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Organophosphonate Utilization by the Wild-Type Strain of *Pseudomonas fluorescens*

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The wild-type strain of *Pseudomonas fluorescens* was found to utilize a range of structurally diverse organophosphonates as its sole carbon or nitrogen sources. Representative compounds included aminoalkylphosphonates, hydroxyalkylphosphonates, oxoalkylphosphonates, and phosphono dipeptides. Among them, amino(phenyl)methylphosphonate, 2-aminoethylphosphonate, aminomethylphosphonate, diisopropyl 9-aminofluoren-9-ylphosphonate, and 2-oxoalkylphosphonates were used by *P. fluorescens* as its sole sources of phosphorus. Only slight growth was observed on the herbicide glyphosate (*N*-phosphonomethylglycine), which was metabolized to aminomethylphosphonate. Neither phosphinothricin nor its dialanyl tripeptide, bialaphos, supported growth of *P. fluorescens*. The possible mechanisms of organophosphonate degradation by this strain are discussed.

Organophosphonates are a class of organic compounds characterized by the presence of one or more carbon-phosphorus (C-P) bonds. The C-P bond is resistant to chemical hydrolysis, thermal decomposition, and photolysis (14, 19). Organophosphonates, which are widely used as pesticides, lubricant additives, flame retardants, plasticizers, corrosion inhibitors, and drugs (19), are potent biocides. The most conspicuous examples include the popular herbicides glyphosate and phosphinothricin (12, 23); ethyl- and phenylphosphonate derivatives commonly used as insecticides (12, 39); Fyrol 76, an oligomer of vinylphosphonate-methylphosphonate representative of flame retardants (19); polyaminopolyphosphonic acids, widely used as corrosion inhibitors (19); bisphosphonates, which have an application for the treatment of bone mineralization disorders (13); the antibiotics alafosfalin and phosphonomycin (12, 23); and cyclic esters of aromatic bisphosphonates used as polymer additives (19). The release of xenobiotic phosphonates into the environment warrants intensive research on their biodegradation pathways and mechanisms.

Up to now, a number of bacterial strains, among them a few strains of *Pseudomonas*, have been found to utilize a wide range of natural and synthetic organophosphonates as their sole sources of phosphorus, nitrogen, and/or carbon for growth (3, 8, 10, 18–21, 33, 35, 36, 40–47, 49–51, 56, 59). So far, two biochemically distinct pathways of phosphonate catabolism in bacteria have been determined. The first one involves direct C-P bond cleavage, caused by the action of an enzyme called carbon-phosphorus lyase (2, 7, 9, 33, 37, 42–44, 46, 51, 56) and followed by stepwise degradation of the organic part of the molecule. The second one involves enzymatic reactions, which modify the organic part of the molecule, whereas the C-P bond is still conserved and may then undergo further enzymatic or chemical cleavage (3, 25, 26, 38, 40, 41, 48).

The wild-type strain of *Pseudomonas fluorescens* isolated from soil (55) appeared to be resistant to the action of antibacterial phosphono dipeptides on the basis of phosphonic acid analogs of alanine and α-methylalanine (58) as well as the phosphonic acid analog of glutamic acid (57). This resistance may be due either to these compounds' inability to cross the bacterial cytoplasmic membrane or to the ability of this strain to metabolize the phosphonates. In order to verify the latter assumption, we have studied the ability of *P. fluorescens* to utilize a range of structurally diverse organophosphonates as its sole sources of phosphorus, nitrogen, or carbon.

MATERIALS AND METHODS

Microorganism. The wild-type strain of *P. fluorescens* was isolated from soil during the studies of lipase-producing microorganisms (55) and was kept as a lyophilized preparation.

Media. The culture medium used contained glucose (28 mM), ammonium sulfate (20 mM), calcium chloride (1 mM), sodium chloride (1.7 mM), magnesium sulfate (1 mM), and ferrous sulfate (50 μM). K_2HPO_4 served as a phosphorus source and was added to the medium to a final concentration of 0.5 mM. When organophosphonates were used as sole sources of phosphorus, P_i was replaced by the phosphonates at a final concentration of 0.5 mM. The medium was buffered with 50 mM Tris (pH 7.2). When the organophosphonates were sole sources of either carbon (5 mM) or nitrogen (2 mM), the medium deficient in glucose or ammonium sulfate, respectively, was used. The pH of the medium was adjusted to 7.2.

Chemicals. The phosphonates used in this study were synthesized according to the procedures described earlier; the names and compound numbers of these chemicals are listed in Table 1. Glyphosate (*N*-phosphonomethylglycine) was supplied by Monsanto Agricultural Products, St. Louis, Mo. Phosphinothricin [L-2-amino-4-(methylphosphinyl)butanoic acid] and bialaphos (phosphinothricylalanylalanine) were kind gifts from Satoshi Imai, Pharmaceutical Development Laboratories, Meiji Seika Kaisha Ltd., Kawasaki, Japan. All of the phosphonates were found to be stable in aqueous media for at least 2 weeks at room temperature, and we have not observed their decomposition during autoclaving.

All other chemicals were purchased either from Sigma

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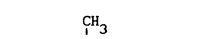
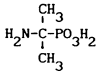
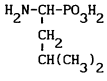
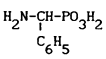
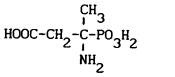
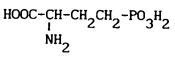
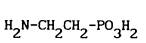
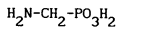
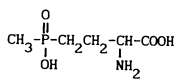
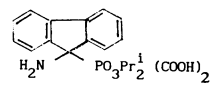
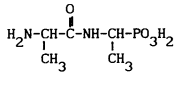
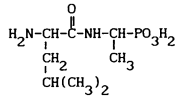
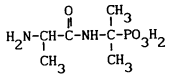
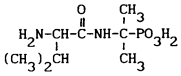
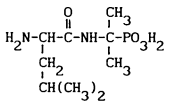
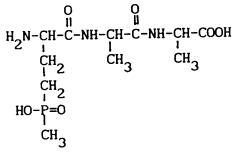
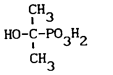
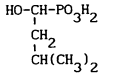
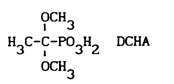
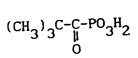
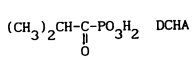
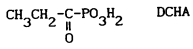
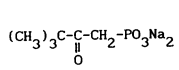
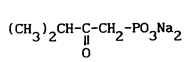
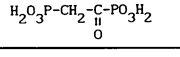
TABLE 1. Organophosphonates used

Compound no. and name	Structure	Origin
1. *N*-Phosphonomethylglycine		Monsanto Chemical Co.
2. 1-Amino-1-methylethylphosphonic acid		52
3. DL-1-Amino-3-methylbutylphosphonic acid		52
4. DL-Amino(phenyl)methylphosphonic acid		52
5. DL-3-Amino-3-phosphonobutanoic acid		34
6. DL-2-Amino-4-phosphonobutanoic acid		34
7. 2-Aminoethylphosphonic acid (ciliatine)		4
8. Aminomethylphosphonic acid		53
9. L-2-Amino-4-(methylphosphinyl)butanoic acid (phosphinothricin)		S. Imai
10. Diisopropyl 9-aminofluoren-9-ylphosphonate oxalate		30
11. DL-1-(*N*-L-Alanylamino)ethylphosphonic acid		31
12. DL-1-(*N*-L-Leucylamino)ethylphosphonic acid		31
13. 1-(*N*-L-Alanylamino)-1-methylethylphosphonic acid		31
14. 1-(*N*-L-Valylamino)-1-methylethylphosphonic acid		31
15. 1-(*N*-L-Leucylamino)-1-methylethylphosphonic acid		31
16. L-Phosphinothricyl-L-alanyl-L-alanine (bialaphos)		S. Imai
17. 1-Hydroxy-1-methylethylphosphonic acid		29
18. DL-1-Hydroxy-3-methylbutylphosphonic acid		32
19. 1,1-Dimethoxyethylphosphonic acid[a]		60
20. 1-Oxo-2,2-dimethylpropylphosphonic acid		60
21. 1-Oxo-2-methylpropylphosphonic acid[a]		60
22. 1-Oxopropylphosphonic acid[a]		60
23. Disodium 2-oxo-3,3-dimethylbutylphosphonate		60
24. Disodium 2-oxo-3-methylbutylphosphonate		60
25. 1-Oxoethyl-1,2-bisphosphonic acid		15

[a] DCHA, dicyclohexylamine (as dicyclohexylammonium salt).

Chemical Co. (St. Louis, Mo.) or from P.O.Ch. (Gliwice, Poland).

Growth conditions. Preliminary studies of the growth of *P. fluorescens* on phosphonates as its sole phosphorus, carbon, or nitrogen sources were carried out with test tubes containing 5 ml of an appropriate medium. They were inoculated with 50 μl of a washed suspension of stationary-phase cells precultured on the medium with glucose, ammonium sulfate, and P_i.

Growth rate determinations were performed with 250-ml Erlenmeyer flasks containing 25 ml of medium, which received 250-μl inocula. All cultures were incubated at 28°C on a shaking platform at 100 rpm. Culture turbidity measurements were made with a Spekol 11 spectrophotometer (Carl Zeiss, Inc., Jena, Germany) at 600 nm.

None of the compounds studied, except for bialaphos (compound 16), was found to be toxic to *P. fluorescens*.

TLC. After removal of bacterial cells by centrifugation, supernatants were examined by thin-layer chromatography (TLC) on high-performance TLC aluminum or plastic sheets precoated with silica gel 60 254-nm-fluorescence detector (F_{254}) for nano-tlc (20 by 20 cm) (Merck, Darmstadt, Germany). Chromatograms were developed by using *n*-butanol–acetic acid–water (12:3:5) as a solvent system. Spots were visualized with UV light and by means of ninhydrin spray reagent.

Gas chromatography. Degradation of 2-oxoalkylphosphonates by the *P. fluorescens* strain was assayed by gas chromatography. *P. fluorescens* cells were cultured on the medium containing 2-oxoalkylphosphonic acids (5

TABLE 2. Growth of *P. fluorescens* on various phosphorus sources

Substrate	Lag phase (h)	Specific growth rate (h^{-1})	Culture turbidity at 600 nm[a]
P_i	8	0.19	1.40
Compound 4	14	0.17	1.20
Compound 7	8	0.23	1.63
Compound 8	11	0.07	0.56
Compound 10	14	0.15	0.81
Compound 23	8	0.20	1.52
Compound 24	8	0.20	1.50
Compound 25	8	0.19	1.38

[a] After growth for 48 h.

mM) as the sole sources of carbon and phosphorus. Culture supernatants were extracted with chloroform, and the chloroform layers were analyzed with a Hewlett-Packard 5890 series II gas chromatograph with a mass spectrum detector.

RESULTS

Growth on phosphonates as the sole phosphorus sources. A phosphorus-free minimal medium that was developed (see Materials and Methods) allowed very good growth of *P. fluorescens* on different phosphorus sources and gave little growth in the absence of phosphorus. Among all organophosphonate compounds tested, *P. fluorescens* grew well only on DL-amino(phenyl)methylphosphonate (compound 4). 2-aminoethylphosphonate (2-AEP [ciliatine], compound 7), aminomethylphosphonate (AMP [compound 8]), diisopropyl 9-aminofluoren-9-ylphosphonate (compound 10), and 2-oxoalkylphosphonates (compounds 23 through 25). The growth characteristics of *P. fluorescens* on these phosphorus sources are shown in Table 2.

2-AEP as well as the 2-oxoalkylphosphonates supported very good growth of *P. fluorescens*. Utilization of these phosphonates required a short lag phase, equal to that observed in the case of P_i. Cellular yields found for the growth on the phosphonate substrates and specific growth rates were comparable to or even slightly higher than those for growth on P_i.

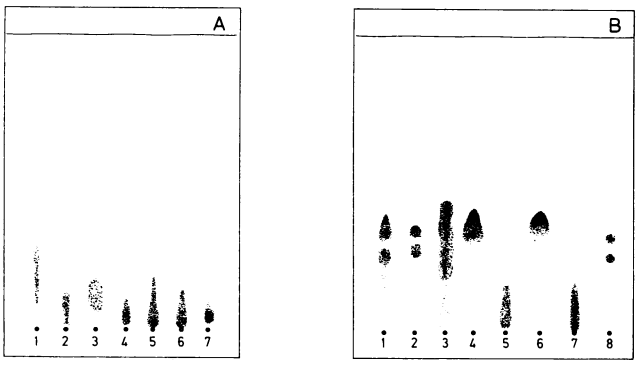


FIG. 1. Representative results of TLC examination of the growth media. (A) Growth on glyphosate. 1, glyphosate; 2, AMP; 3, glycine; 4, medium in which glyphosate was used as a sole phosphorus source; 5, glyphosate as a sole source of phosphorus and carbon; 6, glyphosate as a sole nitrogen source; 7, glyphosate as a sole source of carbon. (B) Growth on phosphonopeptides used as the sole sources of carbon. 1, growth medium with LeuAlaP; 2, LeuAlaP as standard; 3, growth medium with LeuMeAlaP; 4, LeuMeAlaP as standard; 5, growth medium with AlaMeAlaP; 6, AlaMeAlaP as standard; 7, growth medium containing AlaAlaP; 8, AlaAlaP as standard.

TABLE 3. Utilization of aminophosphonates and phosphono peptides by *P. fluorescens* as its sole nitrogen sources

Substrate compound	Growth of bacteria[a]
1	−
2	+++
3	+++
4	+
5	+
6	−
7	++
8	+
9	−
10	−
11	+++
12	+++
13	+++
14	+++
15	+++
16	−

[a] Bacterial growth was scored by comparison of culture turbidity at 600 nm with that of a control culture grown on the standard medium containing ammonium sulfate (2 mM) as a nitrogen source. +++, heavy growth (>70%); ++, 50 to 70% growth; +, 10 to 50% growth; −, <10% growth. Cultures were scored after 4 days.

P. fluorescens grew less efficiently on AMP. Both a longer lag phase and a significantly lower specific growth rate in relation to growth on P_i were noticed with AMP.

Only slight growth was observed on glyphosate (compound 1), a broad-spectrum postemergence herbicide that inhibits 3-enolpyruvylshikimate-5-phosphate synthase (EC 2.5.1.19), the enzyme involved in the biosynthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan (22, 54). Addition of the three aromatic amino acids at 50 mg/liter did not improve growth. As shown in Fig. 1, the transformation of the glyphosate molecule to AMP was documented by means of TLC. The data in Table 2 indicate that AMP was utilized by the *P. fluorescens* strain as the sole phosphorus source. One plausible explanation of these discrepancies is that given by Liu et al. (33). Glyphosate is taken up by the cells but metabolized slowly, which results in the accumulation of a high level of glyphosate inside the cell. Such accumulation of glyphosate may be inhibitory to growth.

Both phosphinothricin (compound 9), a potent inhibitor of glutamine synthetase from *Escherichia coli* (5, 6) and plants (17, 24, 27, 28), and its dialanyl tripeptide, bialaphos (compound 16), did not support growth of *P. fluorescens*.

Because specific growth rates on some organophosphonate substrates are comparable to or even higher than rates of growth on P_i and the observed lag phases are equal to that for growth on P_i, one should not expect the preferential utilization of P_i by *P. fluorescens* in a mixed-incubation experiment. In an attempt to examine such a possibility, *P. fluorescens* was cultured on the medium containing equimolar (0.25 mM) amounts of P_i and either 2-oxo-3,3-dimethylbutylphosphonate (compound 23) or 2-oxo-3-methyl-bu-

TABLE 4. Ability of *P. fluorescens* to utilize phosphonates as its sole carbon source

Substrate compound	Bacterial growth[a]
1	−
2	−
3	++
4	−
5	−
6	−
7	+
8	−
9	−
10	−
11	++
12	++
13	++
14	++
15	++
16	−
17	−
18	++
23	++
24	++
25	++

[a] Bacterial growth was scored by comparison of culture turbidity at 600 nm with that of a control culture grown on the standard medium containing glucose (5 mM) as a carbon source. ++, >50% growth; +, 10 to 50% growth; −, <10% growth. Cultures were scored after 4 days.

tylphosphonate (compound 24). For both substrates, similar results were obtained. Lack of the diauxic lag phase, coincident with P_i depletion, implies that transport of these organophosphonates or their metabolism in the *P. fluorescens* strain, similarly to *Pseudomonas* strain 4ASW (46), is less tightly controlled by P_i than in *Kluyvera ascorbata* (56).

Utilization of aminophosphonates as the sole nitrogen sources for growth. When growing on aminophosphonates as the sole source of nitrogen, *P. fluorescens* was found to utilize a range of substrates (Table 3).

Growth was abundant on all five phosphonic dipeptides (compounds 11 through 15), i.e., on AlaAlaP (alafosfalin), LeuAlaP, AlaMeAlaP, ValMeAlaP, and LeuMeAlaP. Cleavage of the peptide bond, confirmed by TLC, supports the hypothesis that resistance of this strain to the action of antibacterial phosphono peptides may result from its ability to hydrolyze their peptide bonds by extracellular or membrane-bound proteolytic enzymes.

Heavy growth was also observed on phosphonic analogs of α-methylalanine (compound 2) and leucine (compound 3).

P. fluorescens grew apparently less well on 2-AEP (compound 7) and very poorly on DL-amino(phenyl)methylphosphonate (compound 4), AMP (compound 8), and DL-3-amino-3-phosphonobutanoic acid (compound 5).

No growth level higher than that of the control was noticed in the cases of glyphosate (compound 1), phosphinothricin (compound 9), bialaphos (compound 16), and diisopropyl 9-aminofluoren-9-ylphosphonate (compound 10).

Growth of *P. fluorescens* on phosphonates as its sole carbon sources. *P. fluorescens* was able to utilize a number of organophosphonate compounds as its sole carbon sources for growth (Table 4).

Also, in this case, all the phosphono dipeptides (compounds 11 through 15) were easily degraded by the bacterium. As shown by Fig. 1, a TLC examination of the culture

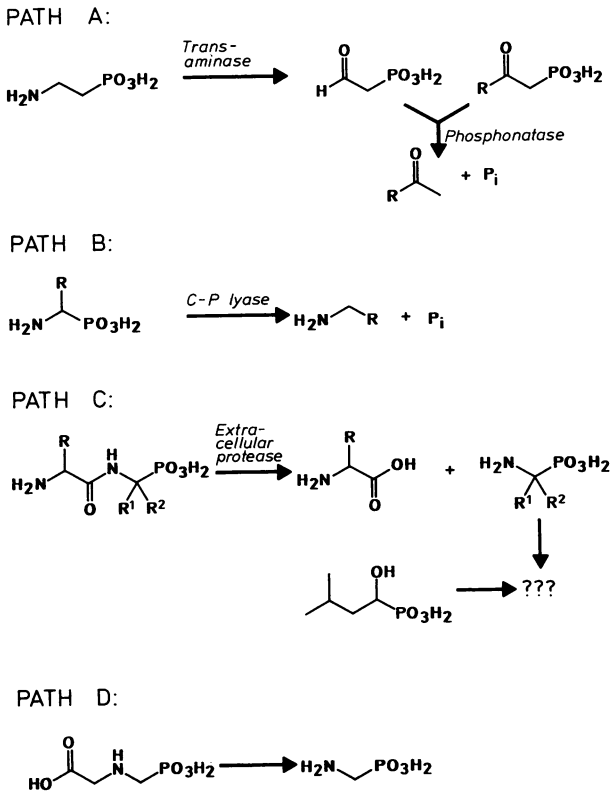


FIG. 2. Possible mechanisms of organophosphonate degradation by *P. fluorescens*. R, R^1, and R^2, substituents; ?, unknown degradation step(s).

media indicated that the bacterial growth was accompanied by hydrolysis of the peptide bonds and liberation of P-terminal 1-aminoalkylphosphonic acids. Growth of *P. fluorescens* on 2-oxoalkylphosphonic acids (compounds 23 through 25) as sole carbon sources was similar to that observed in the case of phosphono dipeptides. Also, DL-1-amino-3-methylbutylphosphonic acid (compound 3) and DL-1-hydroxy-3-methylbutylphosphonic acid (compound 18), compounds structurally very similar to each other, supported heavy growth. It is noteworthy that ciliatine sustained growth of *P. fluorescens*, although to a lesser extent, and thus it is the only compound utilized by this strain as a sole source of phosphorus, carbon, and nitrogen.

DISCUSSION

The present results provide indications for the existence of several possible mechanisms of the degradation of the organophosphonates studied. Although the exact distinction between these mechanisms would require the analysis of intracellular metabolites, the results allow us to suggest three distinct mechanisms of organophosphonate utilization by this strain.

The first one, perhaps most commonly found in bacteria (16), involves the participation of phosphonatase (40), which originally catalyzes the hydrolytic cleavage of 2-phosphonoacetaldehyde into acetaldehyde and P_i. This enzyme is also important for the biodegradation of 2-AEP (ciliatine), which is converted by enzymic transamination into 2-phosphonoacetaldehyde (11). We speculate that this pathway (path A in

Fig. 2) operates in our case during the degradation of 2-AEP (compound 7) and the 2-oxoalkylphosphonic acids (compounds 23 through 25). It is noteworthy that 2-phosphonoacetaldehyde is also representative of the latter class of compounds. This speculation stems from the recognition that ciliatine and 2-oxoalkylphosphonates are efficiently utilized as the sole sources of phosphorus and carbon (and also, in the case of ciliatine, nitrogen) for bacterial growth. Utilization of each of these compounds as the sole phosphorus source is as effective as utilization of P_i. Moreover, no competition between utilization of P_i and that of 2-oxoalkylphosphonates was found. A TLC examination of the growth media containing ciliatine showed complete disappearance of this amino acid during the bacterial growth. Using gas chromatography and mass spectrometry for the analysis of the media containing only 2-oxoalkylphosphonates, we have also not found any remainders of their carbon chain fragments.

Among all the other phosphonates studied in this work, only aminophosphonate compounds 4, 7, and 10 were utilized by *P. fluorescens* as sole sources of phosphorus. We believe that these three compounds were degraded by the action of C-P lyase (path B in Fig. 2), an enzyme catalyzing direct, free-radical cleavage of carbon-to-phosphorus bond (1, 7). This conclusion is supported by the fact that they are not utilized as sole sources of carbon (Table 4) and only slightly sustain growth of *P. fluorescens* as sole sources of nitrogen (Table 3). The slight amount of growth observed on compound 10 as a carbon source should be attributed to the utilization of the oxalate fragment of this molecule. The comparison of the growth of *P. fluorescens* on these three aminophosphonates as the sole phosphorus sources with the growth observed on P_i, ciliatine, and 2-oxoalkylphosphonates reveals significant differences. Thus, a longer lag phase, some reduction of specific growth rates, and incompleteness of growth on compounds 4, 7, and 10 were observed, suggesting the inducible character of their utilization under phosphorus deficiency. It is also worth noticing that the degradation of these 1-aminoalkylphosphonates in a manner similar to that of ciliatine seems to be unlikely since the 1-oxoalkylphosphonate compound 20, an analog of their presumed intermediates, did not as a sole phosphorus source support the growth of the bacterium.

Perhaps the most interesting finding of this work is the indication that *P. fluorescens* is able to use organophosphonates as sole sources of nitrogen or carbon for growth while they are not used as sole phosphorus sources. Clear-cut results were obtained with phosphono dipeptides, which were efficiently degraded by this strain. A TLC analysis of the culture media demonstrated that the hydrolysis of the peptide bond was the first step of their degradation (path C in Fig. 2), with *N*-alanyldipeptides (compounds 11 and 13) being hydrolyzed faster than *N*-leucyl dipeptides (compounds 12 and 15). This process is accompanied by fast utilization of an N-terminal amino acid and accumulation of 1-aminoalkylphosphonate, which probably undergoes further slow degradation. Thus, after 48 h of *P. fluorescens* growth, the total disappearance of compounds 11 (both LL and LD diastereomers) and 13 was observed while their *N*-leucyl counterparts (compounds 12 and 15) were still present in the media (Fig. 1). The finding that *P. fluorescens* is unable to utilize the phosphono peptide bialaphos (phosphinothricyl-L-alanyl-L-alanine [compound 16]) is quite interesting in this respect. However, belonging to the phosphono peptides containing the phosphonic moiety as a side group, bialaphos is structurally different from the peptide compounds 11 through 15. As evidenced by a disc diffusion method (data not shown), only bialaphos exerts antibacterial activity against *P. fluorescens*, which additionally supports the hypothesis that the extracellular hydrolysis of phosphono peptides may contribute to the resistance of this strain to the action of these antibacterial agents.

The most intriguing observation, however, is that *P. fluorescens* is able to utilize two aminophosphonates (compounds 2 and 3) as the sole sources of nitrogen as well as the aminophosphonate compound 3 and the hydroxyphosphonate compound 18 as the sole sources of carbon. These compounds were not degraded when the strain was grown under phosphorus deficiency, suggesting that the bacterium is able to functionalize certain amino- and hydroxyalkylphosphonates and that the C-P bond remains preserved during this process (path C in Fig. 2). The mechanism(s) of this degradation is unclear and requires more detailed study. The decomposition of the organic part of organophosphonate without the cleavage of the C-P bond was also found in the case of glyphosate (path D in Fig. 2). Glyphosate very poorly supported bacterial growth when used as the sole phosphorus, nitrogen, carbon, or phosphorus-plus-carbon source. However, a TLC examination indicated that this herbicide was converted into AMP. This probably reflects the fact that glyphosate is taken up by the cells in amounts sufficient to cause a bacteriostatic effect (33).

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