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Degradation of the Herbicide Glyphosate by Members of the Family *Rhizobiaceae*

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Several strains of the family *Rhizobiaceae* were tested for their ability to degrade the phosphonate herbicide glyphosate (isopropylamine salt of *N*-phosphonomethylglycine). All organisms tested (seven *Rhizobium meliloti* strains, *Rhizobium leguminosarum*, *Rhizobium galega*, *Rhizobium trifolii*, *Agrobacterium rhizogenes*, and *Agrobacterium tumefaciens*) were able to grow on glyphosate as the sole source of phosphorus in the presence of the aromatic amino acids, although growth on glyphosate was not as fast as on P_i. These results suggest that glyphosate degradation ability is widespread in the family *Rhizobiaceae*. Uptake and metabolism of glyphosate were studied by using *R. meliloti* 1021. Sarcosine was found to be the immediate breakdown product, indicating that the initial cleavage of glyphosate was at the C—P bond. Therefore, glyphosate breakdown in *R. meliloti* 1021 is achieved by a C—P lyase activity.

Glyphosate (isopropylamine salt of *N*-phosphonomethylglycine) is the active ingredient in Roundup, a broad-spectrum postemergence herbicide sold worldwide for use in a large number of agricultural crops and industrial sites. It is a potent inhibitor of the enzyme 3-*enol*-pyruvylshikimate-5-phosphate synthase (EPSP synthase, EC 2.5.1.19), which is involved in the biosynthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan (6, 21). It is immobilized by soil and is rapidly degraded by soil organisms (22). Only a few bacteria have been isolated that can utilize phosphonates, which include glyphosate, as their sole source of phosphorus for growth. Two pathways for the breakdown of glyphosate (Fig. 1) have been documented (5, 8, 12, 14, 16, 19). One involves cleavage of the molecule to give aminomethylphosphonate, which is further broken down by subsequent unknown steps. The second pathway is via initial cleavage of the C—P bond to give sarcosine by a C—P lyase activity. Bacteria known to degrade glyphosate via sarcosine include *Pseudomonas* sp. strain PG2982 (8) and *Arthrobacter* sp. strain GLP-1 (14). In this paper, *Rhizobium meliloti* and many other members of the family *Rhizobiaceae* are shown to possess glyphosate-degrading ability. Our efforts have focused on *R. meliloti* 1021 (RM1021), since this strain is well characterized both genetically and biochemically. Metabolism of glyphosate by RM1021 was found to follow the sarcosine pathway, implicating C—P lyase activity.

MATERIALS AND METHODS

Chemicals. *N*-Phosphonomethylglycine (isopropylamine salt; technical grade, 86%) was provided by Monsanto Co., St. Louis, Mo. Methyl- and ethylphosphonic acids and diethylphosphite were obtained from Alfa Products, Danvers, Mass. Sarcosine, aminomethyl phosphonic acid (AMPn), and vitamins were obtained from Sigma Chemical Co., St. Louis, Mo. Formaldehyde (37%) and glycine were purchased from Aldrich Chemical Co., Milwaukee, Wis. 3-(*N*-morpholino)-propanesulfonic acid (MOPS) was obtained from Research Organics, Cleveland, Ohio. Agarose was obtained from International Biotechnology Inc., New Haven, Conn.

Culture of bacteria. Inocula of all rhizobia except *Rhizobium leguminosarum* (strain 300) and ANU843 were grown in LB (1% Bacto tryptone, 0.5% Bacto yeast extract, 0.5% NaCl) at 28 to 32°C for 18 to 30 h. Inocula of agrobacteria, ANU843, and *R. leguminosarum* were grown in TY (0.5% Difco tryptone, 0.3% yeast extract, 0.05% $CaCl_2 \cdot 2H_2O$) at 32°C. A P-free minimal medium was developed which contained 25 mM MOPS/KOH (pH 7.4), 2 mM $MgSO_4$, 100 μM ferric citrate, 1.2 mM $CaCl_2$, 33 mM NH_4Cl, 0.5 mg of thiamine · HCl per liter, 1 mg of nicotinic acid per liter, 0.1 mg of biotin per liter, 0.5 mg of pantothenic acid per liter, and trace elements (0.8 μM $CoCl_2$, 40 μM $CuSO_4$, 14 μM H_3BO_4, 0.1 μM $MnCl_2$, 10 μM Na_2MoO_4, 0.2 μM $NiSO_4$, and 1.5 μM $ZnSO_4$). The P-free medium was made up and stored in disposable polystyrene tissue culture flasks (Falcon) as a sterile 2× solution lacking a C source, vitamins, and P source, and it was diluted for liquid medium or mixed with an equal volume of molten sterile 1.8% agarose for solid medium. Streptomycin sulfate (200 mg/liter; Sigma) was usually added to the medium for RM1021, which is streptomycin resistant, as a precaution against contamination. A phosphate assay (3) indicated that contaminant P_i was <1 μM. Rhizobia were grown on the P-free medium with disodium succinate (50 mM) as a carbon source. ANU843 was grown on the same medium, containing in addition monosodium glutamate (1.1% wt/vol). Agrobacteria were grown in P-free medium with glucose (0.2% wt/vol) as the carbon source. P sources were added at 0.5 mM unless otherwise stated. In experiments in which glyphosate was the P source, the three aromatic amino acids were added at 50 mg/liter. Growth experiments were done either in 250-ml side-arm flasks containing 30 ml of medium or in test tubes (18 by 150 mm) containing 1.5 ml of medium. Inoculation was done by direct transfer of an 18- to 30-h culture in either LB or TY medium. The volume transferred was 1/1,000 that of the medium inoculated. Cultures in side-arm flasks were grown by shaking at 250 rpm for 40 to 48 h, and growth was monitored by measuring the turbidity with a Klett-Summerson colorimeter (green filter). Cultures in test tubes were

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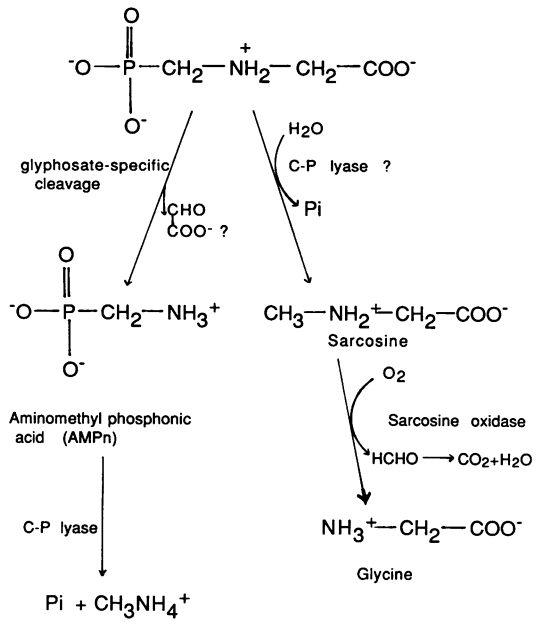

FIG. 1. Glyphosate breakdown pathways.

grown in a roller for 48 h, and growth was determined by measuring the optical density at 600 nm.

Uptake and metabolic experiments. Mid-log-phase RM1021 cells (30 ml) grown in P-free medium with glyphosate for about 48 h were collected by centrifugation (20,000 × *g*, 5 min). The cell pellet was resuspended in P-free medium (4 ml/g [wet weight] of cells), and the suspension was placed on ice. [^{14}C]glyphosate was added to a final concentration of 0.1 mM, and the suspension was incubated at 28°C with shaking in a water bath. In some experiments, either glycine or sarcosine (pH adjusted to neutrality) was added to a final concentration of 2 mM. Aliquots of 75 μl were withdrawn at 30-min intervals. For uptake studies, total radioactive counts were measured on a portion of the withdrawn sample (10 μl) by scintillation counting. A second 10-μl portion was filtered through a 0.22-μm-pore-size membrane (Millipore, GVWP 025 00) under vacuum. The cells on the membrane were washed with P-free medium (2 × 100 μl) and counted. The counts associated with the cells measured the extent of glyphosate uptake. The remainder of the sample was used for metabolic studies. The cells were collected by centrifugation and extracted twice with ice-cold 5% trichloroacetic acid (200 μl, 4°C, 5 min). Both the supernatant and the extract were concentrated in vacuo and resuspended in water (10 μl). A 4-μl portion of the solution was co-spotted on a microcrystalline cellulose TLC plate (J. T. Baker) with 1 μl of a mix of reference compounds containing 10 mM (each) glyphosate, AMPn, glycine, and sarcosine.

TLC. Glyphosate and its metabolites were separated on microcrystalline cellulose plates by using ethanol–water–17 N ammonium hydroxide–trichloroacetic acid–15 N acetic acid (55:35:2.5:3.5:2 vol/vol/vol/wt/vol, solvent A) as the solvent system (20). Two-dimensional thin-layer chromatography (TLC) was carried out by using solvent A for the first dimension and chloroform–methanol–17 N ammonium hydroxide (40:45:20) for the second dimension. The radiolabeled compounds were detected by autoradiography, while the reference compounds were visualized by ninhydrin spray (0.5% [wt/vol] in ethanol; Sigma). The identities of the radiolabeled compounds were determined by superimposing the X-ray film with the TLC plate that was treated with ninhydrin. The radioactive spots corresponding to glyphosate, glycine, and sarcosine were excised and counted.

TABLE 1. Bacterial strains

Bacterial species	Strain	Source	Reference
R. meliloti	RM1021	F. M Ausubel	9
	ATCC4399	A.T.C.C.[a]	
	ATCC4400	A.T.C.C.	
	ATCC7022	A.T.C.C.	
	ATCC9930	A.T.C.C.	
	ATCC10310	A.T.C.C.	
	ATCC35176	A.T.C.C.	
R. leguminosarum	300	C. W. Ronson	7
R. galega	HAMBI 540	F. M. Ausubel	
R. trifolii	ANU843	F. M. Ausubel	15
A. rhizogenes	A4	V. Buchanan-Wollaston	
A. tumefaciens	B6	V. Buchanan-Wollaston	17
	C58	V. Buchanan-Wollaston	23

[a] American Type Culture Collection, Rockville, Md.

Chemical synthesis of [^{14}C]glyphosate. A process for the synthesis of [1-^{14}C]glyphosate was developed on the basis of a published account for the preparation of glyphosate (13). To a solution of [1-^{14}C]glycine (250 μCi, 50 mCi/mmol; ICN Biomedicals) was added 89 μl of a 8.5% K^+-glycinate solution (8.5 mg of glycine and 0.71 mg of KOH in 100 μl of water). The specific activity of the resulting glycine solution was ~2 μCi/μmol. After the addition of a 37% solution of formaldehyde (9.4 μl), the mixture was kept at 4°C overnight. The reaction was worked up by precipitation with ice-cold ethanol. The precipitate was then washed successively with ethanol and diethyl ether. The residue was vacuum-dried and redissolved in water (100 μl). Diethylphosphite (14.5 μl) was then added, and the clear solution was heated to 100°C and kept at that temperature for 3 h. The reaction was cooled, and concentrated HCl (1 ml) was then added. The acid hydrolysis was carried out at 100°C for 16 h. The reaction was purified by a cellulose TLC plate (solvent A). The glyphosate band (R_f, 0.25) was visualized by autoradiography, excised, and extracted with water. The extract was then loaded onto a column of Dowex AG50W-X8 cation exchange resin. The column was washed with water, and the product was eluted with 2 N NH_4OH (3 ml). The eluant was evaporated to dryness and redissolved in 1 ml of water. The overall yield was about 20%. [3-^{14}C]glyphosate was prepared in a similar manner by using [^{14}C]formaldehyde (250 μCi, 50 mCi/mmol; ICN Biomedicals) and glycine.

RESULTS

Growth on phosphonates. A P-free minimal medium which allowed very good growth of *R. meliloti* with diverse P sources but gave little growth in the absence of a P source was developed (see Materials and Methods). We initially examined seven strains of *R. meliloti* and found that they all were able to grow on glyphosate as the sole P source. These observations were subsequently extended to other members of the *Rhizobiaceae* family (Table 1). Without exception, they all showed the ability to utilize glyphosate as the sole source of phosphorus for growth. Their ability to utilize other phosphonate compounds was also studied. The growth curves of some strains on various phosphorus compounds

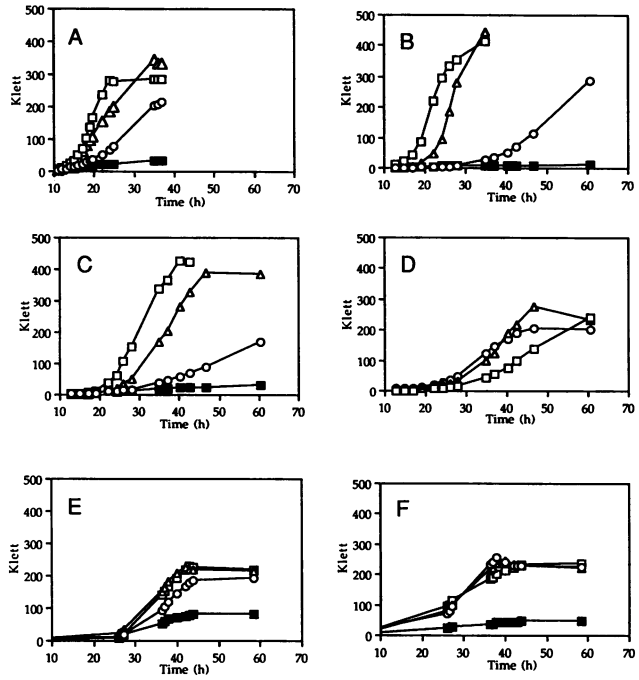

FIG. 2. Growth of *Rhizobiaceae* strains in P-free medium in the absence of a P source (■) and in the presence of 0.5 mM P_i (□), 0.5 mM glyphosate (○), and 0.5 mM AMPn (△). (A) RM1021; (B) *R. leguminosarum* 300; (C) *R. galega*; (D) ANU843; (E) *A. rhizogenes* A4; (F) *A. tumefaciens* B6.

are shown in Fig. 2. In general, the rate of growth on P_i was comparable to that on AMPn for both rhizobia and agrobacteria. With the exception of ANU843, the growth of *Rhizobium* strains on glyphosate, however, was slower. ANU843 and the two *Agrobacterium* strains did not grow as rapidly on all three P sources as the other strains because they were grown at 32°C, which is outside the optimum temperature range of 25 to 28°C. *R. meliloti* grew well on methyl phosphonate, AMPn (Fig. 2A), and aminoethylphosphonate (data not shown). It grew less well on glyphosate (Fig. 2A) and poorly on ethyl phosphonate (data not shown). Growth on glyphosate and ethyl phosphonate was not truly logarithmic and stopped at a lower optical density than on the other phosphonates. It was found necessary to add the aromatic amino acids in order to get reproducible growth on glyphosate. The order of growth for RM1021 was aminoethylphosphonate ≥ AMPn ≥ P_i > methyl phosphonate > glyphosate > ethyl phosphonate. Thus, the ability to degrade glyphosate and many other phosphonates is widespread in the family *Rhizobiaceae*.

Growth of RM1021 on glyphosate. In an effort to obtain mid-log-phase cells for metabolic studies, the growth of RM1021 in P-free medium containing glyphosate was studied. The growth of RM1021 on glyphosate was rather sluggish. In order to improve the growth, the concentrations of streptomycin, NaCl, aromatic amino acids, and vitamins in the P-free medium were varied. The results showed that the levels of all these ingredients in the medium were optimal. It was also found that growth was more rapid with increasing levels of glyphosate. However, growth was retarded and the culture usually turned greenish-yellow when the glyphosate level exceeded 1 mM in the medium. Under this constraint, the best growth was observed with 100 mM succinate and 1 mM glyphosate (Table 2). However, even under the best conditions, growth was never truly logarithmic (Fig. 2A). Uptake studies with [1-^{14}C]glyphosate revealed that glyphosate was efficiently taken up by the cells but was metabolized slowly, resulting in the accumulation of glyphosate inside the cell (see below). It was suspected that such accumulation of glyphosate might be detrimental to the growth of the cell. Therefore, RM1021 cells were grown on 0.5 mM glyphosate to a cell density of about 30 Klett units and collected by centrifugation. The cell pellet was resuspended to the same volume as before in P-free medium that was devoid of glyphosate. The optical density of the resuspended cells was checked and found to be unchanged. The cells were then allowed to grow on the stored glyphosate, and it was observed that the growth rate increased significantly after a 5- to 10-h lag (Fig. 3A). The maximum growth rate for glyphosate-grown cells was obtained at Klett readings between 50 to 100 (mid-log phase; see Fig. 3A), giving doubling times of 10 h without the removal of glyphosate and 4.5 h with the removal of glyphosate. The most linear section of the semilog plots of the growth curves with or without the removal of glyphosate fell within the late-log phase (Klett readings, 100 to 200), giving doubling times of 17 h without the removal of glyphosate and 10 h with the removal of glyphosate. Thus, the removal of glyphosate resulted in an approximately 50% decrease in doubling time. Control cells which were treated similarly but were resuspended in the original medium containing glyphosate gave a growth curve virtually indistinguishable from that of undisturbed cells. Furthermore, the effect was unique to glyphosate. When cells grown on P_i or AMPn were treated in the same manner (i.e., with the removal of P_i and AMPn), the opposite effect was observed, i.e., the growth rate dropped off markedly because of the depletion of the P source (Fig. 3B).

TABLE 2. Growth of RM1021 at various succinate and glyphosate concentrations[a]

Succinate (mM)	Glyphosate (mM)	OD (600 nm)[b]
50	0.05	0.35
100	0.05	1.05
200	0.05	0.38
1	0.5	0.15
5	0.5	0.48
50	0.5	2.70
250	0.5	0.08
500	0.5	0.03
10	1	1.00
100	1	3.26
250	1	0.37
500	1	0.01

[a] Growth in test tube at 28°C for 48 h.
[b] Optical density at 600 nm.

Uptake and breakdown of glyphosate by RM1021. The uptake of glyphosate by RM1021 was followed by monitoring the radioactivity associated with mid-log-phase cells incubated with [1-^{14}C]glyphosate. The radioactive substrate was taken up and metabolized. As shown in Fig. 4, the RM1021 cells transported nearly 85% of the glyphosate into the cells within 30 min, after which the percentage started to decrease. TLC analysis of the trichloroacetic acid extract of the cells sampled at 30-min intervals showed the appearance of sarcosine and glycine (Fig. 5). No AMPn was detectable when [3-^{14}C]glyphosate was used. Similar analysis of the medium also showed the presence of these two compounds.

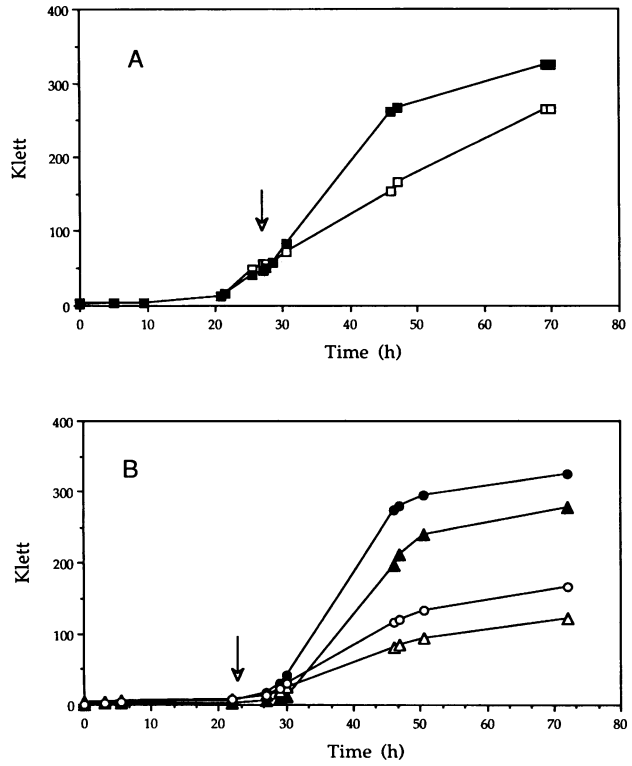

FIG. 3. Effect of P source removal on the growth of RM1021 with glyphosate (A) and P_i or AMPn (B) as the P source. (A) The cells were centrifuged and resuspended (at the point marked by arrow) in P-free medium containing either no glyphosate (■) or 1 mM glyphosate (□). (B) The cells were treated as described for panel A and resuspended in P-free medium containing no P_i (○), 0.1 mM P_i (●), no AMPn (△), or 0.1 mM AMPn (▲).

The identity of glycine and sarcosine was further confirmed by two-dimensional TLC analysis as described in Materials and Methods. In order to rule out the possibility that these products were derived from nonspecific chemical or biochemical reactions, cells grown on P_i were used as a control. We have evidence from our genetic study of RM1021 (unpublished data) that P_i suppresses the expression of genes coding for the C—P lyase system and renders the cells

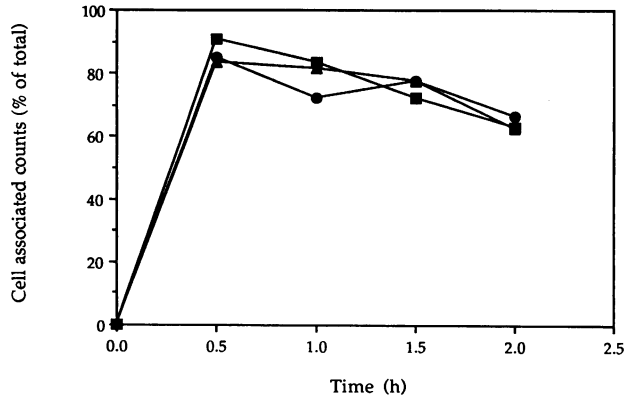

FIG. 4. [1-^{14}C]glyphosate uptake in the absence (▲) and in the presence (2 mM) of either sarcosine (●) or glycine (■).

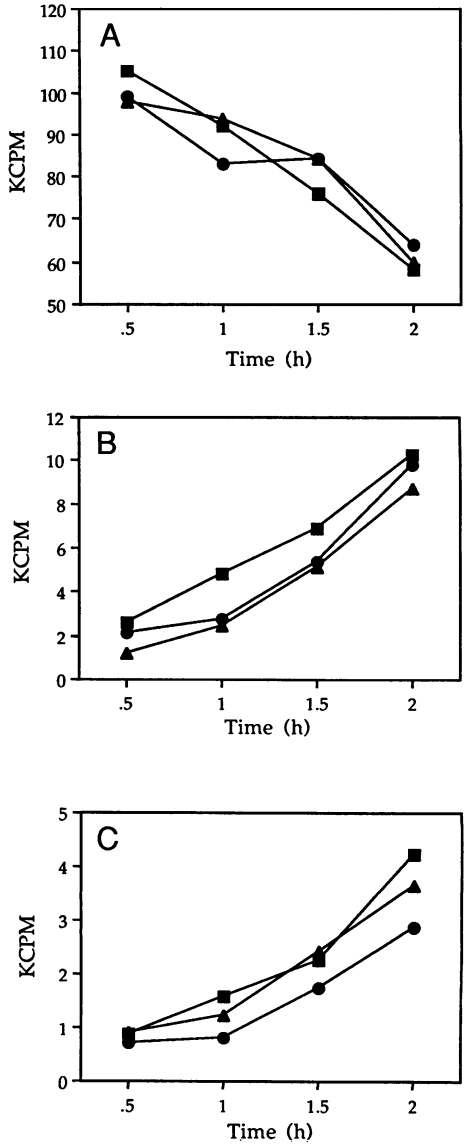

FIG. 5. Distribution of cell-associated radioactivity in glyphosate (A), sarcosine (B), and glycine (C) in the absence of sarcosine and glycine (▲) and in the presence of 2 mM sarcosine (●) and 2 mM glycine (■). KCPM, counts per minute × 10^3.

unable to metabolize glyphosate. Cells grown on P_i accumulated glyphosate (>90% of added counts), but no breakdown products were detected (data not shown). The same results were obtained when glyphosate-grown cells were incubated under nitrogen. This is consistent with the results of our metabolic studies, which indicate that energy metabolism is required for C—P bond cleavage in *R. meliloti* with methyl phosphonate as substrate (unpublished results).

The pathway for glyphosate degradation by RM1021 was studied by performing radiotracer experiments with [^{14}C] glyphosate. [^{14}C]sarcosine and [^{14}C]glycine were readily identified as metabolic intermediates by two-dimensional TLC analysis. Pulse-chase experiments with either glycine or sarcosine were also carried out. The results showed that, at the level (2 mM) used, neither glycine nor sarcosine interfered with the normal glyphosate uptake by RM1021 cells

(Fig. 4). In all three cases, the level of glyphosate decreased while those of sarcosine and glycine increased with time (Fig. 5). However, their rates of accumulation differed. In the presence of sarcosine, the level of radioactive sarcosine was higher whereas that of glycine was lower compared with the corresponding levels at which no sarcosine was added. For example, at the end of 2 h, the level of sarcosine was 13% higher (Fig. 5B) and that of glycine was 26% lower (Fig. 5C). In the presence of glycine, the levels of both intermediates were higher. This is consistent with the previously proposed pathway (Fig. 1) in which glyphosate is first converted into sarcosine by a C—P lyase activity, followed by conversion into glycine. It was also observed that a large proportion of the breakdown products sarcosine and glycine were released into the medium. It can be concluded that the breakdown of glyphosate in RM1021 follows the same pathway as that proposed for *Pseudomonas* sp. strain PG2982 (8) and *Arthrobacter* sp. strain GLP-1 (14).

DISCUSSION

Although it is well documented that glyphosate is readily metabolized by soil microorganisms (12, 16, 19), surprisingly few glyphosate-degrading bacteria have been isolated. Altogether, one *Flavobacterium* strain (2), one *Arthrobacter* strain (14), one *Agrobacterium* strain (24), and two *Pseudomonas* strains (5, 18) have been reported in the literature. This project started with the goal of finding suitable soil bacteria which degraded the herbicide glyphosate readily. Screening for glyphosate-degrading organisms from soil samples previously exposed to the herbicide had been carried out at our affiliate, BioTechnica Limited (Cardiff, Wales). Three independent isolates, all of which turned out to be different *Agrobacterium* strains, were obtained when selection for growth on glyphosate as the sole P source was carried out. No organism which could use glyphosate as a carbon or nitrogen source was found. We initially sought to use a related species, *R. meliloti*, as a possible recipient for selecting glyphosate-utilization genes from a clone bank of the *Agrobacterium* strain and discovered that it too degraded glyphosate. That prompted us to investigate whether this property was widespread in the family *Rhizobiaceae*. In our efforts to grow these strains for further characterization, it became apparent that refinement of the growth medium for optimal growth was necessary. The result was the general-purpose P-free medium described in this paper. By using the P-free medium, it was soon discovered that many *Rhizobium* and *Agrobacterium* strains were able to utilize glyphosate and other phosphonates as their sole P source. Therefore, glyphosate degradation seems to be a general property of the *Rhizobiaceae* family.

Our goal was to clone the genes associated with the degradation of glyphosate and possibly its breakdown products. Our efforts were focused on RM1021 because it is well characterized and extensive studies on its genetics have been done. Growth of RM1021 on glyphosate was slower in the P-free medium with succinate as the carbon source than was growth on P_i or AMPn. The growth rate can be improved by increasing the glyphosate concentration in the medium. However, at a concentration of greater than 1 mM, growth was inhibited, the culture usually turned greenish-yellow, and cells were less viable as judged by the impairment of their ability to take up glyphosate. The best growth rate and highest optical density were achieved at 1 mM glyphosate. A remarkable increase in growth rate was observed, however, by removing glyphosate from the growth medium 24 h after inoculation. The doubling time was reduced by approximately 50%. Uptake studies showed that glyphosate was taken up rapidly by the cells but metabolized rather slowly. The net result was the accumulation of a high level of glyphosate inside the cell, with an insignificant amount being released back into the medium. One plausible explanation of the observed increase in growth is that the level of glyphosate accumulation was inhibitory to growth and that by removing the glyphosate from the medium, the cells were forced to grow on the stored glyphosate. The internal level of glyphosate was lowered through glyphosate metabolism and cell division to such an extent that it was no longer inhibitory. Subsequently, after a lag of 5 to 10 h the growth rate rose sharply. A similar but less dramatic effect on RM1021 growth was observed when glucose or mannitol was used as the carbon source. This effect is unique to glyphosate and is observed in glyphosate-grown cells collected from cultures ranging from 24 to 60 h old. No such effect was observed when P_i or AMPn was used in place of glyphosate.

The results of this report indicate that RM1021 degrades glyphosate by cleaving the carbon-phosphorus bond. The resulting sarcosine is then converted into glycine, presumably by oxidative degradation involving an enzyme such as sarcosine oxidase. The key evidence is the identification of [^{14}C]sarcosine as the intermediate in the degradation of glyphosate by RM1021. This was readily achieved by simply incubating the RM1021 cells with radiolabeled glyphosate. This is in contrast to other glyphosate-degrading bacteria, for which the addition of unlabeled sarcosine or glycine as a trapping agent is required (8) to trap the intermediate to a detectable level. Our results strongly indicate the involvement of a carbon-phosphorus bond cleavage activity. The ultimate proof of the involvement of such an enzyme would be its isolation and characterization, but all reported attempts (8, 24), including ours, to get in vitro activity of the enzyme have been unsuccessful. Recently, Murata and coworkers (10, 11) have reported that they detected C—P lyase activity in an extract of *Enterobacter aerogenes* and that the activity requires two protein components. It is yet to be established that the C—P lyase activity in *E. aerogenes* is the same as the in vivo activity reported in the literature and the same as the activity discussed here. Recent advances have been made in several laboratories in the characterization of the genes coding for C—P lyase activity in *Escherichia coli* (1, 4). The C—P lyase genes in RM1021 have been isolated and characterized in our laboratory (unpublished data). This new information on the genetic system of C—P lyase will be helpful in achieving the isolation of C—P lyase activity.

In summary, we conclude that glyphosate utilization ability is widespread in the family *Rhizobiaceae*. This is achieved by a C—P lyase activity capable of degrading a broad spectrum of phosphonates. Rhizobia and agrobacteria are common in the rhizosphere and may contribute to the observed rapid degradation of glyphosate residues in soil. It remains to be seen whether this contribution is of ecological significance, since their activity may be limited to the rhizosphere.

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