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Isolation of a *Pseudomonas* sp. Which Utilizes the Phosphonate Herbicide Glyphosate

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A strain of bacteria has been isolated which rapidly and efficiently utilizes the herbicide glyphosate (*N*-phosphonomethylglycine) as its sole phosphorus source in a synthetic medium. The strain (PG2982) was isolated by subculturing *Pseudomonas aeruginosa* ATCC 9027 in a synthetic broth medium containing glyphosate as the sole phosphorus source. Strain PG2982 differs from the culture of *P. aeruginosa* in that it is nonflagellated, does not produce pyocyanin, and has an absolute requirement for thiamine. Strain PG2982 has been tentatively identified as a *Pseudomonas* sp. strain by its biochemical activities and moles percent guanine plus cytosine. Measurements of glyphosate with an amino acid analyzer show that glyphosate rapidly disappears from the medium during exponential growth of strain PG2982. In batch culture at 30°C, this isolate completely utilized 1.0 mM glyphosate in 96 h and yielded a cell density equal to that obtained with 1.0 mM phosphate as the phosphorus source. However, a longer lag phase and greater generation time were noted in the glyphosate-containing medium. Strain PG2982 can efficiently utilize glyphosate as an alternate phosphorus source.

Natural organophosphonates have been isolated from a variety of organisms. They are most often found as components of membrane lipids, but have also been found in proteins and in membrane polysaccharides (13). One naturally occurring organophosponate, 2-amino-ethylphosphonate, is incorporated into the liver lipids of rats (12) and goats (11). Another natural organophosphonate, phosphonoalanine, has been shown to be degraded to either acetaldehyde or 2-amino-ethylphosphonate by rat liver homogenates (9). An increasing number of synthetic organophosphonates are being introduced into the environment in the form of insecticides, herbicides, and flame retardants. The metabolism of both synthetic and natural organophosphonates by bacteria isolated from soil and sewage samples is well documented and generally involves the utilization of the organophosphonate as a phosphorus source.

The stable carbon-phosphorus (C-P) bond is resistant to chemical hydrolysis, thermal decomposition, and photolysis. In 1963, the first indirect evidence for the biological cleavage of a C-P bond by bacteria was reported by Zeleznick et al. (23). They showed that *Escherichia coli* could sustain growth on the synthetic organophosphonates, methylphosphonate and ethyl phosphonate, as sole sources of phosphorus. Since then, there have been a number of reports of bacteria utilizing organophosphonates as sole phosphorus sources (2–4, 8, 19) or as the sole carbon, nitrogen, or phosphorus source (3). LaNauze et al. (14, 15) were the first to find direct evidence for the enzymatic cleavage of a C-P bond. They have purified and characterized a "phosphonatase" from *Bacillus cereus* that produces inorganic phosphate from 2-phosphonoacetaldehyde, which is the transamination product of 2-amino-ethylphosphonate.

Glyphosate (*N*-phosphonomethylglycine) is the active ingredient in Roundup, a herbicide manufactured by Monsanto. It is a postemergent, broad-range herbicide used to control both perennial and annual weeds. The mode of action of glyphosate appears to be an inhibition of aromatic amino acid biosynthesis by direct inhibition of the shikimic pathway (7, 10, 22). The broad range, good translocation in plants, high unit activity, and favorable toxicology (20) of glyphosate explain its widespread use. Glyphosate has been shown to be completely degraded by microbes in the soil (18, 20, 21), with aminomethylphosphonate (Amph) as one of the major metabolites (18, 20). The degradation is thought to occur through cometabolism (21), and until the present report no pure culture of bacteria had been shown to degrade glyphosate.

We have isolated a thiamine-requiring *Pseudomonas* sp. from a stock culture of *Pseudomonas aeruginosa* ATCC 9027 that can utilize the phosphonate moiety of glyphosate as a sole

phosphorus source. The growth rate of this isolate on glyphosate is slower than the growth rate on inorganic phosphate, and there is a longer lag phase when it is grown in a medium with glyphosate as the phosphorus source. Equivalent cellular yields can be obtained with equimolar amounts of either inorganic phosphate or glyphosate as the phosphorus source. The glyphosate appears to be completely degraded by the isolate, because no amine-containing metabolites were found in the culture supernatant fluids or cellular extracts prepared from cells harvested during exponential growth. Although the isolate appears to completely degrade glyphosate, it cannot utilize glyphosate as both a carbon and phosphorus source.

MATERIALS AND METHODS

Chemicals. The free acid form of glyphosate (99.7% purity) was a gift from the Monsanto Co., St. Louis, Mo. When assayed for inorganic phosphate (1) at a concentration of 0.02 M, no inorganic phosphate was detectable. Potassium D-gluconate, Amph, and methylamine-hydrochloride were obtained from Sigma Chemical Co., St. Louis, Mo. Sarcosine was purchased from Eastman Chemical Co., Rochester, N.Y. Sodium citrate buffer concentrates used for the amino acid analysis were acquired from Beckman Instruments, Inc., Palo Alto, Calif. The ninhydrin used in this analysis was supplied by Pierce Chemical Co., Rockford, Ill. All other chemicals were either analytical grade or of the highest purity available.

Glassware. To remove contaminating phosphate ions, all glassware was washed with a solution composed of 0.2 N HCl and 0.2 N HNO_3 and then rinsed repeatedly with deionized water.

Media, culture conditions, and bacteria. A synthetic broth was used in all growth experiments so that the phosphorus component could be easily regulated. The basic broth consisted of a Dworkin-Foster salt mixture (5) with the inorganic phosphate deleted from the recipe and 1% potassium D-gluconate as a carbon source. Either glyphosate or inorganic phosphate was added as a phosphorus source, and the broths were designated Gly broth and Pi broth, respectively. When the thiamine-requiring isolate was cultured, thiamine-hydrochloride (5 μg/ml) was also added to the medium. All media were adjusted to pH 7.0 before autoclaving. All cultures were incubated at 30°C, and broth cultures were incubated on a rotary shaker at 300 rpm. Slanted medium of 1% mannitol–1% peptone (Difco Laboratories, Detroit, Mich.)–1.5% agar was used to maintain the stock culture of *P. aeruginosa* ATCC 9027 by biweekly transfer. The thiamine-requiring *Pseudomonas* sp. glyphosate degrader, called PG2982 for simplicity, was maintained on glyphosate-thiamine slants, which were composed of Gly broth containing 5 μg of thiamine-hydrochloride per ml and 1.5% washed Nobel agar (Difco).

Analytical methods. Growth turbidity in broth cultures was read as absorbance at 490 nm on a spectrophotometer. When inorganic phosphate and glyphosate levels in broth cultures were to be measured, 1-ml samples were taken at desired time periods, and the cells were sedimented by centrifugation at 8,740 × *g* in a microfuge. Then 0.9 ml of the resulting supernatant fluid was transferred to a second microfuge tube, and 0.1 ml of 75% trichloroacetic acid was added. The capped microfuge tube was placed in a freezer at −20°C for storage. When an assay was to be run, each sample was thawed, the protein precipitate was sedimented, and the supernatant fluid was used in the appropriate assay. Inorganic phosphate concentration was measured by the method of Ames (1). We modified this assay procedure slightly in that the absorbance of the phosphomolybdate complex was read at 750 nm rather than at 820 nm. Glyphosate levels and other amine-containing metabolites were assayed on a Beckman Amino Acid Analyzer, model 120C. Samples were diluted in sodium citrate buffer (pH 2.2). An accurate measurement of glyphosate was accomplished by running at 20-min program according to parameter recommendations by Beckman for accelerated single-column protein hydrolyzate analysis, with a 10.0- by 0.9-cm column of W1 type resin.

Samples were run in triplicate for quantitative analysis. To check qualitatively for possible metabolites, a full 220-min program was used on a 69.0- by 0.9-cm column of W1 type resin.

Determination of mol% G+C. The moles percent guanine plus cytosine (mol% G+C) was calculated from the melting temperature of purified DNA by the method of Mandel and Marmur (16). The melting temperature was measured on a Gilford spectrophotometer fitted with a thermal programmer. All DNA samples were dissolved in standard saline citrate buffer, and *E. coli* DNA was used as a standard. DNA was precipitated from lysed cells of the appropriate *Pseudomonas* isolate by the method of Fennewald et al. (6). The DNA was further purified to homogeneity and prepared for analysis by the method of Marmur (17).

RESULTS AND DISCUSSION

A culture was developed in this laboratory that could utilize glyphosate as a sole phosphorus source. The culture was obtained by subculturing a stock culture of *P. aeruginosa* ATCC 9027 in Gly broth. Utilization of glyphosate as a phosphorus source was determined by comparing growth between a Gly broth, which contains glyphosate as the sole phosphorus source, and a control broth that contained the same salts and carbon source but had no phosphorus source. Both broths were inoculated with the same volume (0.1 ml) of a cell suspension (absorbance at 490 nm = 0.1) which was prepared by dilution of a Pi broth starter culture (2 mM inorganic phosphate as phosphorus source). A slight inorganic phosphate carryover was detected but did not support significant growth in the control broth. The utilization of glyphosate was verified by monitoring glyphosate levels in the culture supernatant fluid with an amino acid analyzer.

The culture eventually lost its ability to utilize glyphosate after numerous weekly transfers on Gly slants. At the time, we speculated that contaminating inorganic phosphates prevented us from maintaining proper selection pressures. However, the glyphosate-utilizing culture has

been repeatedly isolated by subculturing in Gly broth from our stock culture of *P. aeruginosa* ATCC 9027 maintained on a complex medium. Glyphosate utilization always occurred after a lag period of 2 weeks. The observed disappearance of glyphosate was not due to the chemical or physical breakage of the stable C-P bond, because coincubated, uninoculated Gly broths showed no increase in inorganic phosphate or decrease in glyphosate.

A closer examination of the glyphosate-utilizing culture was made by streaking brain heart infusion agar plates (Difco). The plates revealed a mixed culture with two distinctly different colony types. One was a fast-growing pale-brown colony, and the other was a slow-growing white colony. An attempt to determine the relationship of the two colony types to the utilization of glyphosate was made by isolating the two colony types, inoculating each type into a separate Gly broth and control broth, and then reconstructing the mixture by inoculating both into the same Gly broth. The reconstructed mixture showed glyphosate utilization after a long lag phase, but neither separated colony type showed utilization. In fact, the white colony type did not grow at all on the minimal contaminating inorganic phosphate, and it was also found later not to grow in Pi broth. A number of chemically defined carbon sources were tried as alternatives for potassium D-gluconate in Pi broth, but none resulted in growth unless supplemented with 0.01% yeast extract (Difco). Even potassium D-gluconate was a sufficient carbon source when supplemented with 0.01% yeast extract. Through a process of elimination, thiamine was determined to be the essential growth factor in the yeast extract for the white colony type. When thiamine was used to supplement Gly broth, the white colony type was able to utilize glyphosate as a phosphorus source, whereas the pale-brown colony type could not.

The pale-brown colony has been identified as *P. aeruginosa*, because it was a gram-negative rod with a polar monotrichous flagellum, as determined by electron microscopy of negatively stained preparations. It also excreted the characteristic pyocyanine pigment and exhibited other biochemical characteristics of its genus and species (Table 1). The white colony was not motile (no flagella were seen by electron microscopy), did not excrete pyocyanine, required thiamine as a growth factor, and utilized glyphosate as a phosphorus source. However, based on evidence to date (Table 1), we have tentatively identified the white colony as belonging to the genus *Pseudomonas*. With the differences listed in Table 1 and the 6% difference in mole% G+C, we do not feel at this time that the white colony type is a mutant of *P. aeruginosa* ATCC 9027. Therefore, we have designated the white colony type PG2982.

TABLE 1. Characteristics of the two isolates from the glyphosate-utilizing mixture

Characteristic	*P. aeruginosa*	PG2982
Gram reaction	−	−
Morphology	Rod	Short rod
Motility[a]	+	−
Pyocyanine	+	−
Growth factor	None	Thiamine
Growth on MacConkey agar	+	+
Growth at 42°C	+	+
Oxidase reaction	+	+
Catalase reaction	+	+
Indole reaction	−	−
Urease reaction	+	+
Acid from:[b]		
Glucose (O/F)	+/−	+/−
Lactose	−	−
Mannitol	+	−
Salicin	−	−
Sucrose	−	−
Xylose	+	+
Rhamnose	−	−
Penicillin	Resistant	Resistant
Tetracyline	Resistant	Sensitive
Mol% G+C	66 ± 1.0	60 ± 1.0
Utilize glyphosate	−	+

[a] Electron microscopy of negatively stained preparations, Craigie tube, hanging drop.

[b] O/F (oxidation-fermentation) medium, 1% filter-sterilized carbohydrate.

PG2982 must be present in our stock culture of ATCC 9027 in very low numbers, because the glyphosate-utilizing culture has been repeatedly isolated from the stock culture, but when single isolated colonies of *P. aeruginosa* were tested, no glyphosate utilization occurred. The thiamine requirement of PG2982 would not be a handicap in the stock culture, because the stock culture was maintained on peptone-mannitol slants. However, it would be a handicap on Gly slants not supplemented with thiamine. This reasoning explains why the original glyphosate-degrading cultures would lose their ability to degrade glyphosate after numerous weekly transfers. The thiamine requirement and the low numbers of PG2982 in the stock culture also would explain why such a long lag period was required before glyphosate utilization appeared in subculturing experiments. For the mixed culture to develop and maintain glyphosate utilization, cross feeding between the two organisms must have occurred. *P. aeruginosa* would supply the thiamine and PG2982 would provide the inorganic phosphate by degrading the glyphosate.

PG2982 can utilize glyphosate as a phosphorus source as well as it utilizes inorganic phosphate. Equivalent cellular yields were obtained

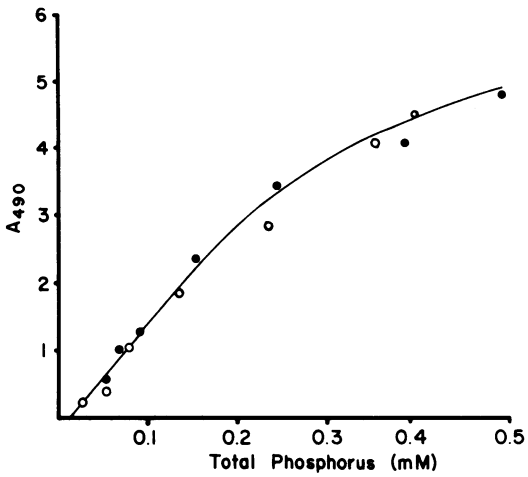

FIG. 1. The stationary phase cell yield obtained when PG2982 was inoculated into chemically defined medium (1% gluconate, 5 μg of thiamine per ml, and Dworkin-Foster salts minus inorganic phosphate) supplemented with either inorganic phosphate (○) or glyphosate (●) as the phosphorus source. The concentration of contaminating phosphate in the glyphosate media (~20 μM) as determined by assay was added to the concentration of glyphosate and is reported as total phosphorus in the medium. A_{490}, Absorbance at 490 nm.

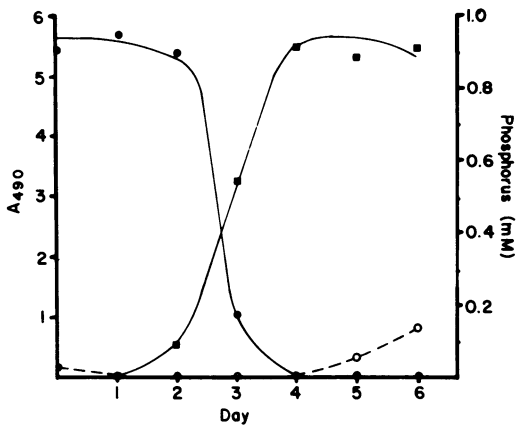

FIG. 3. Growth of PG2982 on chemically defined medium (1% gluconate, 5 μg of thiamine per ml, and Dworkin-Foster salts minus inorganic phosphate) with glyphosate as the source of phosphorus (■). Also shown are glyphosate (●) and inorganic phosphate (○) concentrations in the culture medium. A_{490}, Absorbance at 490 nm.

from equimolar quantities of the appropriate phosphorus source (Fig. 1). However, there is a slightly longer lag phase for growth on glyphosate and a doubling time twice as long as the 4-h doubling time for PG2982 grown on inorganic phosphate (Fig. 2). PG2982 can also utilize Amph as a phosphorus source with a growth rate similar but slightly slower than that obtained with glyphosate (Fig. 2). The slower growth rate suggests that Amph is not a preferred substrate, but this growth rate may be due to the fact that Amph acts as a glycine analog. The growth rate of PG2982 on complex media is not slow (Fig. 2), so our defined broth system is apparently not ideal for growth but for our studies appears to suffice. Measurements of glyphosate with an amino acid analyzer showed that glyphosate rapidly disappeared from the medium during exponential growth, and assays for inorganic phosphate showed increased levels during stationary phase (Fig. 3). The glyphosate is apparently metabolized quickly and completely during the exponential phase of growth. When a comparison was made between PG2982 grown in Pi broth and in Gly broth using both cell extracts and broth supernatant fluid obtained during exponential growth, there were no differences in the amino acid analysis except the glyphosate peak.

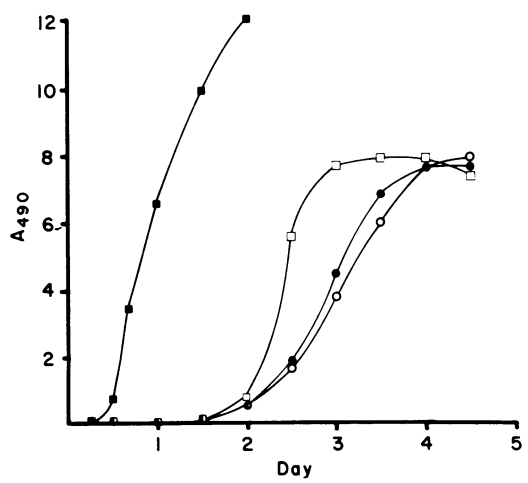

FIG. 2. Growth of PG2982 on the complex medium brain heart infusion (Difco) (■) and on chemically defined medium (1% gluconate, 5 μg of thiamine per ml, and Dworkin-Foster salts minus the inorganic phosphate) with the phosphorus as inorganic phosphate (□), glyphosate (●), or Amph (○). A_{490}, Absorbance at 490 nm.

We are interested in elucidating how PG2982 breaks down glyphosate and whether a "phosphonatase" enzyme similar to that characterized in *B. cereus* by LaNauze et al. (14, 15) is involved in the degradation. Assays for the degradation of glyphosate and Amph with undialyzed and dialyzed cell-free extracts have been unsuccessful. We did not find any degradation of glyphosate or Amph when we used the assay system for enhanced transamination necessary for *B. cereus*. It appears that the enzymatic

system in our isolate is either different from that of LaNauze et al. or is sensitive to cell disruption.

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