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Molecular genetics of carbon-phosphorus bond cleavage in bacteria

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Abstract

Phosphonates (Pn) are a large class of organophosphorus molecules that have direct carbon-phosphorus (C – P) bonds in place of the carbon-oxygen-phosphorus ester bond. In bacteria two pathways exist for Pn breakdown for use as a P source: the phosphonatase and C – P lyase pathways. These pathways differ both in regard to their substrate specificity and their cleavage mechanism. The phosphonatase pathway acts on the natural Pn α-aminoethylphosphonate (AEPn). In a two-step process it leads to cleavage of the C – P bond by a hydrolysis reaction requiring an adjacent carbonyl group. In contrast the C – P lyase pathway has a broad substrate specificity. It leads to cleavage of substituted Pn (such as AEPn) as well as unsubstituted Pn by a mechanism involving redox or radical chemistry. Due to its broad substrate specificity, the C – P lyase pathway is generally thought to be responsible for the breakdown of Pn herbicides (such as glyphosate) by bacteria. As a way to gain a more in-depth understanding of these Pn degradative pathways, their respective genes have been isolated and characterized. In the absence of a biochemical assay for the C – P lyase pathway such molecular approaches have been especially valuable. The roles of individual genes have been inferred from DNA sequence analysis and mutational effects. Genes for the C – P lyase pathway exist in a fourteen-gene operon that appears to encode both a binding protein-dependent Pn transporter and a C – P lyase. Genes for the phosphonatase pathway also exist in a gene cluster containing Pn uptake and degradative genes. A combination of biochemistry, molecular biology, and molecular genetics approaches has provided more detailed understanding of the mechanisms of C – P bond cleavage. Such basic information may provide a new handle for improvement of Pn degradation capabilities in bacteria, or in other cells in which the respective genes may be introduced and expressed.

Abbreviations: AEPn – α-aminoethylphosphonate, C – carbon, kbp – kilobase pair, kDa – kilodalton, MPn – methylphosphonate, P – phosphorus, P_i – inorganic phosphate, Pn – phosphonate, *psi* – phosphate starvation inducible

Introduction

Phosphonates (Pn) are similar to phosphates except Pn have a carbon-phosphorus (C – P) bond in place of the more familiar carbon-oxygen-phosphorus linkage of an organophosphate ester. Due to their structural similarity to phosphate esters, Pn often act as inhibitors of enzymes involving phosphoryl transfer reactions. Since phosphoryl transfer reactions are so prevalent in biology, numerous Pn inhibitors have been developed. Pn inhibitors are commonly used as antibacterial, antiviral, and antitumor agents, and as herbicides. Their effectiveness is due in part to the stability of the C – P bond. With the exception of Pn containing a substitution on the αC, the C – P bond is quite stable and it is not subject to hydrolysis. Indeed, early evidence for natural C – P compounds was based upon finding organophosphorus that resisted strong-acid and strong-base hydrolysis which was released by combustion. Nevertheless, Pn are readily broken down in the environment by both biotic and abiotic transformations. Indeed, many commonly used Pn herbicides (such as glyphosate) are considered "safe" in part due to their rapid biodegradation in the environment.

Pn biotransformations can occur on the C moiety or can result in breakage of the C – P bond. The for-

mer are specific to the C moiety; the latter may be quite nonspecific. In nature bacteria play a major role in Pn biodegradation. Apparently due to the presence of natural Pn in the environment, bacteria have evolved the ability to metabolize Pn as nutrient sources. Those bacteria capable of cleaving the C – P bond are able to use Pn as a phosphorus (P) source for growth. Two pathways for C – P bond cleavage appear to exist for use of Pn as a P source. These are called the "phosphonatase" and the "C – P lyase" pathways (Lee et al. 1992a). A third pathway for C – P bond cleavage (which is specific for phosphonoacetate and involves an apparent phosphonoacetate hydrolase) has been recently found (McMullan et al. 1992; McMullan & Quinn, 1992; McMullan & Quinn 1994). However, this pathway appears to be for Pn use as a C source since it is induced by phosphonoacetate in a manner that is independent of P_i limitation.

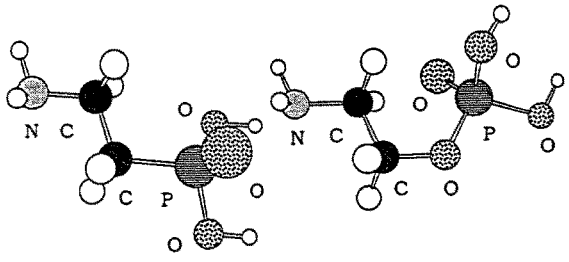
Pn degradation by environmental bacteria

Considerable efforts have been made towards understanding the basis of biodegradation of synthetic Pn, especially the herbicide glyphosate. Since microbial degradation of glyphosate was first noted (Sprankle et al. 1975; Torstensson & Aamisepp 1977), several bacteria capable of glyphosate breakdown have been isolated. Moore et al. (1983) isolated a *Pseudomonas* sp. capable of Pn break down, for its use as a P source. Studies of this strain (*Pseudomonas* sp. PG2982) showed that glyphosate degradation led to formation of glycine as a by-product (Jacob et al. 1985). Thus, C – P bond cleavage occurred via a direct dephosphonation, which is characteristic of the C – P lyase pathway. Also, Pipke et al. (1987) isolated an *Arthrobacter* sp. GLP-1 which metabolizes glyphosate in a similar fashion. There appears to be a sole case in which the genetic determinants of glyphosate degradation have been reported (Liu et al. 1991), however. Importantly, the genes required for glyphosate degradation by *Rhizobium meliloti* appear to be similar to genes for the *Escherichia coli* C – P lyase pathway.

In this article, I will briefly summarize what is understood about Pn breakdown by bacteria. I will focus my attention on recent studies involving identification of the genes and gene products for the phosphonatase and C – P lyase pathways. An understanding of these genes may be of value in the development of improved methods of Pn biodegradation in biotechnology. A treatise on natural Pn has been published about ten years ago (Hori et al. 1984). Also, some material in this article has been included in a recent article on the *E. coli* C – P lyase gene system (Wanner & Metcalf 1992). It is now fairly clear that genes for Pn breakdown have evolved so that bacteria may use Pn as an alternative P source. This is because genes for C – P bond cleavage for Pn use as a P source are members of the phosphate (PHO) regulon. A review on the PHO regulon has been recently published (Wanner 1993).

Occurrence in nature

Pn are quite common (though not universal) among different organisms. They have been found in organisms as diverse as *Bdellovibrio* (a procaryote that lives within a eubacterium), *Streptomycetes* (a filamentous eubacteria), *Tetrahymena* and *Trypanosoma* (eucaryotic protozoans), mollusk, insects, and others. Pn are principally found in the form of phosphonolipids, where α-aminoethylphosphonate (AEPn) often exists in place of its analog ethanolamine phosphate (Fig. 1). Pn are also found as constituents of glycolipids, glycoproteins, and polysaccharides (Hori et al. 1984). Pn have been identified as a component of lipopeptidophosphoglycan, the major cell surface glycoconjugate of *Trypanosoma cruzi* (de Lederkremer et al. 1991), as a carbohydrate-linked constituent of a glycoprotein in locust (Hård et al. 1993), and as a component of capsular polysaccharide in *Bacteroides fragilis* (Baumann et al. 1992). Also, natural Pn include certain antibiotics made by *Streptomycetes* such as fosfomycin (Fig. 2). In addition, Pn may be quite abundant. In *Tetrahymena*, Pn may account for as much as 30% of the P content of phospholipids (Kennedy & Thompson 1970). In locust, AEPn is the main P compound in hemolymph (Kilby & Radda 1991). In a sea anemone the P in Pn accounts for 50% of the total P content (Quin 1965). In many of these organisms, Pn have been shown to be synthesized de novo. In others (and particularly in mammals), Pn are thought to be derived primarily (and perhaps solely) from the diet (Hori et al. 1984). In spite of the widespread occurrence of Pn in nature, the biological role of natural Pn is poorly understood (Hilderbrand & Henderson 1982).



α-aminoethylphosphonate ethanolamine phosphate

Fig. 1. Structures of a natural Pn and its analog.

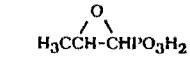
Name	Formula	Description
2-Aminoethylphosphonate	$NH_2CH_2CH_2PO_3H_2$	Lipids (Phosphonolipids) *Tetrahymena*
Fosfomycin		Antibiotic *Streptomyces*
Glyphosate (Roundup™)	$HOOCH_2NHCH_2PO_3H_2$	Herbicide Synthetic
Methylphosphonate	$CH_3PO_3H_2$	Substrate Synthetic

Fig. 2. Some representative Pn.

Pathways for C – P bond cleavage

The phosphonatase pathway for Pn degradation involves a transamination-mediated dephosphonation (Fig. 3). This pathway has a narrow substrate specificity, as it acts only on a Pn containing a substitution on the αC which can be converted to an aldehyde. AEPn is a natural substrate of this pathway. First, AEPn is converted to phosphonoacetaldehyde by an AEPn transaminase, and phosphonoacetaldehyde is subsequently converted to acetaldehyde and P_i by a phosphonoacetaldehyde phosphonohydrolase (trivial name phosphonatase). In this pathway an aldolase-like imine of phosphonoacetaldehyde is formed with an active site lysine on the phosphonatase. This leads to destabilization of the C – P bond, thus allowing for hydrolytic cleavage of the C – P bond (Olsen et al. 1988). C – P bond fission by the phosphonatase pathway leads to retention of stereospecificity at the P center (Lee et al. 1992b). Enzymes carrying out both steps have been purified and characterized from *Bacillus cereus* and *Pseudomonas aeruginosa*. In addition, an AEPn-specific uptake system has been demonstrated in *Bacillus cereus* (Rosenberg & La Nauze 1967). Genes for the

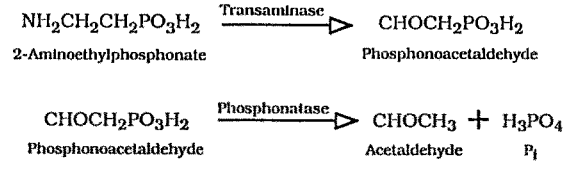


Fig. 3. C – P bond cleavage by transamination-mediated phosphonatase pathway.

phosphonatase pathway have been isolated from *Enterobacter aerogenes* (Lee et al. 1992a) and *Salmonella typhimurium* (WW Metcalf, W Jiang, K-S Lee, & BL Wanner, unpublished results), as described later.

The C – P lyase pathway for Pn biodegradation involves a direct dephosphonation (Fig. 4). The biochemical characterization of this pathway has remained elusive due to the inability to detect a C – P lyase activity in a cell-free system. The only reports of an in vitro C – P lyase activity were later proven to be incorrect, for reasons discussed previously (Metcalf & Wanner 1993a). The inability to detect an in vitro activity may due to the existence of the C – P lyase as a fragile membrane-associated enzyme complex. The C – P lyase pathway has a broad specificity. It acts on both unsubstituted and substituted Pn. On the basis of *in vivo* studies, the C products from degradation of alkyl- or phenylphosphonates are the corresponding hydrocarbons. Biodegradation of methylphosphonate (MPn), ethylphosphonate, and benzylphosphonate leads to formation of methane, ethane, and benzene, respectively. Because these products are not normally made by these bacteria, it is fairly certain that they are the direct C products of C – P bond cleavage by a lyase. C – P bond cleavage by a lyase leads to racemization of stereospecificity at the C center (Ahn et al. 1992). This is compatible with proposed mechanisms of C – P bond fission involving radical or redox chemistry. On the basis of genetic studies (Metcalf & Wanner 1993a), *E. coli* C – P lyase activity requires seven to nine proteins, including one apparent integral membrane protein. Curiously, *Arthrobacter* sp. GLP-1 may contain two distinct C – P lyases, on the basis of in vivo product formation (Kertesz et al. 1991).

Due to the absence of a cell-free system, the P product of C – P bond cleavage by a lyase has not been determined. While it may be P_i, it should be pointed out that the P product need not be P_i in order for a Pn to serve as a P source. This is because the transfer of a phosphoryl group from a Pn to a nucleotide (or to

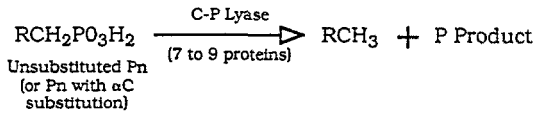


Fig. 4. C – P bond cleavage by direct dephosphonation via a C – P lyase. The catalytic mechanism is unknown. As indicated in the text, seven to nine proteins appear to constitute the C – P lyase (which may exist in a complex in the membrane). Also, the P product of C – P bond cleavage has not been determined.

another compound to form a phosphate ester) would allow for Pn use as a P source without the release of P_i. Further, physiological results have shown that the amount of P_i released was undetectable during growth of bacteria on a Pn broken down by the C – P lyase pathway (Lee et al. 1992a). This implies either that P_i is not the immediate cleavage product or that the P_i released is rapidly incorporated into another P compound. In contrast, the amount of P_i released was in sufficient excess to cause gene repression during growth of the same bacteria on a Pn broken down by the phosphonatase pathway. With the exception of AEPn (which is also a substrate of the phosphonatase pathway), no natural Pn substrate is known that is specific to the C – P lyase pathway. Genes for the C – P lyase pathway have been characterized from *E. coli* B (Chen et al. 1990), *E. coli* K-12 (Makino et al. 1991), *Ent. aerogenes* (Lee et al. 1992a), and *Rhizobium meliloti* (PA McLean, CM Liu, CC Sookdeo, & FC Cannon, unpublished results according to GenBank™ Accession M96263), as described later.

P assimilation and the PHO regulon

Three kinds of P compounds can be used as a P source for growth: P_i, organophosphate esters, and Pn (Wanner 1990). Since P_i is the preferred P source, the ability to use an alternative P source is usually induced only under conditions of P_i limitation. The use of a Pn as a P source requires cleavage of the C – P bond. It has been shown that activities for Pn breakdown are induced under conditions of P_i limitation in a variety of bacteria. Pn degradation by *Agrobacterium radiobacter* (Wackett et al. 1987a), *Arthrobacter* s sp. GLP-1 (Kertesz et al. 1991; Yakovleva & Blackburn, 1993), *E. coli* (Wackett et al. 1987b), *Ent. aerogenes* (Lee et al. 1992a) and various related members of the *Enterobacteriaceae* (Wanner & Boline 1990), *Phytophthora* spp. (Barchietto et al. 1989), *Pseudomonas paucimobilis* and other soil bacteria (Schowanek & Verstraete 1990a; Schowanek & Verstraete 1990b), *Pseudomonas* GS (Albrecht et al. 1991), *Pseudomonas fluorescens* (Zboinska et al. 1992), *Rhizobiaceae* (Liu et al. 1991), *S. typhimurium* ((Wanner & Boline 1990); WWM, WJ, K-SL, & BLW, unpublished results), and other bacteria has been shown to be induced by P_i limitation. This is to be expected if these activities are for Pn utilization as an alternative P source. Further, the finding that the amount of methane produced from MPn breakdown was increased during P_i limitation led to the discovery that *E. coli* genes for Pn degradation were members of the PHO regulon in this model organism (Wackett et al. 1987b).

All PHO regulon genes probably have a role in P assimilation because they are turned on by P_i limitation (Wanner 1987, 1990, 1993). The process of P assimilation involves at least two steps. The environmental P source is first taken up by a transport system, and the P is then incorporated into ATP (the primary phosphoryl donor in metabolism) by one of several central pathways in carbon and energy metabolism (Wanner, 1990, 1992). If P_i is the P source, P_i is taken up either by a low affinity or high affinity P_i transporter (depending upon the extracellular P_i concentration), and then internal P_i is incorporated into ATP by substrate-level phosphorylation, as well as by other central pathways. If an organophosphate ester is the P source, then (depending upon the P compound) three schemes are possible. The ester may be broken down outside and the P_i released may be taken up intact and enter metabolism as P_i. An ester such as *sn*-glycerol-3-phosphate may be taken up by an *sn*-glycerol-3-phosphate transport system (whose synthesis is induced by P_i limitation), and its phosphoryl group may be (eventually) transferred to ATP. Or, the ester may taken up intact after which the ester is broken down and the P_i released inside the cell may then be incorporated into ATP by substrate-level phosphorylation, or other pathways. If a Pn is the P source, then the Pn is taken up before it is metabolized since Pn utilization by both the phosphonatase and C – P lyase pathways require a Pn uptake system. If the Pn is broken down by the phosphonatase pathway, then P_i is released inside the cell and this P_i is then incorporated into ATP by substrate-level phosphorylation, or other pathways. If the Pn is broken down by the C – P lyase pathway, then either P_i is released and this P_i is rapidly incorporated into ATP (or another organophosphate) or no P_i is released and instead a

phosphoryl group is somehow transferred to ATP (or another organophosphate) by the C – P lyase.

Genes belonging to the PHO regulon are called *psi* genes because they are phosphate-starvation-inducible (Wanner, 1987, 1993). These *psi* genes include: the *pstSCAB-phoU* operon for the high-affinity P_i-specific transport (Pst) system and a protein called PhoU, the *phoA* operon for the *phoA* gene product bacterial alkaline phosphatase and one or more downstream genes of unknown function, the *ugpBAECQ* operon for an *sn*-glycerol-3-phosphate transport (Ugp) system and the UgpQ phosphodiesterase, and the *phoE* gene for an outer membrane anion-specific porin, in addition to ones for Pn utilization and several *psi* genes of unknown function (Metcalf et al. 1990; Kim et al. 1993). Typically, a *psi* gene is induced more than 100-fold by P_i limitation. This control of *psi* genes involves a two-component regulatory system (Nixon et al. 1986) comprised of the PhoB and the PhoR proteins.

The PhoB protein is a DNA-binding protein and a transcriptional activator. It recognizes the consensus "PHO" box sequence CTGTCA-TA(AT)A(TA)CTGT(CA)A(CT) in the −35 promoter region, in which frequent alternative bases are in parentheses. The PhoB protein activates transcription only when phosphorylated (Makino et al. 1989) by a mechanism that may involve contact between the phospho-PhoB protein and the sigma-70 factor of RNA polymerase (Makino et al. 1993). The PhoR protein is called the P_i sensor. It is required both for induction during P_i limitation and for P_i repression when P_i is in excess (Wanner, 1987). It activates the PhoB protein by phosphorylation under conditions of P_i limitation, and it inactivates the PhoB protein (presumably by dephosphorylation) when P_i is in excess.

The PHO regulon is a paradigm for a signal transduction pathway in bacteria (Wanner, 1993) in which a cell surface protein (s) regulates gene expression by phosphorylation and dephosphorylation of a regulatory protein (the PhoB protein). P_i control of the PHO regulon requires the Pst P_i transport system, an accessory protein called PhoU, and the PhoR protein. The Pst system is a cell surface receptor complex for P_i uptake. Even though P_i control involves the Pst system, P_i control is independent of transport per se. Rather P_i control involves detection of the extracellular P_i level. The Pst system together with the PhoU protein somehow regulates the activity of the PhoR protein as a protein kinase and (presumably) as a phospho-PhoB protein phosphatase, which in turn determines the amount of phospho-PhoB protein. In addition, the PHO regulon appears to be subject to cross regulation by two other cell signaling pathways. Controls involving carbon and energy metabolism lead to phosphorylation of the PhoB protein by a membrane protein called CreC (the catabolite sensor) and by acetyl phosphate (Wanner, 1992; Wanner & Wilmes-Riesenberg, 1992).

Cloning genes for the C – P lyase pathway

The finding that the *E. coli* C – P lyase activity was induced by P_i limitation and was subject to PHO regulon mutational effects led to a search for *psi* genes that might encode the C – P lyase (Wackett et al. 1987b). This was feasible because a collection of mutants was available in which individual *psi* genes had been interrupted by mutation. These *psi* mutants resulted from random insertion of a transposon carrying the *lacZ* gene (for β-galactosidase) into the *E. coli* chromosome. In those mutants containing the *lacZ* reporter gene (which was devoid of its own regulatory sequences) in the proper orientation, the *lacZ* gene was controlled by the regulatory sequences of the interrupted gene (Fig. 5). The *psi*::*lacZ* mutants had been identified several years earlier by screening mutants carrying an insertion of the *lacZ* transposon Mu d1 for ones that showed phosphate-starvation-inducible synthesis of β-galactosidase (Wanner et al. 1981; Wanner & McSharry, 1982). By testing fifty-five independent *psi*::*lacZ* mutants for their ability to use MPn as a sole P source, three Pn nonutilizing mutants were found. All three had lesions in the *psiD* locus, whose function had previously not been determined. A search for additional Pn nonutilizing mutants (which was not restricted to *psi* genes) led to the isolation and characterization of nine other mutants. These also had lesions in the *psiD* locus (Metcalf & Wanner 1991), and this locus was therefore renamed the *phn* locus. In addition, mutants with large deletions of the *psiD* (*phn*) locus were isolated, and these mutants were especially useful in subsequent studies of this locus (Wanner & Boline 1990).

The availability of Pn nonutilizing mutants allowed for the molecular cloning of genes for Pn utilization by complementation of such mutants for growth on a Pn as a sole P source. Plasmid libraries containing insertions of random DNA fragments from a Pn utilizing strain were introduced into an appropriate *E. coli* host having a deletion of the *phn* locus, and these bacteria were screened for ones that now grew with a Pn as a sole P source (Wanner & Boline 1990). Unexpectedly, the size of the *phn* locus was proven to be

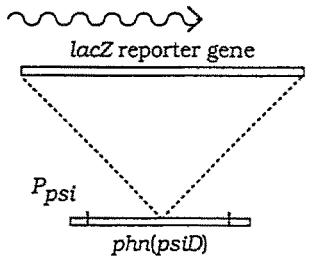


Fig. 5. Physical structure of *psiD(phn)::lacZ* insertion mutation. The wavy line indicates the mRNA.

quite large. Genetic mapping experiments showed that the Pn-negative insertion mutations spanned a region of approximately 10-kbp. In agreement, the minimal size DNA fragment necessary for complementation of a *phn* deletion mutant was greater than 10-kbp (Wanner & Boline 1990; Metcalf & Wanner 1991). With the goal of understanding the gene organization of the *phn* locus, its complete DNA sequence was determined (Chen et al. 1990). This revealed that as many as seventeen *phn* genes might be arranged in a single large operon. Subsequent studies showed that the *phnA* and *phnB* genes were unnecessary, that a PhoB-dependent *psi* promoter preceded the *phnC* gene, and that the most distal gene was the *phnP* gene (Metcalf & Wanner 1993b). Therefore fourteen genes that were (fortuitously) named *phnC* to *phnP* spanned a region that was both necessary and sufficient for Pn utilization (Metcalf & Wanner 1993a). Studies on the expression of these genes indicate that they are transcribed as a fourteen-gene operon from the *phnC* promoter (Metcalf & Wanner 1993a; WWM, WJ, & BLW, unpublished results). It has also been shown that phospho-PhoB protein binds a PHO Box sequence overlapping the *phnC* −35 promoter region (Makino et al. 1991).

Structure and function of the *phnC-phnP* gene cluster

In the absence of a biochemical assay for the C – P lyase, our understanding of the role of protein products of the *phnC*-to-*phnP* gene cluster is based on mutational effects and protein sequence comparisons. The effects of mutations in all fourteen genes have been examined. Because these genes are transcribed from a single promoter (Fig. 6), each gene was mutated in a way that allowed monitoring expression of downstream

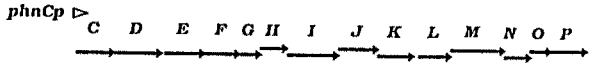


Fig. 6. Organization of the *phnC*-to-*phnP* gene cluster. Arrows show the gene orientation. Some genes share short overlapping DNA sequences, as indicated by the raised arrows.

genes. In this way, mutational effects could be attributed to disruption of individual genes as polarity effects had been ruled out (Metcalf & Wanner 1993a). The results of these analyses are summarized in Table 1.

The PhnC, PhnD, and PhnE proteins probably make up a binding protein-dependent Pn transporter in which the PhnC protein is the ATPase permease component (also called the ABC permease component), the PhnD protein is the periplasmic binding protein component, and the PhnE protein is the integral membrane protein component (Metcalf & Wanner 1993a). Although binding protein-dependent transporters often contain two integral membrane proteins, some others also contain only one. In those that contain one, it is thought that the integral membrane protein acts as a dimer (Maloney et al. 1990). Further, the *phnCDE*-encoded Pn transporter appears to be (largely) responsible for the substrate specificity of the C – P lyase pathway. Attempts to broaden the specificity of this pathway have so far yielded mutations only in the transport genes (Wanner & Metcalf 1992). The genes for the *E. coli* C – P lyase pathway had been cloned from plasmid libraries containing *E. coli* B *phn* genes (Wanner & Boline 1990). This was done because Pn utilization was shown to be cryptic in the laboratory strain *E. coli* K-12, while Pn utilization was shown to be functional in *E. coli* B (as it is in most of *E. coli* strains (Wanner & Boline 1990)). DNA sequence analysis of the *E. coli* K-12 *phnC*-to-*phnP* gene cluster revealed that the cryptic phenotype of *E. coli* K-12 was due to a frameshift mutation in the *phnE* (EcoK) gene (Makino et al. 1991).

Seven proteins (PhnG, PhnH, PhnI, PhnJ, PhnK, PhnL, and PhnM) are absolutely required for catalysis, and these proteins are therefore necessary components of the C – P lyase (Metcalf & Wanner 1993a). These proteins are likely to comprise a membrane-associated C – P lyase enzyme complex. A membrane association is inferred from the hydrophobic nature of the PhnM protein, and an absolute requirement for the PhnM protein in catalysis. An enzyme complex is inferred by the large number of required proteins. A membrane-associated enzyme complex is consistent with the pro-

Table 1. Roles of the gene products for the *E. coli* C – P lyase pathway.

Protein	M_r	Description*
PhnC	29.5 kDa	ABC permease component of Pn transporter
PhnD	37.3 kDa	Periplasmic binding protein component of Pn transporter
PhnE	30.3 kDa	Membrane protein component of Pn transporter
PhnF	27.5 kDa	Unnecessary. PhnF may have a role in gene regulation
PhnG	16.5 kDa	C – P lyase component
PhnH	21.0 kDa	C – P lyase component
PhnI	38.7 kDa	C – P lyase component
PhnJ	31.9 kDa	C – P lyase component
PhnK	27.8 kDa	C – P lyase component. PhnK contains an ABC motif
PhnL	24.8 kDa	C – P lyase component. PhnL contains an ABC motif
PhnM	41.7 kDa	Membrane protein component of C – P lyase
PhnN	20.7 kDa	C – P lyase component. PhnN may be accessory protein
PhnO	16.2 kDa	Unnecessary. PhnO may have a role in gene regulation
PhnP	27.9 kDa	C – P lyase component. PhnP may be accessory protein

*ABC, ATP-binding cassette. This motif is shared by many transporters that use ATP as an energy source, and in some cases it has been shown to be necessary for ATP binding or hydrolysis (Higgins 1992).

posed involvement of redox or radical chemistry in C – P bond cleavage by a lyase. The occurrences of ATP-binding cassette motifs in the PhnK and PhnL proteins (Table 1) suggest a role for ATP (or another nucleotide) in C – P bond cleavage. Genes for Pn utilization have also been cloned from *Rhizobium meliloti* (PAM et al., unpublished results). They include ones that are homologous to the *E. coli phnG*, *phnH*, *phnI*, *phnJ*, and *phnK* genes.

Two proteins (PhnN and PhnP) are conditionally required for catalysis, and these proteins are therefore likely accessory components of the C – P lyase (Metcalf & Wanner 1993a). The PhnN protein has no absolute requirement since a mutant lacking the PhnN protein grows (albeit poorly) on Pn (regardless of the Pn used as substrate). The PhnP protein may be necessary only in the presence of the PhnN protein, because a mutant that appears to be lacking the PhnN, PhnO, and PhnP proteins grows poorly on Pn (like a *phnN* mutant), while a mutant lacking only the PhnP protein is Pn-negative (Metcalf & Wanner 1993a; Metcalf & Wanner 1991).

Two additional proteins (PhnF and PhnO) have no obligatory role in Pn utilization. Both the PhnF and PhnO proteins may be regulatory proteins (Metcalf & Wanner 1993a; Metcalf & Wanner 1991). Although mutational effects due to *phnF* and *phnO* lesions are observed, those effects may be entirely attributable to polarity. A *phnF* mutation leads to somewhat reduced growth on a Pn (which is independent of polarity), while a nonpolar *phnO* mutation is without effect. While it is possible that the PhnF protein (like the PhnN protein) may have an accessory role, it seems more likely that the PhnF protein has a regulatory role. On the basis of sequence similarity at the protein level, the PhnF protein may be a member of a new family of helix-turn-helix regulatory proteins together with the FadR, GntR, HutC, KorA, GenA, and P30 proteins (Haydon & Guest, 1991; Yoshida et al. 1993). Interestingly, some members of the PhnF (GntR) family contain a consensus regulatory site that closely matches a sequence in the 5′ end of the *phnF* structural gene. The PhnO protein also contains a helix-turn-helix motif that is commonly found in regulatory proteins. Since the PhnO protein has no apparent role in Pn utilization, a regulatory role is inferred from the absence of a *phnO* mutational effect and the presence of its helix-turn-helix motif.

Cloning genes for the phosphonatase pathway

In the same way that genes for the *E. coli* C – P lyase were cloned, genes for Pn utilization were cloned from *Ent. aerogenes* (Lee et al. 1992a) and *S. typhimurium* (WWM, WJ, S-KL, & BLW, unpublished results) by complementation of Pn-negative *E. coli* hosts by using plasmid libraries containing insertions of ran-

dom DNA fragments from these bacteria. Genes for Pn utilization were cloned from these strains because these bacteria displayed different growth characteristics in regard to Pn utilization. Two kinds of complementing plasmids were isolated from *Ent. aerogenes* plasmid libraries. One kind encoded genes for an *Ent. aerogenes* C – P lyase which were homologous to the *E. coli* C – P lyase genes, except that no plasmid containing *Ent. aerogenes* C – P lyase genes carried also Pn transport genes. Other data suggested that the C – P lyase and Pn transport genes may be located in separate regions on the *Ent. aerogenes* chromosome, which would have prevented finding plasmids carrying them together. The second kind of complementing plasmid encoded genes for a phosphonatase pathway. Such plasmids were isolated from both *Ent. aerogenes* and *S. typhimurium* plasmid libraries. These plasmids also carried Pn uptake genes, however these uptake genes apparently encode a transport system that is specific for AEPn (the substrate for the phosphonatase pathway). The absence of homology between genes for the C – P lyase and phosphonatase pathways provides further evidence for the existence of two distinct biochemical pathways whose enzymes are unrelated. Also, the finding of genes for two Pn degradative pathways under PHO regulon control in the same strain highlights the importance of Pn as a nutrient source in bacteria.

Like the *E. coli phnC*-to-*phnP* gene cluster, genes for the *Ent. aerogenes* C – P lyase, and genes for both the *Ent. aerogenes* and *S. typhimurium* phosphonatase pathway and AEPn-specific uptake systems are under PHO regulon control (Lee et al. 1992a; WWM, WJ, S-KL, & BLW, unpublished results). Preliminary data indicate that a 7.2-kbp fragment (an amount sufficient to encode about seven average size bacterial proteins) contains all genes necessary for AEPn uptake and breakdown by the phosphonatase pathway. Also, genes for the *Ent. aerogenes* and *S. typhimurium* phosphonatase pathways are homologous (WWM, WJ, S-KL, & BLW, unpublished results). Due to the size of the DNA fragment necessary for uptake, it appears that several uptake genes are present on these plasmids. Therefore AEPn is probably taken up by a binding protein-dependent transport system (which is also known as an ABC transporter). This is because such transporters in bacteria usually require genes for three or four protein components, while other transporters often require only one gene for a single protein component. While it is clear that these plasmids also carry genes for Pn breakdown, further studies are necessary to show whether they carry genes for both the AEPn transaminase and the phosphonoacetaldehyde phosphonohydrolase. It is also unknown whether these plasmids carry regulatory genes. Mutational studies that are similar to ones carried out on the*E. coli* C – P lyase pathway are now underway on genes for the phosphonatase pathway (WWM, WJ, & BLW, unpublished results). By using strategies similar to those used in the cloning of genes for the C – P lyase and phosphonatase pathways, it may also be possible to isolate genes for C – P bond cleavage by the phosphonoacetate-inducible phosphonoacetate hydrolase pathway. It will be interesting to examine the sequence and functional similarities among these genes.

Summary and prospectus

Studies of two Pn degradation pathways have revealed that both pathways involve genes for Pn uptake and genes for Pn breakdown, that these genes are induced by P_i limitation, and that they are under PHO regulon control. The phosphonatase pathway has a narrow substrate specificity and appears to be associated with an AEPn-specific transporter and enzymes for AEPn breakdown. In contrast, the C – P lyase pathway has a broad substrate specificity. It appears to be associated with both an uptake system and C – P lyase of broad substrate specificity. Also, due to its broad substrate specificity, the C – P lyase pathway may be the better target for development of new Pn degradative capabilities. Further, the inability of *E. coli* to degrade a Pn such as glyphosate may be due to an inability to take up glyphosate (when present at a concentration too low to inhibit growth) or an inability to breakdown glyphosate. Therefore alteration of the substrate specificity of the C – P lyase pathway may involve changing the specificity of its associated transporter, the C – P lyase, or both. Molecular genetic approaches may be invaluable in new studies aimed at manipulation of the substrate specificity of the phosphonatase or C – P lyase pathways by altering transport or catalysis genes of that pathway. For example, by determining which gene(s) limit the substrate specificity of the C – P lyase pathway may indicate what kinds of mutational change(s) may be necessary to extend the substrate specificity of the C – P lyase pathway. Also, it may be worthwhile to examine the evolutionary relatedness between genes for the same C – P bond cleavage pathway in different bacteria as well as between genes for C – P bond cleavage by different pathways, as a way

to design a C – P bond cleavage pathway with a different substrate specificity. Such studies may provide an additional guide on how to alter a particular pathway.

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