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Carbon-phosphorus bond cleavage by Gram-positive and Gram-negative soil bacteria

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Summary. Five soil bacterial isolates, originally selected for their ability to utilize the herbicide glyphosate as sole phosphorus source, were characterized with respect to their ability to use a range of other structurally-diverse phosphonates. Most showed broad substrate specificity and strains of *Pseudomonas* and of *Bacillus megaterium* were capable of degrading 14 of the other 15 phosphonates investigated. However no isolate was able to utilize isopropyl phosphonate, nor the phosphinate herbicide phosphinothricin. Growth rates on most phosphonates were significantly lower than those sustained by inorganic phosphate, and evidence was obtained for preferential utilization of the latter. In addition, the length of lag phase preceding growth on phosphonates varied widely. These characteristics are believed to reflect the diversity of routes by which such molecules enter bacterial cells and are metabolized.

Introduction

The existence of natural products containing a covalent carbon-phosphorus bond has been known for some two decades and many examples have now been described (Horiguchi 1984), although most are of relatively limited occurrence. By contrast, the production of synthetic organophosphonates and -phosphinates, which contain one and two C-P bonds respectively, has greatly increased in recent times because of their extensive use as pesticides, flame retardants, antibiotics, lubricant additives, plasticizers and agents of chemical warfare (Daughton et al. 1979; Hilderbrand 1983). This has led to fears of their persistence in soils and natural waters. Such concerns are heightened by the stability of the C-P bond to chemical, photolytic and thermal decomposition (Freedman and Doak 1957), by the fact that many phosphonates and phosphinates are potent biocides, and by the apparent recalcitrance of several phosphonates widely used as chelating agents in household products (Egli 1988).

At least two chemically-distinct pathways of phosphonate catabolism are known to exist. The mechanism for the biodegradation of phosphonoacetaldehyde, described by La Nauze et al. (1977) for *Bacillus cereus* involves a hydrolytic cleavage catalysed by phosphonoacetaldehyde hydrolase (phosphonatase), with the production of acetaldehyde and orthophosphate via a covalent imine intermediate formed between the substrate carbonyl group and the side chain of a lysine residue in the enzyme. Such a mechanism, however, cannot be responsible for the cleavage of the unactivated carbon-to-phosphorus bonds of, for example, alkyl- and phenylphosphonic acids which have been shown to serve as the sole phosphorus source for a number of Gram-negative bacteria (Smith 1983; Wackett et al. 1987). The failure of numerous attempts to demonstrate unactivated carbon-to-phosphorus bond cleavage in cell-free extracts of these oganisms (Daughton et al. 1979; Avila et al. 1987; Shames et al. 1987) has led both to the development of biomimetic models of the "C-P lyase" activity involved (Cordeiro et al. 1986; Gohre et al. 1987), and also to a detailed analysis of physiological parameters associated with the reaction in vivo (Wackett et al. 1987; Pipke and Amrhein 1988a). In addition Pipke and Amrhein (1988b) have successfully obtained in situ C-P lyase activity in permeabilized cells of *Arthrobacter* sp. in the presence of the phosphonate

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herbicide glyphosate [N-(phosphonomethyl) glycine].

We have previously reported the isolation of a number of soil bacteria capable of the utilization of glyphosate as sole phosphorus source (Quinn et al. 1988). Here we examine the substrate specificity of five of these isolates and the kinetics and regulation of phosphonate degradation, in an attempt to gain further insight into the mechanism of C-P bond cleavage.

Materials and methods

Isolation and identification of bacteria. Eight soil isolates of apparently distinct morphology, from the 32 obtained by enrichment culture on mineral salts medium containing 0.5 m*M* glyphosate as sole phosphorus source (Quinn et al. 1988) were selected. After preliminary biochemical screening they were sent for identification to the National Collections of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland.

Media and growth conditions. The basal mineral salts medium used was buffered with 50 m*M* TRIS (pH 7.2) and contained (per litre): 5.0 g sodium gluconate, 5.0 g sodium pyruvate, 5.0 g NH_4Cl, 0.008 g $CaCl_2$, 0.16 g $MgSO_4$, 0.007 g $FeSO_4 \cdot 7H_2O$, 0.001 g ethylenediaminetetraacetate disodium salt, 1.0 ml trace element solution (Krieg 1981), 0.01 g thiamine HCl. It was sterilized by filtration. All phosphorus sources were added to a final concentration of 0.5 m*M*, unless otherwise stated, from 20 m*M* filter-sterilized stock solutions, buffered with 50 m*M* TRIS/HCl, pH 7.2.

Preliminary growth studies were carried out using 25-ml Universal bottles containing 5 ml medium, and were inoculated with 50 µl of a washed suspension of stationary phase cells pre-cultured on the same medium but with 0.2 m*M* inorganic phosphate (P_i) as sole phosphorus source. They were incubated at 29° C on a rotary shaker at 100 rpm.

Subsequent experiments to determine rates of growth and substrate disappearance employed 250-ml Erlenmeyer flasks containing 25 ml medium, which received 1% inocula and were incubated as described above; confirmation of each result was obtained in a separate experiment. All glassware was rigorously washed with Decon (Decon Laboratories, Hove, UK) and subsequently rinsed with double-distilled water.

Culture turbidity measurements were made using a Corning EEL portable colorimeter equipped with a no. OR1 red filter (EEL, Halstead, UK). Values were expressed in arbitrary EEL units.

Chemicals Phenylphosphonate was supplied by Aldrich Chemical Co., Dorset, UK, and methyl-, ethyl- and isopropylphosponates by Ventron, Karlsruhe, FRG. Alafosfalin (L-alanyl-L-1-aminoethylphosphonic acid) was a gift from Roche Products, Welwyn Garden City, UK, and phosphinothricin (D,L-homoalanine-4-yl (methyl)-phosphinic acid) a gift from Hoechst Aktiengesellschaft, Frankfurt am Main, FRG. Glyphosate [N-(phosphonomethyl)-glycine] was supplied by Monsanto Agricultural Products, St Louis, Mo, USA. All other phosphonates were purchased from Sigma Chemical Company, Poole, UK. With the exception, to a minor degree, of phosphonoformate, no phosphonate was found to contain detectable levels of P_i when assayed at a concentration of 0.5 m*M* by the method of Fiske and SubbaRow (1925). Nor did any release P_i when incubated in uninoculated growth medium for a period of 10 days. Other chemicals, all of Analar grade, were obtained from BDH, Poole, UK.

Analytical methods. Levels of phenylphosphonate and 2-aminoethylphosphonate in culture supernatants were determined as total phosphorus by the method of Fiske and SubbaRow (1925) after digestion of 0.1 ml aliquots in the presence of $Mg(NO_3)_2$ as described by Ames (1966). Inorganic phosphate levels in these samples were determined by the same method, but without prior digestion.

Results and discussion

Eight glyphosate-degrading bacterial strains whose isolation was previously reported (Quinn et al. 1988), were identified. Three were regarded as being closely-related *Pseudomonas* strains having similarities to the members of RNA Homology Group III. Strain 4ASW was selected for further work. Two other isolates were considered to be identical strains of *Pseudomonas* but could not be assigned to any well-characterized group within the genus; of these 7B strain was selected. The three remaining isolates were considered to be an *Escherichia hermannii,* a strain of *Bacillus megaterium,* and an actinomycete which was not further characterized.

Each strain was subsequently tested for the ability to utilize a variety of phosphonates and the phosphinate phosphinothricin, as sole phosphorus source (Table 1); control experiments with uninoculated media served to confirm that none of these substrates contained inorganic phosphate or released it during incubation. The results indicate that the two *Pseudomonas* strains and *B. megaterium* strain 2BLW possess a C-P bond cleavage activity of broad specificity; only isopropylphosphonate, whose structure indicates possible steric constraints on C-P bond cleavage (Wackett et al. 1987), and phosphinothricin, which has potent antimicrobial properties (Smith 1983) failed to support growth. Such broad substrate specificity, comparable to the *Agrobacterium radiobacter* of Wackett et al. (1987), contrasts with the narrow specificities of a range of unidentified phosphonate-utilizing bacteria isolated by Cook et al. (1978).

The range of substrates utilized by *E. hermannii* strain 4AY and *Actinomyces* sp. strain 2BSW proved much more limited, however (Table 1). In the case of the slow-growing actinomycete a prolonged incubation period might well have produced a greater number of positive results, but the failure of *E. hermannii* to utilize the majority of phosphonate substrates, including glyphosate on which it was originally isolated, is surprising.

Table 1. Ability of bacterial isolates to utilize phosphonates as sole phosphorus source

Substrate	Growth of bacteria[a]				
	Escherichia hermannii strain 4AY	*Actinomyces* sp. strain 2BSW	*Bacillus megaterium* strain 2BLW	*Pseudomonas* sp. strain 4ASW	*Pseudomonas* sp. strain 7B
Glyphosate	−	+*	++	++	++
Phosphonomycin	−	−	++	++	++
Alafosfalin	++*	−	++	++	++
Phenylphosphonate	+	−	++	++	++
Phosphonoacetate	−	−	++	++	++
Phosphonoformate	−	−	++	++	++
Methylphosphonate	−	−	++	++	++
Aminomethylphosphonate	−	−	++	++	−
Ethylphosphonate	−	−	++*	++	++
1-Aminoethylphosphonate	−	−	++*	++*	++*
2-Aminoethylphosphonate	−	++	++	++	++
1-Aminopropylphosphonate	−	++*	++	++	++
3-Aminopropylphosphonate	−	+*	++	++	++
1-Aminobutylphosphonate	−	+*	++	++	++
4-Aminobutylphosphonate	−	++*	++	++	++
Isopropylphosphonate	−	−	−	−	−
Phosphinothricin	−	−	−	−	−

[a] Bacterial growth was scored by comparison of culture turbidity with that of a control culture grown in the absence of a phosphorus source: ++, heavy growth; +, some growth above that of control; −, no growth above that of control. Cultures were normally scored after 3 days; an *asterisk* indicates that growth was not complete until after 7 days

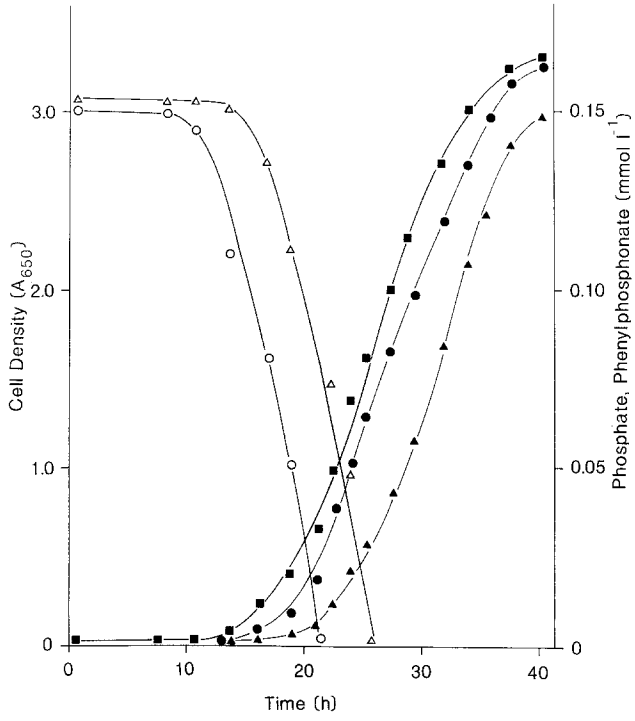

Fig. 1. Growth of *Pseudomonas* sp. 4ASW in mineral salts medium containing 0.3 m*M* PO_4 (●—●), 0.3 m*M* phenylphosphonate (▲—▲) or 0.15 m*M* PO_4 plus 0.15 m*M* phenylphosphonate (■—■) as sole phosphorus sources, and PO_4 concentration (○—○) and phenylphosphonate concentration (△—△) during growth of the organism on 0.15 m*M* PO_4 plus 0.15 m*M* phenylphosphonate as sole phosphorus sources

In view of their metabolic versatility, *Pseudomonas* sp. strain 4ASW and *B. megaterium* strain 2BLW were chosen for further investigation. With the exceptions of phosphonoformate and methyl-, ethyl-, and 1-aminoethylphosphonates, cellular yields on the majority of substrates were found to be similar to those obtained on equimolar P_i (e.g. for phenylphosphonate: Fig. 1), and when specific growth rates are compared (Table 2) it is clear that in the case of *Pseudomonas* sp. 4ASW many of the phosphonates supplied also supported rates comparable to that on P_i. In all instances, however, a longer lag phase for growth of *Pseudomonas* sp. 4ASW on phosphonates was observed (Table 2); its length showed some correlation with the subsequent maximum specific growth rate sustained. Compounds whose utilization required a short lag phase and supported relatively high growth rates included glyphosate, aminomethylphosphonate, alafosfalin, phenylphosphonate, phosphonoacetate and — strikingly — those aminoalkylphosphonates not substituted at position 1.

The significance of these findings for our understanding of the mechanism of C-P bond cleavage activity is unclear, however, since they may simply reflect another rate-limiting process such as uptake by the cell. Bacterial transport of phosphonates has not yet been fully elucidated, but

Table 2. Growth characteristics of bacterial isolates on various phosphorus sources

Substrate	*Bacillus megaterium* strain 2BLW		*Pseudomonas* sp. strain 4ASW	
	Lag phase (h)	Specific growth rate (h^{-1})	Lag phase (h)	Specific growth rate (h^{-1})
Inorganic phosphate	4	0.38	8	0.23
Glyphosate	73	0.17	12	0.22
Phosphonomycin	86	0.10	19	0.20
Alafosfalin	81	0.14	13	0.20
Phenylphosphonate	78	0.14	11	0.21
Phosphonoacetate	74	0.12	10	0.18
Phosphonoformate	91	0.05	31	0.06
Methylphosphonate	22	0.03	21	0.11
Aminomethylphosphonate	63	0.20	11	0.18
Ethylphosphonate	31	0.03	41	0.11
1-Aminoethylphosphonate	13	0.03	114	0.11
2-Aminoethylphosphonate	10	0.10	13	0.23
1-Aminopropylphosphonate	64	0.10	20	0.14
3-Aminopropylphosphonate	69	0.12	13	0.22
1-Aminobutylphosphonate	92	0.09	21	0.16
4-Aminobutylphosphonate	67	0.12	11	0.21

appears to involve both specific, inducible systems (e.g. for 2-aminoethylphosphonate) and the normal uptake systems of other related compounds (Smith 1983), as well as systems subject to inhibition and suppression by orthophosphate (e.g. for glyphosate; Pipke et al. 1987a). Phosphonopeptides are generally transported into bacterial cells more readily than are free phosphonates (Allen et al. 1978) and this might well be reflected in the ninefold greater lag phase and much-reduced growth rate of *Pseudomonas* sp. 4ASW on 1-aminoethylphosphonate in comparison with its peptide analogue alafosfalin (Table 2).

Growth of *B. megaterium* 2BLW on the majority of phosphonates was characterized by long lag phases and low specific growth rates with respect to inorganic phosphate (Table 2). The shortest lag phase occurred on 2-aminoethylphosphonate which was shown by Rosenberg and La Nauze (1967) to have a specific transport system in *B. cereus,* whilst highest growth rates were obtained on glyphosate and its analogue aminomethylphosphonate. What seems clear, however, is that this Gram-positive organism does possess a biological activity capable of the dephosphorylation of unfunctionalized alkyl phosphonic acids, which is distinct from the phosphonatase activity described in *B. cereus* by La Nauze et al. (1977). Until the recent report of a glyphosate-degrading *Arthrobacter* (Pipke et al. 1987b), the ability to directly cleave C-P bonds had appeared to be confined to Gram-negative bacteria and was thought to be possibly associated with a periplasmic component (Wackett et al. 1987).

To gain some insight into the regulation of this activity, phosphorus-limited mineral salts medium containing equimolar (0.15 m*M*) amounts of inorganic phosphate and either phenylphosphonate or 2-aminoethylphosphonate were inoculated with *Pseudomonas* sp. 4ASW and growth rates and rates of substrate disappearance monitored. Similar results were obtained for both substrate combinations; those for P_i/phenylphosphonate are shown in Fig. 1. Whilst providing some evidence of preferential utilization of inorganic phosphate by the organism, there is no indication of the 'tight' regulation by phosphate of either organophosphonate transport or C-P bond cleavage that a diauxic lag coincident with P_i depletion would have implied. Thus the metabolism of organophosphonates is less stringently controlled by P_i levels in *Pseudomonas* sp. 4ASW than in *Kluyvera ascorbata* (Wackett et al. 1987); regulation in the pseudomonad is perhaps more analogous to that found in *Agrobacterium radiobacter* by the same authors.

In the absence of any demonstration of stable in vitro activity the characterization of the enzyme(s) catalysing unactivated C-P bond cleavage must remain uncertain. It is hoped, however, that both *Pseudomonas* sp. 4ASW and *B. megaterium* 2BLW will in future provide useful tools for the further study of this activity in bacteria.

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