JOURNAL OF BACTERIOLOGY, Feb. 1987, p. 710–717
0021-9193/87/020710-08$02.00/0

Vol. 169, No. 2

Bacterial Carbon-Phosphorus Lyase: Products, Rates, and Regulation of Phosphonic and Phosphinic Acid Metabolism

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Received 7 July 1986/Accepted 28 October 1986

Carbon-phosphorus bond cleavage activity, found in bacteria that utilize alkyl- and phenylphosphonic acids, has not yet been obtained in a cell-free system. Given this constraint, a systematic examination of in vivo C-P lyase activity has been conducted to develop insight into the C-P cleavage reaction. Six bacterial strains were obtained by enrichment culture, identified, and characterized with respect to their phosphonic acid substrate specificity. One isolate, *Agrobacterium radiobacter*, was shown to cleave the carbon-phosphorus bond of a wide range of substrates, including fosfomycin, glyphosate, and dialkyl phosphinic acids. Furthermore, this organism processed vinyl-, propenyl-, and propynylphosphonic acids, a previously uninvestigated group, to ethylene, propene, and propyne, respectively. A determination of product stoichiometries revealed that both C-P bonds of dimethylphosphinic acid are cleaved quantitatively to methane and, furthermore, that the extent of C-P bond cleavage correlated linearly with the specific growth rate for a range of substrates. The broad substrate specificity of *Agrobacterium* C-P lyase and the comprehensive characterization of the in vivo activity make this an attractive system for further biochemical and mechanistic experiments. In addition, the failure to observe the activity in a group of gram-positive bacteria holds open the possibility that a periplasmic component may be required for in vivo expression of C-P lyase activity.

Living systems contain organophosphorus mostly in the form of oxygen esters, diesters, and anhydrides of phosphoric acid. However, phosphonic acids, a class of organophosphorus compounds containing a direct carbon-phosphorus bond, are also found in nature (14, 21). The initial discovery of a natural phosphonic acid product, 2-aminoethylphosphonic acid, in 1959 (18) is relatively recent compared with the history of research in biological organophosphate esters. Since that time dozens of naturally occurring C-P compounds have been discovered (17). The biosynthesis of the C-P bond has been documented in protozoa, fungi, and molluscs (16, 37). The importance of C-P bonds in these invertebrates is underscored by the observation that 50 to 75% of the cilia phospholipids of *Tetrahymena* are phosphonolipids (22).

Given the biosynthetic accessibility of the C-P linkage it is almost axiomatic that bacteria would have evolved the ability to catabolize phosphonic acids. Indeed, by enrichment culture bacteria have been obtained that utilize 2-aminoethylphosphonic acid, the most abundant naturally occurring phosphonic acid, as the sole source of phosphorus, nitrogen, and carbon (4). The biochemistry of this process was first investigated in *Bacillus cereus*, where 2-aminoethylphosphonic acid was shown to undergo transamination to yield phosphonoacetaldehyde followed by hydrolysis to acetaldehyde and P_i (25). The hydrolytic cleavage step is catalyzed by phosphonoacetaldehyde hydrolyase (26), an enzyme given the trivial name of phosphonatase (equation 1).

$$OHCCH_2PO_3^{2-} + H_2O \xrightarrow{\text{phosphonatase}} OHCCH_3 + P_i \quad (1)$$

Additionally, bacterial strains have been described that are able to utilize alkyl- and phenylphosphonic acids as their source of phosphorus for growth (4, 5, 7–9, 35, 41). This is an apparent direct C-P bond cleavage in which the detected products are inorganic phosphate and the respective hydrocarbon (equation 2).

$$R\text{—}PO_3^{2-} + H_2O \xrightarrow{\text{C-P lyase}} R\text{—}H + P_i \quad (2)$$

R is methyl or phenyl, for example.

This carbon-phosphorus lyase activity is functionally distinct from phosphonoacetaldehyde hydrolase, which is unable to hydrolyze alkylphosphonic acids (26). Furthermore, hydrolysis of the C-P bond of phosphonacetaldehyde occurs via the formation of an enzyme-bound imine (24; T. W. Hepburn, D. B. Olsen, D. Dunaway-Mariano, and P. S. Mariano, Fed. Proc. **45**:1650, 1986), a reaction pathway that is not accessible to alkyl- and phenylphosphonic acids.

Inherent interest in bacterial C-P lyase is heightened by organochemical precedents that show phosphonic acids to be strongly resistant to acid- or base-catalyzed hydrolysis of the C-P bond (15). In the only model system involving dephosphonylation of alkylphosphonic acids, lead tetraacetate has been shown to catalyze the formation of the respective hydrocarbon, albeit at yields on the order of 1% with respect to the phosphonic acid (6). Efforts to delve into the more efficient biochemical C-P cleavage mechanism have been thwarted by the instability of the enzyme activity. To date, no cell-free C-P lyase activity has been obtained despite documented efforts (7, 29).

In view of both the lack of a suitable biomimetic model and the reported inability to fractionate and characterize enzyme components (also experienced by us), we have conducted an extensive and systematic investigation of in vivo C-P lyase activity. In this context, various phosphonic acid-catabolizing bacteria were isolated and identified, and their substrate specificities were examined. The strain showing the broadest-spectrum C-P lyase activity, *Agrobacterium radiobacter*, was used in further studies involving growth rate determinations, organic product elucidation, and the determination of product stoichiometries. Also examined was the effect of exogenous P_i on the amount of phosphonic

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acid processed and on the time course of P_i versus ethylphosphonic acid utilization. *A. radiobacter* has further proven useful in a directed study on the mechanism of C-P bond cleavage (S. L. Shames, L. P. Wackett, C. P. Venditti, and C. T. Walsh, Fed. Proc. **45**:1865, 1986).

This report represents the most comprehensive examination of in vivo C-P lyase to date and should serve as an important prelude to mechanistic enzymology, to future studies on genetics and regulation, and to further attempts to stabilize and define cell-free activity.

MATERIALS AND METHODS

Isolation and identification of bacteria. Sludge from the Lawrence, Mass., water treatment plant was used as a source of bacteria for enrichment culture by the protocol of Cook et al. (4). After four successive enrichment cultures, bacteria were streaked onto nutrient agar plates. Each distinctive isolate was transferred to fresh agar plates and subsequently retested on the respective phosphonic acid used for its enrichment. Those strains selected for taxonomic identification were examined by Gram staining and then inoculated into API-E and API-NFT test strips according to the instructions of the manufacturer (Analytab Products, Plainview, N.Y.). Initial identifications were obtained from the API computer index (all excellent fits) and confirmed by test results described in the literature (3, 23, 28, 32, 39). The identification of *A. radiobacter* was confirmed by its uniquely positive result on 3-ketolactose indicator plates (2).

Bacterial growth. With the exception of *A. radiobacter*, the isolates were grown on the enrichment medium of Cook et al. (4), except that 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer was used. *Bacillus brevis*, *Bacillus cereus* ATCC 14579, and *B. cereus* ATCC 13061 were grown on the enrichment medium supplemented with a mixture of amino acids (33). In substrate specificity studies, the enrichment medium was used for *A. radiobacter*. However, in all other studies this isolate was grown on a mineral salts medium (38) with 40 mM HEPES buffer substituted for phosphate and containing 1% (wt/vol) sucrose and 0.2% (wt/vol) L-glutamic acid at a final pH of 7.0.

In an effort to minimize background phosphate contamination, glassware was rinsed with nitric acid before use. Before supplementation with a phosphorus source, growth media contained less than 2 μM P_i as determined by assay with malachite green (27). Additionally, no bacterial growth was observed on these media without added phosphorus. P_i, phosphonic acids, or phosphinic acids were added to growth media from filter-sterilized 50 mM stock solutions to a final concentration of 500 μM unless otherwise indicated. After the addition of phosphonic or phosphinic acids, growth media contained a maximum of 10 μM P_i, and most phosphonic acids yielded a background of less than 3 μM P_i. Thus, the possibility of bacterial growth on contaminating P_i could be excluded.

Small-scale growth studies were conducted in previously unused 4-dram (ca. 14.8-ml) vials containing 5 ml of medium or, when monitoring gaseous products, in new 14-ml heavy glass vials containing 4 ml of medium and fitted with crimp-sealed rubber septa (American Scientific Products, Bedford, Mass.). Growth rate determinations were performed with 150-ml sidearm flasks containing 25 ml of growth medium. Small inoculation volumes (10 μl) were taken from cultures grown overnight in the sucrose-glutamate growth medium supplemented with 250 μM P_i. Growth flasks were incubated at 30°C and 200 rpm. Turbidity determinations were taken hourly with a Klett-Summerson photometer equipped with a red filter. In experiments that required the detection of gaseous products, the sidearm flasks were fitted with rubber septa.

Phosphonic and phosphinic acid substrates and standard gases. The following phosphonic and phosphinic acids were obtained from Alfa Products (Danvers, Mass.): methyl-, ethyl-, propyl-, butyl-, vinyl-, carboxymethyl-, carboxyethyl-, hydroxymethyl-, and isopropylphosphonic acids and dimethyl-, diethyl-, dihydydroxymethyl-, and methylbutylphosphinic acids. Free acids or their salts were used as supplied. Phosphonate esters were hydrolyzed with 6 N HCl overnight and isolated as the sodium salts. 2-Aminoethylphosphonic acid and fosfomycin (phosphonomycin) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Chloromethylphosphonic acid was kindly provided by Larry Byers, Tulane University (New Orleans, La.). Glyphosate was generously supplied by Laurence Hallis of Monsanto Co. (St. Louis, Mo.). 1-Propenylphosphonic acid was a kind gift from Merck Sharpe & Dohme (Rahway, N.J.). 1-Propynylphosphonic acid was synthesized by published methods (36) and deblocked with trimethylsilyliodide. Previously published procedures (12, 19) were followed in the syntheses of 1-oxo-1-hydroxy-3-methyl-3-phospholene and 1-oxo-1-hydroxy-3-methyl-2-phospholene. All standard gases were purchased from Matheson Gas Products, Gloucester, Mass., with the exception of *n*-butane, which was prepared by the addition of *n*-butyllithium to a vented water-filled tube fitted with a rubber septum.

Gaseous product identification and quantification. Gas chromatography was performed with a Carle AGC series 100 chromatograph (Hach-Carle, Loveland, Colo.). Gases were separated with a 7-ft (ca. 2.1-m) steel column containing VZ-10 packing material (Applied Science Laboratories, Deerfield, Ill.) and operating at 76°C with a nitrogen carrier gas flow of 30 ml/min. Peaks were identified by retention time and by coinjection with authentic standards. Gaseous products were quantitatively determined by peak height relative to stock solutions of the corresponding standard gases.

RESULTS

Isolation and identification of phosphonic acid-utilizing bacteria. Enrichment cultures were performed with methyl-, phenyl-, carboxymethyl-, carboxyethyl-, and 2-aminoethylphosphonic acids as the sole source of phosphorus; from these enrichments six, five, four, five, and two strains were isolated on the respective phosphonic acids. To facilitate subsequent biochemical studies, the identification of six of the isolates was undertaken. All were gram-negative rods and were identified as indicated in Table 1.

Substrate specificity of isolates. Each isolate had been obtained by enrichment on a single phosphonic acid as the sole phosphorus source (Table 1). The most useful strain(s) for further metabolic and mechanistic studies would contain a broad-specificity C-P lyase activity. In this context, each bacterial strain was tested for its ability to grow on a variety of phosphonic acids and on dimethylphosphinic acid (Table 1). Due to inherent growth rate differences on the growth medium used, the incubation time selected was relatively long (96 h), and growth was scored as a percentage of the final absorbance attained during the same period by a P_i control. In several cases slight growth was observed that could not be explained by phosphate contamination in the

TABLE 1. Identity and substrate specificity of bacterial isolates

Phosphorus source	Growth of bacteria[a]					
	Agrobacterium radiobacter (PA)	*Kluyvera ascorbata* (Ph)	*Kluyvera cryocrescens* (MP)	*Klebsiella oxytoca* (Pr)	*Klebsiella pneumoniae* (Ph)	*Pseudomonas* sp. (MP)
Phosphonic acids: R PO_3^{2-}, R =						
$H_3N^+CH_2CH_2$ —	++	++	++	++	++	++
CH_3 —	++	++	++	++	++	++
$CH_2=CH$ —	++	++	++	++	++	++
C_6H_5 —	++	++	++	++	++	−
$HOCH_2$ —	++	++	++	+	+	+
$^-O_2CCH_2$ —	++	++	+	+	−	+
$^-O_2CCH_2NHCH_2$ — (glyphosate)	++	−	−	−	−	−
$H_3CCH(O)CH$ — (fosfomycin)	+	−	−	−	−	−
$(CH_3)_2CH$ —	−	−	−	−	−	−
Phosphinic acid: R_2 PO_2^-, R =						
H_3C —	++	−	−	−	−	−

[a] The letters PA, MP, Ph, or Pr in parentheses after the species name indicate that the strain was isolated by enrichment on phosphonoacetic acid, methylphosphonic acid, phenylphosphonic acid, or phosphonopropionic acid, respectively. Growth was scored by comparison of A_{600} to that of P_i a control culture: (++) >50%, (+) 10 to 50%, (−) <10%.

growth medium. However, only substantial growth was scored as positive in light of our goal of discerning substrates and strains that showed levels of phosphonic acid turnover suitable for metabolic and mechanistic studies.

Some general trends were evident from this survey. All of the strains grew well with 2-aminoethyl-, methyl-, and vinylphosphonic acids, whereas hydroxymethyl- and carboxymethyl-phosphonic acids supported growth to varied degrees. Growth was more restricted on the herbicide glyphosate and on the antibiotic fosfomycin. Since the latter compound shows broad-spectrum antibacterial activity (20), it is not clear whether its utilization by *A. radiobacter* was a function of inherent resistance or due to antibiotic detoxification conferred on this bacterium by the C-P lyase activity or both. Isopropylphosphonic acid failed to support growth of any of the isolates over a 96-h period suggesting that steric factors imposed a constraint on C-P bond cleavage at this secondary sp^3 carbon atom. However, it should be noted that *A. radiobacter* showed growth after incubation for 10 days on this substrate, with the concomitant formation of propane. It is of further interest that this strain was also unique due to its ability to grow on dimethylphosphinic acid. Overall, *A. radiobacter* showed great versatility with respect to substrate utilization, and so it was selected for use in the majority of experiments chronicled below.

Cell-free C-P lyase activity. To examine C-P lyase activity in greater detail, experiments to obtain cell-free activity were attempted. Concentrated cell suspensions of *A. radiobacter* and *Kluyvera ascorbata* were each prepared in 50 mM HEPES buffer containing the potential cosubstrates or cofactors NAD(P)H, $NAD(P)^+$, ATP, GTP, and Mg^{2+}. Cell-free activity was not observed under any circumstances with a detection sensitivity as low as 1 pmol of product formed per min.

Growth rate determinations. Given the inability to obtain cell-free activity by ourselves and by previous investigators (7, 29), it has not yet been possible to investigate the C-P lyase reaction mechanism by a classical approach of in vitro rate discrimination with different phosphonic acid substrates. However, identification of the bacterial strains allowed the use of optimized growth media with the objective of attaining minimal doubling times. Under these conditions, C-P bond cleavage might be rate determining in microbial phosphonic acid utilization. If this were the case, differences in the rates of this step with different phosphonic acids would be expressed in the specific growth rates obtained with *A. radiobacter*. In this manner, mechanistic information pertaining to C-P lyase could be derived from a comparison of growth rate data.

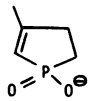
Indeed, a discrimination between growth rates on P_i and between various phosphonic acids was observed (Table 2). P_i supported the most rapid growth of *A. radiobacter*. The majority of the phosphonic acids examined sustained growth at moderately reduced specific growth rates of 0.23 to 0.30 h^{-1} relative to P_i at 0.39 doublings per h. Ethyl-, propyl-, and butylphosphonic acids supported growth at significantly lower rates, as did glyphosphate and fosfomycin. However, a comparison of the logarithm of specific growth rates on various phosphonic acids with the respective aliphatic substituent constant (11) or Taft steric parameter (40) yielded no apparent correlation.

Phosphinic acids, which contain two C-P bonds, also served as the sole phosphorus source for growth of *A.*

TABLE 2. Specific growth rate of *A. radiobacter* with 0.5 mM phosphorus source

Phosphorus source: R-PO_3^{2-}, R =	Specific growth rate (h^{-1})
HO —	0.39
$H_3N^+CH_2CH_2$—	0.30
$H_3CC\equiv C$ —	0.29
$HOCH_2$ —	0.29
$H_2C=CH$ —	0.29
C_6H_5 —	0.29
H_3C —	0.26
$H_3CCH=CH$ —	0.26
$ClCH_2$ —	0.25
$^-O_2CCH_2$ —	0.23
$H_3C(CH_2)_3$ —	0.16
$H_3C(CH_2)_2$ —	0.13
H_3CCH_2 —	0.12
$H_3CCH(O)CH$ — (fosfomycin)	0.09
$^-O_2CCH_2NHCH_2$ — (glyphosate)	0.07

TABLE 3. Specific growth rate of *A. radiobacter* with 0.5 mM phosphinic acid as a sole phosphorus source

Phosphinic acid	Specific growth rate (h^{-1})
	0.20
$(H_3C)_2PO_2^-$	0.10
$(H_3CCH_2)_2PO_2^-$	<0.07
$(HOCH_2)_2PO_2^-$	<0.07
$(H_3C)(H_3CCH_2CH_2CH_2)PO_2^-$	<0.07
	<0.07

radiobacter. However, the phosphinic acids examined in this study supported growth at significantly lower rates than did the majority of the phosphonic acids (Table 3). At the slowest rates observed, cell clumping precluded an accurate turbidimetric determination of cell growth. Thus, with these substrates only an upper limit of the specific growth rate of 0.07 h^{-1} could be confidently affixed. Although the slow growth on phosphinic acids could be reflective of the first C-P bond cleavage being rate determining in growth, another process such as transport may be the limiting step.

Product identification. Previous investigators have observed that biological processing of alkyl- and phenylphosphonic acids yields the corresponding alkanes and benzene, respectively (4, 5, 7, 8, 41). We have repeated these earlier studies with a range of alkylphosphonic acids as well as with vinyl-, propenyl-, propynyl-, and chloromethylphosphonic acids (Table 4). In all cases the major detectable organic product was the respective alkane, alkene, alkyne, benzene, or chloromethane.

As with the phosphonic acids, the C-P bond of dialkylphosphinic acids were cleaved to form the corresponding alkanes (Table 4). With methylbutylphosphinic acid, both methane and butane were detected in the culture headspace. It is worth noting here that, similar to alkylphosphonic acids that yield alkanes upon treatment with lead tetraacetate (6), dialkylphosphinic acids yielded alkanes with this reagent.

Stoichiometry of organic products. The C-P lyase catalyzed reaction yields 1 mol of P_i and 1 mol of organic product per mol of phosphonic acid processed (equation 2). Thus, it was possible to quantitatively monitor C-P cleavage events by sampling an insoluble gas in the headspace of a bacterial culture. Product stoichiometries were examined in cultures of *A. radiobacter* under conditions of substrate limitation and in the presence of excess phosphonic acid. Experiments designed to provide substrate at limiting concentrations were based on the requirement for approximately 100 μM phosphate to bring a bacterial culture to a turbidimetric density of 1.0 absorbance unit at 600 nm (30). By analogy, use of the appropriate phosphonic or phosphinic acid at less than or equal to 100 μM would allow bacterial growth to proceed until the substrate was completely consumed. The lack of a stoichiometric quantity of a particular gaseous product after cessation of growth would indicate the occurrence of side products in the C-P lyase reaction or metabolism of the substrate by another enzyme system.

For example, the determination of total gas production with limiting substrate proved decisive in investigating the mode of phosphinic acid utilization. If both C-P bonds were cleaved by the same mechanism, the anticipated result would be the production of 2 mol of alkane per mol of dialkylphosphinic acid consumed. A comparison of the amount of gaseous product formed after the cessation of growth with dimethylphosphinic acid versus methylphosphonic acid clearly established methane product stoichiometries of 2.0 and 1.0, respectively (Fig. 1).

A second dividend of stoichiometry determinations accrued from the observation that different phosphonic acids, when present at nonlimiting concentrations, gave rise to different quantities of products. For example, the amount of ethylene evolved from vinylphosphonic acid (249 nmol/ml) was substantially greater than that of ethane from ethylphosphonic acid (84 nmol/ml), although in each case the growth vials were sampled at the endpoint of gas production and at similar culture turbidities. Thus, the quantity of P_i released from vinylphosphonic acid (249 nmol/ml) represents an amount greater than that required for maximal growth. It appears that, with certain substrates, *A. radiobacter* will execute more C-P bond cleavage than is necessary to attain its P_i requirements. The excess phosphate may be stored as polyphosphate as previously demonstrated (7), but this was not investigated in the present study. To examine the relationship between the extent of C-P cleavage and the observed specific growth rate, final gas stoichiometries were determined for a range of phosphonic acid substrates (Fig. 2). Indeed, greater gas production was observed with substrates that supported higher growth rates, and the linear relationship that existed between these data showed a correlation coefficient of 0.97. Extrapolation of the experimental points gave a line that passed through the origin of the plot, demonstrating that cell growth was directly correlated with the extent of phosphonic or phosphinic acid utilization.

Effects of P_i on C-P lyase activity. In addition to the paucity of available biochemical data, C-P lyase regulation and gene expression have been largely uninvestigated. For this reason, and also to develop methods for suppressing in vivo C-P lyase activity for control experiments in mechanistic studies (S. L. Shames, L. P. Wackett, M. S. LeBarge, R. L. Kuczkowski, and C. T. Walsh, submitted for publication), the effect of P_i on the expression of activity was investigated.

TABLE 4. Major products in culture headspace of *A. radiobacter* grown on phosphonic and phosphinic acids

Phosphonic or phosphinic acid	Major gaseous product
$R-PO_3^{2-}$, R =	
CH_3-	CH_4
H_3CCH_2-	H_3CCH_3
$CH_3CCH_2CH_2-$	$H_3CCH_2CH_3$
$H_3CCH_2CH_2CH_2-$	$H_3CCH_2CH_2CH_3$
$H_2C=CH-$	$H_2C=CH_2$
$H_3CCH=CH-$	$H_3CCH=CH_2$
$H_3CC\equiv C-$	$H_3CC\equiv CH$
C_6H_5-	C_6H_6
$ClCH_2-$	$ClCH_3$
$R_1R_2PO_2^-$, R_1 and R_2 =	
$(CH_3)_2-$	CH_4
$(H_3CCH_2)_2-$	H_3CCH_3
$(CH_3)(H_3CCH_2CH_2CH_2)-$	CH_4, $H_3CCH_2CH_2CH_3$

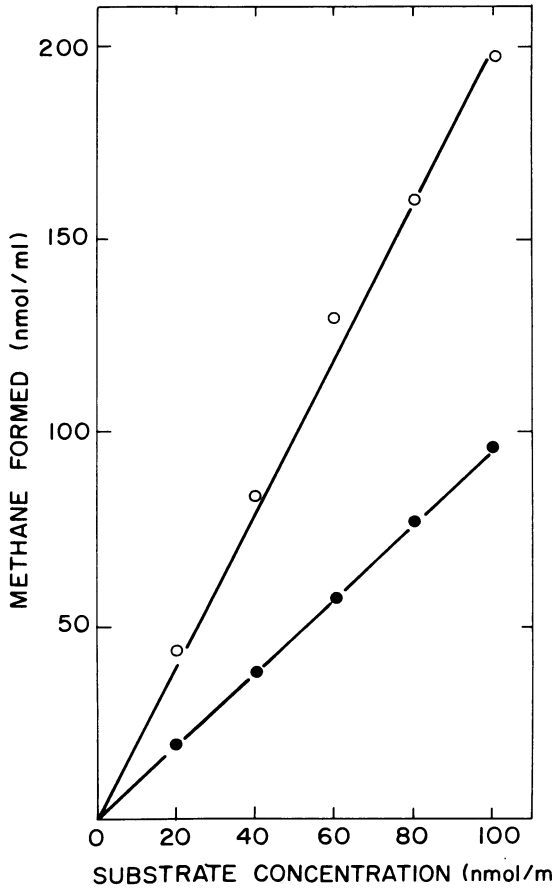


FIG. 1. Methane in culture headspace of *A. radiobacter* grown on limiting concentrations of methylphosphonic acid (●) or dimethylphosphinic acid (○). The amount of gas is expressed as the nanomoles per milliliter of culture medium to allow direct comparison with the amount of substrate at time zero.

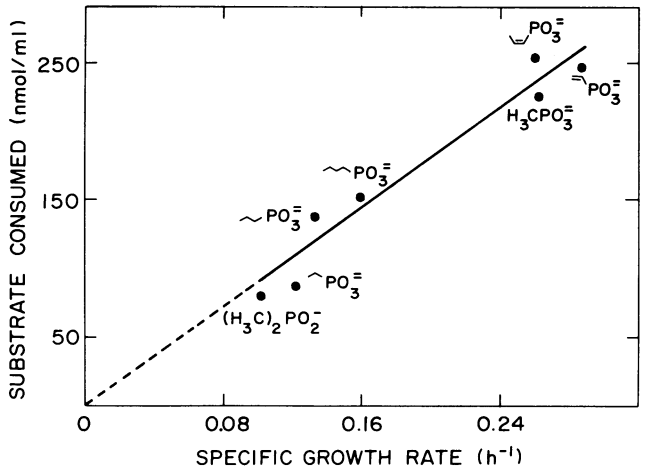


FIG. 2. Relationship between maximal conversion of substrate phosphonic and phosphinic acids to gaseous products during the growth of *A. radiobacter* and the specific growth rate sustained by that substrate. The endpoint of substrate consumption was determined by monitoring product formation to completion and is expressed as nanomoles of substrate utilized per milliliter of culture medium.

In the growth experiments depicted in Table 2, *A. radiobacter* showed a faster growth rate on P_i than on any of the phosphonic acids examined. This suggested that phosphate may be preferentially utilized in a mixed incubation experiment and, furthermore, that we might expect C-P lyase activity to be repressed under these conditions. In light of this, we extended the determination of product stoichiometries described previously by examining the effect of different concentrations of P_i on final gaseous product yield (Fig. 3). With the phosphonic acids at a starting concentration of 500 μM, increasing the concentration of P_i led to a marked suppression of organic product formation with greater than 85% inhibition at 500 μM P_i. It should be pointed out that *A. radiobacter* executes C-P bond cleavage even at a phosphate concentration of 350 μM, a level that will sustain complete growth. Thus, phosphonic acid utilization cannot be attributed to a switchover to phosphonic acids after depletion of P_i. Interestingly, *Kluyvera ascorbata* showed a somewhat more stringent response to P_i, with C-P bond cleavage inhibited greater than 99.9% at 350 μM P_i, suggestive of tighter regulatory control.

These experiments, however, do not address the kinetic course of phosphonic acid processing, which could serve to give insight into possible C-P lyase induction (as opposed to phosphate competition in phosphonic acid uptake, for example). In this context, mixed growth was conducted with 50 μM ethylphosphonic acid, the phosphonic acid substrate that yielded the slowest growth rate, and 50 μM P_i. Growth was initially sustained by P_i (Fig. 4), as evidenced by the lack of detectable ethane (<0.8% of substrate ethylphosphonic acid consumed). Thereafter, the growth rate fell off and a short, but distinct, lag phase set in during which ethane formation commenced. These results might be indicative of induction of a C-P lyase component(s) during the switchover from P_i to ethylphosphonic acid utilization.

Screening gram-positive bacteria for C-P lyase. It is interesting that all 20 alkyl- or phenylphosphonic acid-utilizing bacteria described in the literature and this report are gram

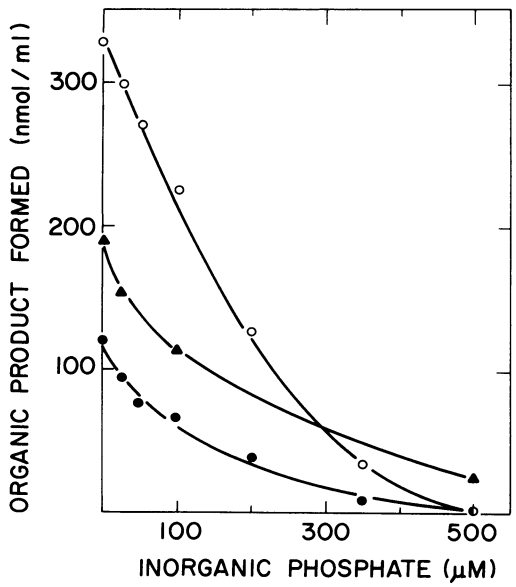


FIG. 3. Effect of P_i on alkane and alkene formation by *A. radiobacter* with methyl- (▲), vinyl- (○), and butyl- (●) phosphonic acids. The amount of gaseous product is expressed as nanomoles per milliliter of culture medium.

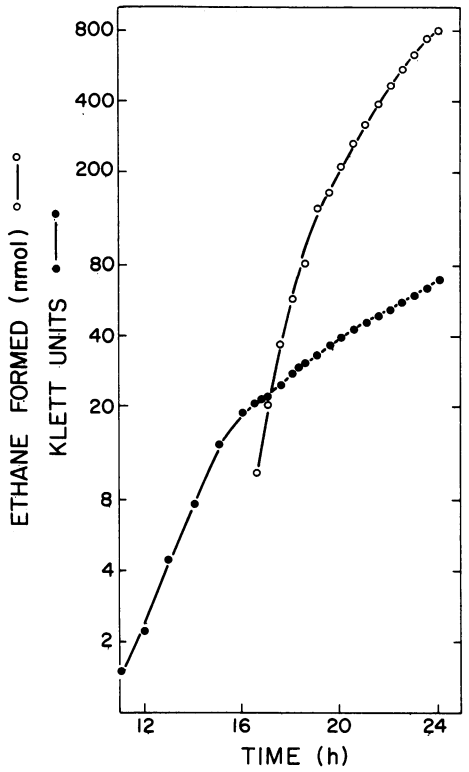


FIG. 4. Growth and ethane formation by *A. radiobacter* at limiting concentrations of 50 μM P_i and 50 μM ethylphosphonic acid.

negative. The gram-positive bacteria that are reported to utilize phosphonic acids grow on 2-aminoethylphosphonic acid (33) or 2,3-dihydroxypropylphosphonic acid (29), both of which might be metabolized through β-ketophosphonic acids and an enzyme similar to phosphonoacetaldehyde hydrolase. In this light, several gram-positive strains were screened for growth on methyl- and phenylphosphonic acids to look for potential C-P lyase cultures. Those screened were *Corynebacterium glutamicum* MB-1789, *Arthrobacter globiformis* ATCC 8010, *Bacillus subtilis*, *B. brevis* B-17N2, *B. cereus* ATCC 14579, and *B. cereus* ATCC 13061. None of these bacteria grew with methyl- or phenylphosphonic acid as the phosphorus source, although all grew on their respective medium supplemented with P_i. *B. brevis* and the two *B. cereus* strains grew with 2-aminoethylphosphonic acid as phosphorus source. The latter two were previously documented to contain phosphonatase activity (33). Note that several *Escherichia coli* laboratory strains examined grew on methylphosphonic acid, suggesting that C-P lyase activity is not a trait that is readily lost during laboratory culture.

DISCUSSION

Along with methanogen methyl-*S*-coenzyme M reductase (10) and eubacterial organomercurial lyase (34), C-P lyase belongs to a class of microbial enzymes that catalyze the formation of alkanes. In general, bacteria and organic chemists have developed similar strategies to carry out these steps. In the first two reactions above, nickel-catalyzed desulfurization and protonolysis, respectively, serve to liberate an alkane product (1a; L. P. Wackett, J. F. Honek, T. P. Begley, S. L. Shames, E. C. Niederhoffer, R. P. Hausinger, W. H. Orme-Johnson, and C. T. Walsh, *in* J. Lancaster, ed., *Bioinorganic Chemistry of Nickel*, in press). By contrast, the C-P lyase-catalyzed liberation of alkanes is much less well characterized by both biochemical and biomimetic probes. The present investigation was undertaken to develop further insight into microbially catalyzed C-P bond cleavage chemistry by carefully defining the microbial physiology relevant to this reaction.

As a starting point, bacteria that expressed C-P lyase activity were isolated with the hope of obtaining a unique organism that would prove valuable in studies on enzyme mechanism. The bacteria isolated were reminiscent of previously described organisms in that they were all gram-negative rods. The lack of a gram-positive C-P lyase-producing strain in the literature and in this study points to the possibility that a periplasmic component(s) is requisite for C-P lyase activity. This observation suggests that concentrated osmotic shock fluids (31) be used in future experiments aimed at reconstituting a cell-free activity.

A. radiobacter has proven to be a unique strain that has served as a versatile tool for studying bacterial C-P bond cleavage. This organism displayed the broadest specificity C-P lyase activity reported to date, processing substrates characterized by disparate electronic and steric parameters. For example, the herbicide glyphosate is relatively recalcitrant to pure culture biodegradation, with only two strains previously described that utilize this phosphonic acid as a phosphorus source (1, 30). However, glyphosate supported luxuriant growth of *A. radiobacter*. Fosfomycin, an antibiotic produced by three species of *Streptomyces* (13), was also observed to serve as sole phosphorus source for *A. radiobacter*. Note that the concentration of fosfomycin used in these growth studies, 500 μM, is markedly higher than the inhibitory concentration range of 1 to 200 μM that affects susceptible bacteria (20), suggesting a potential detoxification role for C-P lyase.

The wide substrate tolerance of *A. radiobacter* prompted studies on growth rates with the hope of observing a correlation between the natural logarithm of the specific growth rate versus the polar aliphatic substituent constant (11), s^*, or the Taft steric parameter (40), E_s. However, no correlation was observed between these parameters and the specific growth rates shown in Table 2. The strong correlation between observed growth rates and the quantity of phosphonic or phosphinic acid consumed indicates that one or more processes involved in the utilization of these compounds is completely growth rate determining. This conclusion is buttressed by the observation that extrapolation of the curve of Fig. 2 yields a line that goes through the origin, demonstrating a theoretical zero growth rate at zero phosphonic acid concentration. It is likely that C-P cleavage rates, phosphonic acid transport, energy requirements in C-P bond cleavage, or other processes may contribute to the observed growth rates. With respect to transport, it is known that fosfomycin enters a number of bacteria via the L-*a*-glycerophosphate uptake system (20), but other phosphonic acids might travel by other transporters.

This study contains the first report of bacterial cleavage of the C-P bond of dialkyl phosphinic acids. Potentially, phosphinic acid utilization provides greater flexibility in designing mechanistic experiments to probe C-P lyase. The same activity is implicated in the cleavage of both C-P bonds by data showing that both methyl moieties of dimethylphosphinic acid are cleaved stoichiometrically to methane (Fig. 5). It is of interest that phosphinothricin is a natural product phosphinic acid antibiotic, and so the possible detoxification

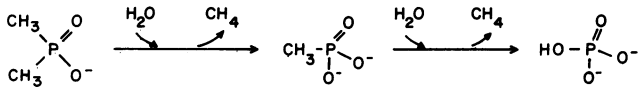


FIG. 5. Metabolism of dimethylphosphinic acid by *A. radiobacter*.

function of C-P lyases might also extend to this antibiotic group.

The effect of P_i on C-P lyase activity was studied to learn more about the genetics and regulation of this enzyme. The expression of C-P lyase activity at P_i concentrations that support growth will allow a negative enrichment strategy for obtaining C-P lyase mutants. At a limiting concentration of phosphonic acid, P_i completely shut off C-P lyase in *A. radiobacter*, a result similar to those obtained by Daughton et al. (9) with *Pseudomonas testosteroni*. However, in that study the specific growth rates with phosphate and methylphosphonic acid were sufficiently close (0.54 versus 0.47 h^{-1}, respectively), such that a distinct switchover was not apparent by monitoring cell density. In the present investigation specific growth rates were sufficiently different (0.39 h^{-1} for P_i versus 0.12 h^{-1} for ethylphosphonic acid) that a switchover punctuated by a lag phase was observed, suggesting enzyme induction. A more conclusive demonstration of C-P lyase inducibility will be possible after the successful attainment of cell-free activity or the assignment of specific polypeptides to the C-P lyase function by genetic or other methods. In general, further progress on this fascinating enzyme system will be dependent on developments in obtaining cell-free activity, developing genetic approaches, and conducting experiments designed to elucidate the mechanism of C-P bond cleavage.

ACKNOWLEDGMENTS

We thank Ganesh Kishore and Laurence Hallis of Monsanto Company for helpful discussion. We are grateful to the members of Arnold Demain's laboratory at the Massachusetts Institute of Technology for providing some of the gram-positive bacteria.

ADDENDUM IN PROOF

Recent work has demonstrated that the C-P lyase activity in *Escherichia coli* K-12 is induced by P_i starvation (L. P. Wackett, B. L. Wanner, C. P. Vinditti, and C. T. Walsh, submitted for publication). Furthermore, we have found that this activity is absent in *psiD* mutants.

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