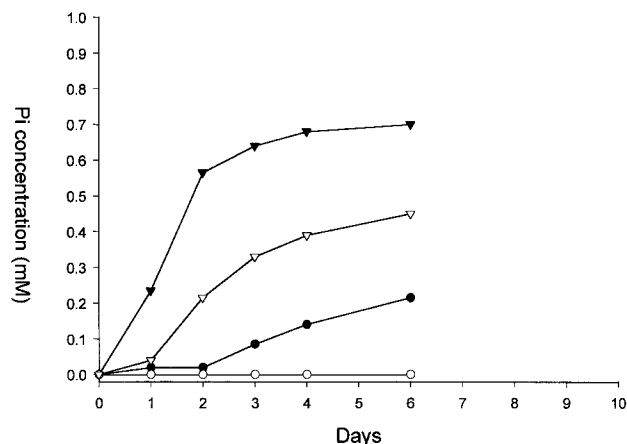


FIG. 5. Phosphate release during growth of *Pseudomonas* strains on 2PP. Strains were grown in batch cultures. 2PP (2 mM) was used as a source of phosphorus and sodium gluconate (2 g l⁻¹) as a source of carbon. P_i release was measured as described in Materials and Methods. ●, *P. fluorescens* 23F; ○, *P. putida* AC577; ▽, *P. putida* AC577(pLA45); ▼, *P. putida* AC577(pLA82).

activity against PA (Table 2). To prove that the degradation of 2PP is determined by the enzymatic activity of the PA hydrolase encoded by the *phnA* gene, P_i release and accumulation of propionate were analyzed when 2PP was incubated with cell extract known to contain PA-hydrolase activity (Table 3). Although only low levels of C-P cleavage activity were found, near equimolar release of P_i and propionate from 2PP was obtained. These results confirmed that the hydrolase encoded by *phnA* was also active against 2PP.

Regulation of PA-hydrolase activity by the *phnR* gene. It has been shown above that the *phnR* gene encodes a protein similar to a number of positive regulators belonging to the LysR family. To further investigate a possible role for PhnR in the expression of PA hydrolase, plasmids containing either *phnR* and *phnA* or only *phnA* were constructed. Plasmid pLA45, in which *phnR* was deleted, still contained the whole of the *phnA* gene and 120 nt of upstream region which included a promoter

TABLE 2. Dependence of PA-hydrolase activity in *P. putida* AC577 and *E. coli* DH5 α on the presence of the *phnR* gene

Strain	Plasmid (substrate) ^a	<i>phnR</i> gene	PA-hydrolase activity \pm SD ^b		
			No inducer	PA induction	2PP induction
<i>P. putida</i>					
AC577	pLA82 (PA)	+	0.31 \pm 0.03	26.5 \pm 1.7	35.25 \pm 2.2
AC577	pLA82 (2PP)	+	<0.01	0.65 \pm 0.06	0.35 \pm 0.05
AC577	pLA45 (PA)	-	31 \pm 1.8	36 \pm 1.4	39.7 \pm 1.4
AC577	pLA45 (2PP)	-	0.28 \pm 0.06	0.34 \pm 0.04	0.10 \pm 0.01
<i>E. coli</i>					
DH5 α	pKK6-4 (PA)	+	0.22 \pm 0.02	2.5 \pm 0.12	1.90 \pm 0.16
DH5 α	pKK2-26 (PA)	-	0.24 \pm 0.03	0.27 \pm 0.02	0.25 \pm 0.04
DH5 α	PKK38 (PA)	+	0.14 \pm 0.03	2.1 \pm 0.15	<0.01

^a Identities of the inserts containing the *phnA* gene region are shown in Fig. 1.

^b Specific activity of PA hydrolase given as P_i release \pm SD in nanomoles per minute per milligram. For this analysis, cultures of *P. putida* and *E. coli* were grown and prepared as described in Materials and Methods. Cell-free extracts were then prepared, and PA-hydrolase activity was assayed. The results are expressed as an average of three experiments.

TABLE 3. Phosphate release and accumulation of propionate in cell-free extracts of *P. putida* AC577(pLA82)^a

Substrate	Concn (mM)	P _i release ± SD	Propionate ± SD ^b
2PP	2	410 ± 12.5	400 ± 10.3
	5	620 ± 21	560 ± 12.5

^a P_i release and propionate accumulation are given in nanomoles per milligram of protein measured in the samples after 12 h of incubation of cell-free extracts with 2PP at 30°C.

^b Control samples from which either 2PP or cell-free extract was omitted contained no measurable amounts of propionate after 12 h of incubation.

sequence (Fig. 1 and 4). Plasmid pLA82 contained both *phnR* and *phnA*. Plasmids pLA82 and pLA45 (pLAFR5 vector) were transferred into *P. putida* AC577 using the helper plasmid pRK2013.

Phosphate release was assayed in the corresponding cell extracts using PA and 2PP as substrates after the induction of *phnA* with PA (2 mM) or 2PP (2 mM) or in the absence of any inducer (Table 2). These results showed that PA and 2PP were similarly effective inducers of expression of the *phnA* gene in clones of *P. putida* that contained both *phnA* and *phnR*. Uninduced activities of PA hydrolase in these clones were 30 to 100 times lower. In those clones in which the *phnR* gene was deleted, induction with either PA or 2PP was not required for the effective expression of the PA hydrolase (Table 2). Transcription of the *phnA* gene in these cases was probably achieved from the *lac* promoter of the pLAFR5 vector. Similar results were obtained with these plasmids in *E. coli* DH5α cells (data not shown). To distinguish between possible expression from the *lac* promoter and expression regulated by *phn*-regulatory elements, a promoter probe vector pKK232-8 was used for analysis of the expression of *phn* genes in *E. coli*. For the latter analysis, two clones were constructed: pKK6-4 (Fig. 1), containing the *phnR*, *phnA*, and *phnB* genes with a putative promoter region, and pKK2-26, which lacked most of the putative transcriptional-regulator gene sequence *phnR* but contained *phnA* and *phnB* genes and a putative promoter region. *E. coli* DH5α strains containing these plasmids were analyzed for the ability to grow in liquid medium with PA as phosphorus source. Only *E. coli* DH5α(pKK6-4) showed the ability to grow in this medium and release P_i. In vitro activity of the PA-hydrolase gene (*phnA*) was measured using cell extracts from induced and uninduced strains. The results of these experiments showed that, without induction, PA-hydrolase activity is low (0.22 to 0.25 nmol min⁻¹ mg⁻¹). Induction by PA led to an 11-fold increase in PA-hydrolase activity (2.5 nmol min⁻¹ mg⁻¹) and by 2PP to a 9-fold increase (1.9 nmol min⁻¹ mg⁻¹) (Table 2). Since this effect was observed only in the clone with the *phnR* gene, it is clear that the *phnR* gene is essential as a positive regulator for the PA- or 2PP-dependent induction of PA hydrolase.

Role of *phnB* in the utilization of 2PP. As discussed above, the putative product of the *phnB* gene showed similarity to several transport proteins. To analyze the possible involvement of *phnB* in the transport of PA and/or 2PP into bacterial cells, the activity of PA hydrolase towards these substrates was analyzed using both cell extracts and resting cell cultures. For these experiments clones were constructed (Fig. 1) in which both the *phnA* and *phnB* genes were present (plasmid pCA455) or in which the *phnB* gene was deleted (plasmid pCA452). The

results are summarized in Table 4. It is clear that PA-hydrolase activity against PA is present in both cell extracts and resting cell cultures induced with PA, irrespective of the presence of the *phnB* gene. By contrast, P_i release from 2PP was dependent on the presence of the *phnB* gene in the case of the resting cell cultures but not of cell extracts. These results suggest that the *phnB* gene product was needed for the transport of 2PP into the bacterial cell. Induction of PA-hydrolase activity by PA but not by 2PP was observed in clones with plasmid pKK38 which lacked the *phnB* gene (Fig. 1, Table 2). This observation supported the conclusion that the *phnB* gene product was involved in 2PP transport.

DISCUSSION

PA hydrolase has been reported to be a C-P bond cleavage enzyme of unique specificity; the purified enzyme showed no detectable activity towards 39 organophosphonate and analogous compounds tested (17, 15). The cloning of the PA-hydrolase structural gene *phnA* and adjacent genes has, however, allowed us to analyze expression of PA hydrolase in different genetic environments using *E. coli* and *P. putida* host strains. We have now demonstrated that the *phnA* gene product from *P. fluorescens* 23F can hydrolyze the C-P bond not only of PA but also of 2-phosphonopropionate (Table 3). Activity against 2PP was nevertheless very low, constituting no more than 2% of that on PA; the compound did not serve as sole carbon and energy source for either *P. fluorescens* 23F or any recombinant strain containing the cloned *phnA* gene. However, both PA and 2PP served as similarly effective inducers of the *phnA* gene in the original *P. fluorescens* 23F strain and in hybrid plasmids expressed in both *E. coli* DH5α and *P. putida* AC577 (Table 2). This induction was dependent on the presence in the clones of the *phnR* gene. *phnR* is located immediately upstream of the *phnA* gene and shows remarkable similarity to the group of transcriptional regulators belonging to the LysR family (24): (i) *phnR* encodes a putative protein of 299 amino acids, typical of the members of the LysR family which range from 276 to 324 residues; (ii) its direction of transcription is divergent to that of *phnA*, and it is located 140 bp upstream of the structural gene; (iii) since regulation of the expression of *phnA* is dependent on the presence of PA or 2PP, each of these may be

TABLE 4. Phosphate release from PA and 2PP in cell-free extracts and resting cells of *E. coli* DH5α with different plasmids, preinduced with PA

Plasmid ^a	<i>phnB</i> gene	Phosphate release ± SD in			
		Cell-free extracts ^b (nmol min ⁻¹ mg ⁻¹) with		Resting cells ^c with	
		PA	2PP	PA	2PP
pCA455	+	128.6 ± 1.6	1.03 ± 0.01	2.30 ± 0.03	0.45 ± 0.04
pCA452	-	85.5 ± 1.2	0.90 ± 0.1	2.40 ± 0.1	0.05 ± 0.02

^a Identities of the inserts containing the *phnA* gene region are shown in Fig. 1.

^b Specific activity of PA hydrolase given as P_i release in nanomoles per minute per milligram. For this analysis, cultures of *E. coli* were grown and prepared as described in Materials and Methods. Cell-free extracts were then prepared and PA-hydrolase activity was assayed.

^c Activity of PA hydrolase in resting cell cultures (OD₆₀₀ = 0.5) was assayed by P_i release (millimoles) after 16 h of incubation with the corresponding substrate (PA or 2PP) at 30°C. The results are expressed as an average of three experiments.

considered as a coinducer essential for the PhnR regulator protein; (iv) the amino acid sequence of PhnR shows significant homology to other members of the LysR family; this homology is almost entirely restricted to the N-terminal part of the protein, which is characteristic of the homology distribution among LysR family regulators (Fig. 2); (v) a putative site for PhnR recognition was found in the P_A promoter at position -65 (Fig. 4); this structure included dyadic symmetry and a T-N₁₁-A motif which has been proven to be critical for binding of the NodD, NahR, and MetR transcriptional regulators (9).

Our finding was not unusual, since transcriptional regulators belonging to the LysR family are the most common group in bacteria, with more than 50 reported members that regulate a diversity of genes and complex regulons in many prokaryotic genera (24). However, the involvement of a transcriptional regulator of this type in the regulation of the expression of PA hydrolase, a unique enzyme of its class, is of particular interest; all other C-P cleavage enzymes studied have been shown to be under Pho regulon control (10).

Three ORFs, which may encode proteins involved in phosphonate utilization, were found downstream of the *phnA* gene. Of these, only the role of the *phnB* gene has been analyzed; it seems probable that its product is involved in the transportation of 2PP into the bacterial cell. The data obtained, however, do not rule out the possibility that PA can also be transported by *phnB*. Deletion of *phnB* cannot be used to prove this because *E. coli* DH5 α can use PA but not 2PP as a source of phosphorus (A. Kulakova, data not shown). Significantly, in view of the 25% homology between *phnB* and the glycerol-3-phosphate transporter in *B. subtilis* (20) (Table 1), it is known that the antibacterial antibiotic fosfomycin (phosphonomycin; *cis*-1,2-epoxypropylphosphonic acid) which has close structural similarity to 2PP, is transported into bacterial cells by the glycerol-3-phosphate pathway (11). It should be noted that expression of the *phnB* gene has been investigated using *E. coli* DH5 α as a host. Accordingly, extrapolation of these results to the original *P. fluorescens* 23F host should be made with care. However, it is significant that cloned PA hydrolase was expressed to equally high levels in *P. putida* and *E. coli* hosts (results not shown). The inducible nature of this expression (Table 2) is likely to be due to the function of the positive regulator encoded by the *phnR* locus.

In the course of Northern blot analysis (Fig. 3), a transcript of ~2.6 kb was identified in RNA preparations obtained from induced cells, using a DNA probe specific to *phnA*. In terms of its size, this transcript could represent mRNA for *phnA* and *phnB*. Transcripts of similar lengths could be synthesized if termination of transcription occurred in the intergenic region between the *phnB* gene and ORF2. There were two inverted repeats in this region (positions 6742 to 6773 and 6923 to 6942). The presence of these repeats might lead to formation of stem and loop structures in RNA with free energies (ΔG_{25}) of -8.0 kcal and -9.4 kcal, respectively. It is likely that one or both of these repeats serve as a terminator(s) of transcription.

Analysis of the expression of different clones containing the *phn* region (Tables 1 and 2) in *P. putida* and *E. coli* cells indicated that neither ORF2 nor ORF3 was necessary for the degradation of PA and 2PP. Furthermore the results of Northern hybridization experiments (Fig. 3) did not reveal transcripts of lengths greater than that of a dimeric transcript for

phnA and *phnB*. Therefore, we could suppose that ORF2 and ORF3 do not belong to the *phnAB* operon and are transcribed independently.

It was also evident from Northern blot analysis that *phnR* was transcribed in the form of monocistronic mRNA. A region of tandem inverted repeats localized at the 3' end of ORF1 (positions 2610 to 2828) might serve for termination of its transcription.

Northern blot analysis of the *phnA* region also revealed two transcripts of ~2.0 and ~1.4 kb as the major products. The former may represent the whole *phnA* ORF and the 5' part of the *phnB* ORF, and the latter may represent *phnA* only. These transcripts might be produced as a result of the termination of the transcription inside the *phnB* gene and in the *phnA* and *phnB* intergenic region, respectively. We also can not exclude the possibility that these transcripts originated from the processing of a *phnAB* transcript.

In conclusion, our study has indicated a probable mechanism by which the inducible expression of PA hydrolase occurs in *P. fluorescens* 23F. In light of other studies in this laboratory (16) which show that 2-aminoethylphosphonic acid, phosphonomycin, and phosphonoalanine can also be degraded by environmental microorganisms in a P-insensitive manner, it is likely that the expression of a number of other C-P cleavage enzymes may prove to be similarly regulated.

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