

Biodegradation of Phosphonomycin by *Rhizobium huakuii* PMY1

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The biodegradation by *Rhizobium huakuii* PMY1 of up to 10 mM phosphonomycin as a carbon, energy, and phosphorus source with accompanying P_i release is described. This biodegradation represents a further mechanism of resistance to this antibiotic and a novel, phosphate-deregulated route for organophosphonate metabolism by *Rhizobium* spp.

Phosphonomycin [fosfomycin; *cis*-(1*R*,2*S*)-1,2-epoxypropylphosphonic acid] is a broad-spectrum, cell wall-active, organophosphonate antibiotic, produced as a product of secondary metabolism by strains of *Streptomyces fradiae*, *Streptomyces wedmorensis*, *Streptomyces viridochromogenes*, and *Pseudomonas syringae* (7, 26, 27). Structurally, phosphonomycin is characterized by both an epoxide ring and a highly stable, covalent, carbon-phosphorus bond (Fig. 1) which is resistant to, for example, acid and base hydrolysis, thermolysis, and photolysis, and to phosphotransferase-catalyzed cleavage (12).

The pathway leading to the formation of phosphonomycin has been extensively studied (1, 6, 24, 33); however, very little is known about its biodegradation. Previous studies have shown that environmental microorganisms undergoing phosphate starvation are capable of the utilization of phosphonomycin as the sole source of phosphorus. Cleavage of the constituent carbon-to-phosphorus bond is presumed to be catalyzed by a C-P lyase enzyme complex(es) which, being under the direct control of the PHO regulon, is inducible only under conditions of phosphate limitation (9, 22, 23, 31). We now report the isolation of a bacterium capable of utilizing phosphonomycin independently of the phosphate status of the cell, as either a carbon source or as a carbon and phosphorus source, with essentially quantitative extracellular release of organophosphonate-derived orthophosphate. Degradation of phosphonomycin by cleavage of the carbon-phosphorus bond may also provide a novel mechanism of resistance to the antibiotic.

Enrichment was done with a basal mineral salts medium (pH 6.8) which contained the following (per liter): KCl, 0.2 g; MgSO₄ · 7H₂O, 0.2 g; CaCl₂ · 2H₂O, 1.0 mg; NH₄Cl, 1.0 g; ferric ammonium citrate, 1.0 mg; phosphate-free yeast extract (32), 0.05 g; BME essential amino acids solution, 20 ml/liter (Sigma); and 1 ml each of trace element solution (11) and vitamin solution (17). Filter-sterilized (0.22- μ m pore size) phosphonomycin (5 mM) was routinely added as a carbon and phosphorus source.

Enrichment cultures (25 ml in 250-ml Erlenmeyer flasks) were inoculated with a 0.5% (vol/vol) combined inoculum from an activated sludge plant (Dunmurry, Northern Ireland), a laundry effluent disposal lagoon (Summit Lake, Wis.) and a sheep dip disposal site (County Antrim, Northern Ireland); each site was known to have a previous history of exposure to

organophosphonate xenobiotics, although significant exposure to phosphonomycin is unlikely. Cultures were incubated at 30°C on an orbital shaker at 100 rpm. Growth was measured by the increase in optical density at 650 nm with a Pye-Unicam PU 8200 UV/Vis spectrophotometer (Pye-Unicam Ltd., Cambridge, United Kingdom), while phosphate release into the culture supernatant was monitored by the method of Fiske and SubbaRow (4).

After 11 serial transfers, a bacterial isolate, designated PMY1, capable of growth on phosphonomycin (5 mM) as a carbon, energy, and phosphorus source was purified on medium solidified by the addition of 1.2% purified agar (Oxoid) by the plate screening method of McGrath and Quinn (16).

Isolate PMY1 was found to be gram negative, nonmotile, and oxidase and catalase positive and grew poorly on all conventional rich laboratory media tested. Partial 16S rRNA sequencing (ca. 450 nucleotides), performed by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany), revealed 100% 16S rRNA gene similarity to *Rhizobium huakuii* and indicated a close relationship to other *Rhizobium* spp.

Growth of *R. huakuii* PMY1 on 5 mM phosphonomycin as a carbon, energy, and phosphorus source is shown in Fig. 2. Phosphonomycin biodegradation was observed by virtue of its total disappearance from growth medium as detected by the gas chromatography-mass spectrometry method of Longo et al. (15). This strain was unable to utilize hydroxymethylphosphonate, phosphonobutyrate, phosphonoformate, 2-phosphonopropionate, 3-phosphonopropionate, phosphonomethylglycine (glyphosate), methylphosphonate, trimethylphosphonate, ethylphosphonate, dimethylmethylphosphonate, 2-amino-3-phosphonopropionate, 1-aminoethylphosphonate, 2-aminoethylphosphonate, phenylphosphonate, or phosphonoacetate as carbon, energy, and phosphorus sources.

The metabolism of 5 mM phosphonomycin was accompanied by the concomitant release of up to 96% inorganic phosphate (Fig. 2). No spontaneous orthophosphate release was observed either in uninoculated control experiments or from cultures incubated in the absence of substrate.

R. huakuii PMY1 was grown on a range of concentrations (0 to 20 mM) of phosphonomycin as a carbon, energy, and phosphorus source (Fig. 3). Final cell yields were directly proportional to phosphonomycin concentrations within the range of 0 to 10 mM and were accompanied by the extracellular release of >95% phosphonate-phosphorus as P_i. Negligible growth rates and cell yields were observed at phosphonomycin concentrations of 15 and 20 mM (Fig. 3).

Cell extracts of *R. huakuii* PMY1, grown on phosphonomycin (5 mM) as a carbon, energy, and phosphorus source and

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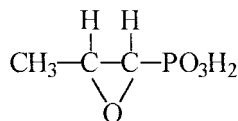


FIG. 1. Structure of phosphonomycin (*cis*-1,2-epoxypropylphosphonic acid; fosfomycin).

prepared by sonication as described by McGrath et al. (17), contained no *in vitro* C—P bond-cleaving activity either on phosphonomycin or any of the other organophosphonate substrates tested. The addition of either Mg^{2+} (1 mM), Fe^{3+} (1 mM), Mn^{2+} (1 mM), Co^{2+} (1 mM), Zn^{2+} (1 mM), or NAD^+ (3 mM) or exhaustive dialysis against 50 mM Tris-HCl buffer (pH 6.8) produced no subsequent detectable *in vitro* C—P bond-cleaving activity. No other previously described C—P bond-cleaving enzyme, neither phosphonoacetate hydrolase nor phosphonatase (assayed by the methods of McGrath et al. [17] and La Nauze et al. [13], respectively) could be detected in cell extracts of the isolate.

This report represents not only the first demonstration of the microbial biodegradation of phosphonomycin but also the first description of the metabolism of an organophosphonate as a source of carbon by any member of the *Rhizobiaceae* family. Other members of the *Rhizobiaceae* have been shown capable of the utilization of organophosphonates, including the herbicide glyphosate, as a source of phosphorus; metabolism occurs only under conditions of strict phosphate limitation with no detectable *in vitro* C—P bond-cleaving activity (14). Indeed, the utilization of compounds containing a direct carbon-to-phosphorus bond as a source of phosphorus is widespread. The utilization of organophosphonates as a source of carbon is more limited; this may be explained by the proposed regulation of both phosphonate uptake and catabolism as part of the PHO regulon, with degradation being repressed and/or inhibited by the excess phosphate released during mineralization of the organophosphonate carbon skeleton. Thus, the utilization

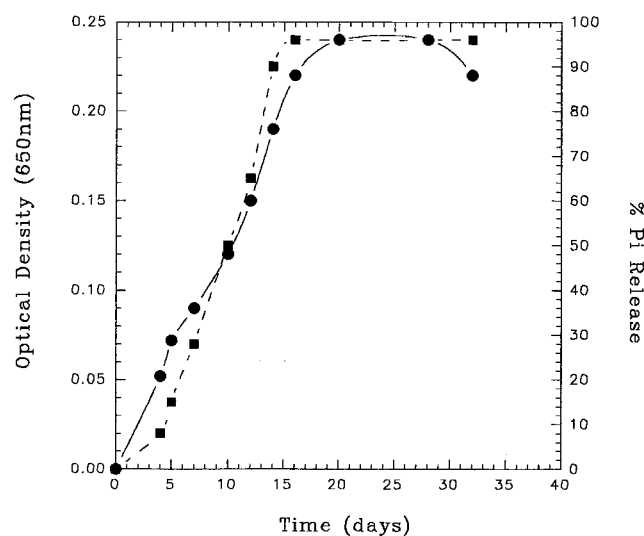


FIG. 2. Growth of isolate PMY1 on phosphonomycin (5 mM) as the sole carbon, energy, and phosphorus source. Cultures were incubated at 30°C on an orbital shaker at 100 rpm. Growth was measured by the increase in optical density at 650 nm, while phosphate release was determined by the method of Fiske and SubbaRow (4). Symbols: ●, growth (optical density at 650 nm); ■, percentage of phosphate released from substrate.

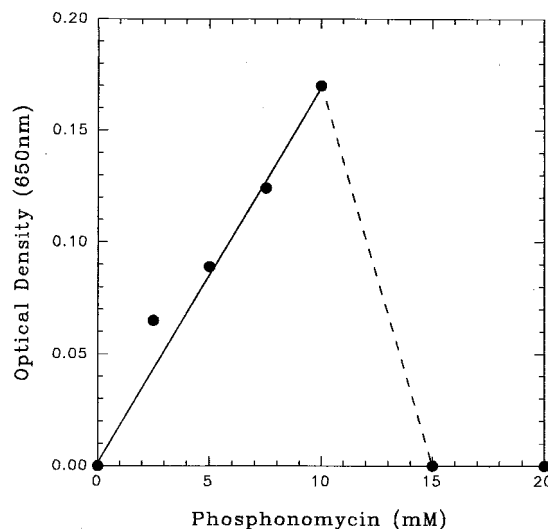


FIG. 3. Stationary-phase cell yields of isolate PMY1 as a function of phosphonomycin concentration (0 to 20 mM).

of phosphonomycin by *R. huakuii* PMY1 as a carbon source is surprising and would suggest the existence and involvement of an enzyme system not under classical PHO regulon control. Only two other organisms have previously been reported to utilize organophosphonates as the sole carbon source; *Pseudomonas fluorescens* 23F mineralized phosphonoacetic acid via acetic acid and orthophosphate (18–20) while *Pseudomonas putida* utilized phosphonoacetaldehyde via C—P bond cleavage to yield acetaldehyde (2). *R. huakuii* PMY1 represents only the third reported exception to the stringent control of microbial organophosphonate uptake and metabolism by inorganic phosphate.

Rhizobia are commonplace in the biosphere and may contribute to the rapid degradation of organophosphonate herbicides and pesticides in the soil (14). Whether this report, detailing phosphate-deregulated organophosphonate metabolism, is of ecological significance to the environmental fate of organophosphonate residues, such as glyphosate in the rhizosphere, remains to be seen.

Our failure to detect any C—P bond-cleaving activity in cell extracts of *R. huakuii* PMY1 leaves the route of phosphonomycin metabolism uncertain. Of the documented C—P bond-cleaving enzymes, only phosphonatase (13) and phosphonoacetate hydrolase (17) (each specific for their respective substrates phosphonoacetaldehyde and phosphonoacetate) have been detected *in vitro*, while cell-free C-P lyase activity has remained elusive despite numerous attempts (3, 5, 9, 10, 14, 21, 25, 31). Neither phosphonatase nor phosphonoacetate hydrolase was demonstrable in cell extracts of *R. huakuii* PMY1, suggesting the likely presence of a previously undescribed C—P bond-cleaving enzyme (although a specific phosphate-deregulated C-P lyase cannot be ruled out; the existence of different classes of C-P lyase, each with a defined organophosphonate substrate range has previously been postulated [9]). Further enzyme characterization may become possible only after elucidation of the phosphonomycin degradative pathway through the synthesis of potential pathway intermediates. In this way, the actual substrate for the enzyme involved in C—P bond cleavage may eventually be identified, thus allowing for further enzymatic characterization *in vitro*.

The degradation of phosphonomycin may also afford *R. hua-*

kuii PMY1 a novel resistance mechanism to the antibiotic, through the cleavage of the constituent carbon-phosphorus bond. This isolate is resistant to antibiotic concentrations many times greater than the inhibitory concentration ranges described for susceptible bacteria (1 to 200 $\mu\text{mol/liter}$ [8]). Three other methods of bacterial fosfomycin resistance have been reported: (i) impermeability owing to chromosome mutations affecting fosfomycin uptake (8, 29); (ii) loss of target enzyme affinity and thus sensitivity to fosfomycin (29, 30); (iii) modification of the molecule through the production of a 16-kDa polypeptide (glutathione *S*-transferase), which catalyzes the formation of a fosfomycin adduct with glutathione, thus inactivating the antibiotic (28). Cleavage of the C—P bond may therefore be regarded as a fourth mechanism of fosfomycin detoxification, confirming the postulated existence of antibiotic resistance mechanisms based on C—P bond cleavage (23, 31).

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