Genetic and Biochemical Characterization of a Pathway for the Degradation of 2-Aminoethylphosphonate in Sinorhizobium meliloti 1021

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A variety of microorganisms have the ability to use phosphonic acids as sole sources of phosphorus. Here, a novel pathway for degradation of 2-aminoethylphosphonate in the bacterium Sinorhizobium meliloti 1021 is proposed based on the analysis of the genome sequence. Gene deletion experiments confirmed the involvement of the locus containing phnW, phnA, and phnY genes in the conversion of 2-aminoethylphosphonate to inorganic phosphate. Biochemical studies of the recombinant PhnY and PhnA proteins verified their roles as phosphonoacetalddehyde dehydrogenase and phosphonoacetate hydrolase, respectively. This pathway is likely not limited to S. meliloti as suggested by the presence of homologous gene clusters in other bacterial genomes.

Phosphonates, organic molecules containing a carbon-phosphorus (C-P) bond, can be utilized by a variety of microorganisms mainly as a source of phosphorus and, in some cases, as sources of carbon and nitrogen via several pathways (1–3). The broad specificity carbon-phosphorus lyase (C-P lyase) enzyme is widespread among bacteria. C-P lyase allows catabolism of a wide variety of structurally diverse phosphonates containing activated or unactivated C-P bonds to yield inorganic phosphate and the corresponding hydrocarbons (Fig. 1A). Consistent with a role in providing phosphorus for the cell, the C-P lyase pathway is expressed only under phosphate-limiting growth conditions. The mechanistic details of the C-P lyase-catalyzed transformation have yet to be established but are believed to involve a direct scission of the C-P bond in a redox-dependent manner that involves radical intermediates (3, 4).

In addition to C-P lyase, at least three other catabolic pathways have been identified that allow use of individual phosphonate substrates chemically activated at the β-position relative to the C-P bond. One such compound is 2-aminoethylphosphonate (2-AEPn), the most common of the naturally occurring phosphonates. Whereas 2-AEPn can also be degraded by C-P lyase, it is often catalyzed via the phosphonatase pathway, which involves hydrolytic cleavage of the C-P bond (Fig. 1B). The phosphonatase pathway involves an initial transamination of 2-AEPn to yield phosphonoacetaldehyde (PnAA), followed by hydrolysis using the enzyme PnAA hydrolase, also known as phosphonatase, to yield phosphate and acetaldehyde. Phosphonatase belongs to the haloacid dehalogenase enzyme superfamily (5). The reaction mechanism of phosphonatase involves the formation of a Schiff base intermediate between an active site lysine and a carbonyl group of PnAA followed by Mg2+-dependent hydrolysis of the C-P bond (6). Expression of the phosphonatase pathway is regulated by phosphate starvation in some organisms (7) and by the presence of 2-AEPn in others (8). Although the biogenic source of phosphonoacetate (PnA) is unknown, this activated C-P compound can also be utilized as a source of P and C by a number of microorganisms (Fig. 1C). The key enzyme required for this process, phosphonoacetate hydrolase, has been purified from Pseudomonas fluorescens 23F, where it catalyzes the divalent metal-dependent hydrolysis of PnA to acetate and inorganic phosphate (9, 10). A third activated phosphate, phosphonooalanine (PnAla, or 2-amino-3-phosphonopropionic acid), can be found as a structural component in some bacterial membranes. This compound can be utilized via transamination to phosphonyruvate followed by a metal-dependent hydrolysis of the C-P bond catalyzed by phosphonyruvate hydrolase and resulting in the formation of inorganic phosphate and pyruvic acid (Fig. 1D) (11, 12).

Finally, a variety of data suggest that additional pathways for phosphonate catabolism await discovery. For example, Martinez et al. (13) discovered both known and novel pathways for 2-AEPn catabolism by expression of genes encoded in a marine metagenomic library in Escherichia coli. This finding underscores the importance of phosphonates, which are especially prevalent in marine environments, in the global phosphorus cycle (14). Genomic and genetic data suggest that other novel pathways can be found in well studied organisms, such as a Gram-negative, symbiotic nitrogen-fixing bacterium S. meliloti 1021.

S. meliloti can use a number of phosphonates via the C-P lyase pathway and the corresponding phnFGHIJKL gene cluster.
has been identified (15, 16). The phn genes in S. meliloti are induced under phosphate-limiting growth conditions. However, when C-P lyase was genetically inactivated, S. meliloti retained the ability to grow on 2-AEPn as a sole phosphorus source. Therefore, it was suggested that S. meliloti encoded genes for both a C-P lyase and phosphonatase pathway (16), as had previously been shown for Enterobacter aerogenes (17). Later, the complete genome sequence of S. meliloti was determined, revealing a chromosome (3.65 Mbp) and two megaplasmids, pSymA (1.35 Mbp) and pSymB (1.68 Mbp) (15, 18–20). Surprisingly, genes for a phosphonatase pathway were absent. Instead, the genetic complement suggests that S. meliloti catabolizes 2-AEPn via a novel pathway involving (i) conversion of 2-AEPn to PnAA, (ii) oxidation of PnAA to PnA, and (iii) hydrolysis of PnA to acetate and inorganic phosphate by a metal-dependent phosphonoacetate hydrolase similar to the P. fluorescens enzyme described above. Here we report the genetic and biochemical characterization of this novel pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chemical reagents used in this study were obtained from Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Pittsburgh, PA) and were used without further purification. Media components were purchased from Thermo Fisher Scientific or VWR (West Chester, PA).

**Bacterial Strains, Plasmids, and Culture Conditions**—The strains and plasmids used in this study are listed in supplemental Table S1. E. coli strains were grown at 37 °C unless indicated otherwise. S. meliloti strains were grown at 30 °C. Luria-Bertani (LB) liquid or solid media were used for most purposes with the addition of appropriate antibiotics at the following concentrations: 200 μg/ml neomycin, 100 μg/ml ampicillin, 50 μg/ml kanamycin. SOC media used for transformation of E. coli DH5α Apir and E. coli BL21 (DE3) cells with plasmid DNA was prepared as previously described (21). To test for utilization of various phosphorus sources, strains were first grown to saturation at 30 °C in 0.2% (w/v) glucose-MOPS medium (22) containing phosphate (50 μM), biotin (100 ng/ml) and L-methionine (5 μg/ml). Subcultures were then inoculated into 0.2% (w/v) glucose-MOPS medium containing biotin and L-methionine and the desired phosphorus source at 500 μM. Growth was measured by monitoring optical density at 410 nm using a Bausch & Lomb Spectronic 21 spectrophotometer.

**DNA Isolation and Manipulation**—All cloning procedures were done using established cloning methods (23). Restriction endonucleases and T4 DNA ligase were purchased from Invitrogen (Carlsbad, CA). Plasmid DNA was isolated using the Qia-gen (Valencia, CA) Miniprep kit. The Qiagen QIAquick kit was used for the purification of DNA fragments from enzymatic reactions and agarose gels. PCR amplifications of DNA fragments were done using high-fidelity KOD polymerase (Novagen, EMD Chemicals, Inc., Gibbstown, NJ). FailSafe PCR 2×J premix buffer purchased from Epicentre (Madison, WI) was used in all of the PCR amplifications. Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). The recombinant plasmids were verified by DNA sequencing at the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois, Urbana-Champaign.

S. meliloti 1021 (WM5130) crude DNA was prepared by scraping a single colony into 100 μl of sterile water followed by incubation at 100 °C for 5 min. The cell debris was removed by centrifugation at 14,000 × g for 5 min.

**NMR Spectroscopy and Mass Spectrometry (MS) Instrumentation**—All NMR experiments were performed at the Varian Oxford Center for Excellence in NMR laboratory at the University of Oxford.
University of Illinois, Urbana-Champaign. The presence of phosphorus-containing compounds was detected using $^3$P-decoupled $^3$P NMR spectroscopy. All of the spectra were collected in H$_2$O supplemented with 20% (v/v) D$_2$O as a lock solvent. The $^3$P NMR spectra were externally referenced to an 85% (w/w) phosphoric acid standard (0 ppm). Spectra were acquired at room temperature on a Varian Unity Inova-600 spectrometer. The spectrometer was equipped with a 5-mm Varian 600DB AutoX probe with ProTune accessory tuned for phosphorus at 242.789 MHz and proton at 599.764 MHz.

For MS analysis samples were diluted 1:10 in 80% methanol/0.1% NH$_4$OH and directly infused into a custom 11T LTQ-FT mass spectrometer (Thermo Fisher Scientific) using an Advion NanoMate 100 nanospray source (Advion Biosciences, Ithaca, NY). Mass spectra were acquired manually from the mass spectrometer (Thermo Fisher Scientific) using an Advion 600DB AutoX probe with ProTune accessory tuned for phosphorus containing compounds was detected using $^1$H-decoupled $^1$H NMR spectroscopy. All of the spectra were collected in T-3 GACACGCTGAGCTCTCCGGCGTCTGCACCAATGATTAG-1’-3’ and 5’-GGGGCCGAGTTTCTGGCACTCGGCCG-3’ (SacI site is underlined) and 5’-GGGGCCGACCTTCAGTTATGGAGATATACATATGACGAACGCGGAGG-3’ (BamHI site is underlined) and ligated into PETDuet-1 vector at XbaI and EcoRV restriction sites to give plasmid pHCO04. The pHCO04 plasmid DNA was isolated and the phnY gene fragment was removed via NdeI and XhoI digestion. The resulting fragment was then ligated into PET28a at the same sites to give plasmid pHnY-N-His. The phnA gene was amplified by PCR using primers 5’-GGGGCCGCCATATGAACAGATGTAGG-3’ (Ndel) and 5’-GGGGCCGCCAGCTTTCATAAGCCCGCCTCCGCAC-3’ ( HindIII). The PCR-amplified phnA gene fragment was digested with NdeI and HindIII restriction endonucleases and ligated into the same sites of PET28a vector to give plasmid pHnA-N-His.

PhnY-N-His and PhnA-N-His Protein Purification—Plasmids pHnY-N-His and pHnA-N-His were used to transform the expression strain E. coli BL21 (DE3). E. coli BL21/pPhnY-N-His (MMG431) and E. coli BL21/pPhnA-N-His (MMG433) were grown at 37 °C in LB media containing 50 μg/ml of kanamycin to an A$_{600}$ of ~0.6. The cultures were then transferred to an 18 °C incubator, and the protein production was induced with 0.2 mM IPTG overnight. Cells were pelleted by centrifugation and stored at ~80 °C until further use.

The PhnY-N-His and PhnA-N-His proteins were purified using affinity purification with Ni-NTA resin (QIAGen). The cell pellet was thawed and resuspended in 30 ml of lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 10% (v/v) glycerol, 10 mM imidazole, pH 8.0). Lysozyme (1 mg/ml) was added and the suspension was incubated on ice for 30 min. The cells were disrupted by two passages through a French pressure cell (20,000 psi) and the cell debris was removed by centrifugation (35,000 × g, 4 °C, 30 min). The resulting supernatant was agitated gently with 6 ml of Ni-NTA resin at 4 °C for 3 h. The suspension was loaded onto an empty column and the flow through fraction was collected. The unbound proteins were removed by washing with lysis buffer containing 20 mM imidazole until the concentration of the protein in eluent decreased to the background level, as monitored by the visual test with Bradford reagent. The bound proteins were eluted with lysis buffer containing 250 mM imidazole. The elution fractions containing desired protein (as detected by SDS-PAGE) were concentrated to 2.5 ml using an Amicon Ultra YM-30 centrifugal filter unit (Millipore, Billerica, MA). The concentrated protein was loaded onto a PD-10 desalting column (GE Healthcare, Piscataway, NY), and eluted with 3.5 ml of 50 mM HEPES-K, 0.2 M NaCl, 10% (v/v) glycerol, pH 7.5 according to the column manufacturer’s instructions. The purified protein sample was...
2-Aminoethylphosphonate Degradation in Sinorhizobium meliloti

concentrated to ~0.5 ml using a YM-30 centrifugal filter unit, centrifuged to remove precipitated particles, aliquoted, and flash frozen with liquid nitrogen prior to storing at ~80 °C. Protein concentrations were determined using an absorbance at 280 nm and extinction coefficients estimated by Geneious Software (Auckland, New Zealand). SDS-PAGE analysis of purified proteins was consistent with the expected molecular masses of 55,076 Da for PhnY-N-His and 48,419 Da for PhnA-N-His.

Preparation of PnAA Substrate for PhnY Activity Assay—The PnAA substrate was enzymatically prepared immediately prior to use in PhnY activity assays as previously described (29). The reaction mixture contained 50 mM HEPES (pH 7.5), 1 mM MgCl₂, 10 mM phosphoenolpyruvic acid monopotassium salt (PEP), and 1.5 mM thiamine pyrophosphate chloride (TPP) in a total volume of 500 μl. The formation of PnAA was furnished by the addition of RhH-N-His and Ppd-Bf-His enzymes at 0.08 mM and 0.04 mM, respectively, and incubation at 30 °C for 40 min. The proteins were removed using a Microcon YM-30 centrifugal unit (Millipore, Billerica, MA). The conversion of PEP to PnAA was complete as assessed by 31P NMR analysis. The filtrate was estimated to contain 10 mM PnAA and was used without further purification.

PhnY-N-His Reaction Analysis by 31P NMR Spectroscopy—PnAA stock solution (500 μl) was prepared as described above except that 80 mM sodium cacodylate buffer (pH 7.5) was used in place of HEPES to simplify the analysis of 1H NMR spectra. NAD⁺ (10 mM) and PhnY-N-His (14 μM) were added and the reaction mixture was incubated at 30 °C for 1 h. The proteins were removed using Microcon YM-30 centrifugal unit which was pre-washed with the assay buffer to remove traces of glycerol. Chelex 100 resin (Bio-Rad, 142–2822) was added, and sample was incubated at room temperature for 20 min to remove Mg⁺⁺ ions causing broadening of NMR signals. Resin was removed, D₂O (20%, v/v) was added to the supernatant, and the sample was subjected to analysis by 31P, 1H, and 1H-31P gHMBC NMR spectroscopy. A quantitative conversion of PnAA (31P chemical shift (δ) of 9.9 ppm, 1H δ: 9.37 [t, 4.5 Hz, 1H], 2.70 [dd, 4.5, 20.1 Hz]) to a product with a 31P resonance at 14.8 ppm and 1H resonance at 2.34 ppm [d, 20.4 Hz] was confirmed by spiking of the sample with PnA authentic standard followed by 31P and 1H NMR analysis. High resolution MS analysis was used to verify that PhnAA (calculated for C₁₀H₁₉O₃P (M-H)⁻ 122.9853, found 122.9852) was converted to PnA (calculated for C₇H₁₈O₁₅P (M-H)⁻ 138.9802, found 138.9801) in the presence of PhnY-N-His.

PhnY-N-His Enzyme Kinetics—The assay mixtures (1 ml total volume) containing 50 mM HEPES-K (pH 7.5), 1 mM MgCl₂, 0.29 μM PhnY-N-His, and NAD⁺ with final concentrations of 0–460 μM were preincubated at 30 °C for 5 min. The reaction was initiated by the addition of 50 μl PnAA stock solution to 0.5 mM and the absorbance at 340 nm was measured using a Cary 4000 spectrophotometer (Varian, Palo Alto, CA). The assay was maintained at 30 °C throughout the measurement. The assays were done in triplicate. The initial rates were calculated using an extinction coefficient ε (NADH) = 6200 M⁻¹ cm⁻¹ and were fit to the Michaelis-Menten equation (V₀ = ([S]·Vₘₐₓ)/(Kₘ + [S])) using the IGOR Pro 6.1 software package (WaveMetrics, Portland, OR). The assays with variable amounts of PnAA were done in a similar manner except that 0.25 μM PhnY-N-His and 1 mM NAD⁺ was used and the reaction was initiated by the addition of PnAA stock solutions to final concentrations of 0–100 μM (20 μl to 980 μl of prewarmed assay mix). The assays containing nicotinamide adenine dinucleotide phosphate (NADP⁺, 5 mM) were run as above.

PhnA-N-His Enzyme Kinetics—The formation of inorganic phosphate from PnA by the action of PhnA-N-His was detected by a discontinuous assay using the Malachite Green phosphate assay kit (BioAssay Systems, Hayward, CA). Assay mixtures (500 μl total volume) containing 50 mM HEPES-K (pH 7.5) and 0.42 μM PhnA-N-His were preincubated at 30 °C for 8 min, and the reaction was initiated by the addition of PnAA stock solutions to final concentrations of 0–400 μM. Aliquots of the reaction mixture (80 μl) were taken out every 20 s for 2 min, added to 20 μl of Malachite Green reagent prepared as per the manufacturer’s instruction, and incubated at room temperature for 30 min. The assays were done in triplicate. The absorbance at 620 nm was plotted against the reaction time; and the rate of A₆₃₀ increase was converted to the rate of phosphate formation using a linear calibration curve prepared with known concentrations of inorganic phosphate standard (0–40 μM). The initial rates of phosphate formation were fit to the Michaelis-Menten equation as described above to determine steady state kinetic parameters of PhnA-N-His.

To evaluate divalent metal dependence of PhnA-N-His, 10 μM ZnCl₂ was added to the assay mixture prior to the preincubation, and assays were performed as described above. Metal-free PhnA-N-His was prepared by the treatment of the protein with 8.3 mM EDTA at 4 °C for 3 h with gentle agitation followed by size exclusion chromatography using a PD-10 column. The resulting metal-free PhnA-N-His was tested in the activity assay as described above with and without addition of ZnCl₂.

RESULTS

Identification of a Putative Gene Cluster for 2-AEPn Degradation in S. meliloti—Analysis of the S. meliloti 1021 genome sequence (NCBI accession number NC_003078) led to the identification of a putative gene cluster for the degradation of 2-AEPn (Fig. 2C). Based on the annotation of individual open reading frames (ORFs), we hypothesize that enzymatic degradation of 2-AEPn proceeds as depicted in Fig. 3. Extracellular 2-AEPn could be taken up either by a putative phosphate transporter (ORF2), or an ABC-type transporter (encoded by ORF3). PhnA would then be oxidized to PnA by phosphonoacetate dehydrogenase (encoded by ORF5, also named phnW). PhnA would then be oxidized to PnA by phosphonoacetate dehydrogenase (encoded by ORF5, also named phnW). PhnA would then be oxidized to PnA by phosphonoacetate dehydrogenase (encoded by ORF5, also named phnW).
F** FIGURE 2.** Operons for phosphonate utilization in *S. meliloti* and *E. coli.* A, *S. meliloti* C-P lyase genes, occupy two different loci on pSymB plasmid; B, C-P lyase operon in *E. coli.* C, Putative gene cluster for 2-AEPn degradation in *S. meliloti* 1021 and bioinformatic-based annotation of its components. The regions of the *S. meliloti* genome deleted in ΔphnF-L and ΔphnW-Y mutants are indicated in the panels A and C, respectively.

**FIGURE 3.** Proposed pathway for 2-AEPn degradation in *S. meliloti* 1021.

Construction and Characterization of *S. meliloti* phn Mutants—To assess the in vivo role of the putative 2-AEPn catabolism genes we constructed a set of *S. meliloti* mutants lacking the phn genes and examined their ability to utilize various phosphonates as sole phosphorus sources (Fig. 4). A negative control without any phosphorus source and a positive control lacking the C-P catabolism genes we constructed a set of mutants—To assess the putative 2-AEPn catabolic pathway (Fig. 2). A negative control without any phosphorus source and a positive control without any phosphorus source and a positive control lacking the C-P catabolism genes we constructed a set of mutants lacking the genes needed to synthesize C-P lyase (Fig. 2A), a ΔphnW-Y strain lacking genes encoding the putative 2-AEPn catabolic pathway (Fig. 2C), and a double ΔphnF-L ΔphnW-Y mutant lacking both sets of genes.

Consistent with previous reports (16), the wild-type strain is capable of growth on all phosphorus sources tested. The ΔphnF-L mutant is incapable of using methylphosphonate (MnP), a substrate that can only be used via the C-P lyase pathway, but remains capable of using 2-AEPn. We also observed that the ΔphnF-L mutant is able to utilize PnA after prolonged incubation, suggesting that the organism has an alternate catabolic route for this compound as well. The phenotype of the ΔphnW-Y strain is indistinguishable from the wild type. Thus, these genes are not required for use of phosphonic acids when the C-P lyase genes are intact. In contrast, the ΔphnF-L, ΔphnW-Y double mutant is incapable of using any phosphonic acids, although it grows if P is provided. This result shows that the phnWAY locus is required for the use of 2-AEPn in the absence of C-P lyase. Moreover, the double mutant did not grow on PnA, even after extended incubation times. Thus, the phnWAY locus is also required for the alternate PnA catabolic pathway.

**NMR and Steady State Kinetics Analysis of the Reaction Catalyzed by Recombinant PhnY**—To confirm the proposed function of phnY gene (ORF5), it was cloned into the *E. coli* expression vector pET28a. The hexahistidine-tagged fusion protein PhnY-N-His was expressed and purified using Ni-NTA affinity chromatography. SDS-PAGE analysis of the purified protein was consistent with the expected monomer molecular weight of 55.1 kDa for PhnY-N-His. Enzymatic activity of PhnY-N-His was assayed in the presence of PnAA and NAD using 31P and 1H NMR spectroscopy and high resolution MS. A quantitative conversion of PnAA to PnA was observed supporting the assignment of PhnY as a PnAA dehydrogenase (Fig. 5).

The steady state kinetic parameters of the PhnY-N-His-catalyzed reaction were determined using a continuous spectrophotometric assay detecting the formation of NADH product at 340 nm. The initial rate data were fit to the Michaelis-Menten equation and are shown in Fig. 6, A and B. The kinetic parameters obtained are summarized in Table 1. The PhnY-N-His reaction has $K_m$ values of $103.9 \pm 7.5 \mu M$ and $2.6 \pm 0.4 \mu M$ for NAD and PnAA, respectively, and a turnover number ($k_{cat}$) value of $2 \times 10^3$ s$^{-1}$. The $k_{cat}/K_m$ values were $2.2 \times 10^4$ M$^{-1}$ s$^{-1}$ and $7.5 \times 10^5$ M$^{-1}$ s$^{-1}$ for NAD and PnAA, respectively. When NAD cofactor was replaced with NADP, no NADPH formation was detected in the spectrophotometric assay indicating the co-substrate specificity of PhnY-N-His for NAD$^+$.

**Steady State Kinetics Analysis of the Reaction Catalyzed by Recombinant PhnA**—The phnA (ORF4) gene was expressed in *E. coli* and the hexahistidine-tagged fusion protein PhnA-N-His was purified in a similar manner. SDS-PAGE analysis of purified PhnA-N-His was consistent with the expected monomer molecular mass of 48.4 kDa. The initial rates of PnA hydrolysis catalyzed by PhnA-N-His were determined using a discontinuous colorimetric assay based on the formation of a green complex with an absorbance at 620 nm between one of the products of the reaction, inorganic phosphate, and a Malachite Green reagent. The steady state kinetics analysis (Fig. 6C and Table 1) established a $K_m$ value for PnA of $22.1 \pm 2.3 \mu M$, a $k_{cat}$ value of 0.9 s$^{-1}$, and $k_{cat}/K_m$ of $4.1 \times 10^5$ M$^{-1}$ s$^{-1}$. When 10 $\mu M$ ZnCl$_2$ was added to the PhnA-N-His assay mixture, there was a small increase in $k_{cat}$ (1.1 s$^{-1}$), but due to a small increase in $K_m$, the overall catalytic efficiency $k_{cat}/K_m$ was lower (1.4-fold, Table 1). However, the preincubation of as-isolated PhnA-N-His with EDTA followed by the removal of chelating agent resulted in inactive protein indicating that enzymatic activity of PhnA-N-His is metal-dependent. When 10 $\mu M$ ZnCl$_2$ was
added to the assay with EDTA-treated PhnA-N-His, the catalytic activity was restored to $k_{\text{cat}} = 1.2 \text{ s}^{-1}$ (Table 1).

**DISCUSSION**

The genetic and biochemical data reported above fully support the presence in *S. meliloti* 1021 of the novel 2-AEPn catabolic pathway shown in Fig. 3. Thus, the *phnWAY* locus is required for 2-AEPn catabolism in C-P lyase-deficient mutants, while the PhnY and PhnA enzymes were shown to possess the predicted biochemical activities. Moreover, the *phnWAY* locus also allows use of PnA when C-P lyase is absent; however *phnWAY*-dependent growth on this substrate is poor relative to C-P lyase-dependent growth. Thus, it is clear that the *phnWAY* locus has evolved specifically for use of 2-AEPn as a substrate.

![FIGURE 4. Growth of *S. meliloti* strains using various phosphorus sources. Triplicate cultures of the indicated strains were grown and monitored as described under “Experimental Procedures.” Strains used were *S. meliloti* 1021 (wild type), *S. meliloti* 6154 (Δ*phnF-L*), *S. meliloti* 7341 (Δ*phnW-Y*), and *S. meliloti* 7345 (Δ*phnF-L, ΔphnW-Y*). Growth curves for various phosphorus sources are color-coded as follows: inorganic phosphate (red, ■), 2-AEPn (maroon, ▲), MPn (green, ●), PnA (purple, ×), absence of phosphorous (blue, ○).](image)

![FIGURE 5. $^{31}$P NMR analysis of the reaction catalyzed by PhnY-N-His.](image)

A. PnAA substrate used in the assay; B. NAD and PhnY-N-His were reacted with PnAA. A quantitative conversion of PnAA to PnA was observed in the presence of NAD and PhnY-N-His.

![FIGURE 6. Steady state kinetics analysis of reactions catalyzed by PhnY-N-His (panel A: variable NAD concentration, panel B: variable PnAA) and PhnA-N-His (panel C: in the absence (○) and presence (●) of Zn$^{2+}$).](image)

Although a number of bacterial genes in the NCBI data base encoding homologs of aldehyde dehydrogenases (30, 31) have been annotated as PnAA dehydrogenases (presumably based...
on the genomic context), no biochemical characterization of these enzymes has been reported prior to this study. We have established that PhnY-N-His is proficient in converting PnAA to PnA in the presence of NAD⁺ as cofactor. Steady state kinetic parameters of PhnY-N-His (Kₑₚ NAD⁺ = 103.9 ± 7.5 μM, Kₑₚ PhnAA = 2.6 ± 0.4 μM, Kₑᵥ = 2 s⁻¹, kₑKat/Kₑₚ NAD⁺ = 2.2 × 10⁴ M⁻¹ s⁻¹, kₑKat/Kₑₚ PhnAA = 7.5 × 10⁵ M⁻¹ s⁻¹) are comparable to those of its characterized bacterial homologs. For example, an NAD⁺-dependent glyceraldehyde-3-phosphate dehydrogenase GAPN from Streptococcus mutans (34% amino acid sequence identity to PhnY) catalyzes oxidation of d-glyceraldehyde-3-phosphate (d-G3P) with Michaelis-Menten parameters Kₑₑₚ NAD⁺ = 24.5 μM, Kₑₑₚ d-G3P = 50 μM, and kₑₑKat = 67 s⁻¹ (32). An NAD⁺-dependent aldehyde dehydrogenase AldA from E. coli (32% identity) accepts a variety of aldehyde substrates, such as glycolaldehyde, l-glyceraldehyde, and l-lactaldehyde, with turnover numbers (kₑₑKat) and Kₑₑ values in the ranges 2–17 s⁻¹ and 40–1000 μM, respectively, and Kₑₑₑₑ NAD⁺ = 120–280 μM, depending on the substrate tested (33). It remains to be investigated whether PhnY possesses similar relaxed substrate specificity, but we have determined that PhnY is only active in the presence of an NAD⁺ and not NAD⁺⁺ as cofactor. This finding is consistent with the presence of Glu-184 in the amino acid sequence of PhnY, as this residue is thought to be involved in the binding of the 2'- and 3'-hydroxyls of the adenine ribose, thereby providing cofactor specificity for NAD⁺ over NAD⁺⁺.

PhnA, a PhnA hydrolase from S. meliloti and a homolog of PhnA from P. fluorescens 23F (amino acid sequence identity of 56%, NCBI accession number AAC15507), belongs to the alkaline phosphatase superfamily of enzymes that includes phosphatases (e.g. phosphatases and sulfatases), isomerases, and transfersases (35, 36). Enzymes of this superfamily share a conserved active site containing two metal ions (usually zinc) essential for the substrate binding and catalysis. We established that PhnA from S. meliloti efficiently catalyzes the hydrolysis of PnA to acetate and phosphate with a Kₑₑₚ PhnA value of 22.1 ± 2.3 μM and a kₑₑKat value of 0.9 s⁻¹. The activity of PhnA from P. fluorescens is dependent on the presence of various metal ions with Zn²⁺ providing the highest (2-fold at 1 mM ZnCl₂) increase in activity as compared with the as-isolated form (10). We tested the effect of Zn²⁺ (at 10 μM) on the activity of the enzyme from S. meliloti and found that it only provided a small (1.2-fold) increase in the turnover number. However, the treatment of S. meliloti PhnA with chelating agent rendered it inactive confirming its dependence on the presence of metal ion(s). Reconstitution of the apo protein with Zn²⁺ (at 10 μM) resulted in a complete restoration of the activity to as-isolated level. Hence, metal-dependence of S. meliloti PhnA is consistent with its assignment to the alkaline phosphatase superfamily. The studies of the effect of other metal ions on the activity of PhnA from S. meliloti are to be published elsewhere.

Finally, it should be noted that gene clusters similar to the phnWAY locus can be found in dozens of prokaryotic genomes, mostly within the α-proteobacteria (data not shown). This finding suggests that the alternate pathway for 2-AEPn catabolism described herein is common in bacteria and highlights the importance of this C-P compound in the global phosphorus cycle.

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TABLE 1

| Substrate | kₑₑKat | Kₑₑ | kₑₑKat/Kₑₑ |
|-----------|--------|-----|-------------|
| PhnY-N-His: NAD⁺⁺ | 2.26 ± 0.05 | 103.9 ± 7.5 | 2.2 × 10⁴ |
| PhnY-N-His: PhnAA | 1.92 ± 0.06 | 2.6 ± 0.4 | 7.5 × 10² |
| PhnA-N-His: PhnAA | 0.91 ± 0.03 | 22.1 ± 2.3 | 4.1 × 10⁴ |
| PhnA-N-His + Zn²⁺: PhnAA | 1.06 ± 0.02 | 36.8 ± 1.8 | 2.9 × 10⁴ |
| PhnA-N-His/EDTA: product not detected | 1.16 ± 0.03 | 30.3 ± 3.0 | 3.8 × 10⁴ |
| PhnA | 3 | 1250 | 2.4 × 10² |
| PhnA-P. fluorescens | | | |

JUNE 24, 2011 • VOLUME 286 • NUMBER 25

JOURNAL OF BIOLOGICAL CHEMISTRY

22289
2-Aminoethylphosphonate Degradation in Sinorhizobium meliloti

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