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Utilization of Glyphosate as Phosphate Source: Biochemistry and Genetics of Bacterial Carbon-Phosphorus Lyase

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SUMMARY

After several decades of use of glyphosate, the active ingredient in weed killers such as Roundup, in fields, forests, and gardens, the biochemical pathway of transformation of glyphosate phosphorus to a useful phosphorus source for microorganisms has been disclosed. Glyphosate is a member of a large group of chemicals, phosphonic acids or phosphonates, which are characterized by a carbon-phosphorus bond. This is in contrast to the general phosphorus compounds utilized and metabolized by microorganisms. Here phosphorus is found as phosphoric acid or phosphate ion, phosphoric acid esters, or phosphoric acid anhydrides. The latter compounds contain phosphorus that is bound only to oxygen. Hydrolytic, oxidative, and radical-based mechanisms for carbon-phosphorus bond cleavage have been described. This review deals with the radical-based mechanism employed by the carbon-phosphorus lyase of the carbon-phosphorus lyase pathway, which involves reactions for activation of phosphonate, carbon-phosphorus bond cleavage, and further chemical transformation before a useful phosphate ion is generated in a series of seven or eight enzyme-catalyzed reactions. The *phn* genes, encoding the enzymes for this pathway, are widespread among bacterial species. The processes are described with emphasis on glyphosate as a substrate. Additionally, the catabolism of glyphosate is intimately connected with that of aminomethylphosphonate, which is also treated in this review. Results of physiological and genetic analyses are combined with those of bioinformatics analyses.

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INTRODUCTION

N-Phosphonomethylglycine (glyphosate) has become the most widely used herbicide in agriculture, in silviculture, and in urban environments as the active ingredient of weed-killer formulations such as Roundup. Glyphosate is spread on foliage, where it is taken up and transported to growth areas for exertion of inhibition (1). The compound is a competitive inhibitor of phosphoenolpyruvate of plant 5-enolpyruvylshikimate 3-phosphate synthase and thus prevents the biosynthesis of the aromatic amino acids phenylalanine, tryptophan, and tyrosine as well as some benzoic acid derivatives, thereby killing the plant. The versatility of the compound is based on the acquisition of either of two mechanisms in plants: (i) resistance or (ii) tolerance to glyphosate. A review of the mechanisms of resistance and tolerance has been published (2). Both mechanisms depend on the genetic engineering of bacterial genes into plant cells.

In contrast to the enzyme of plant origin, 5-enolpyruvylshikimate 3-phosphate synthase activity (encoded by *aroA*) of many bacterial species is insensitive to glyphosate. Thus, insertion and expression in a plant cell of an *aroA* gene from *Agrobacterium* render the cell resistant to glyphosate (3, 4). Similar results were obtained with mutant variants of the *aroA* gene of *Salmonella enterica* serovar Typhimurium (5) or *Escherichia coli* (6). Tolerance to glyphosate is obtained by different mechanisms. Here the compound is enzymatically modified or removed from the cell, whereas the target of inhibitory action remains unaltered. In general, glyphosate catabolism by microorganisms proceeds by either of two pathways. First, cleavage of the carbon-phosphorus (C-P) bond by an enzyme with the trivial name C-P lyase results in the formation of *N*-methylglycine, also known as sarcosine, and a phosphorus-containing molecule (7, 8). The methyl moiety of *N*-methylglycine may be transferred to tetrahydrofolic acid derivatives for further metabolism through one-carbon metabolic pathways and with the simultaneous formation of glycine (9) or, in the presence of dioxygen, *N*-methylglycine may be converted to hydrogen peroxide, formaldehyde, and glycine (10). Second, cleavage of a carboxymethylene-nitrogen bond results in the formation of aminomethylphosphonic acid (AMPA) and glyoxylate in a reaction catalyzed by an oxidase, i.e., an enzyme, which utilizes dioxygen as an oxidizing agent. Glyoxylate in turn is oxidized to carbon dioxide by the glyoxylic acid cycle, whereas AMPA is further catabolized by acetylation followed by cleavage of the C-P bond through reactions, which include C-P lyase. Figure 1 shows the two pathways: C-P lyase catalyzed versus oxidase catalyzed. Insertion and expression of the *Ochrobactrum anthropi* strain LBAA *gox* gene, encoding an oxidase with affinity for glyphosate in a plant cell, render the cell tolerant to glyphosate by removal of the compound (11, 12). A variant of the tolerance mechanism involves the *Bacillus licheniformis yitI*-encoded enzyme, which acetylates glyphosate at the nitrogen. *N*-Acetylglyphosate does not inhibit 5-enolpyruvylshikimate 3-phosphate synthase (13). The physiological function of neither the *gox* gene nor the *yitI* gene product is known. Often, an *aroA* gene or a *gox* gene has been manipulated *in vitro* to specify enzymes with improved kinetic parameters before insertion into plants (2). Finally, glyphosate tolerance in *E. coli* may be acquired by overexpressing a gene, *yhhS*, presumably encoding a membrane-located efflux transporter (14). The physiological ligand for this transporter is currently unknown, but pentose phosphates have been suggested as possible ligands (15). It remains to be seen if this efflux transporter may contribute to glyphosate tolerance in plants.

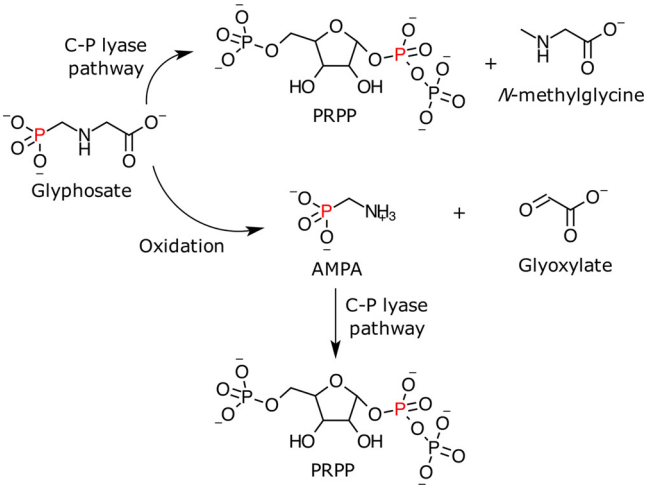

FIG 1 Pathways for biological extraction of glyphosate-phosphorus. Glyphosate may be cleaved through the *phn*-specified C-P lyase pathway with the formation of PRPP and *N*-methylglycine or by the oxidation of C_2 by glyphosate oxidase with the formation of AMPA and glyoxylate. AMPA-phosphorus in turn may be converted to PRPP through the *phn*-specified C-P lyase pathway, which also results in the formation of *N*-methylacetamide (see the text for details).

Although plants in general are sensitive to glyphosate, exceptions to this have also been observed, as limited uptake and translocation and enhanced metabolic degradation may contribute to glyphosate tolerance (16). In addition, glyphosate resistance by various mechanisms has evolved in natural weed populations due to the extensive use of the herbicide for crop protection (reviewed in reference 17).

Glyphosate is readily removed from soil by microbial metabolic activity (18). These processes result in the formation of phosphorus-, carbon-, or nitrogen-containing compounds, some of which microbes can use in their metabolism. In spite of 40 years of glyphosate utilization, detailed knowledge of the biochemical mechanism of conversion of glyphosate-phosphorus to phosphoric acid or phosphate ion (P_i) is only presently emerging. The present review deals with the transformation of glyphosate-phosphorus to usable phosphorus compounds, a process harbored by a number of microbial species. As AMPA is a prominent catabolic intermediate of glyphosate, a description of the catabolism of AMPA is included as well.

TRANSPORT AND ACCUMULATION OF GLYPHOSATE AND AMPA

The utilization of glyphosate depends on the specificity of the catabolic enzyme system as well as that of the polypeptides responsible for the internalization of the compound. Transport of glyphosate has been analyzed in two instances. Thus, quantitative measurements with [3-^{14}C]glyphosate have been conducted in *Arthrobacter* sp. strain GLP-1 and *Pseudomonas* sp. strain PG2982, both of which were isolated by enrichment with glyphosate as a P_i source. In both cases, catabolism functioned simultaneously with transport. The former therefore very likely affected the latter. Additionally, the uptake of glyphosate was genetically regulated by the phosphorus source, as described further below. The K_m values

for glyphosate uptake were 23 and 125 μM for *Pseudomonas* sp. strain PG2982 and *Arthrobacter* sp. strain GLP-1, respectively (19, 20). Furthermore, measurements of intracellular pool sizes of metabolites revealed that *O. anthropi* strain GPK3 and *Achromobacter* sp. strain MPS contained detectable amounts of glyphosate following growth of the species in the presence of glyphosate, providing evidence for the existence of a transport mechanism for the compound. Both of these strains were isolated from phosphonate-contaminated soil (21).

In general, phosphonic acids are internalized by an ATP-binding cassette (ABC) transport system consisting of a periplasmic binding protein for phosphonate (encoded by *phnD* in *E. coli*) (22), an ATP-binding polypeptide (*phnC*), as well as a membrane-spanning transporter polypeptide (*phnE*). The *phnD* gene specifies a polypeptide that contains a signal sequence for transmembrane export to the periplasm, and indeed, the PhnD polypeptide of *E. coli* has been shown to be able to bind phosphonate (22). Thus, by engineering the *phnD* gene to specify the Q267C variant, containing a single cysteine, and subsequently adding acrylodan to the cysteinyl residue as a fluorophore, the dissociation constant (K_d) values were determined for a number of phosphonates. The values obtained for methylphosphonate, 2-aminethylphosphonate, AMPA, and glyphosate were 1 μM, 5 nM, 5 μM, and 0.65 mM, respectively. For comparison, the K_d value for P_i was 50 μM (22). These data show variations over >5 orders of magnitude for the efficiency of phosphonate binding and, presumably, uptake of phosphonate. In particular, the binding of glyphosate to PhnD is poor, and the compound may not be transported at all. The *E. coli* K-12 wild-type *phnE* allele has been shown to contain an 8-bp duplication, which renders phosphonate utilization cryptic (23, 24). A genetic switch mechanism has been shown to be responsible for the removal and reversible reinsertion of this 8-bp duplication in *E. coli* K-12 (25, 26). Amino acid sequence alignments with known ABC transport system components were originally used to assign a function of PhnC, PhnD, and PhnE (23). Additional evidence for *phnCDE* specifying a transport system was provided by analyses of phosphoserine as a P_i source in *phn* mutants. Thus, in *E. coli*, phosphoserine may be dephosphorylated by phosphoserine phosphatase (specified by *serB*) (27) or by periplasmic alkaline phosphatase (specified by *phoA*) (28). Additionally, *phoA* Δ*phn* strains are unable to use phosphoserine as a P_i source, whereas *phoA* Δ*phn* strains provided with the *phnCDE* genes in *trans* may utilize phosphoserine as a P_i source. Apparently, PhnC, PhnD, and PhnE are able to transport phosphoserine and make the compound available to cytoplasmic phosphoserine phosphatase (29). Figure 2 shows the genetic organization of *phn* operons from a variety of species and strains. The cistrons encoding transport functions are labeled in red, and the genes have been assigned letters similar to those of *E. coli* genes (*phnC*, *phnD*, and *phnE*). A survey of these operons in organisms containing C-P lyase-coding capacity reveals two possible organizations of the cistrons specifying the transport polypeptides, either *phnCDE*, as in *E. coli*, *Burkholderia pseudomallei*, and the *Pseudomonas stutzeri phn* operon, or *phnCDEE*, i.e., with two distinct transmembrane polypeptides, as in *Sinorhizobium meliloti*, *O. anthropi*, *Agrobacterium radiobacter*, *Mesorhizobium loti*, *Bradyrhizobium japonicum*, *Nostoc* sp. strain PCC7120, and *Trichodesmium erythraeum*. In addition, the transport polypeptide-encoding cistrons of the *P. stutzeri htx* operon are organized somewhat differently. However, the *htx* operon is involved in uptake and oxidation of hypophosphite in addition to phosphonate (30). The actual position of the *phnCDE*(*E*) cistrons may vary from species to species. Thus, the *E. coli phnCDE* cistrons are cotranscribed with and located upstream of the remaining C-P lyase pathway-encoding cistrons. In *O. anthropi*, *M. loti*, and *B. pseudomallei*, the *phnCDEE* cistrons are transcribed in the same direction as the C-P lyase pathway-encoding cistrons, but they are located downstream of these. In *S. meliloti*, the *phnCDEE* cistrons are located far away from the C-P lyase pathway-encoding cistrons *phnFGHIKLO*. Finally, in *Nostoc* sp. PCC7120, the *phnCDE*(*E*) cistrons are located close to the C-P lyase pathway-encoding cistrons but are transcribed divergently.

CARBON-PHOSPHORUS LYASE PATHWAY OF *E. COLI* AND *P. STUTZERI*

The C-P lyase enzyme system is widespread among bacteria. Thus, analyses of the sequenced genomes for phosphonate degradation pathways revealed that C-P lyase was the most abundant pathway next to the phosphonate phosphohydrolase pathway (31). In general, a bacterial species contains a single C-P lyase pathway-encoding operon. Exceptions to this generality are *P. stutzeri*, which contains two distinct operons, *phn* and *htx*, both of which specify polypeptides involved in catabolism of phosphonate (30); *M. loti*, which contains coding capability for three distinct C-P lyases (Fig. 2) (32); and *Marinobacter aquaeolei* VT8 (33). The latter organism contains four putative C-P lyase-encoding operons, with two located on the chromosome (*phnFDCEGHIJKLMNP* and *phnDCEMGHIJKLMNP*) (GenBank accession no. CP000514.1) and the other two located on two plasmids, pMAQU01 (*phnDCEMGHIJKLMNP*) (accession no. CP000515.1) and pMAQU02 (*phnFDCEGHIJKLMNP*) (accession no. CP000516.1). Remarkably, the nucleotide sequences of a DNA fragment of 18,589 bp, encompassing the *phnFDCEGHIJKLMNP* operon as well as four genes upstream of *phnF* and one gene downstream of *phnP* in the chromosome and in pMAQU02, are identical. Another exception is *Arthrobacter* sp. GLP-1, which has been shown to contain two C-P lyase systems, as evaluated on the basis of differential induction of the synthesis of C-P lyase activities for methylphosphonate and glyphosate and, similarly, differential inhibition by organophosphonates of C-P lyase activities for methylphosphonate and glyphosate (34). BLAST analysis (35) with a variety of *phn* gene sequences as a query revealed no C-P lyase-encoding genes within the sequenced genomes of the genus *Arthrobacter*. Additionally, methylphosphonate growth-proficient *Achromobacter* sp. strain MPS12 could be adapted to growth with glyphosate as the sole P_i source, apparently by synthesizing a second glyphosate-specific C-P lyase, and *O. anthropi* strain GPK3 has been postulated to contain two C-P lyases, one specific for methylphosphonate and the other specific for glyphosate (21). The C-P lyase-encoding genes and their organization in a number of organisms are described in detail below. First, the *phn* operon as well as the C-P lyase of *E. coli*, followed by that of *P. stutzeri*, are described. Second, data for organisms which utilize glyphosate as a P_i source are reviewed, i.e., *O. anthropi*, *S. meliloti*, *A. radiobacter*, *B. pseudomallei*, and *Nostoc* sp. PCC7120, followed by data for the soil bacteria *M. loti* and *B. japonicum*. Finally, data are reviewed for the two phosphonic acid growth-proficient species *T. erythraeum* and *Acidithiobacillus ferrooxidans*. Operon structures were established by using program packages at the Integrated Microbial Genomes website (http://img.jgi.doe.gov/) (36) and the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) (37).

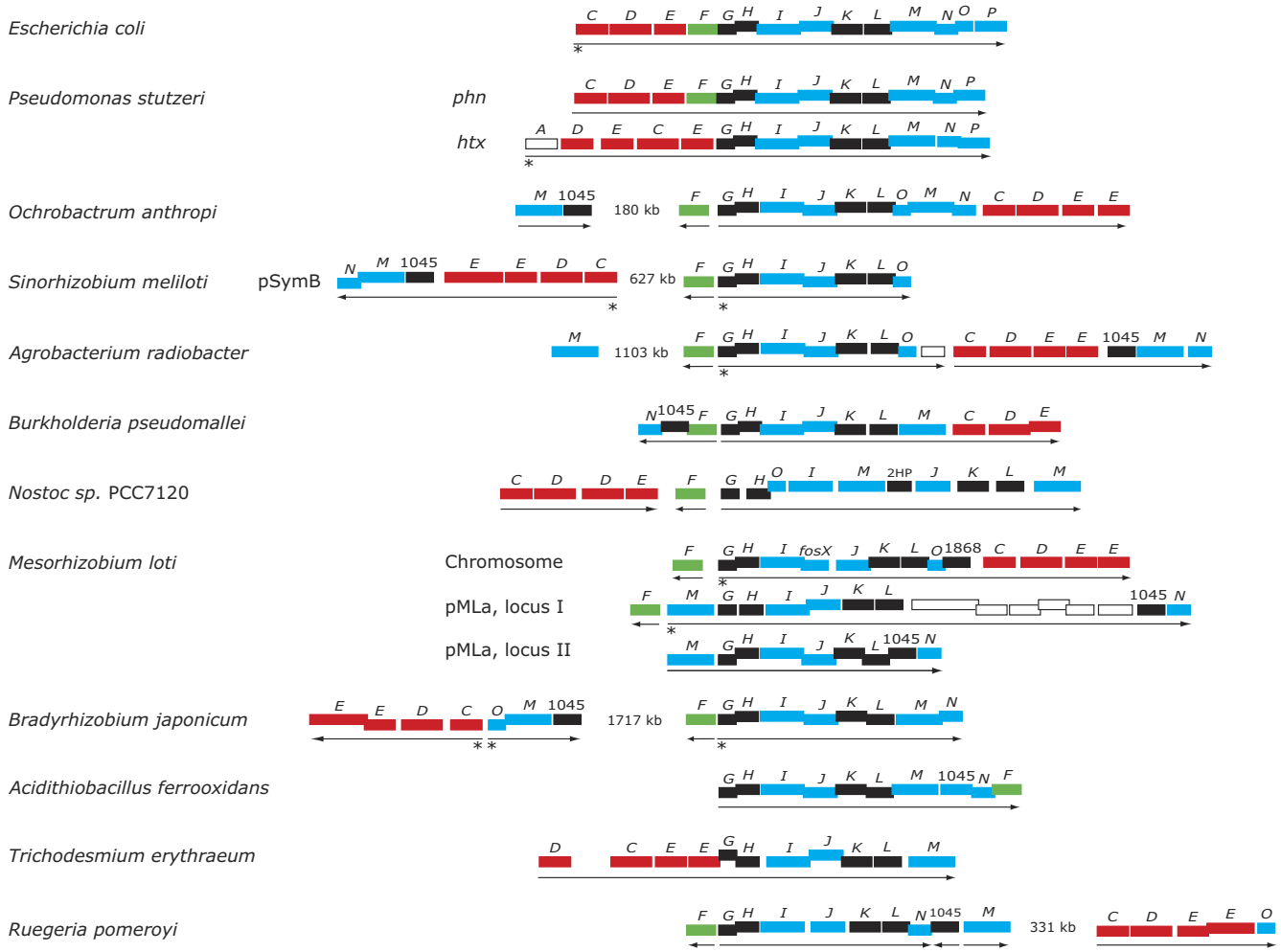

FIG 2 Organization of C-P lyase pathway-encoding operons in various microbial species and strains. Each open reading frame is shown as a rectangle, with the *phn* gene designation shown above in italics. Displacement of an open reading frame relative to the previous open reading frame indicates overlapping reading frames. Genes for transport proteins are labeled in red. A nomenclature has been adopted so that genes encoding periplasmic binding proteins are labeled *D*, and genes encoding a nucleotide-binding protein are labeled *C*, whereas genes encoding a membrane-spanning protein are labeled *E*. In some cases, this nomenclature may deviate from the originally published gene designations. Genes for products with known enzymatic activity in phosphonate catabolism are labeled in blue, and genes specifying putative regulatory proteins are labeled in green, whereas genes specifying auxiliary polypeptides involved in phosphonate catabolism are labeled in black. Genes apparently not involved in phosphonate catabolism are unfilled and unlabeled. In some cases, more than one cistron appears to encode homologous proteins, e.g., *phnM* of *O. anthropi*. 1045 indicates the domain of unknown function 1045 gene (encoding DUF1045). It is included as phosphonate degradation relevant, as it is usually located in proximity to *phn* genes (129). Similarly, 1868 indicates the gene encoding DUF1868, whereas 2HP designates a gene that may specify a member of the two-histidine phosphodiesterase superfamily (see the text for details). The designations of the *P. stutzeri htx* operon cistrons have been altered (except for *htxA*) to underline the similarity with other phosphonate catabolism operons. The *htx* operon was originally designated *htxABCDEFGHIJKLMN* (30). Similarly, the *S. meliloti* and *B. japonicum phnCDEE* genes were originally designated *phoCDET* (119, 130, 131). Numbers expressed in kilobases indicate interoperon distances. Arrows beneath the genes indicate the direction of transcription and attempts to estimate the boundaries of individual transcripts. Contiguous arrows do not necessarily indicate operon expression, except for the *E. coli phnC-P*, *A. ferrooxidans phnG-M*, and *P. stutzeri htxA-P* genes, where operon expression has been experimentally confirmed (29, 30, 76). An asterisk under an arrow indicates that a Pho box has been identified in the DNA sequence and, thus, that transcription may be regulated by the P_i supply. Unless otherwise indicated, operons are located in the chromosomes. The GenBank accession numbers of the nucleotide sequences employed are U00096.2 for *E. coli* (132), AF061267 (*htx*) and AY505177 (*phn*) for *P. stutzeri* (117), CP000758.1 for *O. anthropi* ATCC 49188 (133), AL591985 (plasmid pSymB) and AL591688 (chromosome) for *S. meliloti* 1021 (57, 58), CP000628.1 for *A. radiobacter* K84 (63), BA000012.4 (chromosome) and BA000013.4 (plasmid pMLa) for *M. loti* MAFF303099 (32, 134), BA000040.2 for *B. japonicum* USDA 110 (130, 135), CP002833.1 (chromosome 1) for *B. pseudomallei* 1026b (136), BA000019.2 for *Nostoc* sp. PCC7120 (70), CP001219.1 for *A. ferrooxidans* ATCC 23270 (77), CP000393.1 for *T. erythraeum* IMS101, and CP000031.2 for *R. pomeroyi* DSS-3 (137).

Escherichia coli

The *phn* operon. Although *E. coli* is unable to use glyphosate as a P_i source, it is included here because the C-P lyase pathway from this organism may be regarded as the "standard" C-P lyase pathway. Phosphonate utilization requires the expression of 14 polypeptides, specified by the *phnCDEFGHIJKLMNOP* operon (Fig. 2) (23). A summary of the assigned biochemical functions of each of these 14 polypeptides is given in Table 1. As mentioned above, 3 of the 14 cistrons (*phnCDE*) encode an ATP-binding cassette transport system, where PhnD is a periplasmic binding protein for phosphonic acids (22), PhnE is believed to be a membrane-spanning polypeptide, and PhnC is believed to be an ATP-

TABLE 1 Biochemical function of *E. coli phn* operon-specified polypeptides

Phn polypeptide	Activity and/or function	Reference(s)
PhnC	ABC transport system, nucleotide-binding protein	23, 24
PhnD	ABC transport system, periplasmic binding protein	22–24
PhnE	ABC transport system, membrane-spanning protein	23, 24
PhnF	Regulatory protein	23, 24, 38
PhnG	Purine ribonucleoside triphosphate phosphonylase component, component of PhnGHIJK	40, 41
PhnH	Purine ribonucleoside triphosphate phosphonylase component, component of PhnGHIJK	40, 41
PhnI	Purine ribonucleoside triphosphate phosphonylase component, component of PhnGHIJK	40, 41
PhnJ	C-P lyase, component of PhnGHIJK	40, 41
PhnK	Component of PhnGHIJK, accessory protein, nucleotide-binding protein of an ABC transport system	23, 40
PhnL	Purine ribonucleoside triphosphate phosphonylase component or nucleotide-binding protein of an ABC transport system	23, 41
PhnM	5′-Triphosphoribosyl 1′-phosphonate diphosphohydrolase	41
PhnN	Ribosyl bisphosphate phosphokinase	52
PhnO	Aminoalkylphosphonate *N*-acetyltransferase	39, 101
PhnP	Phosphoribosyl cyclic phosphodiesterase	42, 49

binding polypeptide (23, 24). *phnF* presumably is a regulatory gene. This function was assigned by amino acid sequence comparison of *E. coli* PhnF with *Mycobacterium smegmatis* PhnF, which negatively regulates the expression of a P_i transport system encoded by *phnDCE* (38). The remaining 10 polypeptides are either enzymes responsible for the transformation of phosphonic acid phosphorus to the product 5-phosphoribosyl 1-diphosphate (PRPP) or auxiliary but essential polypeptides in phosphonate utilization, as described further below. The catabolism of 1-aminoalkylphosphonic acids such as AMPA requires all 10 polypeptides (PhnG, PhnH, PhnI, PhnJ, PhnK, PhnL, PhnM, PhnN, PhnO, and PhnP), whereas the catabolism of alkylphosphonates, arylphosphonates, and aminoalkylphosphonates with the amino group at carbon 2 or higher does not require PhnO (39). Thus, rather than specifying a single enzyme, C-P lyase, the *phn* operon specifies enzymes for a small pathway, hence the C-P lyase pathway.

The C-P lyase pathway. Recent research has shed light on most of the biochemical pathways by which phosphonate is transformed to phosphate. The chemical reactions of the pathway with glyphosate and AMPA as examples are shown in Fig. 3. A multisubunit protein complex, PhnGHIJK, was identified (40). No enzymatic activity of PhnGHIJK was demonstrated, but *in vitro* analysis has shown that PhnI has ribonucleoside 5′-triphosphate nucleosidase activity (ATP/GTP + H_2O → ribosyl 5-triphosphate + adenine/guanine), whereas PhnI in the presence of PhnG, PhnH, and PhnL has ribonucleoside 5′-triphosphate phosphonylase activity (ATP/GTP + methylphosphonic acid → 5′-triphosphoribosyl 1′-methylphosphonate + adenine/guanine) (EC 2.7.8.37 [α-D-ribose 1-methylphosphonate 5-triphosphate synthase]). The enzyme uses ATP and GTP as phosphonyl acceptors, with almost identical k_{cat}/K_m values (3.4×10^5 M^{-1} s^{-1}) (41). The phosphonylase activity is dependent on the presence of PhnG, PhnH, PhnI, and PhnL, whereas PhnK is not necessary. Further research is required to clarify the role of each of the polypeptides in this phosphonylase reaction. The reaction mechanism of the phosphonylase may bear some mechanistic resemblance to that of (deoxy)ribonucleoside phosphorylases, which convert (deoxy)ribonucleoside and P_i to ribosyl 1-phosphate and the corresponding nucleic acid base. In the following step of the C-P lyase pathway, the β,γ-diphosphoryl group of 5′-triphosphoribosyl 1′-methylphosphonate is hydrolyzed, and 5′-phosphoribosyl 1′-methylphosphonate is generated in a reaction catalyzed by PhnM, hence 5′-triphosphoribosyl 1′-phosphonate diphosphohydrolase (EC 3.6.1.63 [α-D-ribose 1-methylphosphonate 5-triphosphate diphosphatase]) (41). Similar reactions occur in the catabolism of glyphosate or AMPA (Fig. 3).

5′-Phosphoribosyl 1′-phosphonate was previously suggested to be the substrate for C-P lyase, the product being an alkane and 5-phosphoribosyl 1,2-cyclic phosphate (42). Indeed, *in vitro* analysis showed that this was the case, as the purified *phnJ* gene product is able to catalyze the cleavage of 5′-phosphoribosyl 1′-methylphosphonate with the formation of methane and 5-phosphoribosyl 1,2-cyclic phosphate as the products. Thus, *phnJ* specifies C-P lyase (EC 4.7.1.1 [α-D-ribose 1-methylphosphonate 5-phosphate C-P lyase]). Furthermore, the compound 5′-phosphoribosyl 1′-(*N*-2-acetamidoethylphosphonate) was isolated from a culture of a *phnP* mutant strain grown in the presence of 2-aminoethylphosphonate. 5′-Phosphoribosyl 1′-(*N*-2-acetamidoethylphosphonate) is analogous to the C-P lyase substrate of cells grown with methylphosphonate. The PhnJ-catalyzed reaction requires anaerobic conditions and *S*-adenosylmethionine, which is converted to 5′-deoxyadenosine and methionine. Spectroscopy revealed the possibility of a 4Fe-4S cluster, and with the observation of the presence of four cysteine residues within PhnJ, the data are consistent with a radical reaction mechanism typical of the radical *S*-adenosylmethionine/4Fe-4S enzyme superfamily. Three cysteine residues (Cys241, Cys244, and Cys266) are involved in the formation of the 4Fe-4S cluster, whereas Cys272 is believed to take part in the catalytic cycle as a thiyl radical (41, 43). Additionally, these data are consistent with results obtained >2 decades ago by Frost and coworkers, who demonstrated that C-P bond cleavage by C-P lyase occurs by a radical reaction mechanism (44–46). Also, amino acid sequence alignments of Phn polypeptides revealed a remarkable evolutionary conservation of PhnJ compared to other Phn polypeptides, consistent with the critical enzymatic function of PhnJ (40, 47). The 2-hydroxyl of the phosphoribosyl moiety plays a crucial role in C-P lyase catalysis, possibly in turning over a covalent PhnJ-phosphoribosyl intermediate. Thus, the reactions leading from the free phosphonate to the C-P lyase substrate 5′-phosphoribosyl 1′-phosphonate may be regarded as steps necessary for activation of the substrate (48).

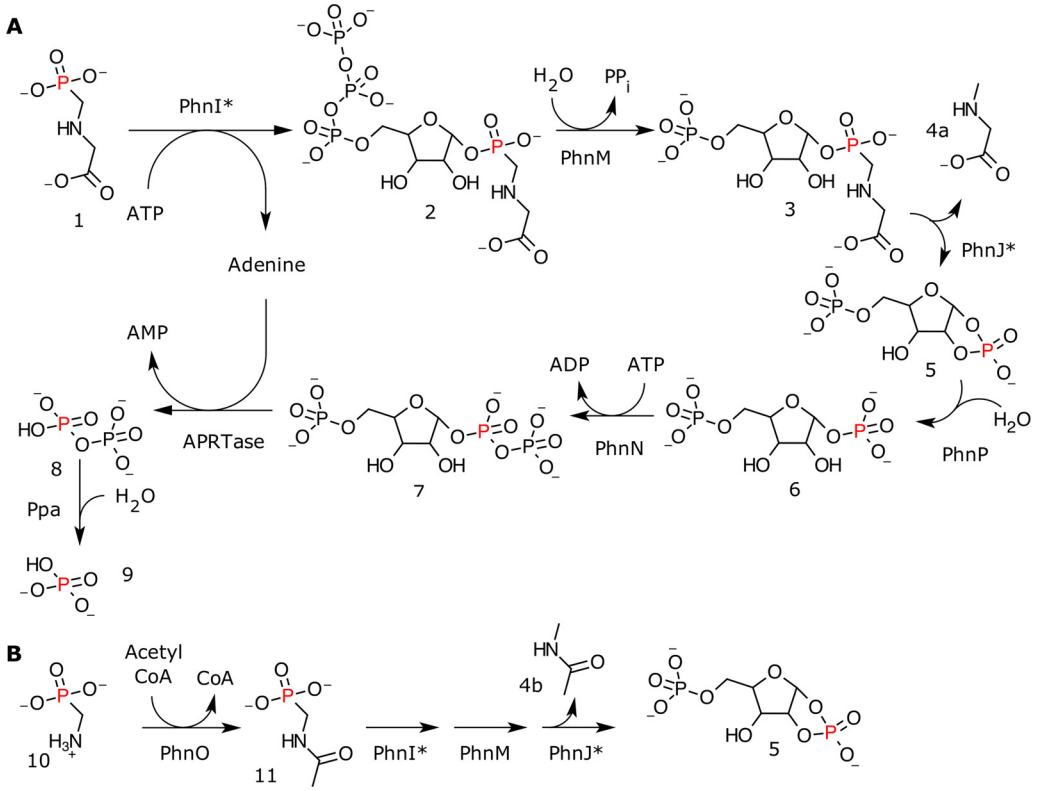

FIG 3 Pathways for the conversion of phosphorus of glyphosate and AMPA to P_i. (A) Conversion of glyphosate. Compounds: 1, glyphosate; 2, 5′-triphospho-α-D-ribosyl 1′-(*N*-phosphonomethylglycine); 3, 5′-phospho-α-D-ribosyl 1′-(*N*-phosphonomethylglycine); 4a, *N*-methylglycine; 5, 5-phospho-α-D-ribosyl 1,2-cyclic phosphate; 6, α-D-ribosyl 1,5-bisphosphate; 7, 5-phospho-α-D-ribosyl 1-diphosphate (PRPP); 8, diphosphate ion (PP_i); 9, phosphate ion (P_i). Reactions are indicated by their enzymes as follows. PhnI* indicates purine ribonucleoside 5′-tri/diphosphate phosphonylase, an enzyme complex where PhnI plays a crucial catalytic role and which may also involve PhnG, PhnH, PhnJ, PhnK, and/or PhnL. PhnM is 5′-triphospho-α-D-ribosyl 1′-phosphonate diphosphohydrolase. PhnJ* indicates *S*-adenosylmethionine-dependent carbon-phosphorus lyase. PhnJ* may constitute a protein complex also containing PhnG, PhnH, PhnI, PhnK, and/or PhnL. PhnI* and PhnJ* may be the same protein complex. PhnP is the *phnP*-specified phosphoribosyl cyclic phosphodiesterase. PhnN is the *phnN*-specified ribosyl bisphosphate phosphokinase. APRTase is the *apt*-specified adenine phosphoribosyltransferase. Ppa is the *ppa*-specified inorganic diphosphate hydrolase (138). The enzymes catalyzing the latter two reactions are not specified by the *phn* operon. APRTase was arbitrarily chosen among the 10 phosphoribosyltransferases of *E. coli* (53). Any of these 10 enzymes may participate in the process. (B) Conversion of AMPA. Compounds: 10, AMPA; 11, *N*-acetamidomethylphosphonate; 4b, *N*-methylacetamide; compound 5 is described above for panel A. Compound 5 is further catabolized as described above for panel A. PhnO is aminoalkylphosphonate *N*-acetyltransferase, and PhnI*, PhnM, and PhnJ* are described above for panel A. The pathways were established on the basis of previously reported data (39–41, 52, 139, 140).

The formation of 5-phosphoribosyl 1,2-cyclic phosphate completes the conversion of the phosphonate phosphorus to a phosphate ester, where the phosphonate phosphorus is the 1,2-cyclic phosphate. Next, 5-phosphoribosyl 1,2-cyclic phosphate is hydrolyzed to ribosyl 1,5-bisphosphate in a reaction catalyzed by *phnP*-specified phosphoribosyl cyclic phosphodiesterase (EC 3.1.4.55 [phosphoribosyl 1,2-cyclic phosphate phosphodiesterase]) (42). This enzyme is a member of the β-lactamase family of metal ion-dependent hydrolases, and, typically for this family of enzymes, it is a homodimer and contains a dinuclear active site with two Mn^{2+} ions as well as a structural Zn^{2+} ion. The enzyme has activity toward a number of phosphodiesters besides the physiological substrate (49, 50). Mutants defective in *phnP* have proven valuable in elucidating the structure of the components of the C-P lyase pathway. *phnP* mutants not only accumulate substrates of phosphoribosyl cyclic phosphodiesterase (42, 51) but also accumulate substrates of the previous enzyme of the pathway, i.e., C-P lyase. An example is 5′-phosphoribosyl 1′-(2-*N*-acetamidoethylphosphonate), which is formed in cells thriving on 2-aminoethylphosphonate. In addition, the dephosphorylated derivatives ribosyl 1,2-cyclic phosphate, α-D-ribosyl 1′-(2-*N*-acetamidoethylphosphonate), and α-D-ribosyl 1′-ethylphosphonate have been identified in the growth medium of a *phnP* mutant strain. The latter compound accumulates in a *phnP* mutant strain supplemented with ethylphosphonate (42). The dephosphorylated compounds very likely appear in the growth medium after intracellular buildup of the 5- or 5′-phosphorylated compounds and subsequent excretion with simultaneous hydrolysis of the 5- or 5′-phosphate ester.

Ribosyl 1,5-bisphosphate in turn is a substrate for *phnN*-specified ribosyl bisphosphate phosphokinase (EC 2.7.4.23), whose reaction product is PRPP (52). Genetic analysis has shown that *phnN* is dispensable for phosphonate utilization in *E. coli*, although the growth of *phnN* mutant strains on methylphosphonate as a P_i source is impaired compared to the growth of *phn*$^+$ strains (29). The fact that *phnN* mutant strains grow with phosphonate at all may be explained by the presence of a *phnN*-redundant activity (Fig. 4). The physiology of ribosyl 1,5-bisphosphate metabolism in *E. coli* is only poorly understood. However, the compound is also synthesized in cells that are not exposed to

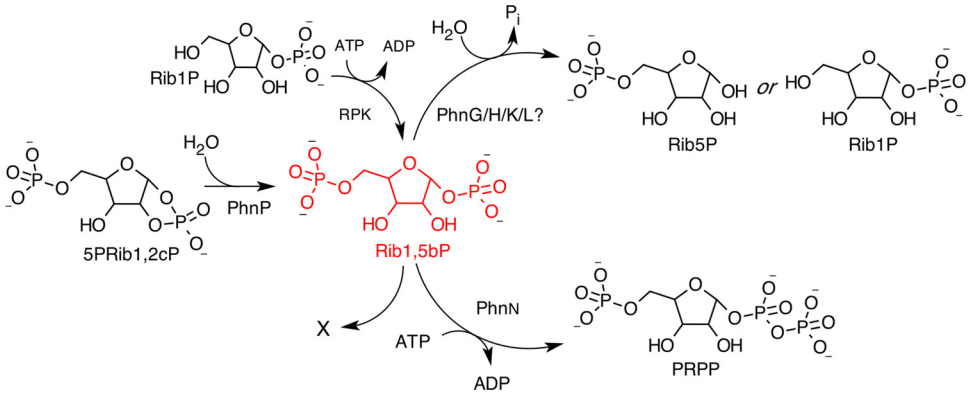

FIG 4 Metabolic pathways of ribosyl 1,5-bisphosphate of *E. coli*. α-D-Ribosyl 1,5-bisphosphate (Rib1,5bP) is shown at the center in red. The compound is generated by the activity of phosphoribosyl cyclic phosphodiesterase (PhnP) (42) and presumably by the activity of ribosyl 1-phosphate kinase (RPK) (52). Ribosyl 1,5-bisphosphate may be further converted to PRPP by ribosyl 1,5-bisphosphate phosphokinase (PhnN) and presumably to some unknown metabolite, metabolite X (52). The possibility of hydrolysis with the formation of P_i by a *phn* operon-specified gene product (PhnG, PhnH, PhnK, or PhnL or a combination thereof) is indicated by PhnG/H/K/L?. Presently, there is no evidence for this activity of any *phn*-specified polypeptide.

phosphonate, presumably by the activity of ribosyl 1-phosphate kinase, and similarly, the compound can also be removed by enzymatic activity in a reaction leading from ribosyl 1,5-bisphosphate to an unknown compound, compound "X" (Fig. 4) (52). Additionally, the possibility of the existence of a *phn*-specified phosphohydrolase highly specific for ribosyl 1,5-bisphosphate is indicated in Fig. 4 by the reaction leading from ribosyl 1,5-bisphosphate to ribosyl 1- or 5-phosphate. Candidates for this phosphohydrolase are PhnG, PhnH, PhnK, and PhnL, to which a biochemical function has not yet been assigned. However, this hypothesis seems less attractive, as a ribosyl 1,5-bisphosphate phosphohydrolase activity would also remove ribosyl 1,5-bisphosphate synthesized for other purposes, at least under conditions where phosphonate catabolism is active.

A biochemical function of *phnN*-specified ribosyl bisphosphate phosphokinase was assigned as follows. A PRPP-less strain of *E. coli* deleted for the *prs* gene (Δ*prs*), encoding PRPP synthase, requires purine and pyrimidine compounds, the amino acids histidine and tryptophan, as well as NAD. NAD requirement suppression mutants were isolated and found to have lesions in the *pstSCAB-phoU* operon, i.e., constitutive in Pho regulon expression. Thus, the mutants had the genotype Δ*prs pstSCAB-phoU*. Genetic analysis revealed that the NAD requirement suppression phenotype resulted from elevated expression levels of the *phnN* gene. Additionally, expression of the *phnN* gene in the parent strain (i.e., Δ*prs*) was sufficient to create the NAD requirement suppression phenotype. It was therefore concluded that the substrate for PhnN, ribosyl 1,5-bisphosphate, is a metabolite of *E. coli* also in cells which do not catabolize phosphonate (52). Presumably, therefore, ribosyl 1,5-bisphosphate is synthesized from some unknown source and is metabolized to some unknown product(s). This may explain why the *phnN* gene is dispensable for phosphonate utilization: a different metabolic pathway handles the substrate of the *phnN* gene product, ribosyl 1,5-bisphosphate. Although a biochemical function has now been assigned to the vast majority of the polypeptides specified by the *phn* operon, an alternative pathway for P_i formation cannot be ruled out. Thus, there is no doubt that the product of C-P lyase is 5-phosphoribosyl 1,2-cyclic phosphate and that this compound is hydrolyzed to ribosyl 1,5-bisphosphate by PhnP. Subsequently, a *phn*-specified substrate-specific phosphohydrolase might hydrolyze ribosyl 1,5-bisphosphate to P_i and ribosyl 1- or 5-phosphate, followed by salvage of the ribosyl phosphate. If this were the case, *phn*-specified phosphonate degradation would generate P_i by two different pathways, one of which was PhnN directed and the other of which was directed by an unidentified *phn* operon-specified enzyme. *phnG*, *phnH*, *phnK*, or *phnL* might be a candidate for this activity. Thus, a double mutant (*phnN phnG*, *phnN phnH*, *phnN phnK*, or *phnN phnL*) would be even more impaired in phosphonate utilization than a *phnN* strain. Although *phnG*, *phnH*, *phnK*, *phnL*, and *phnN* mutants exist and can be combined, the outcome may be predicted as a phosphonate-negative phenotype. The mutations are knockout mutations, and presumably, deletion of either *phnG*, *phnH*, or *phnK* may result in the formation of an abortive PhnGHIJK protein complex.

The *phnO* gene encodes an aminoalkylphosphonate *N*-acetyltransferase (EC 2.4.2.—). The *phnO* cistron is essential for utilization of 1-aminoalkylphosphonates such as AMPA and *S*-1-aminoethylphosphonate, whereas *phnO* is dispensable for utilization of alkylphosphonate. Aminoalkylphosphonate *N*-acetyltransferase is furthermore necessary for the detoxification of the cell wall synthesis inhibitor (*S*)-1-aminoethylphosphonate (39). Aminoalkylphosphonate *N*-acetyltransferase is dealt with further below.

Although a specific function has not been assigned to all of the Phn polypeptides, it is evident from the description above that the PhnJ polypeptide is C-P lyase and that there are quite a number of auxiliary proteins and enzymes, all of which are necessary for a complete transformation of phosphonate phosphorus.

Formation of P_i. Contrary to general belief, *phn*-specified enzymes of *E. coli* likely do not convert phosphonic acid-phosphorus to P_i. Rather, the product of the activities of the *phn*-specified enzymes appears to be PRPP, where the phosphorus of the α-phosphate at the C_1 hydroxyl originates from phosphonic acid-phosphorus. The sum of these reactions with methylphosphonate as the substrate is methylphosphonate + 2ATP + $2H_2O \rightarrow CH_4$ + PRP*P + inorganic diphosphate (PP_i) + ADP + adenine, where the asterisk is placed after the phosphorus originating from phosphonate. *S*-Adenosylmethionine and an electron of unknown origin as the substrates and methionine and 5′-deoxyadenosine as the products are omitted from the sum of the reaction. The gen-

eration of P_i may be accomplished by reactions that are catalyzed by non-*phn*-specified enzymes. First, PRPP is used by a phosphoribosyltransferase (PRTase), which phosphoribosylates aromatic bases (or glutamine-derived ammonia) with the formation of β-*N*-glycosidic bonds of ribonucleoside 5′-monophosphates (or 5-phosphoribosyl β-1-amine) and PP_i according to the following general scheme: aromatic base + PRPP → ribonucleoside 5′-monophosphate + PP_i. This PP_i contains the original phosphonic acid phosphorus. PRTases are ubiquitous among microorganisms. Organisms such as *E. coli* contain 10 enzymes that utilize PRPP as a substrate. The nitrogen-containing substrate for these reactions, with the gene(s) encoding the PRTases given in parentheses, is adenine (*apt*), guanine (*gpt* and *hpt*), xanthine (*gpt*), hypoxanthine (*hpt* and *gpt*), uracil (*upp*), orotic acid (*pyrE*), quinolinic acid (*nadC*), nicotinamide (*pncB*), anthranilic acid (*trpD*), or ATP (*hisG*). A variant utilizes glutamine-derived ammonia (*purF*). These PRTases are constituents of the *de novo* as well as the so-called salvage pathways for purine, pyrimidine, and pyridine nucleotide biosynthesis and the *de novo* pathways for biosynthesis of the amino acids tryptophan and histidine (reviewed in reference 53). Second, the formation of P_i is completed by the activity of *ppa*-specified inorganic diphosphatase, which hydrolyzes PP_i with the formation of P_i. The inclusion of adenine PRTase and inorganic diphosphatase in the conversion of methylphosphonate results in the following sum of reactions: methylphosphonate + 2ATP + $4H_2O$ → CH_4 + $4P_i$ + 5′-AMP + ADP. As before, *S*-adenosylmethionine, an electron, methionine, and 5′-deoxyadenosine are omitted from the sum of the reaction. Thus, C-P lyase may have evolved so as to take advantage of existing metabolic pathways within the cell. The utilization of PRTases and diphosphatase for the terminal steps of the C-P lyase pathway is attractive because these enzymes are present at all times. At least the PRTases involved in purine, pyrimidine, and pyridine nucleotide salvage (i.e., those encoded by *apt*, *hpt*, *gpt*, *upp*, and *pncB*) are believed to be synthesized constitutively. Likewise, in the absence of purine, pyrimidine, pyridine, histidine, or tryptophan, PRTase activity will be present (53). Also, transcriptome analysis revealed that the expression level of the *E. coli purF* gene, encoding the PRTase glutamine PRPP amidotransferase, increases following phosphate exhaustion (54). Although PRTases are dispensable for *E. coli*, the hypothesis that they are necessary for phosphonate catabolism predicts that the knockout of all 10 PRTase genes renders *E. coli* phosphonate growth deficient. Finally, diphosphatase is essential for *E. coli* (138).

An exception to this general pathway with PRPP as the product of a *phn*-specified C-P lyase pathway is represented by the enzyme 5-phosphoribosyl 1,2-cyclic phosphate dihydrolase, which catalyzes the sequential hydrolytic reactions of the C-P lyase product: 5-phosphoribosyl 1,2-cyclic phosphate → ribosyl 2,5-bisphosphate → ribosyl 5-phosphate + P_i. The gene encoding 5-phosphoribosyl 1,2-cyclic phosphate dihydrolase has been isolated from the Gram-positive organisms *Eggerthella lenta* and *Clostridium difficile*, and the enzyme has been characterized (55). BLAST analysis showed that the enzyme has limited evolutionary dissemination.

Pseudomonas stutzeri

Similarly to *E. coli*, *P. stutzeri* is unable to use glyphosate as a P_i source. However, *P. stutzeri* is included here as an organism in which two different operons specifying C-P lyase, *phnCDEFGHIJKLMNP* and *htxABCDEFGHIJKLMN*, have been genetically identified. *phnCDE* and *htxBCD* likely encode ABC transport systems for phosphonate, whereas the remaining 10 cistrons of each operon (*phnFGHIJKLMNP* and *htxEFGHIJKLMN*) encode polypeptides that are homologous to those encoded by *E. coli phnFGHIJKLMNP*, i.e., a regulatory protein (*phnF* or *htxE*); polypeptides constituting a protein complex that includes C-P lyase activity (*phnJ* or *htxI*); a ribonucleoside 5′-triphosphate phosphonylase (*phnI* or *htxH*); some auxiliary polypeptides (*phnGHK* or *htxFGJ*); as well as three additional enzymes necessary for phosphonate utilization, 5′-triphosphoribosyl 1′-phosphonate diphosphohydrolase (*phnM* or *htxL*), phosphoribosyl cyclic phosphodiesterase (*phnP* or *htxN*), and ribosylbisphosphate phosphokinase (*phnN* or *htxM*). Neither the *phn* operon nor the *htx* operon contains a *phnO* homolog (30). The *P. stutzeri htxA* gene encodes the enzyme hypophosphite:2-oxoglutarate dioxygenase, which catalyzes the oxidation of hypophosphite to phosphite. Phosphite in turn may be converted to P_i by C-P lyase pathway enzymes. In spite of these two C-P lyase-specifying operons, *P. stutzeri* is unable to utilize glyphosate as a P_i source. Furthermore, there is a marked difference in substrate specificity among the two enzyme systems, where *htx*-specified enzymes catabolize only methylphosphonate and ethylphosphonate, whereas *phn*-specified enzymes catabolize a broad spectrum of phosphonates similar to that of *E. coli* C-P lyase. However, as pointed out by the authors of that study, this differential substrate specificity may rely on the specificity of the transport system as well as that of C-P lyase (30). Studies with mutant variants of *phn* and *htx* were performed with a strain deleted for either operon or both (i.e., Δ*htx*, Δ*phn*, or Δ*htx* Δ*phn*).

TABLE 2 Effect of AMPA on utilization of methylphosphonate as a P_i source in *P. stutzeri*[a]

Strain	Presence of operon		Growth response with phosphorus-containing compound(s)				
	hxt	*phn*	None	MePn	AMPA	AMPA + MePn	MePn + AMPA + P_i
WM567	+	+	−	++	−	−	++
WM1614	Δ	+	−	++	−	−	++
WM3514	+	Δ	−	+	−	+	++
WM3516	Δ	Δ	−	−	−	−	++

[a] Cells were streaked on phosphate-free morpholinepropanesulfonic acid (MOPS) minimal agar containing glucose (0.2%) (141), and the indicated phosphorus-containing compounds were added at 0.2 mM (methylphosphonate [MePN] and P_i) or 2 mM (AMPA) and incubated at 37°C for 48 h. Growth was recorded as follows: −, no growth; +, poor growth; ++, almost wild-type growth.

Interestingly, the polypeptides specified by the *phn* and *htx* operons showed lower similarity to one another than to C-P lyase polypeptides of other organisms. Thus, *phn*-specified polypeptides showed the highest identity to those of other pseudomonads, whereas the *htx*-specified polypeptides showed the highest identity to those of *Rhizobiaceae*, which suggests that the two operons originate from different organisms (30).

P. stutzeri is able to use methylphosphonate as the P_i source, provided that either the *phn* or the *htx* operon is functional (30). In contrast, AMPA could not be used as a P_i source by *P. stutzeri* (Table 2). When both methylphosphonate and AMPA were present, with the latter compound at a 10-fold surplus, the two strains with a functional *phn* operon (WM567 and WM1614) did not

TABLE 3 Effect of glyphosate on utilization of methylphosphonate as a P_i source in *P. stutzeri*[a]

Strain	Presence of operon		Growth response with phosphorus-containing compound(s)			
	hxt	*phn*	MePn	Glyphosate	Glyphosate + MePn	MePn + glyphosate + P_i
WM567	+	+	++	−	++	++
WM1614	Δ	+	++	−	++	++
WM3514	+	Δ	+	−	+	++
WM3516	Δ	Δ	−	−	−	++

[a] Medium and incubation are described in Table 2, with the inclusion of phenylalanine, tryptophan, and tyrosine (0.2 mM each) as well as 2-aminobenzoic acid, 2,3-dihydroxybenzoic acid, and *p*-aminobenzoic acid (1 μM each). The concentration of glyphosate was 2 mM. MePn, methylphosphonate. Growth was recorded as described in footnote *a* to Table 2.

grow. In contrast, the presence of AMPA had no effect on the utilization of methylphosphonate by the *htx*$^+$ Δ*phn* strain (WM3514). These data are most simply interpreted as follows. AMPA can be taken up by the *phn*-specified phosphonate transporter but not by that specified by *htx*. Neither the *phn*- nor the *htx*-specified C-P lyase pathway is able to handle AMPA, and finally, AMPA or a derivative of AMPA inhibits one or more enzymes of the C-P lyase pathway. These findings, i.e., the lack of growth of *P. stutzeri* with AMPA as a P_i source, are consistent with studies of aminoalkylphosphonate degradation in *E. coli*, where it was shown that catabolism of AMPA requires a wild-type allele of the *phnO* cistron and, thus, acetylation of AMPA (39). Neither the *phn* nor the *htx* operon of *P. stutzeri* contains a *phnO* ortholog. Thus, the necessity of acetylation of AMPA for catabolism may be prevalent among microorganisms. As shown in Table 3, *P. stutzeri* could not utilize glyphosate as a P_i source. When both methylphosphonate and glyphosate were present, with the latter compound at a 10-fold surplus, the strains responded as when methylphosphonate was present alone; i.e., growth was observed when either the *phn* or the *htx* operon was functional, indicating that glyphosate could not be transported by any of the strains. In conclusion, *P. stutzeri* is (i) unable to transport glyphosate and (ii) able to take up but unable to catabolize AMPA. These data are valid, at least for *P. stutzeri* strains derived from WM88 (56).

ORGANISMS THAT UTILIZE GLYPHOSATE AS A P_i SOURCE

In contrast to *E. coli* and *P. stutzeri*, several microbial species or strains have been shown to be able to utilize glyphosate as a P_i source. These organisms are *S. meliloti*, *O. anthropi*, *A. radiobacter*, *B. pseudomallei*, and *Nostoc* sp. PCC7120. The data for these organisms and strains are described below.

Ochrobactrum anthropi

Genome sequence analysis of *O. anthropi* strain ATCC 49188 revealed three possible *phn* transcriptional units: a *phnGHIJKLOMNCDEE* gene cluster or operon, a *phnM*-domain of unknown function 1045 (duf_{1045}) gene cluster or operon, as well as a monocistronic *phnF* operon located next to *phnG* but transcribed in the opposite direction relative to *phnG*. The *phnGHIJKLOMNCDEE* gene cluster and the *phnM-*duf_{1045} gene cluster are separated by 180 kb (approximately 270 open reading frames [ORFs]) (Fig. 2). All of these *phn* genes are located on chromosome 1. A similar organization is found in *O. anthropi* strain LMG 3301. Thus, these strains appear to contain a complete set of genes for a C-P lyase pathway similar to that of *E. coli*. Apparently, there are two distinct *phnE* genes, similar to what was observed for *S. meliloti*. A duf_{1045} gene is frequently found among *phn* genes, most often next to *phnN*, *phnM*, or both. The gene product DUF1045 is a member of the two-histidine phosphodiesterase superfamily, and it remains to be established if the function of DUF1045 is related to that of *phnP*-specified phosphoribosyl cyclic phosphodiesterase (31).

O. anthropi strain GPK3, isolated from glyphosate-contaminated soil, was shown to be able to grow with glyphosate as a P_i source. Small amounts of *N*-methylglycine were detected in cells grown with glyphosate, indicating C-P lyase-mediated cleavage of glyphosate (Fig. 1). It was claimed that this activity is a property of C-P lyase II (methylphosphonate cleavage being a property of C-P lyase I). No genome sequence information is available for this particular strain. It is presently unclear why strain GPK3 evolved or acquired a second C-P lyase pathway under laboratory conditions. Additionally, an oxidoreductase is responsible for the formation of AMPA and glyoxylate. Data for AMPA utilization by *O. anthropi* strain GPK3 are not available. However, the authors of that study favored an aminotransferase-phosphonate phosphohydrolase pathway rather than a C-P lyase pathway for AMPA catabolism. This conclusion was based on the observation that AMPA could be removed from cell extracts by the addition of pyruvate, which was converted to alanine, indicating the presence of an aminotransferase activity. The remaining part of AMPA subsequently was expected to be phosphonoformaldehyde, for which an enzymatic cleavage activity has not been identified (21). Perhaps the presence of glyphosate rather altered the uptake system for glyphosate and simultaneously altered the properties for the uptake of other phosphonates as well.

Sinorhizobium meliloti

The utilization of glyphosate as P_i source appears to be a natural property of *S. meliloti* 1021, as it grows with glyphosate without prior exposure to the compound. A variety of other phosphonates, such as methylphosphonate, ethylphosphonate, 2-aminoethylphosphonate, and AMPA, are also P_i sources for *S. meliloti* 1021 (47). Analysis of the genome sequence revealed that *S. meliloti* 1021 contains two *phn* loci, both of which are located on the extrachromosomal DNA molecule pSymB (57). There are a least three transcriptional units: a *phnGHIJKLO* gene cluster, which very likely is expressed as an operon; a *phnCDEE-*duf_{1045}*-phnMN* gene cluster, which may also be expressed as an operon; and, finally, a monocistronic operon, *phnF*, which is located next to the *phnGHIJKLO* gene cluster but transcribed divergently. *phnCDEE* may constitute an ABC transport system for phosphonate, where PhnC is an ATP-binding protein, PhnD is a periplasmic phosphonate-binding protein, and the two PhnE polypeptides are membrane-spanning polypeptides and constitute a permease for phosphonate. A *phnP* homolog may be present, as a gene encoding a metal ion-dependent hydrolase of β-lactamase superfamily I has been identified in the chromosome (GenBank accession no. NP_385705.1) (58), although the similarity of this amino acid sequence with that of *E. coli* PhnP is modest, <25%. Alternatively, with DUF1045 replacing PhnP, *S. meliloti* 1021 appears to contain a C-P lyase pathway similar to that of *E. coli*. It is noteworthy that *S. meliloti* contains a *phnO* gene. In accordance with the presence

of PhnO, *S. meliloti* is able to utilize AMPA as a P_i source (47). Δ*phnIJ* and Δ*phnFGHIJKL* deletion mutants were isolated and shown to be phosphonate negative. Both deletion strains retained the ability to grow on 2-aminoethylphosphonate, as *S. meliloti* 1021 contains an alternative pathway for 2-aminoethylphosphonate catabolism, as described later (47, 59).

Agrobacterium radiobacter

Agrobacterium radiobacter was isolated by enrichment cultivation with phosphonoacetic acid. It utilizes a variety of phosphonates as P_i sources, including glyphosate; methyl-, hydroxymethyl-, chloromethyl-, ethyl-, 2-aminoethyl-, vinyl-, propyl-, propenyl-, propynyl-, butyl-, and phenylphosphonate; phosphonoacetic acid; and fosfomycin. Growth of *A. radiobacter* with AMPA as a P_i source was not analyzed (60).

Genome sequence and BLAST analyses of *A. radiobacter* K84 revealed two *phn* loci. Locus 1 comprises *phnFGHIJKLO*-ORF-*phnCDEE-duf*$_{1045}$-*phnMN*, whereas locus 2 comprises a monocistronic operon, *phnM*. *phnF* is transcribed divergently from the other genes of locus 1 (Fig. 2). Thus, the genome of this organism appears to encode two polypeptides each of PhnM and PhnE. Similar to *S. meliloti* and *O. anthropi*, *A. radiobacter* contains a gene encoding DUF1045, which may be consistent with an apparent lack of a *phnP* homolog. Altogether, the coding capacity of *A. radiobacter* resembles that of *S. meliloti* and *O. anthropi*, i.e., a complete set of *phnCDEFGHIJKLMNO* (*phnC-O*) genes, a possible phosphoribosyl cyclic phosphodiesterase-encoding gene (*duf*$_{1045}$), as well as some redundancy in coding capacity (*phnE* and *phnM*). The organization of *phn* genes in *A. radiobacter* appears to be similar to that of other species of the genus *Agrobacterium*. Thus, inspection of the genomes of *Agrobacterium vitis* S4 (GenBank accession no. CP000633.1), *A. tumefaciens* strain C58 (accession no. AE007869), and *Agrobacterium* sp. strain H13-3 (accession no. CP002248) revealed identical *phn* gene orders for the four organisms, except that the latter three did not contain the open reading frame located between *phnO* and *phnC* of *A. radiobacter* (61–63). It remains to be established if the latter three strains are able to grow with glyphosate as the P_i source. The amino acid sequence specified by the ORF bears some similarity to the enzyme pyridoxamine 5-phosphate oxidase (64). A relevance of this ORF to phosphonate degradation remains to be established.

Additionally, some members of the *Rhizobiaceae* are glyphosate growth proficient (65), one example of which is *Rhizobium leguminosarum*. Strain WSM1325 contains a single *phn* locus, located on the chromosome, with the structure *phnGHIJKLOCDEE-duf*$_{1045}$-*phnMN*. These genes are transcribed in the same direction. Immediately upstream of *phnG*, a *phnF* gene is located and transcribed divergently from the other *phn* genes (GenBank accession no. CP001622.1) (66). Finally, *Rhizobium etli* strain CFN42 also has a *phnFGHIJKLOCDEE-duf*$_{1045}$-*phnMN* gene organization similar to that of *R. leguminosarum* (accession no. CP000133.1) (67).

Burkholderia pseudomallei

Burkholderia (*Pseudomonas*) *pseudomallei* strain 22, isolated from glyphosate-treated soil, is able to utilize glyphosate or AMPA as a phosphate source (68). The nucleotide sequence of the genome of this particular strain is not known. However, inspection of the genome nucleotide sequence of *B. pseudomallei* type strain 1026b reveals some properties of a possible C-P lyase pathway in this organism. A single locus contains 13 genes or cistrons organized as a *phnGHIJKLMCDE* operon and, transcribed divergently, a *phnF-duf*$_{1045}$-*phnN* operon (Fig. 2). With *duf*$_{1045}$ replacing *phnP*, *B. pseudomallei* contains a complete set of genes necessary for catabolism of alkylphosphonate. Notably, the *phn* locus of this organism does not contain a *phnO* cistron. Additionally, BLAST analysis with the deduced amino acid sequence of *E. coli* or *S. meliloti phnO* did not reveal a putative *phnO* ortholog in this organism. Thus, *B. pseudomallei* 1026b, in contrast to strain 22, is predicted to be unable to catabolize AMPA.

Nostoc

Analysis of the phylum *Cyanobacteria* for the ability to utilize glyphosate as a source of P_i revealed four species. These species are *Nostoc* (or *Anabaena*) sp. PCC7120, *Leptolyngbya boryana*, *Microcystis aeruginosa*, and *Nostoc punctiforme* (69). The genetic organization of the *phn* genes of *Nostoc* sp. PCC7120 is shown in Fig. 2. There is a single locus consisting of at least three transcriptional units. One putative transcriptional unit consists of the enzyme-specifying *phnGHOIM*-ORF-*phnJKLM*, the second putative transcriptional unit consists of repressor-specifying *phnF*, and the third putative transcriptional unit consists of ABC transporter-specifying *phnCDEE* (70). Contrary to most other C-P lyase-containing organisms, there is very little overlap of *phn* open reading frames in *Nostoc* sp. PCC7120. Additionally, the *phnO* gene is located at an unusual position, between *phnH* and *phnI*, and with a potential, unprecedented overlap of the coding sequences of *phnH* and *phnO*. The open reading frame located between *phnM* and *phnJ* (designated 2HP in Fig. 2) may encode a polypeptide that is a member of the two-histidine phosphodiesterase superfamily (71). BLAST analysis of *Nostoc* sp. PCC7120 did not reveal orthologs of *phnN*, *phnP*, or *duf*$_{1045}$. Thus, hydrolysis of the C-P lyase product 5-phosphoribosyl 1,2-cyclic phosphate to ribosyl 1,5-bisphosphate and further conversion of the latter compound must be catalyzed by other enzymes. One of the activities might be replaced by the two-histidine phosphodiesterase specified by the open reading frame located between *phnM* and *phnJ*. BLAST analysis with various *E. coli*, *Nostoc* sp. PCC7120, or *S. meliloti* Phn amino acid sequences did not reveal any *phn* coding capacity among the cyanobacterial species *L. boryana*, *M. aeruginosa*, or *N. punctiforme*.

ORGANISMS WITH UNKNOWN GLYPHOSATE GROWTH PROFICIENCY

Mesorhizobium loti

Mesorhizobium loti appears to contain three *phn* loci, one of which is located on the chromosome and the other two of which are located on plasmid pMLa (32). The chromosomal locus contains *phnFGHI-fosX-phnJKLO-duf*$_{1868}$-*phnCDEE*, where *phnF* constitutes a monocistronic operon transcribed divergently from the remaining 13 genes, which may be expressed as an operon. Thus, the chromosome encodes an ABC transport system for phosphonate (*phnCDEE*). Furthermore, there is a capacity for the coding of an incomplete C-P lyase pathway, with the presence of *phnGHIJKLO-duf*$_{1868}$ but with a lack of *phnM*. The *duf*$_{1868}$ cistron located between *phnO* and *phnC* specifies DUF1868, which is a member of the two-histidine phosphodiesterase superfamily and, thus, may catalyze the hydrolysis of the C-P lyase reaction product 5-phosphoribosyl 1,2-cyclic phosphate in place of a *phnP*-speci-

fied phosphodiesterase (72). The *fosX* cistron specifies 1,2-epoxypropylphosphonic acid (fosfomycin) hydratase: fosfomycin + $H_2O \rightarrow$ 1,2-dihydroxypropylphosphonic acid. Fosfomycin is an antibiotic that is synthesized by some *Streptomyces* species (reviewed in reference 73) and prevents cell wall synthesis in target organisms by inhibiting UDP *N*-acetylglucosamine-3-enolpyruvyltransferase (encoded by *murA* in *E. coli*) (74). Some steady-state kinetic constants of fosfomycin hydratase were determined: a k_{cat} of $0.15 \pm 0.02\ s^{-1}$ and a k_{cat}/K_m of $500 \pm 60\ M^{-1}\ s^{-1}$. The MIC of fosfomycin for *E. coli* expressing *fosX* was 25 mg liter^{-1}. These values led the authors of that study to suggest that the *fosX* gene product may have a biological role in addition to hydrolysis of the epoxide ring of fosfomycin (75). However, it remains to be established if *fosX* confers fosfomycin resistance in *M. loti*. It is furthermore tempting to speculate that this organism, once fosfomycin has been inactivated, degrades the product by the C-P lyase pathway.

The two loci located on pMLa share extensive similarity. Locus II contains *phnMGHIJKL-duf$_{1045}$-phnN* genes, which are likely expressed as an operon. Locus I contains the same genes with the addition of six ORFs between *phnL* and *duf$_{1045}$*. These six ORFs may encode components of ABC transporter systems apparently unrelated to phosphonate catabolism. Locus I furthermore contains a *phnF* cistron, which is transcribed divergently from the remaining genes. Thus, if *duf$_{1045}$* replaces *phnP*, the two loci both contain the coding capacity for a complete C-P lyase pathway for alkylphosphonate degradation.

Collectively, *M. loti* contains a complete set of *phn* genes sufficient for catabolism of alkyl- and aminoalkylphosphonates, a *phnO* ortholog, as well as genes encoding a transport system. Conversely, the plasmid loci supply *phnM* and *phnN* gene products. *M. loti* appears to contain an amount of coding redundancy for phosphonate catabolism. Data on the growth of *M. loti* with glyphosate or AMPA as a P_i source are not available.

Bradyrhizobium japonicum

In *Bradyrhizobium japonicum*, there are four *phn* transcriptional units organized at two loci. One locus consists of a putative polycistronic *phnGHIJKLMN* operon and a second divergently transcribed monocistronic *phnF* operon. A second locus also consists of two apparently polycistronic operons, the divergently transcribed *phnOM-duf$_{1045}$* and *phnCDEE* operons. Again, the genome of this organism appears to encode polypeptides for a full complement for a C-P lyase pathway, provided that *duf$_{1045}$* replaces *phnP*. Additionally, redundancy is observed for *phnM* and *phnE*. Data for the growth of *B. japonicum* with phosphonate as a P_i source are missing.

Acidithiobacillus ferrooxidans

Acidithiobacillus ferrooxidans has been shown to thrive on methyl- and ethylphosphonate as the P_i source. Growth with glyphosate or AMPA as a P_i source has not been reported. A *phn* gene cluster has been identified, which consists of *phnGHIJKLM-duf$_{1045}$-phnNF*. With *duf$_{1045}$* replacing *phnP*, *A. ferrooxidans* contains a full complement for alkylphosphonate catabolism. In comparison with *E. coli*, *A. ferrooxidans* lacks only a *phnO* cistron, and the organism is therefore predicted to lack the ability to utilize 1-aminoalkylphosphonates such as AMPA. Genes encoding transport proteins have not been identified, which indicates a lack of similarity with other phosphonate transport system-encoding genes or, alternatively, that phosphonate is transported as a secondary ligand of a different transport system. Additionally, the *phnGHIJKLM* genes are expressed as an operon whose expression is derepressed during P_i limitation. An unusual feature of the *A. ferrooxidans phn* gene cluster is the location of the *phnF* ortholog as the terminal gene or cistron in the gene cluster, possibly cotranscribed with the remaining genes, rather than being located next to and divergently transcribed from the *phnG* cistron (76, 77).

Trichodesmium erythraeum

The cyanobacterial organism *Trichodesmium erythraeum* contains a single *phn* locus, which consists of *phnDCEEGHIJKLM*. All of these genes are transcribed in the same direction and may constitute a hendecacistronic operon, although there is a gap of >800 apparently untranslated nucleotides between *phnD* and *phnC*. Transcription analysis showed that *phnD* and *phnJ* expression levels increased during P_i depletion. The genome of *T. erythraeum* apparently lacks orthologs of *phnF*, *phnN*, *phnP*, *duf$_{1045}$*, and *phnO* (78) (Fig. 2). At least *phnP* or *duf$_{1045}$* is found among all organisms known to be able to catabolize phosphonate. At first, this suggests that *T. erythraeum* might be unable to utilize phosphonate as a P_i source. However, this was found to be incorrect, as *T. erythraeum* indeed utilizes methylphosphonate, ethylphosphonate, and 2-aminoethylphosphonate as P_i sources and produces methane and ethane from the two former compounds, respectively (79). The biochemical pathway for phosphonate utilization of this organism may therefore deviate from the "standard" C-P lyase pathway. The presence of *phnGHIJKLM* suggests the formation of 5-phosphoribosyl 1,2-cyclic phosphate, the product of C-P lyase activity. Transformation of the 1,2-cyclic phosphate to a useful phosphate source may occur by a mechanism different from that of *E. coli* and other *phnP*- and *phnN*-harboring organisms. For example, C-P lyase may utilize water as a nucleophile, as opposed to the 2-hydroxyl of ribose, to directly hydrolyze the putative covalent ribosyl 1-phosphate–enzyme intermediate. BLAST analysis of *T. erythraeum* with the amino acid sequence of *E. lenta* 5-phosphoribosyl 1,2-cyclic phosphate dihydrolase (locus tag Elen_0235 of GenBank accession number CP001726.1) (142) as the query revealed no significant identity.

C-P Lyase of Archaeal Organisms

C-P lyase is not limited to *Eubacteria*. A few archaeal species contain genes with the coding capacity for a C-P lyase pathway for phosphonate catabolism. Thus, the chromosomes of *Halorhabdus tiamatea* SARL4B (GenBank accession no. HF571520.1) (80), *Haloquadratum walsbyi* C23 (accession no. FR746099.1) (81), and *Natronomonas moolapensis* 8.8.11 (accession no. HF582854.1) (82) all contain a putative *phnGHIJKLM* operon. Next to the *phnG* cistron, but transcribed divergently, the three organisms contain a putative *phnDCE* operon (*H. tiamatea*) or a putative *phnDCEO* operon (*H. walsbyi* and *N. moolapensis*). Neither species apparently contains a *phnN* or a *phnP* homolog. There are no data available with respect to the physiological importance of the *phn* genes.

SPECIES VARIATION OF THE SPECIFICITY OF C-P LYASE

Among the bacterial species described above, five have been shown to be able to use glyphosate as a P_i source (*S. meliloti*, *O. anthropi*, *A. radiobacter*, *B. pseudomallei*, and *Nostoc* sp. PCC7120), and two are unable to use glyphosate as a P_i source (*E. coli* and *P. stutzeri*),

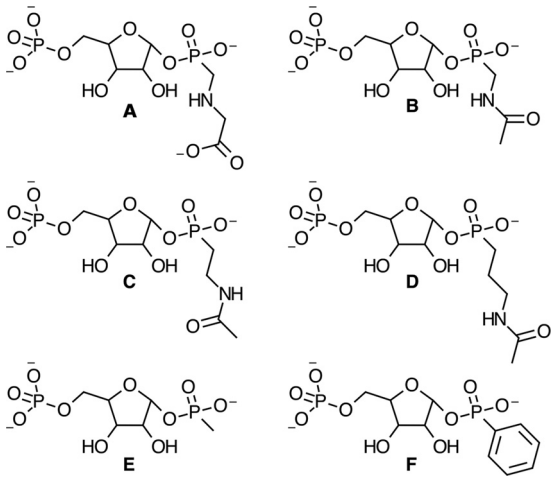

FIG 5 Putative substrates for C-P lyase of cells grown with various phosphonates. (A) Cells grown with glyphosate; (B) cells grown with AMPA; (C) cells grown with 2-aminoethylphosphonate; (D) cells grown with 3-aminopropylphosphonate; (E) cells grown with methylphosphonate; (F) cells grown with phenylphosphonate.

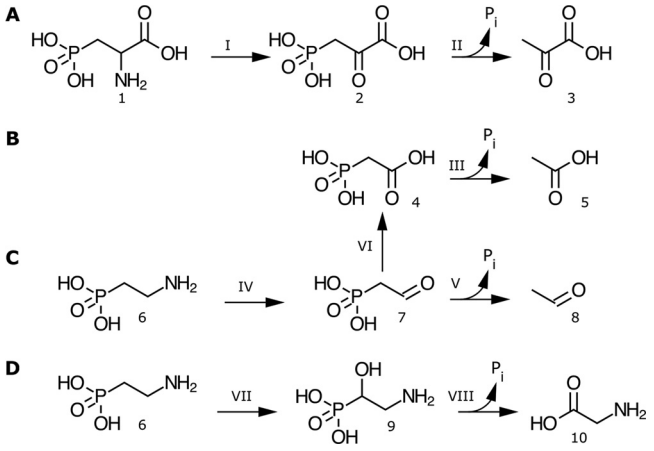

FIG 6 Hydrolytic and oxidative cleavage of C-P bonds. (A) Transformation of phosphonoalanine (compound 1) to P_i and pyruvate (compound 3) via phosphonopyruvate (compound 2). Enzymes: I, phosphonoalanine:α-ketoglutarate transaminase; II, phosphonopyruvate phosphohydrolase. (B) Transformation of phosphonoacetate (compound 4) to P_i and acetate (compound 5) catalyzed by phosphonoacetate phosphohydrolase (enzyme III). (C) Transformation of 2-aminoethylphosphonate (compound 6) to P_i and acetaldehyde (compound 8) via phosphonoacetaldehyde (compound 7). Enzymes: IV, 2-aminoethylphosphonate:pyruvate transaminase; V, phosphonoacetaldehyde phosphohydrolase; VI, phosphonoacetaldehyde dehydrogenase. (D) Oxidative transformation of 2-aminoethylphosphonate (compound 6) to P_i and glycine (compound 10) via 2-amino-1-hydroxyethylphosphonate (compound 9). Enzymes: VII, α-ketoglutarate-dependent 2-aminoethylphosphonate dioxygenase (PhnY); VIII, 2-amino-1-hydroxyethylphosphonate oxygenase (PhnZ).

whereas data for the utilization of glyphosate as a P_i source are not available for four species (*M. loti*, *B. japonicum*, *T. erythraeum*, and *A. ferrooxidans*). As mentioned above, the ability to use glyphosate depends on the availability of a transport system for uptake of the compound as well as an enzyme system with affinity for the compound. The C-P lyase pathway in general has relatively broad substrate specificity compared to those of other phosphonate degradation enzymes or pathways (see below). In addition, it is evident from the description of the organization of the *phn* operons given above and in Fig. 2 that there appears to be an overall unifying structure of C-P lyases. Thus, the organisms included all contain a set of *phnGHIJKLM* genes although not always located as a contiguous gene cluster. In particular, there is some deviation in the location of the *phnM* gene, which is sometimes located away from the *phnGHIJKL* genes, as seen in *S. meliloti*, or there appear to be two copies of *phnM*, as seen in *O. anthropi* and *A. radiobacter*. Also, the locations of the *phnO*, *phnN*, and *phnP* genes vary to some extent, if they are present at all. In *E. coli*, the *phnO* gene is located between *phnN* and *phnP*; in *S. meliloti*, *O. anthropi*, *A. radiobacter*, and *M. loti*, it is located downstream of and overlapping the *phnL* cistron; and in *Nostoc* sp. PCC7120, it is located at yet a different position relative to the remaining *phn* genes. As mentioned above, a *phnP* gene is frequently missing, but in all the *phnP*-less strains, it may be replaced by the duf_{1045} gene. Finally, a *phnN* gene is present in all the organisms except for *Nostoc* sp. PCC7120.

In view of the broad substrate specificity, it comes as no surprise that C-P lyase is able to cleave the C-P bond of glyphosate. Figure 5 shows the structure of substrates for C-P lyase in proficient cells supplemented with various phosphonates. Rather, in view of the similarity of the genetic organization of the *phn* operons of various organisms, and, therefore, apparently similar enzymatic activities, it may seem surprising that not all C-P lyases apparently possess affinity toward glyphosate. In addition to the internalization process and C-P lyase, two enzymatic activities are necessary for conversion of phosphonate, namely, the activation steps catalyzed by purine ribonucleoside triphosphate phosphonylase (apparently an activity of the PhnGHIJK protein complex) and by 5′-triphosphoribosyl 1′-phosphonate diphosphohydrolase (specified by *phnM*) (Fig. 3). Thus, there are quite a few activities that may contribute to variations in the specificity of the overall C-P lyase pathway.

Enzymes or pathways with narrow substrate specificity are responsible for the conversion of phosphonoalanine, phosphonoacetate, and 2-aminoethylphosphonate, as summarized in Fig. 6. 2-Aminoethylphosphonate is converted to phosphonoacetaldehyde by transamination catalyzed by 2-aminoethylphosphonate:pyruvate transaminase and subsequent hydrolysis of phosphonoacetaldehyde to P_i and acetaldehyde catalyzed by phosphonoacetaldehyde phosphohydrolase (83–85). Alternatively, phosphonoacetaldehyde is oxidized to phosphonoacetate catalyzed by phosphonoacetaldehyde dehydrogenase (59). Also, hydrolytic activities for phosphonoacetate (59, 86–90) as well as for phosphonopyruvate (91) have been described, and a hydrolytic activity for phosphonoformaldehyde has been suggested (21). Phosphonopyruvate in turn may be formed by the activities of phosphoenolpyruvate phosphomutase or phosphoalanine:2-oxoglutarate transaminase (91–93). Finally, 2-aminoethylphosphonate may be oxidatively converted to P_i and glycine with the intermediate formation of 2-amino-1-hydroxyethylphosphonate (94, 95). Accounts of these highly specific enzymes in microbial metabolism have been reported previously (96, 97).

AMINOALKYLPHOSPHONATE *N*-ACETYLTRANSFERASES

Acetylation has been shown to be crucial for the utilization of AMPA by *E. coli*. Thus, *phnO* strains are unable to grow with

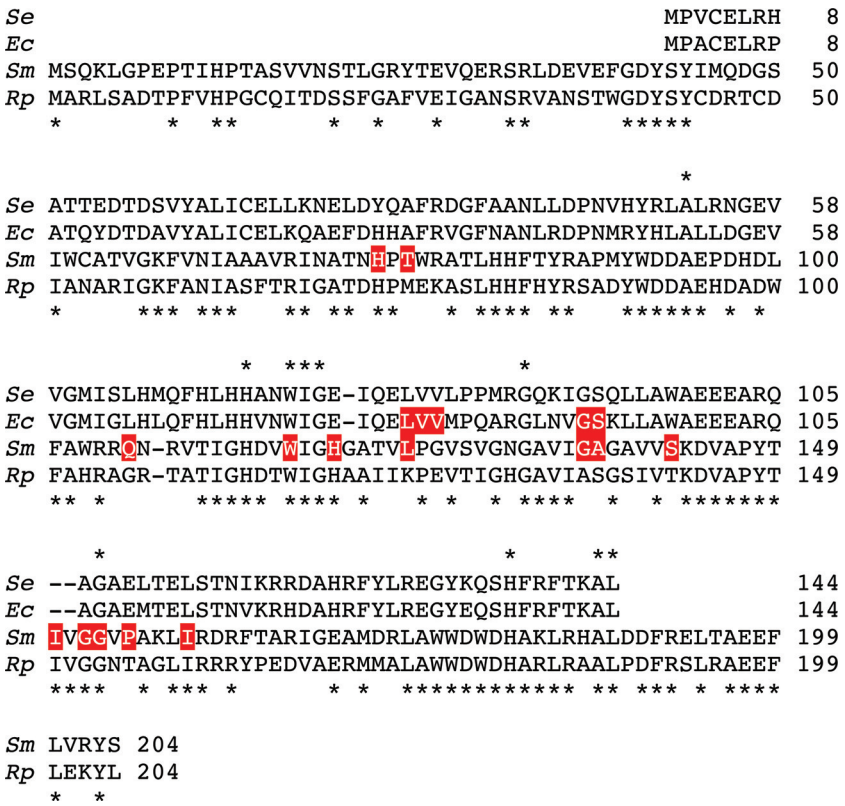

FIG 7 Alignment of amino acid sequences of *E. coli* (*Ec*) and *S. enterica* (*Se*) aminoalkylphosphonate *N*-acetyltransferases and putative *S. meliloti* (*Sm*) and *R. pomeroyi* (*Rp*) aminoalkylphosphonate *N*-acetyltransferases. Asterisks above the sequences indicate identical amino acid residues in all four sequences, whereas asterisks below the sequences indicate identical amino acids in *S. meliloti* and *R. pomeroyi* aminoalkylphosphonate *N*-acetyltransferase sequences. Amino acid residues highlighted in white on a red background are believed to be involved in the binding of the substrate acetyl coenzyme A (98, 99).

AMPA as the sole P_i source. Additionally, the catabolism of the *S*-enantiomer of 1-aminoethylphosphonate requires acetylation. It has been suggested that the presence of a formal positive charge on the amino group might destabilize a carbon-centered radical formed during C-P bond cleavage and that this positive charge might be alleviated by acetylation (39). Given the broad and similar specificities among C-P lyase pathways of various bacterial species, it is likely that acetylation of 1-aminoalkylphosphonates is a requisite for catabolism of these compounds by all C-P lyases. BLAST analysis with the *E. coli* and *S. enterica phnO*-specified amino acid sequences, which share 78% identity, as queries revealed that this sequence is found only among *Enterobacteriaceae*. A large number of bacterial species revealed aminoalkylphosphonate *N*-acetyltransferase amino acid sequences with identities of approximately 30% or lower. Examples of these are the amino acid sequence of the *S. meliloti* 1021 *phnO* open reading frame, i.e., the cistron located immediately downstream of and overlapping *S. meliloti phnL*, and the *Ruegeria* (*Silicibacter*) *pomeroyi phnO* open reading frame, i.e., the cistron located immediately downstream of *R. pomeroyi phnE* (Fig. 2). The *S. meliloti* and *R. pomeroyi phnO*-specified amino acid sequences share 52% identity. Conversely, BLAST analysis with this *S. meliloti phnO*-specified amino acid sequence revealed a relatively high identity (50% or more) of *N*-acetyltransferases in a large number of species other than *Enterobacteriaceae*. Thus, there appear to be two different classes of aminoalkylphosphonate *N*-acetyltransferases. Representatives of one class are *S. enterica* and *E. coli* aminoalkylphosphonate *N*-acetyltransferases, whose activities have been confirmed experimentally, whereas representatives of the other class of aminoalkylphosphonate *N*-acetyltransferases are those specified by *S. meliloti* and *R. pomeroyi phnO*, whose function is assessed by sequence comparison with other acetyltransferases. An alignment of the amino acid sequences specified by the *phnO* genes of the four organisms is shown in Fig. 7. The overall sequence identity is poor (7%). Amino acid residues involved in the binding of acetyl coenzyme A are highlighted in the *E. coli* and *S. meliloti* PhnO amino acid sequences. These amino acid residues were assigned upon sequence alignments with *Tetrahymena* GCN5, an enzyme with acetyltransferase activity, and *Enterococcus faecium* streptogramin acetyltransferase, both of which have known three-dimensional structures (98, 99). The amino acid sequence of the *S. meliloti phnO* gene is a member of the so-called hexapeptide repeat-transferase family, which uses acyl coenzyme A as the substrate and a variety of acceptors (100). Genes for this hexapeptide repeat-transferase family are frequently located among phosphonic acid catabolic genes.

Results of a detailed kinetic analysis of aminoalkylphosphonate *N*-acetyltransferase from *S. enterica* have been published. The enzyme accepts a variety of acetyl acceptors, including AMPA, 2-aminoethyl- and 3-aminopropylphosphonate, (*S*)-1-aminoethyl- and 1-aminopropylphosphonate, and, less efficiently, 1- and 4-aminobutylphosphonate. In contrast, (*R*)-1-aminoethylphosphonate is not a substrate. The k_{cat}/K_m values of the various acetyl acceptors range between 38 M^{-1} s^{-1} (4-aminobutylphos-

phonate) and 7.8×10^4 M^{-1} s^{-1} [(*S*)-1-aminoethylphosphonate]. Additionally, the enzyme accepts acetyl coenzyme A, propionyl coenzyme A, and, less efficiently, malonyl coenzyme A as acyl donors. A divalent metal ion, preferably Mn^{2+}, Ni^{2+}, or Co^{2+}, is required for activity. The physiological role of *S. enterica* aminoalkylphosphonate *N*-acetyltransferase is not fully understood, partially due to the lack of mutant *phnO* alleles. *S. enterica* does not harbor the C-P lyase pathway. It has been suggested, however, that the enzyme serves to protect the organism from the bactericidal effect of the D-alanine analog (*S*)-1-aminoethylphosphonate (101). Curiously, *S. enterica* contains the enzymes 2-aminoethylphosphonate transaminase (encoded by *phnW*) and phosphonoacetaldehyde phosphohydrolase (encoded by *phnX*), which are responsible for extraction of P_i from 2-aminoethylphosphonate. The activity of *phnO*-specified aminoalkylphosphonate *N*-acetyltransferase converts 2-aminoethylphosphonate to 2-*N*-acetamidoethylphosphonate and, thus, makes it unavailable for 2-aminoethylphosphonate transaminase. Presumably, in *S. enterica*, the *phnO* gene and the *phnWX* operon are not expressed simultaneously.

The properties of *E. coli* aminoalkylphosphonate *N*-acetyltransferase resemble those of the *S. enterica* enzyme. The specificity of the two enzymes is essentially the same, except that the *E. coli* enzyme accepts (*R*)-1-aminoethylphosphonate as an acetyl acceptor even though the organism cannot use this compound as a P_i source (39).

C-P LYASE CATABOLISM VERSUS OXIDATION OF GLYPHOSATE

C-P Lyase Activity with Glyphosate as the Substrate

As mentioned above and as indicated in Fig. 1, glyphosate may be catabolized by either of two pathways for utilization of the phosphonyl moiety as a P_i source: (i) by the C-P lyase pathway, resulting in the transfer of the phosphorus to PRPP, or (ii) by oxidative cleavage of a carbon-nitrogen bond, resulting in the formation of AMPA. The phosphorus of AMPA is similarly transferred to PRPP by the C-P lyase pathway. In addition to the organisms described above and in Fig. 2, a number of observations with other species have been made.

A total of 133 environmental bacterial strains were isolated from glyphosate-treated as well as nontreated soil. Among these strains, 26 were able to utilize glyphosate as a P_i source (5 of the strains were isolated from untreated soil, 15 were isolated from glyphosate-treated soil, and 6 were isolated from the river Rhine). After incubation with [3-^{14}C]glyphosate, all 26 strains produced the C-P lyase reaction product *N*-methylglycine, indicating that C-P lyase acted on glyphosate (or a derivative thereof) and, thus, without the involvement of glyphosate oxidase. The data furthermore excluded the presence of glyphosate oxidase activity, as no radiolabeled AMPA was observed. Twenty-four of the 26 strains also utilized AMPA as a P_i source (102).

In addition, for *Pseudomonas* sp. PG2982, a derivative of *Pseudomonas aeruginosa* ATCC 9027 obtained as a subculture after glyphosate treatment (103), solid-state ^{15}N and ^{13}C nuclear magnetic resonance (NMR) performed on cells grown in the presence of [2-^{13}C,^{15}N]glyphosate, [3-^{13}C,^{15}N]glyphosate, or [1-^{13}C]glyphosate was used to demonstrate the conversion of glyphosate to glycine and, thus, presumably with *N*-methylglycine as an intermediate, consistent with catabolism of glyphosate by the C-P lyase pathway (103). Subsequently, by using [1-^{14}C]glyphosate, [2-^{14}C]methylglycine, [3-^{14}C]glyphosate, [^{3}H]glyphosate, or [1,2-^{14}C]glyphosate, *N*-methylglycine was found as an intermediate, once again consistent with catabolism of glyphosate by the C-P lyase pathway (104, 105).

Furthermore, in a number of species of *Rhizobiaceae*, *N*-methylglycine is the immediate catabolic product after incubation with [1-^{13}C]glyphosate (65), and *Arthrobacter* sp. GLP-1 similarly converted glyphosate to glycine and a one-carbon compound, as shown by solid-state NMR and by radiotracer experiments (106). Similarly, *Alcaligenes* sp. strain GL converted [3-^{14}C]glyphosate to *N*-[^{14}C]methylglycine and [^{14}C]glycine as well as other radiolabeled amino acids derived from glycine, demonstrating the presence of C-P lyase activity with affinity for glyphosate in this organism. *Pseudomonas* sp. strain LBr, which was isolated from a glyphosate waste stream, cleaved the C-P bond of glyphosate in an amount sufficient for the P_i supply, as described further below (107). Also, *Pseudomonas* sp. GS catabolized glyphosate through the C-P lyase pathway (108, 109).

Conversion of Glyphosate to AMPA

AMPA is a substrate for the C-P lyase pathway. Indeed, a number of bacterial species have been shown to be able to use AMPA as a P_i source, including *E. coli* (39), *S. meliloti* (47), *Arthrobacter* sp. GLP-1 (106), *Arthrobacter atrocyaneus* ATCC 13752 (110), *Bacillus megaterium*, *Pseudomonas* sp. strains 4ASW and 7b (111), as well as *Pseudomonas* sp. strain LBr (107). Additionally, among the 133 environmental bacterial strains mentioned above, 55 were able to use AMPA as a P_i source (102). *Pseudomonas* sp. strain LBr efficiently catabolized glyphosate by converting it to AMPA, i.e., by oxidation of the carboxy-methylene-nitrogen bond, as shown by solid-state ^{13}C NMR on cells grown in the presence of [^{13}C,^{15}N]glyphosate. As mentioned above, *Pseudomonas* sp. strain LBr was also able to cleave the C-P bond of glyphosate, as demonstrated by the conversion of [2-^{13}C,^{15}N]glyphosate to [2-^{13}C,^{15}N]glycine. Quantitatively, the glycine formation amounted to approximately 5% of the total glyphosate catabolism; i.e., the C-P bond cleavage/glycine formation process is sufficient to meet the P_i need of the cell. Similarly, AMPA could be cleaved in amounts sufficient to meet the need for P_i (107).

A single strain, *A. atrocyaneus* ATCC 13752, appears to degrade glyphosate solely by oxidation with formation of AMPA. This conclusion is based on the following observations: [^{14}C]glyphosate is converted to [^{14}C]AMPA as well as to $^{14}CO_2$. In addition, no radiolabel is found in cellular components such as proteins or nucleic acids, which would be expected if the metabolite *N*-[^{14}C]methylglycine, [^{14}C]methyltetrahydrofolate, or [^{14}C]glycine had been formed. Additionally, glyphosate is utilized as a P_i source by *A. atrocyaneus* ATCC 13752. When *A. atrocyaneus* ATCC 13752 was grown simultaneously with equimolar quantities of P_i and [^{14}C]glyphosate, [^{14}C]AMPA and $^{14}CO_2$ were generated although only after the complete disappearance of P_i. [^{14}C]AMPA accumulated to 30% of the total radioactivity, consistent with one carbon in AMPA and three in glyphosate. Nevertheless, the cell density simultaneously almost tripled (110). It is therefore plausible that the P_i necessary for this increment in cell mass originated from C-P bond cleavage of glyphosate rather than by C-P bond cleavage of AMPA. Additionally, the amount of $^{14}CO_2$ generated under these conditions apparently did not originate from AMPA. It is likely, therefore, that *N*-methylglycine ca-

tabolism in *A. atrocyaneus* ATCC 13752 is different from that of the other species described.

Glyphosate Oxidation

Organisms that utilize the phosphonate-phosphorus of glyphosate via conversion to AMPA initially cleave glyphosate by means of the activity of an oxidase with specificity for glyphosate (Fig. 1). Two such enzymes have been identified: glyphosate oxidase and glycine oxidase. A glyphosate oxidase-encoding gene (*gox*) was identified in a gene library of *Pseudomonas* sp. strain LBAA, a strain isolated from a glyphosate process waste stream facility, for its ability to provide growth of *E. coli* with glyphosate as a P_i source. As *E. coli* is AMPA growth proficient (and glyphosate growth deficient), uptake of glyphosate followed by cleavage to AMPA was predicted to provide growth of *E. coli*. However, the affinity of glyphosate oxidase, the product of the cloned *gox* gene, was low, as kinetic analyses revealed an apparent K_m value of 27 mM and an apparent V_{max} value of 0.8 μmol (min · mg protein)$^{-1}$. Mutant glyphosate oxidase variants were easily obtained by *in vitro* manipulation of the *gox* gene (11). Similar to glyphosate oxidase, glycine oxidase (encoded by *thiO*) of *Bacillus subtilis* has a low affinity for glyphosate (apparent K_m value of 87 mM), but, as with glyphosate oxidase, mutant glycine oxidase variants with improved kinetic parameters were isolated following *in vitro* manipulation of the *thiO* gene (112). The *gox* and *thiO* genes have been manipulated to improve their kinetic parameters for glyphosate as a substrate, with the aim of inserting them into plant cells to make these plants glyphosate tolerant, as described above. A physiological role of glyphosate oxidase, glycine oxidase, or some other glyphosate cleavage enzyme in glyphosate catabolism remains to be established.

In general, and in contrast to C-P bond-cleaving activities, the regulation of the activity of glyphosate-oxidizing enzymes is independent of the P_i source (107), except for the activity described above for *A. atrocyaneus* ATCC 13752, where the glyphosate-cleaving and AMPA-forming activity appeared to be controlled by the P_i supply (110). It is possible that other hitherto undisclosed enzymes will be able to cleave the carboxy-methylene-nitrogen bond of glyphosate.

Interestingly, at least in one example of the 26 glyphosate-proficient environmental strains mentioned above (see Fig. 2 in reference 102), the conversion of glyphosate (2 mM) in the presence of AMPA or methylphosphonate (0.2 mM each) was much less prominent than that in the absence of AMPA or methylphosphonate, which shows that the entire process (i.e., the transport of phosphonate and the C-P lyase pathway) of this particular strain (shown as an example in reference 102) had a remarkable preference for AMPA or methylphosphonate over glyphosate.

The relative contribution of the C-P lyase pathway versus the glyphosate oxidase (or glycine oxidase)/C-P lyase pathway to the physiological extraction of glyphosate-phosphorus is difficult to assess. However, the general pattern seems to be that the C-P lyase pathway is predominant and that the glyphosate oxidase (or glycine oxidase)/C-P lyase pathway is acquired under conditions of selective pressure for detoxification of glyphosate, i.e., in environments with increased concentrations of glyphosate or other phosphonic acids. Thus, the glyphosate oxidases characterized to date appear to have quite poor kinetic properties for glyphosate, although these properties can be easily improved by *in vitro* manipulation of the encoding genes (reviewed in reference 2). These types of improvements very likely also occur under environmental conditions, and such alterations may account for the occurrence and improvement of glyphosate oxidase activity *in situ* after adaption to detoxification and growth in nature.

Glyphosate-Utilizing Species and Strains without Assignment of a Pathway

A number of bacterial species, such as *A. radiobacter* (60) and *S. meliloti* (47), have been shown to utilize glyphosate as a P_i source without assignment of the pathway. BLAST analysis with the glycine oxidase amino acid sequence, specified by *thiO* of *B. subtilis*, or with the glyphosate oxidase amino acid sequence, specified by *gox* of *O. anthropi*, as queries on *A. radiobacter* K84 and *S. meliloti* 1021 genome sequences revealed neither glycine oxidase- nor glyphosate oxidase-specifying genes in these organisms. Similar results were obtained by BLAST analysis of the genomes of *B. pseudomallei* and *Nostoc* sp. PCC7120. Thus, the pathway for glyphosate catabolism, oxidation or C-P lyase, in these species remains uncharacterized.

The thermophile *Geobacillus caldoxylosilyticus* T20, isolated from central heating system water, is able to utilize glyphosate as a P_i source by converting the compound to AMPA and glyoxylate, and the activity of glyphosate oxidase was measurable *in vitro*. In contrast, exogenously supplied AMPA could not be used as a P_i source. Quantitation of the disappearance of glyphosate and the appearance of AMPA showed that, as expected, glyphosate-phosphorus was consumed by growth. Additionally, glyphosate utilization occurred only under conditions of P_i deprivation. The authors of that study concluded that AMPA formed intracellularly from glyphosate is catabolized by C-P lyase and that AMPA cannot be taken up by the cells (113). Although this organism obviously oxidizes glyphosate, the origin of the P_i extraction capability remains unclear. Although isolated from an apparent glyphosate-less environment, it may have been exposed to glyphosate prior to inclusion in the central heating system water, or alternatively, the central heating system may have been treated with a phosphonic acid derivative, which may have been used to prevent the formation of scales and which may have caused adaption of the organism to utilize phosphonate.

Glyphosate and AMPA Utilization Is Regulated by the P_i Level

Glyphosate catabolism appears to be regulated by the P_i supply. Thus, transport and catabolism of glyphosate are completely abolished by the presence of P_i in cells of *Arthrobacter* sp., presumably by repressing the synthesis of the polypeptides responsible for these processes (19). Similarly, utilization of glyphosate by *Pseudomonas* sp. strain PG2982 was observed only under conditions of P_i limitation (20). Although the mechanism of glyphosate transport in these organisms is unknown, it is likely that glyphosate is taken up by a transport system(s) whose genes are regulated by the P_i supply. Also, the catabolic pathway is regulated by the P_i supply. These results are thus consistent with the regulation of phosphonate catabolism in other organisms.

Numerous microbial species respond to the environmental P_i supply through a two-component signaling pathway. The PhoR and PhoB proteins constitute one such signaling pathway, which is responsible for the regulation of expression of a number of operons, the Pho regulon, whose products are involved in the assimilation of P_i as well as in the acquisition of P_i from alternative

phosphorus sources. The PhoR protein is a sensory histidine kinase and is an integral membrane protein, whereas PhoB is a response regulator and binds to DNA in its active, phosphorylated state (114). In *E. coli*, the sensing of the environmental P_i supply and subsequent mediation of this information to PhoR are believed to also involve the five gene products of the *pstSCAB-phoU* operon, which encode the P_i transporter (Pst) as well as well as a chaperone-like protein (PhoU). Hence, the Pho regulon may be regulated by a seven-component signaling system (115). At a low environmental P_i concentration, i.e., <4 μM, the signaling pathway is activated, resulting in the phosphorylation of PhoR and, subsequently, PhoB. Phosphorylated PhoB binds to the Pho regulon promoters and recruits RNA polymerase, upon which Pho operons are transcribed. PhoB specifically binds to an 18-bp consensus sequence, a Pho box. The *E. coli* Pho regulon currently consists of 31 genes or cistrons organized into nine transcriptional units, one of which is the *phn* operon. In *E. coli* K-12, *phn* operon transcription is initiated 24 nucleotides prior to the adenine nucleotide of the sequence specifying the AUG initiation codon. Additionally, a Pho box is located with a spacing of 59 nucleotides to that particular adenine residue. Binding of the PhoB protein to this Pho box has been demonstrated experimentally (24).

The expression of the *E. coli phn* operon has been studied by whole-transcriptome shotgun sequencing (RNA-Seq) technology (116). These data showed the presence of several transcription units under different growth conditions. Thus, heat shock or growth in rich medium induced low-level expression of the *phnCDE* and *phnNOP* cistrons, whereas P_i starvation induced expression of the entire 14-cistron operon. Furthermore, the *phnCDE* cistrons appeared to be expressed in minimal medium independently of the P_i supply. This expression of *phnCDE* is in accordance with previous studies with *phn-uidA* gene fusions that indicated a regulatory site located between *phnE* and *phnF*. In addition, certain insertion mutations in *phnN* unexpectedly did not exert a polar effect on the expression of *phnP*, which may be explained by the presence of a separate transcriptional unit comprising at least the *phnP* cistron (29).

Although the dynamics of Pho regulon regulation have been studied primarily in *E. coli*, *phoB* orthologs are widespread among microorganisms. BLAST analysis revealed that all of the organisms in Fig. 2 contain *phoR* and *phoB* orthologs. In addition, conserved Pho boxes have been identified in a number of these organisms. These Pho boxes are indicated by asterisks below the arrows representing the transcripts in Fig. 2. Thus, a Pho box is located in front of the *P. stutzeri htx* operon with a spacing of 135 bp to the adenylate residue of the AUG-encoding sequence (117). By using a position weight matrix algorithm, Pho boxes were identified in front of *phnG* and *phnC* of *S. meliloti* (118). Additionally, expression of the *S. meliloti phnCDEE* operon as well as the *phnMN* genes and, thus, presumably also duf_{1045} requires expression of *phoB* (119, 120). The above-mentioned position weight matrix algorithm furthermore revealed a number of Pho boxes in *M. loti*, *B. japonicum*, and *A. tumefaciens*. Thus, Pho boxes are located in front of the *M. loti phnG* cistron in the chromosome and in front of the *phnM* cistron in pMLa locus I. A Pho box is also located in front of the *B. japonicum phnG* cistron, and two Pho boxes are located between *phnC* and *phnO*, presumably one for the *phnCDEE* transcript and another for the $phnOM\text{-}duf_{1045}$ transcript. Finally, Pho boxes were found in *A. tumefaciens* C58 in front of *phnG* and *phnC* (118). It is therefore likely that the genes *phnG* to *phnN* are expressed as two transcriptional units. The genetic arrangement of the *phn* genes of *A. tumefaciens* C58 is similar to that of *A. radiobacter* except for the ORF located between *phnO* and *phnC*. This ORF is absent in *A. tumefaciens* C58 (61).

The *phnF*-specified putative regulatory protein is a member of the widespread GntR superfamily of regulator proteins (121). These proteins, which are active as dimers, consist of an N-terminal domain, sometimes called a the functional output domain, of approximately 75 amino acid residues, where the DNA-binding helix-turn-helix motif is located, and a C-terminal domain, sometimes called the effector domain, where the effector binding sequences as well as sequences necessary for dimerization are located. Among the GntR superfamily members, the C terminus varies, and four subfamilies have been identified, of which PhnF belongs to the HutC subfamily (122). Remarkably, each of the 12 organisms included in Fig. 2 except for *T. erythraeum* contains a *phnF* ortholog. As mentioned above, the *M. smegmatis phnF* gene encodes a repressor of expression of the phosphate transporter-encoding *phnDCE* operon (38). The *M. smegmatis* and *E. coli* PhnF amino acid sequences share 32% identity. The amino acid sequence identity of *E. coli* PhnF with PhnF proteins of the 10 other *phnF*-harboring organisms included in Fig. 2 ranges from 29% (*B. japonicum*) to 41% (*P. stutzeri*). It is likely, therefore, that the *E. coli phnF* gene, as well as the *phnF* genes of the remaining organisms, also specifies repressors of *phn* operon expression. Thus, phosphonate catabolism appears to be controlled by a dual regulatory mechanism: activation of transcription mediated by PhoB and repression mediated by PhnF. The rationale for this dual mechanism is that *phn* operon expression requires P_i deprivation and that *phn* operon expression is necessary only when phosphonate is actually present. Thus, under conditions of P_i limitation in the absence of phosphonate, the *phn* operon remains unexpressed due to PhnF-mediated repression. Consequently, phosphonate would be the logical inducer of *phn* operon expression. However, free phosphonic acid may not be the actual inducer. Instead, a phosphonic acid derivative, such as the phosphonate catabolic pathway intermediate 5′-triphosphoribosyl 1′-phosphonate (similar to compound 2) (Fig. 3) or 5′-phosphoribosyl 1′-phosphonate (similar to compound 3) (Fig. 3), may be the inducer. Alternatively, the C-P lyase reaction product 5-phosphoribosyl 1,2-cyclic phosphate (compound 5) (Fig. 3) may be the inducer. From a mechanistic point of view, the latter compound may seem to be the most attractive inducer, as the compound is a common intermediate in the catabolism of all phosphonates. All of the compounds mentioned above are specific for the C-P lyase pathway. In contrast, the C-P lyase pathway intermediates ribosyl 1,5-bisphosphate and PRPP (compounds 6 and 7, respectively) (Fig. 3) are less likely as candidates for inducers, as these compounds are also constituents of other metabolic pathways. There are several catabolic pathways in microbial metabolism where the inducer is a catabolic pathway intermediate rather than the substrate itself. Thus, the inducer for the *deoR*-specified repressor of the *deoCABD* operon, which specifies enzymes involved in deoxyribonucleoside catabolism, is deoxyribose 5-phosphate, an intermediate of the catabolism of deoxyribonucleosides formed after phosphorylase (encoded by *deoA*)- and phosphomutase (encoded by *deoB*)-catalyzed reactions (123).

A dual activation-and-repression mechanism consisting of the two-component sensor kinase response regulator system SenX3-RegX3 and the PhnF repressor has been proposed for the regulation of the transcription of the *M. smegmatis phnDCE* and *phnF*

operons. The two operons are divergently transcribed, and the *phnD* and *phnF* open reading frames are separated by 202 bp, which contain two presumptive PhnF-binding sites (38). This arrangement, i.e., divergent transcription of *phnF* and *phnD*, resembles that of *phnF* and *phnG* of the majority of the *phn* operons of the organisms described in Fig. 2, the exceptions being the *phn* operons of *E. coli*, where *phnF* is cotranscribed with all of the other *phn* cistrons; *P. stutzeri*, where *phnF* is the fourth cistron of the operon; and *A. ferrooxidans*, where *phnF* is located downstream of the remaining *phn* genes. The intergenic distances (i.e., the distances between the open reading frames) of *phnF* and *phnG* of the organisms in Fig. 2 vary from 62 or 63 bp (*B. japonicum* and *A. radiobacter*, respectively) to 355 or 373 bp (*Nostoc* sp. PCC7120 and *M. loti* pMLa locus I, respectively). The position of the PhnF-binding sites or the nucleotide sequence of these sites is so far unknown, apart from those of *M. smegmatis*.

Attempts to examine an effect of glyphosate on genome-wide transcription in *E. coli* have been conducted. Thus, cells were grown in the presence of a somewhat nonphysiological concentration of glyphosate (0.2 M), and transcriptional activity was found to be altered for >1,000 genes. The vast majority of these alterations was an increase of the transcription level by approximately 2-fold. The transcription of each of the cistrons *phnD*, *phnE*, *phnG*, *phnH*, *phnI*, and *phnJ* was also increased by a factor of 2. An exception to this 2-fold response was the *proVWX* operon, the transcription of which was increased >10-fold (124). Thus, the possibility exists that in addition to a glyphosate shock, the cells were also exposed to an osmotic shock, which has been shown to affect the transcription of *proVWX* (125). Finally, cells were grown in the presence of 0.07 M P_i, which may have hampered the uptake and eventual processing of glyphosate.

FUTURE PERSPECTIVES

Individual Polypeptides and Their Contribution to the C-P Lyase Reaction

The elucidation of the structure of all of the intermediates of the C-P lyase pathway as well as procedures for chemical synthesis or isolation from growth media of some of these intermediates make an *in vitro* analysis of C-P lyase properties with glyphosate and other phosphonates as the substrate possible in the near future. Hitherto, the only C-P lyase substrate synthesized is 5′-phosphoribosyl 1′-methylphosphonate (41), and the only C-P lyase substrate isolated from cells is 5′-phosphoribosyl 1′-(2-*N*-acetamidoethylphosphonate) (39). Such *in vitro* analyses, which are now available, together with the discovery of the multisubunit PhnGHIJK protein complex may answer a longstanding question concerning C-P lyase: why are so many gene products required to cleave a C-P bond? A partial answer is the existence of a whole pathway for phosphonate transformation. In addition, the many polypeptides may constitute the components of a holoenzyme, the core of which is formed by PhnGHIJK. Does each component of this holoenzyme have an active site performing a distinct reaction related to and including C-P bond cleavage, or is there a mix of scaffold, regulatory, and catalytic roles? The elucidation of the crystal structure of the PhnGHIJK protein complex as well as the remaining polypeptides will be helpful in this process as well.

Additionally, an analysis of polypeptides required for glyphosate transport would be desirable, and answers to some of these questions might be straightforward. Thus, the knockout of the transport system-encoding genes of the glyphosate growth-proficient strain *S. meliloti* 1021 would reveal if glyphosate is transported by the phosphonate-specific ABC transport system and, if so, whether the expression of these genes from other organisms may provide glyphosate growth proficiency.

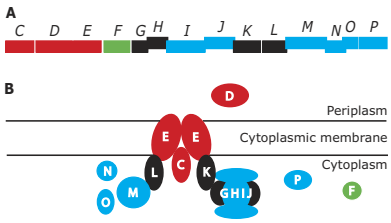

FIG 8 Proposed phosphonate proteome and transport metabolon of *E. coli*. (A) The 14-cistron *phn* operon. The color code is the same as that described in the legend of Fig. 2. (B) Proposed location of *phn*-specified polypeptides. Individual polypeptides are identified by their cistron designation and a color code similar to that of the encoding cistrons in panel A; i.e., red spheres indicate the ABC transport system, blue spheres indicate polypeptides with assigned enzymatic function, the green sphere indicates the putative repressor of *phn* operon expression, and black spheres indicate auxiliary polypeptides without an assigned biochemical function.

The Phosphonate Proteome and Transport Metabolon

Intimately connected to the function of the various Phn polypeptides are their location and organization within the cell. Figure 8 shows a schematic representation of the genetic organization of the *phn* operon as well as a proposal for the cellular location of the various *phn* operon-specified polypeptides in *E. coli*. A number of protein-protein interactions are suggested in Fig. 8. However, experimental interactions are provided only for the PhnGHIJK protein complex. All of the genes of the *phn* operon have been cloned and expressed from various overproducing plasmid vectors, and all of the encoded polypeptides except PhnE and PhnC have been identified by electrophoresis in sodium dodecyl-containing polyacrylamide gels (22, 29, 39–41, 47, 52, 126; B. Hove-Jensen, unpublished results). The *phnE* gene encodes a membrane-spanning protein, and *phnC* encodes an ATP-binding protein of the transporter. Similarly, *phnK* and *phnL* have been assigned ATP-binding-component functions of the phosphonate transporter based on amino acid sequence alignment with known ATP-binding polypeptides (23). The presence of three apparent ATP-binding polypeptides of the *E. coli phn* operon is unique among ABC transporters. Furthermore, none of the three cistrons *phnC*, *phnK*, and *phnL* complements one another, as individual knockout of any of the three cistrons results in a phosphonate growth-deficient phenotype (29). The remaining polypeptides, PhnF, PhnG, PhnH, PhnI, PhnJ, PhnM, PhnN, PhnO, and PhnP, have been postulated to be located in the cytoplasm, and all of them (except for PhnF, which has not been characterized) have been isolated in a soluble form but for some of them only as tagged versions or as part of the PhnGHIJK protein complex (39–41, 52, 101, 126). Of particular interest is the PhnK polypeptide, which is a component of the PhnGHIJK protein complex and which may also be an ATP-binding component of the transporter. Speculations have therefore arisen that the function of PhnK is to attach the protein complex to the transporter and that PhnK therefore is responsible for attachment of the protein complex to the cytoplasmic membrane,

which may cause the coupling of transport and catabolism of phosphonic acids (40). Additionally, the *phnGHIJK* cistrons, i.e., the cistrons encoding the PhnGHIJK protein complex, show translational coupling. Similarly, *phnLMNO* also show translational coupling. Do the products of these cistrons also form a protein complex? It is possible that phosphonate catabolism involves the formation of one or more transport metabolons, i.e., the transient formation of protein complexes responsible for transport and for enzymatic function (127, 128). The obvious advantage of this arrangement for the cell is the efficient transport and catabolism of phosphonate by the channeling of the various intermediates between the reaction centers of the various polypeptides.

Biochemical Function of Additional *phn* Operon Products

In organisms such as *E. coli*, the 14 *phn* cistrons appear to be sufficient for phosphonate transport and catabolism, i.e., the transformation of phosphonate-phosphorus to a compound such as PRPP, which is utilizable by the cell by means of other non-*phn*-encoded enzymes. A survey of the phosphonate catabolism-specifying gene clusters (Fig. 2) reveals additional open reading frames whose gene products do not have an assigned biochemical function. Examples of these gene products are DUF1045 of *S. meliloti*, *O. anthropi*, *A. radiobacter*, *M. loti*, and *B. japonicum* and the two-histidine phosphodiesterase of *Nostoc* sp. PCC7120. Both of these types of genes are quite widespread among *phn* gene clusters, and an elucidation of the biochemical function of their gene products seems pertinent.

PhnF-Mediated Regulation of *phn* Operon Expression

The expression of the *phn* operon in many microbial organisms requires transcriptional activation mediated by PhoB. All of the organisms dealt with in Fig. 2 except for *T. erythraeum* additionally contain a *phnF* homolog believed to specify a repressor protein. This additional level of regulation seems highly reasonable, with the argument being, why express the *phn* operon and synthesize 14 polypeptides if there is no need for them? The regulatory mechanism of PhnF is presently unknown and should be further studied.

CONCLUSION

Several chemical mechanisms exist for the degradation of phosphonic acids. Among the various pathways known to cleave C-P bonds, the C-P lyase pathway has the broadest substrate specificity and is the only known pathway for the extraction of phosphorus from glyphosate. A general pattern for organizing C-P lyase pathway-specifying genes, *phn*, has evolved among microorganisms. Most C-P lyase pathway-containing microorganisms harbor *phnC*, *phnD*, *phnE*, *phnF*, *phnG*, *phnH*, *phnI*, *phnJ*, *phnK*, *phnL*, *phnM*, *phnN*, and *phnP* genes, which include phosphonic acid transport system-specifying genes. In spite of this general gene organization, there is a marked difference in specificity in the utilization of glyphosate among different organisms. Extraction of phosphorus from glyphosate may occur by "direct" C-P bond cleavage catalyzed by C-P lyase or by initial cleavage of glyphosate with the formation of AMPA. Extraction of phosphorus from AMPA also occurs by C-P bond cleavage catalyzed by C-P lyase. The latter process, i.e., cleavage of glyphosate with the formation of AMPA, appears to be a property of cells that have been cultivated in the presence of glyphosate, which, over a prolonged period, may have caused alterations in one or more genes to improve their catalytic capacity for glyphosate cleavage. The ever-increasing use of glyphosate and the release of other phosphonic acids into the environment warrant continued studies of the mechanism of C-P bond cleavage.

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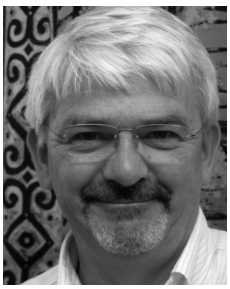

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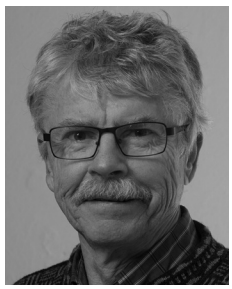

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