APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1986, p. 432–434
0099-2240/86/020432-03$02.00/0

Vol. 51, No. 2

Glyphosate-Degrading Microorganisms from Industrial Activated Sludge

TERRY M. BALTHAZOR AND LAURENCE E. HALLAS*
Monsanto Agricultural Co., St. Louis, Missouri 63166

Received 29 August 1985/Accepted 25 November 1985

A plating medium was developed to isolate *N*-phosphonomethylglycine (glyphosate)-degrading microorganisms, with glyphosate as the sole phosphorus source. Two industrial biosystems treating glyphosate wastes contained elevated microbial counts on the medium. One purified isolate metabolized glyphosate to aminomethylphosphonic acid, mineralizing this accumulating intermediate during log growth. This microorganism has been identified as a *Flavobacterium* species.

N-phosphonomethylglycine (glyphosate) is a widely used, broad-spectrum herbicide. Monsanto Co., St. Louis, Mo., makes and sells Roundup, in which glyphosate is formulated as its isopropylamine salt. Glyphosate biodegradation occurs in soils (7, 8) with aminomethylphosphonic acid (AMPA) and carbon dioxide as the metabolites. Monsanto Co. maintains a glyphosate-degrading activity in its waste treatment activated sludges used for treating glyphosate wastes. The microbial characterizations and modeling of glyphosate-degrading activity are under study to establish activated sludge as the best available technology to meet U.S. Environmental Protection Agency pesticide effluent guidelines.

Isolations of microorganisms which utilize glyphosate as carbon or nitrogen sources have never been reported. Microbes which degrade other phosphonates as phosphorus sources have been isolated and characterized (1), yet only one laboratory has reported a microbe which metabolizes glyphosate (6, 9). This *Pseudomonas* sp. grows with glyphosate as a sole phosphorus source with no accumulating intermediates (3). To date, no microbe which accumulates AMPA as an intermediate has been reported, nor have any glyphosate-degrading microorganisms been isolated from an environmental source. The purpose of this study was (i) to devise a medium for isolation of glyphosate-degrading microorganisms and (ii) to characterize the microbe(s) responsible for glyphosate metabolism in Monsanto activated sludges.

Media and culture conditions. A microbial medium with glyphosate as a sole phosphorus source was developed. Glassware was meticulously cleaned in NOCHROMIX (Godax Laboratories, New York, N.Y.). After 24 to 48 h, the glassware was rinsed in house-deionized water and then in deionized, distilled water to remove any phosphorus contamination. The Leadbetter and Foster (5) basal salts medium (minus Na_2HPO_4 and NaH_2PO_4) was used with gluconate (0.1% [wt/vol]) and pyruvate (0.1 [wt/vol]) as carbon sources and filter-sterilized glyphosate (analytical grade; 99.9% by assay) as a phosphorus source (0.02% [wt/vol]).

Purified agar (Difco Laboratories, Detroit, Mich.) was used for plate enumerations (pH 6.8 to 7.0). For the broth studies, samples were taken at different time intervals, and growth was measured by a spectrophotometer (model 20; Bausch and Lomb, Inc., Rochester, N.Y.) at 660 nm. Samples were then filtered and analyzed for glyphosate, AMPA, and PO_4^{3-} by a modification of the accepted high-pressure liquid chromatographic method for the analyses of industrial effluent (10).

Each sample was diluted, the pH was adjusted to 2 to 3, and each sample was injected onto the ion-exchange column (Brownlee AX-300; mobile-phase trifluoroacetic acid at pH 2.1) for component separation and flow injection analysis. Flow injection was faster than air-segmented injection. The components were then oxidized in a postcolumn reactor, and the free PO_4^{3-} was reacted with molybdate to form a blue complex that was quantitatively detected at 660 nm.

Microbial characterization. An isolate (GD1) from the glyphosate-containing plates was purified by restreaking on fresh plates. It was studied by light microscopy (gram negative, bipolar) and electron microscopy (negative staining revealed a short rod). The isolate was grown in 0.1% nutrient broth and was determined to be catalase and oxidase positive; a yellow pigment was produced in older cultures. The following semiautomated microbial identification test strips were used: API 20E, 20S, An-ident, and ZYM (Analytab products, Plainview, N.Y.); EBC+ (Vitek Systems, St. Louis, Mo.); and DMS rapid CH (anaerobic, aerobic) and rapid NFT (DMS Laboratories, Flemington, N.J.). The isolate was tentatively identified by EBC+ as a nonfermenter. The API index and Bergey's manual (2) suggested the *Flavobacterium* sp.

Microbial enumerations. I1 and I2 are two industrial aerobic biosystems which treat glyphosate-containing wastes. Waste treatment activated sludge samples were collected at three time intervals and stored at 0 to 4°C. Samples were streaked on a glyphosate-containing medium and incubated for 5 days at 25°C before counting.

Glyphosate was the sole source of phosphorus in the medium, and care was taken to remove all other contaminating phosphorus. Thus, it was assumed that the only colonies growing on these plates would be those utilizing glyphosate as a phosphorus source. Table 1 presents the CFU from the biosystems on the three sampling dates. I1 had the highest bacterial counts, averaging 23.5×10^6 CFU/ml. I2 bacterial counts averaged 5.48×10^6 CFU/ml through day 28 but decreased by 50% by day 50. This may have been due to a loss of glyphosate-degrading activity in the I2 biosystem at day 40. Control plates that did not contain glyphosate showed only a few pinpoint colonies on I1 and I2. These results indicate that glyphosate increased total microbial numbers. The growth of some small colonies on the control plates containing no phosphorus source was

* Corresponding author.

TABLE 1. Enumerations of industrial activated sludge on plates containing glyphosate as a sole phosphorus source

Biosystem[a]	CFU (10^6) per ml at time (day)[b]					
	0		28		50	
	G^+	G^-	G^+	G^-	G^+	G^-
I1	24.7	<0.30	20.1	<0.30	25.7	<0.30
I2	5.03	<0.30	5.93	<0.30	2.83	<0.30

[a] Total viable counts at I1 and I2 were 131×10^6 and 24.2×10^6 CFU/ml, respectively. Standard deviation averaged 6%.

[b] Plates were amended with (G^+) and without (G^-) glyphosate. Standard deviations averaged 10%.

presumably due to phosphorus carry-over from the aqueous inoculum or cellular endogenous reserves.

Glyphosate metabolism. A single colony was picked at random from a plate containing glyphosate that had been streaked with I1 activated sludge. This isolate was streaked on fresh glyphosate-containing medium, and a second colony was picked. This isolate (GD1) was identified as a *Flavobacterium* sp. (2).

GD1 was inoculated into glyphosate broth plus a control broth lacking glyphosate; growth of GD1 and the fate of glyphosate were monitored (Fig. 1). Lag phase lasted for approximately 25 h. GD1 then began slow growth as glyphosate was completely metabolized. Nearly stoichiometric amounts of AMPA were produced. Log growth lasted for 50 h. During this exponential phase, about 25% of the AMPA was metabolized, and a small amount of PO_4^{3-} was released. The control medium supported minimal growth. GD1 was also grown with AMPA as a sole phosphorus source (Fig. 2). Growth was similar to that observed in glyphosate-containing broth in that approximately 25% of the AMPA was used as a phosphorus source to support exponential growth. Further experiments (data not shown) indicate that the presence of PO_4^{3-} did not affect glyphosate metabolism to AMPA but did inhibit AMPA degradation.

The metabolism of glyphosate to AMPA by *Flavobacterium* sp. strain GD1 was stoichiometric and occurred during a slow growth phase. AMPA was then mineralized to PO_4^{3-} during log-phase growth. Thus,

$$\underset{\text{glyphosate}}{H_2O_3PCH_2NHCH_2CO_2H} + O_2 \rightarrow \underset{\text{AMPA}}{H_2O_3PCH_2NH_2} \quad (1)$$

$$\underset{\text{AMPA}}{H_2O_3PCH_2NH_2} + 2O_2 \rightarrow PO_4^{3-} + NH_4^+ + CO_2 + H_2O \quad (2)$$

At least two enzymatic steps are involved; a cleavage of glyphosate to AMPA (equation 1) and a phosphonatase activity that mineralizes AMPA (equation 2). In equation 1, the mechanism of carbon cleavage is unclear, with either two C units or one C_2 unit being produced. Ultimately, CO_2 would be produced. There is published precedent for equation 2. LaNauze et al. (4) have suggested the presence of a phosphonatase which would cleave a C-P bond, and Cook et al. (1) have proposed similar enzyme step(s) operating for AMPA degradation. Studies are under way to determine the details of glyphosate metabolism.

This is the first report of a microorganism which simulates the biodegradation of glyphosate in the environment, with AMPA as an initial, observable metabolite. It is also the only isolate which has been found to degrade glyphosate in the presence of PO_4^{3-}, a prerequisite for its use under environmental conditions. Currently, Monsanto Co. uses these microbes in activated sludge to treat glyphosate-containing wastes at its manufacturing facilities. Further work is warranted to enhance, stabilize, and control glyphosate-degrading activity to meet U.S. Environmental Protection Agency pesticide effluent permits.

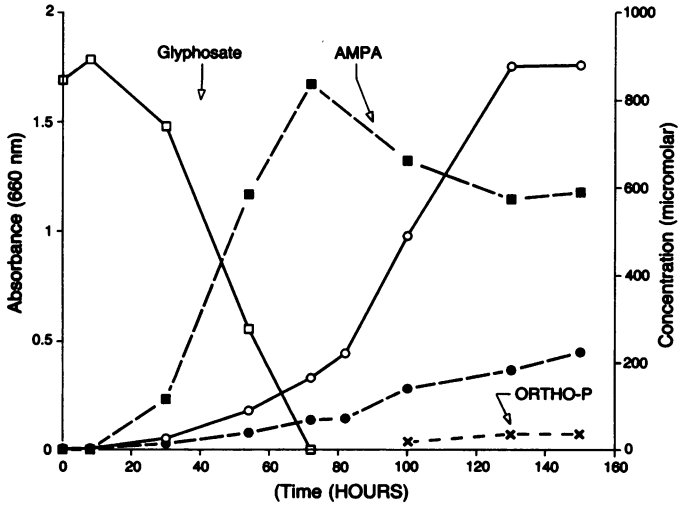

FIG. 1. Growth of *Flavobacterium* sp. in glyphosate broth and the fate of glyphosate, AMPA, and PO_4^{3-}. Symbols: ○, growth in medium containing approximately 0.02% glyphosate as sole phosphorus source; ●, growth in medium containing no glyphosate; □, glyphosate; ■, AMPA; ×, phosphate.

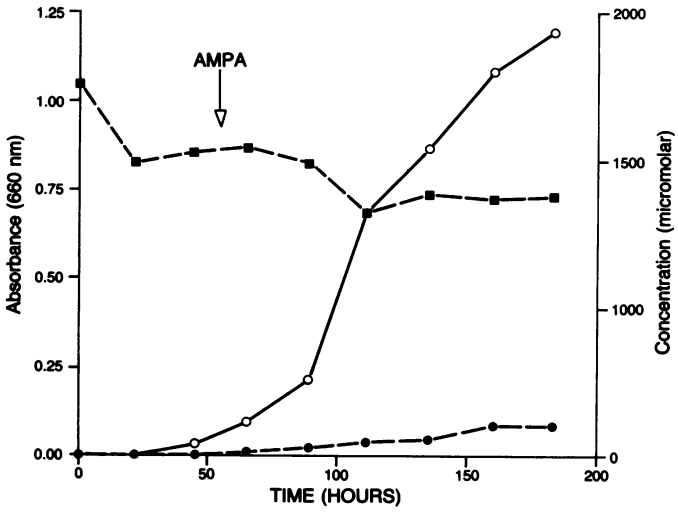

FIG. 2. Growth of *Flavobacterium* sp. in AMPA broth. Symbols: ○, growth in medium containing approximately 0.02% AMPA; ●, growth in medium containing no AMPA; ■, AMPA.

We acknowledge the advice and support of K. Lawrence, P. D. Patel, C. P. Anderson, and C. Korndorfer. T. Roussel and M. Bailey provided samples from the manufacturing plants. C. Ricketts performed all chemical analyses; his skill and patience are hereby acknowledged.

LITERATURE CITED

1. **Cook, A. M., C. G. Daughton, and M. Alexander.** 1978. Phosphonate utilization by bacteria. J. Bacteriol. **133**:85–90.
2. **Holmes, B., R. J. Owen, and T. A. McMeekin.** 1984. Genus *Flavobacterium*, p. 353–361. *In* N. R. Krieg and J. G. Holt

(ed.), Bergey's manual of determinative bacteriology, vol. I. The Williams & Wilkins Co., Baltimore.
3. **Jacob, G. S., J. Schaefer, E. O. Stejskal, and R. A. McKay.** 1985. Solid-state NMR determination of glyphosate metabolism in a *Pseudomonas* sp. J. Biol. Chem. **260**:5899–5905.
4. **LaNauze, J. M., H. Rosenberg, and D. C. Shaw.** 1970. The enzymic cleavage of the carbon-phosphorus bond: purification and properties of phosphonatase. Biochim. Biophys. Acta **212**:332–350.
5. **Leadbetter, E. R., and J. W. Foster.** 1958. Studies on some methane-utilizing bacteria. Arch. für Mikrobiologie **30**:91–118.
6. **Moore, J. K., H. D. Braymer, and A. D. Larson.** 1983. Isolation of a *Pseudomonas* sp. which utilizes the phosphonate herbicide glyphosate. Appl. Environ. Microbiol. **46**:316–320.
7. **Mueller, M. M., C. Rosenberg, H. Siltanen, and T. Wartiovaara.** 1981. Fate of glyphosate and its influence on nitrogen cycling in two Finnish agricultural soils. Bull. Environ. Contam. Toxicol. **27**:724–730.
8. **Rueppel, M. L., B. B. Brightwell, J. Schaefer, and J. T. Marvel.** 1977. Metabolism and degradation of glyphosate in soil and water. J. Agric. Food Chem. **25**:517–522.
9. **Shinabarger, D. L., E. K. Schmitt, H. D. Braymer, and A. D. Larson.** 1984. Phosphonate utilization by the glyphosate-degrading *Pseudomonas* sp. strain PG2982. Appl. Environ. Microbiol. **48**:1049–1050.
10. **U.S. Environmental Protection Agency.** 1983. Methods for nonconventional pesticide analysis of industrial and municipal wastewater: method 127—determination of glyphosate in wastewater. U.S. Environmental Protection Agency publication no. 440/1-83/079-C, p. 1–10. U.S. Environmental Protection Agency, Washington, D.C.