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Phosphonate Utilization by Bacterial Cultures and Enrichments from Environmental Samples

DIEDERIK SCHOWANEK AND WILLY VERSTRAETE*

Laboratory of Microbial Ecology, State University of Ghent, Coupure Links 653, 9000 Ghent, Belgium

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A selection of axenic microbial strains and a variety of environmental samples were investigated with respect to the utilization of a series of natural and xenobiotic phosphonates as the sole phosphorus source for growth. Phosphonate degradation was observed only with bacteria and not with eucaryotic microorganisms. All representatives of the phosphonates examined supported bacterial growth, with the exception of methylphosphonate diethylester. Yet, distinctly different phosphonate utilization patterns were noted between phosphonate-positive strains. C-P bond cleavage by a photosynthetic bacterium is reported for the first time; growing photoheterotrophically, *Rhodobacter capsulatus* ATCC 23782 was able to utilize 2-aminoethylphosphonate and alkylphosphonates. Bacteria with the potential to utilize at least one of the phosphonate moieties from the xenobiotic phosphonates Dequest 2010, Dequest 2041, and Dequest 2060 were detected in all environments, with only two exceptions for Dequest 2010. Phosphonate P utilization to an extent of 94 and 97%, for Dequest 2010 and Dequest 2041, respectively, provided evidence that a complete breakdown of these compounds with respect to the C-P bond cleavage can be achieved by some bacteria. The results suggest that phosphonate-utilizing bacteria are ubiquitous, and that selected strains can degrade phosphonates that are more complex than those described previously.

Phosphonates, in the broad sense of the word, are a class of organophosphorus compounds characterized by the presence of one or more carbon-to-phosphorus (C-P) bonds. The C-P bond(s) provides the molecule with a relative resistance to chemical hydrolysis and thermal decomposition compared with the more reactive N-P, S-P, and O-P linkages (4, 6, 9, 10, 14). Moreover, studies on the biochemistry of phosphonate utilization by microorganisms suggest that the C-P bond is often responsible for recalcitrance to biodegradation (6, 7, 12, 16, 23, 39). In comparison with the wide range of organophosphates, phosphonates are relatively rare in living material. Biodegradation of phosphonates of natural origin like 2-aminoethylphosphonate, as well as some man-made compounds (and including cleavage of the C-P bond) has been demonstrated in a variety of gram-positive and gram-negative bacteria. In the latter studies the compounds were supplied as the sole P source for growth (1, 6, 7, 9, 13, 15, 17, 28, 30, 38, 40). A remarkable aspect of phosphonate degradation is that, so far, only bacteria were found to be capable of enzymatic cleavage of the C-P bond (9, 23). In addition, this trait seems to be restricted to particular strains, rather than to a defined group of microorganisms. The release into the environment of large quantities of xenobiotic phosphonates, used as pesticides, detergent additives, antibiotics, flame retardants, etc. (7, 12, 26), warrants more intensive research on their ecological effects, their biodegradation, and the biochemistry of the C-P bond in general. Very few data have been reported on the (bio)degradation of phosphonates like the herbicide bialaphos or the polyphosphonic acids formulated in a number of laundry detergents (12, 16). Furthermore, the demonstration of in vitro activity and characterization of the C-P lyases (C-P-cleaving enzymes [39, 40]) remains a challenge to the biochemists.

Another poorly documented aspect is the ecology of phosphonate-degrading organisms. Besides standard degradation tests and the more fundamental biochemistry, the study of the abundance and distribution of these strains in an ecosystem is another pertinent aspect in assessing the environmental fate of a pollutant (2, 32).

To our knowledge, no systematic study of the ecology of phosphonate-degrading microorganisms in polluted and nonpolluted environments has been carried out. Therefore, we screened seven different ecosystems and nineteen laboratory microorganisms for growth on a variety of representative natural and xenobiotic phosphonates, based on the strategy of supplying the phosphonates as the sole P source. This study reports the presence of phosphonate-degrading bacteria in a broad range of bacterial genera and in contaminated as well as in uncontaminated environments.

MATERIALS AND METHODS

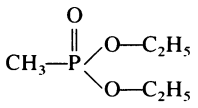
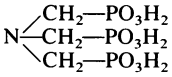
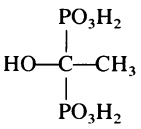
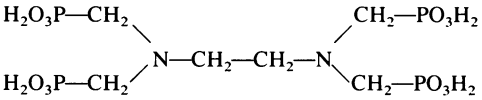
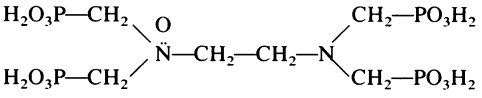
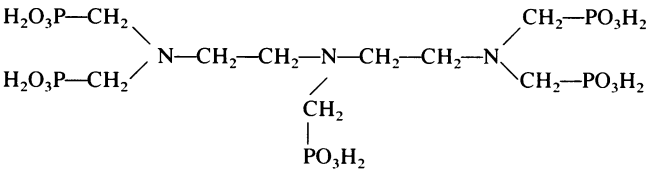
Abbreviations. The following abbreviations are used in this paper: P_n, phosphonate phosphorus; OD_{650}, optical density at 650 nm; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; 2-AEP, 2-aminoethylphosphonate; Φ-P, phenylphosphonate; MeP, methylphosphonate; MePDiEt, MeP diethylester; D2000, Dequest 2000; D2010, Dequest 2010; D2041, Dequest 2041; D2041-Nox, Dequest 2041 mono oxide; D2060, Dequest 2060; APODS, ammonium peroxidisulfate.

Reagents and glassware. The sources, purities, and formulas of the phosphonates used in this study are listed in Table 1. All other reagents were of the highest purity available commercially. Media and stock solutions were prepared with Milli-Q water (Milli-Q System; Waters Associates, Inc.) with a resistance (R) >18 MΩ cm^{-1}. Each piece of glassware was cleaned meticulously by washing with phosphate-free detergent, followed by rinsing with distilled water and soaking for at least 12 h in 10% HNO_3, and finally rinsed four times with Milli-Q water. Background phosphorus in the liquid media never exceeded 2 μg-atoms of P per liter. Accordingly, growth was never observed in phosphorus-free medium (OD_{650}, <0.030).

Media. Several growth media were defined for the screen-

* Corresponding author.

TABLE 1. Characteristics of the phosphonates studied

Phosphonate	Formula	Origin	Active product[a] (%)	% P_i contamination[b]
2-AEP	$H_2N—CH_2—CH_2—PO_3H_2$	Sigma Chemical Co.	98	12.0
Φ-P		Janssen Chemicals, Beerse	98	0.9
MeP	$CH_3—PO_3H_2$	Sigma	98	0.5
MePDiEt		Aldrich Chemical Co., Inc.	97	0.5
Glyphosate	$HOOC—CH_2—NH—CH_2—PO_3H_2$	Monsanto	99	0.6
D2000		Monsanto	>95	7.2
D2010		Monsanto	>95	1.0
D2041		Monsanto	>95	3.5
D2041-Nox		Procter & Gamble	>95	7.7
D2060		Monsanto	92 D2060 5 D2041 1.5 D2000	2.3

[a] Thin-layer chromatograms of freshly prepared stock solutions showed no significant amounts of secondary phosphorus containing products, besides some P_i.
[b] (Microgram-atoms of P_i $liter^{-1}$)/(milligram-atoms of P_n $liter^{-1}$).

ing of various groups of microorganisms. BMM and BAA media were composed for the growth of bacteria, whereas MY, RHO, and GEM media were appropriate, respectively, for yeasts and fungi, members of the family *Rhodospirillaceae*, and cyanobacteria. The C and N sources used in these media are shown in Table 2. All media were buffered with the organic buffer HEPES (normally 15 mM, but 25 mM in GEM medium) instead of a phosphate buffer and contained 1 ml of a solution of trace elements per liter and 1 ml of a solution of vitamins per liter. Each medium contained the following salts (milligrams per liter): KCl, 200; $MgSO_4 \cdot 7H_2O$, 200; $CaCl_2 \cdot 2H_2O$, 25; $FeSO_4 \cdot 7H_2O$, 20. In addition, MY medium contained 50 mg of NaCl $liter^{-1}$, RHO medium contained 25 mg of $NaHCO_3$ $liter^{-1}$ and 100 mg of $MnCl_2 \cdot 4H_2O$ $liter^{-1}$; and GEM medium contained 20 mg of disodium EDTA $liter^{-1}$, 2,000 mg of $NaHCO_3$ $liter^{-1}$, and 75 mg of $CaCl_2 \cdot 2H_2O$ $liter^{-1}$. The amino acid mixture for BAA medium consisted of the following (milligrams per liter): L-alanine, 500; L-arginine, 500; L-aspartic acid, 1,000; L-glutamic acid, 1,000; glycine, 1,000; L-histidine, 500; L-isoleucine, 500; L-leucine, 1,000; L-methionine, 250; L-serine, 500; L-threonine, 500; L-tryptophan, 250; L-valine, 1,000; L-phenylalanine, 500; and L-proline, 250. The trace-elements stock solution contained the following (milligrams per liter): disodium EDTA, 5,000; $ZnSO_4 \cdot 7H_2O$, 100; $NiSO_4 \cdot 6H_2O$, 500; $MnCl_2 \cdot 4H_2O$, 500; $CuSO_4 \cdot 5H_2O$, 30; $Na_2MoO_4 \cdot 2H_2O$, 50; $CoCl_2 \cdot 6H_2O$, 50; H_3BO_4, 100; $Na_2SiO_3 \cdot 9H_2O$, 20; $AlCl_3 \cdot 6H_2O$, 50; $Na_2SeO_3 \cdot xH_2O$, 20. The vitamin stock solution contained the following (milligrams per liter): nicotinamide, 1,000; biotin, 50; thiamine hydrochloride, 1,000; vitamin B_{12}, 50; calcium pantothenate, 100; *p*-aminobenzoic acid, 1,000; pyridoxine hydrochloride, 1,000; riboflavin, 25.

To avoid decomposition, the vitamins and the phospho-

TABLE 2. C and N sources, C/N/P ratio, and pH of the growth media

Medium	C source(s) (mg liter^{-1})	N source(s) (mg liter^{-1})	C/N/P ratio	pH
BMM[a]	Glucose (3,000) Na_2-succinate (3,380) Glycerol (3,070)	NH_4Cl (1,325)	250:25:0.3	7.2
BAA	Mixture of amino acids	Mixture of amino acids	310:44:0.3	7.2
MY	Glucose (15,000)	KNO_3 (1,500) L-Asparagine (1,000)	500:45:0.3	5.5
RHO[b]	DL-Lactic acid (2,700)	Na-L-glutamate (1,300)	250:14:0.2	6.8
GEM		KNO_3 (1,000)	Not determined	6.0

[a] Medium based on reference 6.
[b] Medium based on reference 35.

nates were filter sterilized (0.22-μm-pore-size filter) and added to the liquid and solid media after autoclaving and cooling. Media with phosphonates were never more than 14 days old. Particular attention was paid to the C/N/P ratio of the media to ensure a severe phosphorus limitation, and avoid luxury phosphate uptake. Solid defined media were prepared with purified 2% (wt/wt) agar (Difco Laboratories). No colony growth could be observed without the addition of a supplementary phosphorus source (P total in solid media without P source added, 10 μg-atoms of P liter^{-1}). Nutrient agar was obtained from Oxoid Ltd.

Cultures of microorganisms. *Arthrobacter* sp. strain GLP-1 (28–30) was kindly provided by N. Amrhein (Bochum University, Federal Republic of Germany), *Pseudomonas testosteroni* (7) was supplied by M. Alexander (Cornell University, Ithaca, N.Y.), and *Pseudomonas* sp. strain PG2982 (24, 36) was supplied by H. Braymer (Louisiana State University, Baton Rouge). *Escherichia coli* Crooke strain DSM 1576 (1) was bought from the German Collection of Microorganisms.

The fungi *Cladosporium herbarum*, *Fusarium culmorum*, and *Trichoderma viride* were a gift from J. Poppe (State University Ghent). All other laboratory strains were taken from our own culture collection.

Before the experiments with phosphonates, the optimal growth conditions (type of defined medium, temperature) were studied for each organism. Only strains showing good growth on one of the test media, with P_i as a phosphorus source, were maintained for the tests with phosphonates. The cultures were activated before testing by subculturing them twice on the most appropriate medium with P_i as the P source (1 mg-atom of P_i liter^{-1}). Strains were routinely subcultured every 10 weeks and were stored in the dark at 4°C. Phosphonate-positive bacteria were stored on agar slopes, with a phosphonate as the P source, to ensure continuous selection pressure.

Enrichment procedure and evaluation of phosphonate utilization. The principle of enrichment cultures was used to select for phosphonate-positive isolates. The procedure used in this study was based on the method proposed by Cook et al. (6). For sampling in the environments, 500-g soil or 1-liter water (sludge) samples were taken in various environments in sterile glass vials and stored at 4°C. Microbial enrichment and enumeration were started within 4 days. Enrichment cultures of environmental samples or inocula of laboratory strains were incubated in duplicate in inclined 20-ml test tubes containing 7.5 ml of selective liquid medium, i.e., with one phosphonate as the P source. These tubes were inoculated with 0.5 ml of a fresh culture of a laboratory strain or, alternatively, with 0.5 ml or 0.5 g of an environmental sample. To homogenize the latter samples, the test tubes were vortexed vigorously for 2 min. The cultures were grown in darkness at 28 or 37°C, according to the growth optimum of the strain (28°C for all environmental samples). *Rhodobacter capsulatus* was grown anaerobically in completely filled 25-ml test tubes with screw caps (RHO medium), whereas *Anacystis nidulans* was grown aerobically like the other strains (GEM medium). These photosynthetic organisms were grown in the light at 26°C. Environmental samples were enriched on both BMM and BAA media, to obtain a broader spectrum of carbon and nitrogen sources.

Test tubes were homogenized twice daily by vortexing. The microorganisms were subcultured in fresh selective medium after 7 days for bacteria and yeasts and 10 days for the fungi. Subcultures were inoculated at 6% (vol/vol). For the fungi, a small piece (ca. 2 mm^2) of mycelium was transferred. After four subcultures (28 days), the OD_{650} was measured. Subsequently, the cultures were centrifuged (20 min at 20,000 × *g*) and the P_n and P_i concentrations were determined on the supernatant. OD_{650} and P_n removal were taken as a measure of phosphonate utilization, except for the cyanobacteria and the fungi. An enrichment culture without a P source served as control for background OD_{650}. Moreover, an enrichment culture with the same initial amount of P_i instead of P_n was also maintained to verify effective inoculation.

Purification of isolates resulting from the enrichment procedure. Dilution series in physiological solution were prepared from the last (fourth) enrichment cultures, and 0.1-ml samples of appropriate dilutions were spread out on selective solid medium (BMM; 300 μg-atoms of P_n liter^{-1}). Well-grown colonies were picked from the plates and streaked out twice consecutively on fresh selective medium. A well-grown colony from the last plate was transferred to liquid medium, and the OD_{650} and P_n removal were measured after 7 days. The isolates were coded, and their purity was verified by judging the presence of a single colony type on nutrient agar and by microscopic examination.

Bacterial counts in environmental samples. Bacterial counts in environmental samples were made solely on BMM medium. Four separate dilution series were prepared for each sample, starting from 1 g of solid or 1 ml of liquid material. Samples (0.1 ml) of appropriate dilutions were streaked out on solid selective media with the different phosphonates. Counts of total CFU in the environmental samples were made on BMM with P_i as the phosphorus source (nonselective BMM). CFU were counted after 8 days incubation. The method allowed us to distinguish between normal-sized and pinpoint colonies, the latter resulting from the P_i background and intracellular reserves. Pinpoint colonies were not included in the counts. When these bacteria were streaked out on selective petri dishes, no significant growth was observed in comparison with the broad-spectrum phosphonate-positive strain *Arthrobacter* sp. strain

GLP-1, which was streaked in another lane on the same dish. The four measurements for a combination of sample and phosphonate were transformed into fractions (positive CFU/total CFU), which were used for statistical analysis. Results of the counting were computer analyzed by means of a two-way factorial analysis of variance (11).

Selection of environments. The following environments were examined in this study: peaty soil (PS) from the Hoge Venen natural park; oligotrophic lake (OL), Hoge Venen natural park; activated sludge "Maria Middelares" (ASMM), treated hospital and laundry effluents, Ghent; activated recirculation sludge "Aalter" (ASAR), treated domestic and dairy effluents, Aalter; river water "Leie" (LRW), Drie Leien, Ghent; aerobic compost (AC), from a municipal waste composting plant, Ghent; black soil (BS), i.e., sand-wastewater sludge mixture (50:50, vol/vol) used as the top layer of a landfill (2 years old), Bredene. All samples were taken in Belgium.

Analytical methods. (i) Determination of phosphorus-containing compounds. Total phosphorus and P_i concentrations were determined with a Technicon AAII continuous-flow system by the procedure of Waldhoff and Sladek (41). P_n was calculated from the total phosphorus concentration minus the P_i concentration, in the absence of condensed or organic phosphates. However, the determination of total phosphorus on the autoanalyzer was severely disturbed by the presence of more than ca. 50 mg of organic material liter^{-1} in the samples. This is due to the preferential reaction of the oxidizing agent (APODS) with the organic matter under the given reaction conditions. To determine total phosphorus in samples rich in organic compounds (e.g., bacterial growth medium), a modification of the above-mentioned procedure was used. Samples with substantial amounts of organic compounds were predigested with an excess of concentrated APODS. To 1 ml of centrifuged sample supernatant, 4 ml of APODS (400 g liter^{-1}) was added. The mixture was digested at 95°C for a minimum of 60 min. Under these conditions a complete mineralization of the P_n to P_i was observed for all compounds studied. P_i in the digest was then determined on the AAII system, taking into account a dilution with water by a factor of 30 to 50 (of the original sample) to reduce the remaining concentration of APODS (too-high concentrations of APODS can interfere with the color reaction).

(ii) One-dimensional thin-layer chromatography. Samples (2 to 10 μl) were applied on 0.1-mm cellulose plates (E. Merck AG). The developing solvent consisted of a mixture of *t*-butanol–glacial acetic acid–water–50% NaOH (95:65:60:10, vol/vol). Plates were run at 28°C for some 12 h. P-containing spots were detected without UV exposure on dry plates by using the color reagent of Maile et al. (21). R_f factors were as follows: 2-AEP, 0.64; MeP, 0.60; Φ-P, 0.46; glyphosate, 0.38; D2010, 0.38; D2000, 0.42; D2041, 0.15; D2041-Nox, 0.14; D2060, 0.09; P_i, 0.63.

(iii) Gas chromatography–C-P lyase activity test. Experiments were carried out in 15-ml septum-stoppered test tubes filled with 3 ml of inoculated BMM medium (MeP; 1 mg-atom of P per liter). The headspace was flushed with sterile pure oxygen after inoculation. Methane concentration in the gas phase after growth was analyzed from parts per million levels with a Packard gas chromatograph equipped with a flame ionization detector. The column was 1.8 m by 3 mm (internal diameter), alumina deactivated with NaI, and the carrier gas was He at a flow rate of 30 ml min^{-1}. Temperature settings were 70°C for the oven, 150°C for the detector, and 20°C for the injector. A gas mixture with 490 ppm of methane was used for calibration.

(iv) Miscellaneous. Optical density measurements were performed on a Shimadzu UV 190 spectrophotometer. Cultures incubated in the light were illuminated by Sylvania Gro-Lux neon tubes (Licor quantum sensor; 35 microeinsteins m^{-2} s^{-1}). Growth of *R. capsulatus* was monitored at 380 nm. The chlorophyll content of *A. nidulans* was used to quantify growth and was measured as described by Porra and Grimme (31).

Fungal biomass was assayed by means of the protein content of the mycelium by the method of Lowry et al. (19). The fungi were harvested on Whatman GFC glass fiber filter and washed with demineralized water. Then the cell protein was liberated by digestion of the filter with mycelium in a test tube with 8 ml of 1 N NaOH and placed in a boiling-water bath for 15 min. Protein content was determined on the supernatant after centrifugation (20 min at 6,000 × *g*).

RESULTS

Utilization of phosphonates by different groups of microorganisms. The objective was to study the capacity of a variety of microorganisms, selected from different ecological and systematic groups, for utilization of phosphonates as the sole source of phosphorus for growth. The selection of microorganisms consisted of 14 bacterial strains (including one cyanobacterium), 2 yeast strains, and 3 fungi. Four bacteria, i.e., *Arthrobacter* sp. strain GLP-1 (28, 30), *P. testosteroni* (7), *E. coli* Crooke strain (1), and *Pseudomonas* sp. strain PG2982 (24, 36), have previously been described in the literature as phosphonate positive and were included in this study as a matter of reference. To our knowledge, none of the other organisms had been examined before with respect to phosphonate utilization. For this purpose, five different growth media were formulated.

Ten different phosphonates were selected for the test, including natural (2-AEP) and xenobiotic mono- and polyphosphonic acids (Table 1). For the latter group, the emphasis was put on compounds that, due to their widespread application, are of environmental significance. All phosphorus sources were supplied at the same amount of P; i.e., 300 μg-atoms of P_n or P_i liter^{-1} (100 μg-atoms of P liter^{-1} for RHO medium). The occurrence of spontaneous hydrolysis of P_n to P_i under incubation conditions was monitored for each phosphonate in a parallel experiment with axenic media. No P_i release was found for 2 AEP, Φ-P, MeP, or MePDiEt. A very slow decomposition in the dark of glyphosate and D2010 was found (ca. 0.5‰ P_n converted to P_i day^{-1}), which, however, did not interfere with the experiments. On the other hand, the N-containing Dequests (D2000, D2041, D2041-Nox, and D2060) showed a more significant hydrolysis, which amounted to between 6 and 15‰ P_n hydrolyzed day^{-1}. As a result, some background growth was observed in all tests with D2000, D2041, D2041-Nox, and D2060. To distinguish between biological and nonbiological phosphonate conversion, every strain was grown in medium supplemented with the P_i concentration measured in axenic test tubes at the end of a 7-day incubation period, and the background OD_{650} (the protein and chlorophyll content for the fungi and the cyanobacterium, respectively) was recorded. Each strain was tested in duplicate against all 10 phosphonates by means of the enrichment procedure.

Strains were considered as phosphonate positive if the growth and P_n removal amounted to at least the background level, plus an arbitrary 10% to rule out false-positive results. Furthermore we investigated whether no or limited growth

TABLE 3. Phosphonate utilization as a P source by pure cultures of microorganisms

Microorganisms	Utilization of phosphonate									
	2-AEP	Φ-P	MeP	MePDiEt	Glyphosate	D2000	D2010	D2041	D2041-Nox	D2060
Bacillus subtilis	−	−	−	−	−	−	−	−	−	−
Arthrobacter sp. strain GLP-1	+	+	+	−	+	+	+	+	+	+
Rhizobium sp.	+	+	+	−	−	−	−	−	−	−
Pseudomonas testosteroni DSM 1622	+	−	+	−	−	−	−	−	−	−
Pseudomonas sp. strain 7NSK2	+	+	+	−	−	−	−	−	−	−
Escherichia coli Crooke DSM 1576	+	−	+	−	−	−	−	−	−	−
Pseudomonas sp. strain PG2982	+	+	+	−	+	−	−	−	−	−
Acinetobacter calcoaceticus	−	−	−	−	−	−	−	−	−	−
Micrococcus luteus	−	−	−	−	−	−	−	−	−	−
Alcaligenes eutrophus	+	+	+	−	+	−	−	−	−	−
Salmonella sp.	+	−	−	−	−	−	−	−	−	−
Staphylococcus aureus	−	−	−	−	−	−	−	−	−	−
Rhodobacter capsulatus ATCC 23782	+	+	+	−	−	−	−	−	−	−

could be due to toxicity of the phosphonates toward the test strains. We concluded that negative data were due to P deficiency only and not to toxic effects, since growth could be obtained in all cases by supplementing the culture with equivalent amounts of P_i. The results of the screening procedure are summarized in Table 3. A confirmation for the presence of effective C-P lyase activity in phosphonate-positive strains was obtained by monitoring the C-P lyase activity of whole cells during growth on MeP. Therefore, the release of CH_4 from MeP was checked by gas chromatography in a separate experiment, according to the reaction scheme $CH_3 - PO_3H_2 + H_2O \longrightarrow CH_4 + H_3PO_4$. This method has been used by various authors (8–10, 39) and is a rapid and elegant way to monitor C-P lyase activity. A stoichiometric production of methane was noticed for all MeP-positive strains from Table 3. No or only trace amounts of CH_4 were found for the MeP-negative strains (results not shown). No evidence was found that phosphonate degradation capacity was also present in organisms other than bacteria; no phosphonate utilization was noticed for the cyanobacterium *A. nidulans* UTEX 625 or for the eucaryotes tested. The observation of 2-AEP, Φ-P, and MeP utilization by the photosynthetic bacterium *R. capsulatus* ATCC 23782 is the first report of C-P bond cleavage by a photosynthetic microorganism. In addition, it demonstrates that C-P bond cleavage can also take place under anaerobic circumstances, thus without the requirement for molecular oxygen for C-P bond cleavage. To our knowledge, this is the first demonstration of anaerobic phosphonate degradation by an axenic bacterial culture.

The concept that every phosphonate-positive bacterium can utilize 2-AEP (6, 37) was confirmed by our tests. 2-AEP was found to be the most accessible phosphonate (ca. 50% of strains tested showed phosphonatase activity). Approximately 40% of the bacterial strains also exhibited C-P lyase. Not all C-P lyase-positive strains grew on glyphosate. No Dequest degraders were detected among our strains, besides the reference strain *Arthrobacter* sp. strain GLP-1. MePDiEt, in contrast to MeP, was not available as a P source to any of the test strains.

Screening for phosphonate-utilizing microorganisms in various environments. Environmental samples were inoculated in duplicate in BMM and BAA media (300 μg-atoms of P_n $liter^{-1}$), and the enrichment procedure was used to select for phosphonate-positive microorganisms. Previous contact of the sample with Dequests can be considered very likely for samples ASMM, ASAR, BS, and LRW, possible for AC, and highly unlikely for PS and OL. As to glyphosate, the contamination of PS and OL can be excluded. No precise information is available concerning the possible contact with glyphosate of the other samples. 2-AEP is likely to be found in every environmental soil, water, or sludge sample. Through the choice of the media, the screening focused mainly on bacteria (cfr. screening of laboratory strains). BAA and BMM media are composed of diverse C and N sources and should allow the growth of a relatively broad range of aerobic heterotrophic bacteria. The results of the enrichment procedure are shown in Table 4. A positive result implies the presence of a bacterial strain, or a microbial consortium, in at least one of the duplicates, with more P_n conversion and growth than what is to be expected on the basis of mere physicochemical dark hydrolysis. Again, this P_i background concentration was determined by means of a parallel experiment with uninoculated medium. Potential background growth due to spontaneous hydrolysis of the Dequests to P_i was determined for each enrichment culture (Fig. 1). The amount of P_i released was negligible for 2-AEP, glyphosate, and D2010 (<3 μg-atoms of P_i 300 μg-atoms of P_n^{-1} $week^{-1}$), but reached up to 30 μg-atom of P_i 300 μg-atoms of P_n^{-1} $week^{-1}$ for D2041 and D2060. Including initial amounts up to 10 μg-atoms of P_i $liter^{-1}$, the P_i available for growth in the latter case was a maximum of 40 μg-atoms of

TABLE 4. Results of the screening for phosphonate-positive bacteria in environmental samples enriched on BMM and BAA media

Medium and sample	Utilization of phosphonate				
	2-AEP	Glyphosate	D2010	D2041	D2060
BMM medium					
PS	+	+	+	+	+
OL	+	+	−	+	+
ASMM	+	+	+	+	+
ASAR	+	+	+	+	+
LRW	+	+	+	+	+
AC	+	+	+	+	+
BS	+	+	−	+	+
BAA medium					
PS	+	+	−	+	+
OL	+	+	−	−	+
ASMM	+	+	+	+	+
ASAR	+	+	−	+	+
LRW	+	+	+	+	+
AC	+	+	−	+	+
BS	+	+	−	+	+

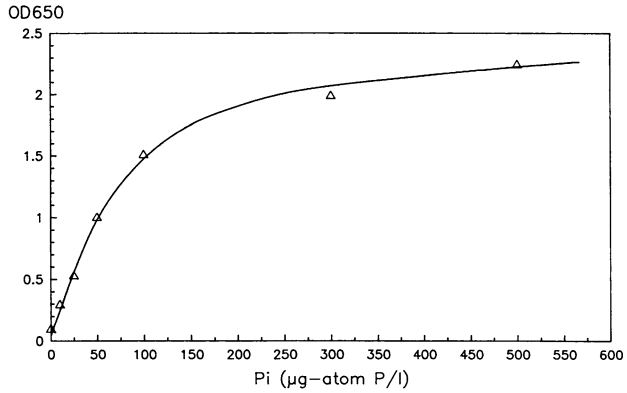


FIG. 1. Typical growth response of a mixed inoculum (environmental sample ASAR) on BMM medium as a function of P_i concentration (microgram-atoms of P per liter).

P_i liter^{-1}. This situation always resulted in a clear-cut background for D2041 and D2060 (OD_{650}, up to ca. 0.800; Fig. 1). Phosphonate-positive strains were recovered on BMM medium for all phosphonates and environments tested, with two exceptions for D2010 in the OL and BS samples. The medium composition apparently influenced the recovery of phosphonate-positive strains. Enrichments were found to be less reliable on BAA than on BMM, but only for the Dequests. The results of the survey point toward a ubiquitous distribution of phosphonate-utilizing microorganisms (although microorganisms utilizing D2010 are probably less widespread).

Extent of phosphonate utilization. The extent of C-P bond cleavage (percent P_n removal), as measured over the last 7 days of incubation of the enrichment procedure, was determined for the different phosphonates and environmental inocula. It must be stressed that, due to a strict P limitation, no luxury phosphonate uptake could be observed; phosphonate removal always corresponded with growth, although not linearly (Fig. 1). Microbial degradation of 2-AEP was invariably fast, with 80 to 100% conversion over 7 days, i.e., average rates of 35 to 45 µg-atoms of P_n converted liter^{-1} day^{-1}. In general, the glyphosate P removal rate and extent were of the same order as those for 2-AEP, with exception of the OL sample, in which only 40 to 45% conversion was recorded over 7 days (results not shown). In the majority of the enrichment cultures, the level of growth and P_n removal for the different Dequest polyphosphonates in the fourth enrichment culture corresponded with the theoretical removal of ca. one phosphonate moiety (i.e., up to 50, 25, and 20% of the theoretical maximum of P_n removal for D2010 [P2], D2041 [P4], and D2060 [P5], respectively). This observation is in agreement with the finding of W. Weissenfels (M.S. thesis, Bochum University, Federal Republic of Germany, 1987), who reported the removal of only one phosphonic acid group from D2041 by *Arthrobacter* sp. strain GLP-1.

However, a few enrichments with a surprisingly higher degree of growth and P_n removal were noted. The best conversions with D2010, D2041, and D2060 are listed in Table 5. These enrichments, which contained different microorganisms, were purified, and the best-growing isolate of each culture was retested. The disappearance of P_n was closely monitored in time in BMM or BAA medium with ca. 150 µg-atoms of P_n liter^{-1} (time courses not shown; these particular experiments were performed with 100 ml of medium in 250-ml Erlenmeyer flasks, which were incubated aerobically in the dark at 28°C on a shaker [150 rpm]). The maximal conversions obtained with these purified strains are presented in Table 6. The isolates were not identified yet. A single organism sufficed to bring about a very significant C-P bond removal. Moreover, the strains were apparently not confined to the phosphonates of their isolation, but could use different types of these complex Dequests. These findings are the first demonstration that a quasi-complete degradation of D2010 and D2041—with respect to the removal of the C-P bonds—can be achieved by microbial action. The pentaphosphonate D2060 was somewhat less accessible to our strains.

TABLE 5. Maximum growth and P_n utilization recorded with enrichment cultures (BMM medium) on Dequests[a]

Phosphonate	Inoculum	OD_{650}	P_n removal (µg-atoms liter^{-1})	% Removed
D2010	ASMM	2.02	279	94
D2041	ASAR	1.93	260	85
D2060	BS	1.82	170	57

[a] The initial P_n concentration was ≈300 µg-atoms liter^{-1}

Utilization of phosphonate nitrogen. Since many phosphonates also contain one or more N-atoms per molecule, we investigated whether this nitrogen is readily biologically available. When the phosphonates 2-AEP, glyphosate, D2041, and D2060 were supplied as both the N and P sources for growth to *Arthrobacter* sp. strain GLP-1 (2.5 mg-atoms of phosphonate N liter^{-1}, BMM medium without NH_4Cl), no significant growth was observed. Also, no P_n removal or NH_4^+ N release could be measured, suggesting that the phosphonate N was not used. This situation could not be alleviated by supplementing the culture with P_i (1,000 µg-atoms of P_i liter^{-1}) or a starter concentration of nitrogen (up to 750 µg-atoms of N liter^{-1} as NH_4Cl). These results stress the importance of P limitation for the initiation of the biodegradation of the phosphonates. Apparently, the action of *Arthrobacter* sp. strain GLP-1 is restricted to the removal of P_n under our experimental conditions.

Quantification of phosphonate-positive microorganisms in the different environments. The number of phosphonate-degrading microorganisms in the environmental samples was examined in an attempt to answer the following questions:

TABLE 6. Dequest utilization by a selection of isolates obtained from environmental samples[a]

Isolate code	Medium and phosphonate of isolation	P source	P_n or P_i conversion over 7 days (%)
MMM101	BMM, D2010	P_i	100
		D2010	88
		D2041	67
		D2060	54
MMM412	BMM, D2041	P_i	100
		D2010	92
		D2041	97
		D2060	64
MAR606	BMM, D2060	P_i	100
		D2010	Not determined
		D2041	80
		D2060	55

[a] P_i and phosphonates were added to 150 µg-atoms of P_n liter^{-1} initially.

TABLE 7. Bacterial counts in environmental samples on nonselective and selective BMM media

Sample	Total CFU (ml^{-1})	% Phosphonate-positive CFU[a] (mean ± SD)				
		2-AEP	Glyphosate	D2010	D2041	D2060
PS	$200 \times 10^5 \pm 113 \times 10^5$	9.77 ± 2.46	4.82 ± 1.94	0.01 ± 0.03	1.66 ± 0.41	0.50 ± 0.12
OL	$49 \times 10^3 \pm 39 \times 10^3$	31.55 ± 20.47	2.70 ± 0.73	0.00 ± 0.00	6.53 ± 0.41	3.60 ± 1.50
ASMM	$125 \times 10^5 \pm 18 \times 10^5$	41.88 ± 8.87	25.55 ± 7.34	1.14 ± 0.14	5.53 ± 0.98	3.00 ± 0.43
ASAR	$230 \times 10^5 \pm 141 \times 10^5$	23.32 ± 8.27	4.24 ± 0.64	0.03 ± 0.07	0.78 ± 0.24	0.48 ± 0.30
LRW	$90 \times 10^3 \pm 36 \times 10^3$	21.76 ± 3.07	3.50 ± 1.64	0.00 ± 0.00	2.05 ± 0.15	0.65 ± 0.23
AC	$426 \times 10^6 \pm 54 \times 10^6$	18.65 ± 2.03	0.09 ± 0.01	0.01 ± 0.003	0.10 ± 0.03	0.16 ± 0.04
BS	$650 \times 10^6 \pm 267 \times 10^6$	30.77 ± 3.33	5.05 ± 2.61	0.00 ± 0.00	1.11 ± 0.35	0.76 ± 0.17

[a] [(Phosphonate-positive CFU $milliliter^{-1}$)/(total CFU $milliliter^{-1}$)] × 100. Mean values for all samples were as follows: 2-AEP, 25.33%; glyphosate, 6.56%; D2010, 0.18%; D2041, 2.49%; D2060, 1.31%. For 2-AEP and glyphosate, the average fractions per phosphonate are significantly different from other values ($\alpha = 0.01$).

does the contact with a particular phosphonate increase the relative number of phosphonate-positive microorganisms for that compound significantly, i.e., does adaptation occur in terms of numbers, and is the relative number of positive CFU over the range of the environments significantly different for different phosphonates? The total number of CFU in a particular environmental sample and the phosphonate-positive fractions for the different phosphonates are shown in Table 7. Relative numbers in the environments were invariably highest for 2-AEP and lowest for D2010. Glyphosate was generally a more common P source than the Dequests. The results of the direct-plate-count method were in good agreement with those of the enrichment procedure (Table 4), except for sample LRW, in which no D2010 degraders were counted. The fractions of phosphonate-positive CFU were computer analyzed by means of a two-way factorial analysis of variance. The effect of phosphonates on the environments and, inversely, the effect of environments on the phosphonates were assessed by means of a Duncan test (11). The analysis of variance revealed a significant effect ($\alpha = 0.01$) on the fraction of positive CFU by the type of phosphonate, as well as by the type of environment. A significant interaction was also observed between the two factors. Over the whole of environments, the average fractions of phosphonate-positive isolates were significantly different ($\alpha = 0.01$) for 2-AEP, glyphosate, and the group of Dequests but not among the Dequests (Table 7). On the other hand, no significant differences in phosphonate-positive fractions were observed between the environments (with exception of the ASMM sample, which yielded particularly high counts for 2-AEP and glyphosate). The enrichments and direct counts did not reveal systematic differences between typically polluted and nonpolluted environments, which could indicate adaptation of the microbial community to phosphonates. Also in the nonpolluted environments (PS and OL samples), a significant fraction of glyphosate-, D2041-, and D2060-utilizing microorganisms was present.

DISCUSSION

The cleavage of the C-P bond can be regarded as a key factor in the degradation of phosphonates. With respect to physicochemistry, photodegradation of the C-P bond has been reported for various phosphonates (16, 20, 22). We have been confronted in our study with a slow hydrolytic degradation that also proceeds in the dark and liberates P_i from the phosphonate moiety. This phenomenon, which particularly acts on N-containing phosphonates, is not well understood. The similarity between the metal-catalyzed hydrolysis reported for a number of organophosphates (25) and the process described in this paper is particularly intriguing, however. From the biological side, it has been documented in literature that P deficiency can induce the synthesis of C-P bond-cleaving enzymes (39, 40) as well as uptake systems for phosphonates (13, 18, 33). Particularly for the uptake of larger and negatively charged polyphosphates (and polyphosphonates?) into the cell, appropriate uptake systems are induced under conditions of P deficiency (27). The potential of P limitation to induce phosphonate biodegradation was also demonstrated in our study. The phosphonate utilization patterns observed for the reference strains are in good agreement with data in the literature (1, 6, 7, 13, 24, 28–30, 34, 36). Moreover, they present additional data on phosphonate utilization by these particular strains and new data for a series of previously unstudied organisms. The detection of C-P bond cleavage among various systematic groups of bacteria suggests a distribution of the respective genes that is wider than what was assumed at first (37). By contrast, N stress failed to induce phosphonate utilization in our tests. The relative imbalance for cell synthesis between N and P in the phosphonate molecule and tight enzyme regulation might account for this (29).

The effects of other more accessible P sources on phosphonate uptake and degradation are of great environmental importance. Many environments (e.g., activated sludge, sediments, soil) that act as a sink for phosphonates are not characterized by a lack of P most of the time. It should therefore be investigated which alternative degradation mechanisms can act on phosphonates, or how phosphonate removal can be maintained even in the presence of ample P. Biological degradation rates were found to be up to 2 orders of magnitude faster than the hydrolysis process and thus can contribute to the degradation process significantly.

Among the phosphonates tested, the large polyphosphonates have received little attention. An indication that polyphosphonic acids were also susceptible to C-P bond cleavage by microorganisms under phosphorus-deficient conditions was only recently presented (12, 28; Weissenfels, M.S. thesis). An *Arthrobacter* strain was reported to use a single phosphonate moiety from Dequest polyphosphonates. A close examination of various environments presented in this study revealed that this *Arthrobacter* sp. strain GLP-1 is not an isolated case. Our screening yielded several strains (or microbial consortia) with analogous and even better capacities in terms of the extent of C-P bond cleavage. It must be added however, that those organisms were present in the environmental samples at low relative numbers, compared with the bacteria growing on simple monophosphonates. These differences in relative numbers between phosphonates and the differences in phosphonate utilization patterns

among laboratory strains are an indication that there might exist more distinct types of C-P bond-cleaving enzymes than currently assumed (phosphonatase and C-P lyase; 18, 39, 40). An enzymatic study in a broad-spectrum phosphonate degrader, which takes into account the aspect of phosphonate uptake, might elucidate this point.

From an ecological viewpoint, it is not evident why, especially in nonpolluted samples, degradation mechanisms for those xenobiotics would be available. In this respect the paper of Cordeiro et al. (8) can be mentioned. It presents some evidence that C-P lyases would use a radical-type reaction to cleave the C-P bond. It is hypothesized that phosphonates like the Dequests can be attacked by such radical-generating proteins. Some of those powerful enzymes (e.g., ligninases) were shown to have a quite broad substrate specificity (5). Such rather unspecific enzymes might account for the ubiquitous presence of phosphonate-positive microorganisms in both polluted and nonpolluted samples. Elevated numbers of phosphonate-positive CFU in phosphonate-contaminated environments have been reported previously, yet without a comparison with unpolluted samples (3). Adaptation in terms of relative numbers toward detergent-derived chemicals has been demonstrated, for instance, by Anderson et al. (2) for alkylsulfates: percentages of alkylsulfatase-positive bacteria were found to be higher at polluted sites in a river. In our survey, we observed marked differences in phosphonate-positive CFU among phosphonates, but not among environments. We therefore tentatively conclude that adaptation, as a result of contact with phosphonates, has not taken place. This might be explained by the absence of a continuous selection pressure (i.e., P stress) in most cases. Furthermore, predicted environmental concentrations in polluted samples are very low (parts per billion to parts per million level), which might not allow active metabolism and competitive growth rates with these substrates. Other degradation phenomena, such as cometabolism or fortuitous metabolism may become of major importance at low concentrations. On the other hand, the spectrum of this survey was somewhat limited by the use of defined growth media—which allow growth of only a fraction of the organisms present—and by the use of plate counts, instead of more specific biochemical tests or DNA probe methods. The latter approach, however, requires a degree of insight into the enzymology and genetics of C-P bond cleavage and phosphonate uptake, which is currently not available. These aspects, as well as aerobic and anaerobic degradation pathways under environmental conditions, are proposed as essential subjects for further research.

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