

Metabolism of Glyphosate in *Pseudomonas* sp. Strain LBr

GARY S. JACOB,^{1†*} JOEL R. GARBOW,^{1‡} LAURENCE E. HALLAS,² NANCEE M. KIMACK,¹
GANESH M. KISHORE,³ AND JACOB SCHAEFER^{1§}

Physical Sciences Centre¹ and Monsanto Agricultural Product Research Department,² Monsanto Co., St. Louis, Missouri 63167, and Plant Molecular Biology Laboratory, Monsanto Co., St. Louis, Missouri 63198³

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Metabolism of glyphosate (*N*-phosphonomethylglycine) by *Pseudomonas* sp. strain LBr, a bacterium isolated from a glyphosate process waste stream, was examined by a combination of solid-state ¹³C nuclear magnetic resonance experiments and analysis of the phosphonate composition of the growth medium. *Pseudomonas* sp. strain LBr was capable of eliminating 20 mM glyphosate from the growth medium, an amount approximately 20-fold greater than that reported for any other microorganism to date. The bacterium degraded high levels of glyphosate, primarily by converting it to aminomethylphosphonate, followed by release into the growth medium. Only a small amount of aminomethylphosphonate (about 0.5 to 0.7 mM), which is needed to supply phosphorus for growth, could be metabolized by the microorganism. Solid-state ¹³C nuclear magnetic resonance analysis of strain LBr grown on 1 mM [2-¹³C, ¹⁵N]glyphosate showed that about 5% of the glyphosate was degraded by a separate pathway involving breakdown of glyphosate to glycine, a pathway first observed in *Pseudomonas* sp. strain PG2982. Thus, *Pseudomonas* sp. strain LBr appears to possess two distinct routes for glyphosate detoxification.

A number of bacteria have been found recently that degrade phosphonates (compounds that contain a C-P bond), including glyphosate (*N*-phosphonomethylglycine), a potent, widely used, broad-spectrum herbicide. The earliest studies of bacterial metabolism of glyphosate were performed with mixed bacterial cultures of soil-water mixtures to simulate the ecological fate of glyphosate in soil (9, 12, 15). More recent studies of glyphosate-degrading bacteria have involved selection for, and isolation of, pure bacterial strains with enhanced or novel detoxification capabilities for potential uses in the biotechnology industry, such as, for example, removal of glyphosate from process waste streams or facilitation of the development of glyphosate-resistant crop plants based on detoxification of glyphosate.

Bacteria degrade glyphosate in two general ways (Fig. 1), leading to the intermediate production of either glycine or aminomethylphosphonate (AMPA). Microorganisms known to degrade glyphosate by way of glycine include *Pseudomonas* sp. strain PG2982 (5, 6) and *Arthrobacter* sp. strain GLP-1 (10). The first step in this pathway has recently been shown to involve cleavage of the C-P bond of glyphosate to produce sarcosine (7, 10, 14), which is then converted to glycine by a sarcosine oxidase-dehydrogenase. A second group of bacteria, represented by a *Flavobacterium* sp. strain GDI (1), as well as the earlier-reported mixed bacterial cultures from soil (9, 12), degrade glyphosate by cleaving its carboxymethyl carbon-nitrogen bond to produce AMPA. Some of the AMPA generated in this way can be further metabolized, providing phosphorus for growth, although the amount eliminated is typically set by the phosphorus requirement of the bacterium in question.

In this paper, we describe the glyphosate-degrading prop-

erties of *Pseudomonas* sp. strain LBr, a bacterium isolated from waste treatment activated sludge by selection for growth in the presence of glyphosate. Metabolism of glyphosate was analyzed by cross-polarization magic-angle spinning (CPMAS) ¹³C nuclear magnetic resonance (NMR) analysis of cells grown on ¹³C, ¹⁵N-labeled glyphosate. The disappearance of glyphosate and appearance of AMPA in the growth medium were monitored by high-performance liquid chromatography (HPLC). From the results of these experiments, we conclude that *Pseudomonas* sp. strain LBr possesses both pathways described above for glyphosate detoxification and that the bacterium can completely degrade glyphosate at concentrations measured as high as 19 mM.

MATERIALS AND METHODS

Chemicals. *N*-Phosphonomethylglycine (99.7% purity) was provided as the free-acid form by Monsanto Agricultural Products, Monsanto Co., St. Louis, Mo. [2-¹³C, ¹⁵N]glyphosate (99 atom% ¹³C:99 atom% ¹⁵N) and [3-¹³C, ¹⁵N]glyphosate (92 atom% ¹³C:99 atom% ¹⁵N) were obtained from Merck Stable Isotopes, Montreal, Canada. The ability of these labeled materials to inhibit the 3-enolpyruvyl-shikimic acid-5-phosphate synthase reaction, a known property of glyphosate (16), was compared with that of natural-abundance glyphosate and found to be identical, which was taken as evidence of chemical authenticity.

Glassware. To eliminate contaminating phosphate from glassware used in *Pseudomonas* sp. strain LBr growth experiments, glassware was suspended overnight in a solution composed of 0.2 N HCl and 0.2 N HNO₃ and then rinsed with glass-distilled water before use.

Culture methods. *Pseudomonas* sp. strain LBr was routinely stored in a lyophilized state on presterilized concentration disks (Difco Laboratories, Detroit, Mich.) within presterilized glass vials. Breakdown of glyphosate required that cells be grown in medium devoid of any source of phosphorus except glyphosate. The medium we used, designated GPI, consisted of a Dworkin-Foster salt mixture (3) without P_i and with 1% potassium D-gluconate as a carbon source and contained varying amounts of glyphosate, de-

* Corresponding author.

† Present address: Department of Biochemistry, University of Oxford, Oxford OX1 3QU, United Kingdom.

‡ Present address: Life Sciences NMR Centre, Monsanto Co., St. Louis, MO 63198.

§ Present address: Department of Chemistry, Washington University, St. Louis, MO 63130.

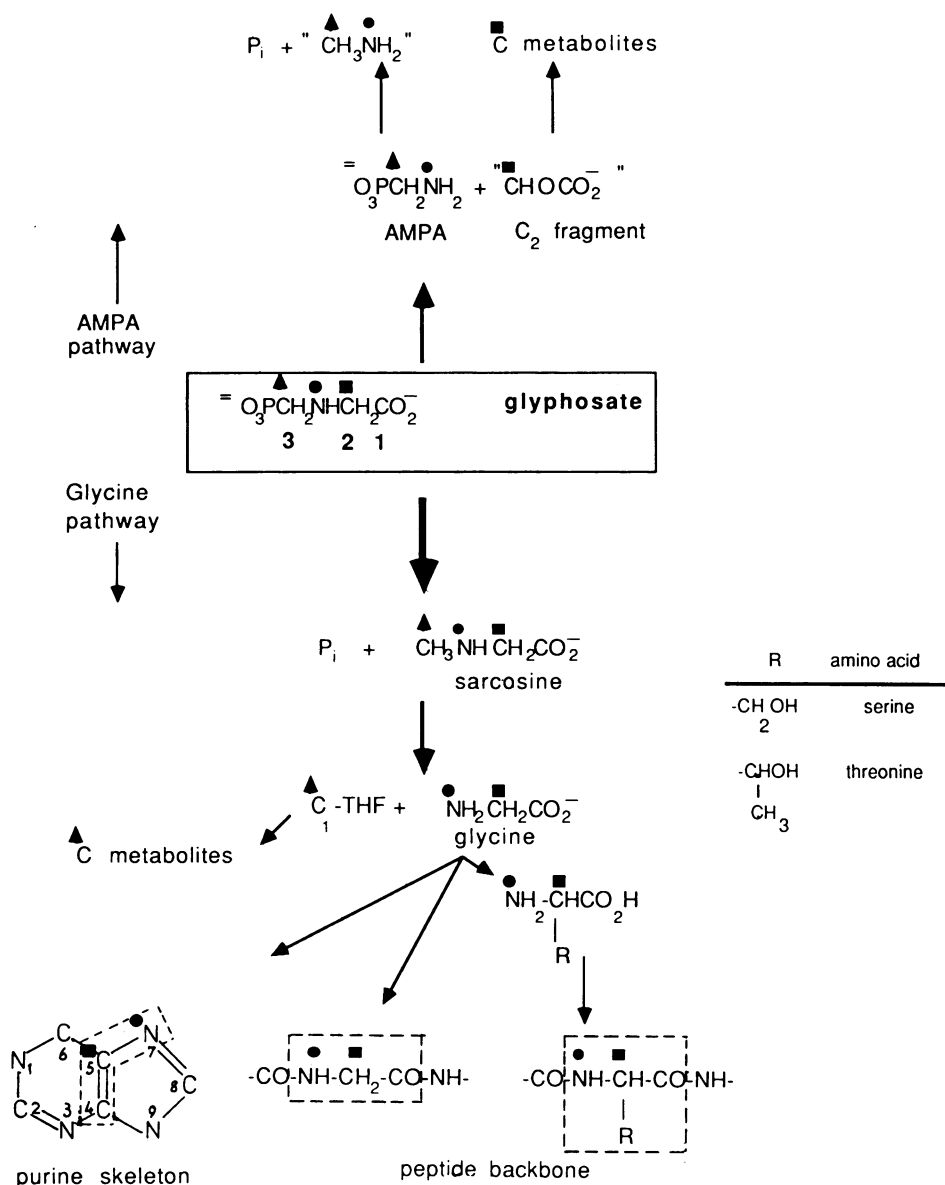


FIG. 1. Pathways for degradation of glyphosate. Numbers in the structural formula of glyphosate indicate the numbering of carbon atoms used in the text. THF, Tetrahydrofolate.

pending on the nature of the experiment. To monitor clearance of high levels of glyphosate, a nominal glyphosate concentration of 20 mM was used, whereas stable-isotope-labeling experiments for the solid-state NMR studies used 1-mM [2-¹³C,¹⁵N]- and [3-¹³C,¹⁵N]glyphosate concentrations. To avoid the possibility of any breakdown of glyphosate by high temperature, glyphosate was added by filter sterilization. The procedure involved dissolving the compound in water, adjusting the pH to 7.0 by addition of NaOH, and sterilizing the preparation with a 0.2 μ m-pore-size filter flask, followed by addition to a separately autoclaved glyphosate-minus medium.

Preliminary experiments on metabolism of glyphosate by *Pseudomonas* sp. strain LBr displayed variation in the amount of glyphosate cleared from the medium. In certain cases, no glyphosate was degraded even though cells grew normally and reached a typical cell density at the end of growth. Since glyphosate was the sole source of phosphorus,

these cells must have used some form of stored phospho compound to provide phosphorus for growth, although this facet of the behavior of *Pseudomonas* sp. strain LBr was not pursued. Rather, reliable glyphosate-degrading conditions, which involved starving cells for phosphorus before the start of the growth experiment, were developed. Typically, this was done by reviving lyophilized cells from a single Difco disk in 10 ml of GPI medium, followed by transfer into a modified GPI medium containing no glyphosate. After growth had ceased, an appropriate amount of the culture was used as an inoculum for the actual growth experiment.

Magic-angle spinning ¹³C NMR. Cells grown on ¹³C,¹⁵N-labeled glyphosate were harvested and prepared for solid-state NMR analysis as reported previously (6). ¹³C NMR spectra of lyophilized cells were obtained at 50.3 MHz by using matched spin-lock cross-polarization transfers with 2-ms contacts and 50-kHz H₁'s (6) under spinning sideband suppression conditions (2). The chemical shift scale is in

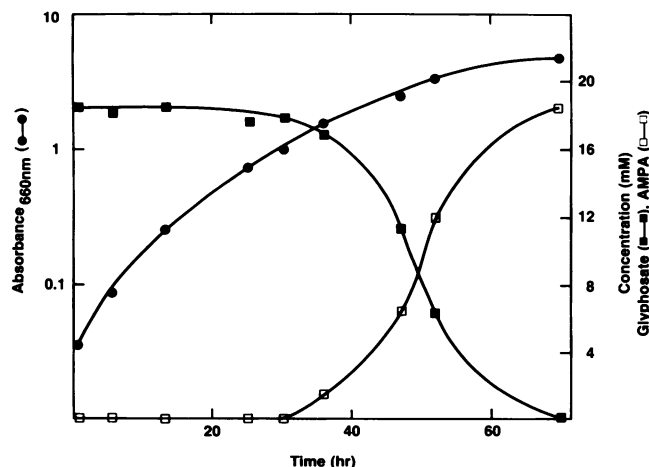


FIG. 2. Growth of *Pseudomonas* sp. strain LBr in medium containing 19 mM glyphosate as the sole phosphorus source. Also shown are glyphosate and AMPA concentrations in the culture medium at various stages of cellular growth.

parts per million downfield from external tetramethylsilane. Technical details of the spinning and cross-polarization procedures are reported elsewhere (13, 17, 18). CPMAS ^{13}C NMR spectra containing only signals for uptake and metabolism of glyphosate were obtained by subtracting a spectrum of cells at ^{13}C natural abundance from that of cells grown on labeled glyphosate after normalizing both spectra for sample weight and number of scans. Double cross-polarization ^{13}C NMR techniques and analysis are described in detail in the supplement to reference 5.

HPLC analysis of glyphosate and AMPA. Concentrations of glyphosate and AMPA in culture media were measured by HPLC as described previously (6).

RESULTS

Glyphosate-degrading properties of *Pseudomonas* sp. strain LBr. A bacterial isolate designated *Pseudomonas* sp. strain LBr was obtained from industrial sludge by selection for growth on glyphosate as the sole source of phosphorus; it was streaked for purity and characterized by morphological and biochemical properties (L. E. Hallas, E. M. Hahn, and C. Korndorfer, *J. Ind. Microbiol.*, in press). Electron micrographs of cells taken from a number of experiments showed all of the cells to be short rods which, depending on growth conditions, contained variable amounts of large inclusion bodies that were shown by CPMAS ^{13}C NMR (4) to consist of poly(β -hydroxybutyrate).

The amount of glyphosate that *Pseudomonas* sp. strain LBr was able to degrade was measured by growing cells in medium containing 19 mM glyphosate and monitoring, by HPLC, glyphosate remaining in the medium throughout growth. Clearance of glyphosate did not begin in earnest until cells had reached the mid- to late log phase of growth (Fig. 2), at which time the disappearance of glyphosate coincided with a buildup of AMPA in the growth medium. At the end of growth, virtually all of the glyphosate present in the growth medium had been replaced with virtually an equimolar amount of AMPA. A separate growth experiment involving a starting glyphosate concentration of 1 mM displayed a similar reciprocal appearance of AMPA with disappearance of glyphosate, although after building to a level of 0.9 mM the AMPA level began to decrease, declining to a

value of 0.3 mM at the end of growth (data not shown). *Pseudomonas* sp. strain LBr was also grown in GPI medium modified to contain only 1 mM AMPA as the sole source of phosphorus and was found to be capable of eliminating about 0.5 to 0.7 mM AMPA from the growth medium.

The appearance of large amounts of AMPA in the 19-mM glyphosate growth experiment showed that *Pseudomonas* sp. strain LBr, in common with *Flavobacterium* sp. strain GDI, was able to cleave the carboxymethyl carbon-nitrogen bond of glyphosate, exporting one product of the reaction, AMPA, back into the growth medium.

Solid-state ^{13}C NMR spectra of *Pseudomonas* sp. strain LBr grown on ^{13}C -labeled glyphosate. Further experiments aimed at elucidating details of the metabolism of glyphosate by *Pseudomonas* sp. strain LBr were done by the method of solid-state ^{13}C NMR, used previously with other glyphosate-degrading bacteria (5, 6, 10). The method involves growing cells on ^{13}C -labeled glyphosate, followed by analysis of lyophilized cellular material by a CPMAS NMR technique which produces high-resolution, liquid-like ^{13}C NMR spectra that can be interpreted in the same way as can standard Fourier-transform ^{13}C NMR spectra. In comparison with solution-state *in vivo* Fourier-transform NMR of living cells, which generally gives only signals for low-molecular-mass metabolites, CPMAS NMR produces quantitatively reliable signals for all components of the cell, including RNA, DNA, membranes, cell walls, and proteins. Consequently, the solid-state NMR experiments can be useful in the study of metabolic pathways which involve specific labelings of macromolecular nucleic acid and protein end products but in which the steady-state concentrations of the metabolites within the pathway are too low to permit their direct observation by NMR. In such cases, the end-product-labeling pattern of the CPMAS spectrum can be used to determine the pathway, whereas a Fourier-transform NMR experiment will give little, if any, information; the metabolites are simply not observed.

Figure 3B (bottom) shows a CPMAS 50-MHz ^{13}C NMR spectrum of lyophilized cells of *Pseudomonas* sp. strain LBr grown in medium containing 1 mM [$2\text{-}^{13}\text{C},^{15}\text{N}$]glyphosate and harvested at an A_{660} of 1.45. (We refer to the carboxymethyl methylene carbon as C-2 and the phosphonomethyl carbon as C-3 in the glyphosate molecule.) Analysis of the growth medium at harvest time showed that 0.86 mM glyphosate had been metabolized and that 0.62 mM AMPA had been generated, which indicated the metabolism of about 0.25 mM AMPA by cells. Four narrow lines appeared in the spectrum at 170, 69, 44, and 23 ppm, which were previously shown to be due to poly(β -hydroxybutyrate) present in the cells (4). The overall appearance of this spectrum was quite similar, in fact, to that of *Pseudomonas* sp. strain LBr grown on ^{13}C -natural-abundance glyphosate (cf. Fig. 1 of reference 4), although the total signal intensity was about 50% greater. (Comparison of signal intensities required that the spectra be normalized for differences in sample weight and number of scans before integration.) Subtraction of the normalized ^{13}C -natural-abundance spectrum (not shown in Fig. 3) of *Pseudomonas* sp. strain LBr from that for cells grown on [$2\text{-}^{13}\text{C},^{15}\text{N}$]glyphosate gave a ^{13}C NMR difference spectrum (Fig. 3B, top) showing only signals for uptake and cellular metabolism of the labeled glyphosate. A difference spectrum (Fig. 3A, top) obtained in a similar fashion for *Pseudomonas* sp. strain PG2982 was previously shown (5, 6) to contain a number of resonances due to breakdown of glyphosate to glycine, followed by its incorporation into proteins and nucleic acids (as depicted in

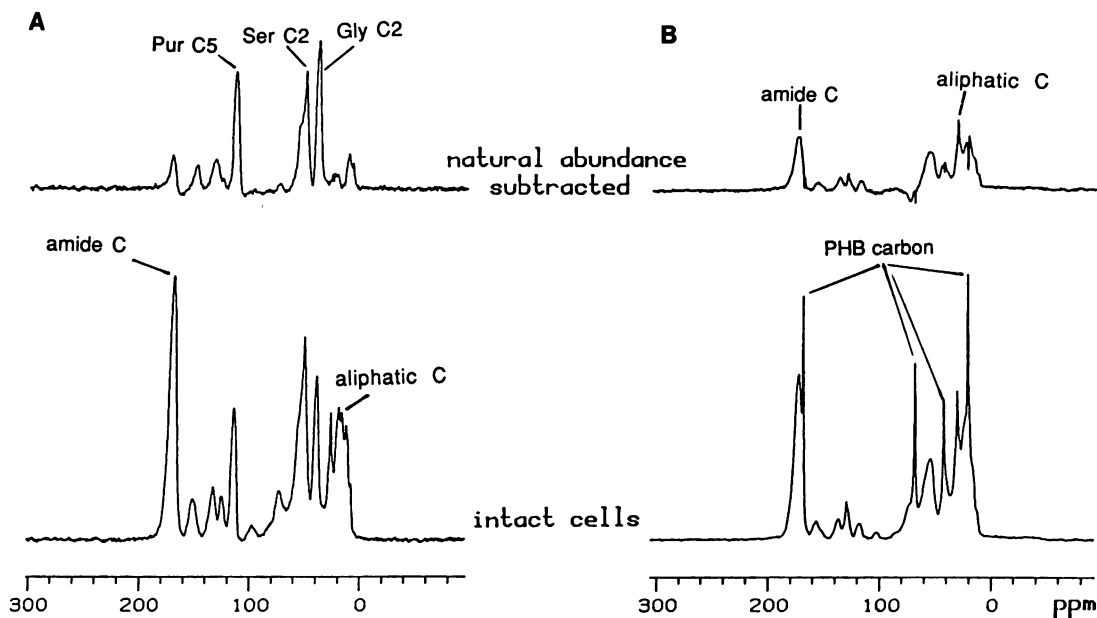


FIG. 3. CPMAS 50.3-MHz ^{13}C NMR spectra of lyophilized cells of *Pseudomonas* sp. strain PG2982 (A) and *Pseudomonas* sp. strain LBr (B) grown in media containing $[2\text{-}^{13}\text{C},^{15}\text{N}]$ glyphosate as the sole source of phosphorus. The spectra at the bottom were obtained directly from the CPMAS NMR experiment and contain signals for metabolism of label plus natural-abundance ^{13}C of cellular material. Spectra shown at the top were generated by subtracting appropriately scaled natural-abundance ^{13}C NMR spectra and therefore contain only signals for uptake and metabolism of labeled glyphosate. PHB, Poly(β -hydroxybutyrate).

Fig. 1). The three major signals were assigned to the C-5 carbon of purines of nucleic acids (119 ppm) and the methylene carbons of glycyl (43 ppm) and seryl (53 ppm) residues. In contrast, the *Pseudomonas* sp. strain LBr difference spectrum contained no major resonance assignable to a specific metabolic pathway. Rather, the low-field carbonyl resonance (175 ppm) and high-field aliphatic resonances (15 to 60 ppm) showed that cellular incorporation of the labeled C-2 methylene carbon primarily involved general scrambling of the label.

Nevertheless, minor resonances in the *Pseudomonas* sp. strain LBr NMR difference spectrum might still be due to metabolism of glyphosate via a glyphosate-to-glycine pathway. To determine whether this was the case, we performed a double CPMAS (DCPMAS) ^{13}C NMR experiment. This experiment gives direct, quantitative information about ^{13}C - ^{15}N dipolar couplings and therefore can be used to determine the routing and flux of metabolites containing ^{13}C - ^{15}N -labeled chemical bonds. (See the supplement to reference 5 for a detailed account of the analysis of DCPMAS ^{13}C NMR spectra.) The 3-ms DCPMAS hold spectrum (Fig. 4B, bottom) was essentially the same as a standard CPMAS ^{13}C NMR spectrum. It contained signals due to metabolism of labeled glyphosate in addition to those attributable to the ^{13}C -natural-abundance level of cells. The DCPMAS difference spectrum (Fig. 4B, top) shows which signals arose from ^{13}C -labeled nuclei directly bonded to ^{15}N . The two weak signals at 119 and 45 ppm had the same positions as those found in a DCPMAS ^{13}C NMR spectrum of *Pseudomonas* sp. strain PG2982 grown on $[2\text{-}^{13}\text{C},^{15}\text{N}]$ glyphosate (5). The latter signals were previously assigned to $[5\text{-}^{13}\text{C}, 7\text{-}^{15}\text{N}]$ purine and $[1\text{-}^{13}\text{C},^{15}\text{N}]$ glycyl residues, respectively, resulting from breakdown of $[2\text{-}^{13}\text{C},^{15}\text{N}]$ glyphosate to $[2\text{-}^{13}\text{C},^{15}\text{N}]$ glycine, followed by its further incorporation into proteins and purines (see Fig. 1 for routing of ^{13}C - ^{15}N -labeled chemical bonds). Any breakdown of $[2\text{-}^{13}\text{C},^{15}\text{N}]$

glyphosate to $[^{15}\text{N}]$ AMPA and a ^{13}C -fragment (as depicted in Fig. 1) could not contribute to the DCPMAS signals observed in Fig. 4B (top) because the initial ^{13}C - ^{15}N -labeled bond would have been broken in the process. Thus, *Pseudomonas* sp. strain LBr must metabolize a small amount of glyphosate (about 5% of the total) to glycine. (Calculation of the percentage was based on a comparison of the integrated area of the 119-ppm signal of Fig. 3B [top] with the overall integrated spectral area.)

A CPMAS ^{13}C NMR spectrum (not shown) of cells grown in medium containing 1 mM $[3\text{-}^{13}\text{C},^{15}\text{N}]$ glyphosate and harvested at the end of growth (A_{660} of 3.1), at which time a total of 0.5 mM AMPA had been degraded, indicated that none of the ^{13}C label originating in the phosphonomethyl methylene carbon of glyphosate was incorporated into cellular material. This finding was confirmed by a DCPMAS ^{13}C NMR experiment on the labeled cells, which showed that no signals for ^{13}C - ^{15}N couplings were present in cellular material (Fig. 4A [top]). These findings on metabolism of glyphosate and AMPA in *Pseudomonas* sp. strain LBr are consistent with earlier results obtained with *Arthrobacter* sp. strain GLP-1 (10) and *Escherichia coli* ATCC 11303 (G. Jacob et al., unpublished data), in which it was shown that small amounts of AMPA were degraded to a material similar, if not identical, to methylamine. The material was not further metabolized but instead was released into the growth medium.

DISCUSSION

Glyphosate metabolism via AMPA formation. *Pseudomonas* sp. strain LBr displays a markedly higher glyphosate-degrading capability than do other bacteria studied to date (5, 6, 10). This capability rests on the ability of *Pseudomonas* sp. strain LBr to shuttle AMPA, the major breakdown product of glyphosate degradation, into the medium, where it continues to build up until all of the glyphosate has been

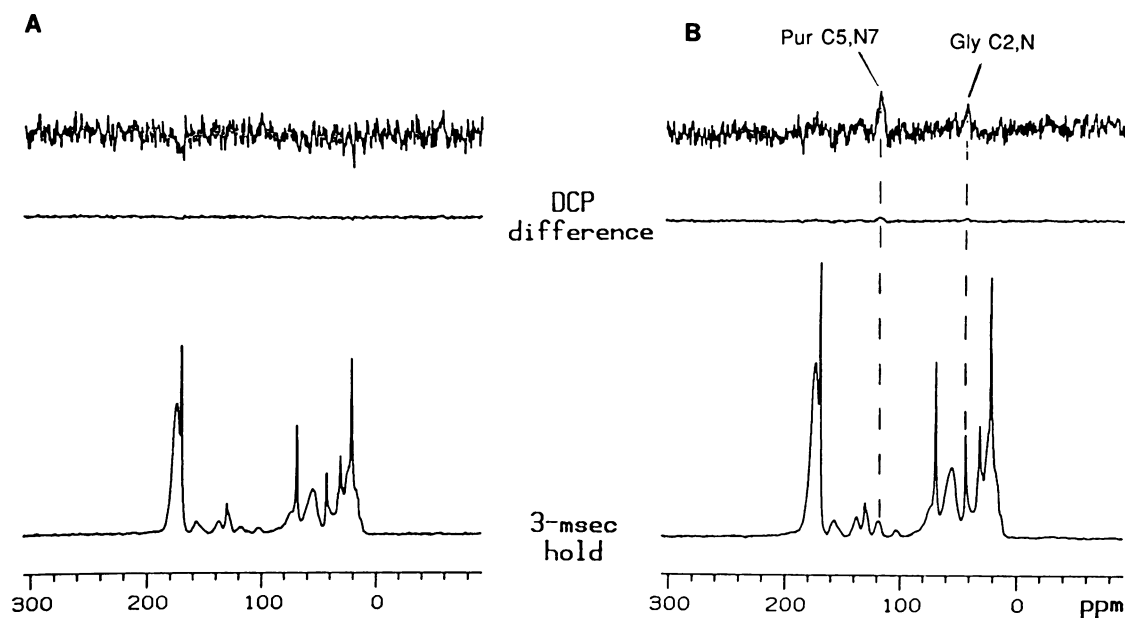


FIG. 4. CPMAS 50.3-MHz ^{13}C NMR spectra of lyophilized cells of *Pseudomonas* sp. strain LBr grown in medium containing 1 mM $[3\text{-}^{13}\text{C},^{15}\text{N}]$ glyphosate (A) and 1 mM $[2\text{-}^{13}\text{C},^{15}\text{N}]$ glyphosate (B). The spectra at the bottom were obtained by using a DCPMAS pulse sequence with a proton-carbon Hartmann-Hahn match of 2 ms, a carbon spin lock of 3 ms, and the nitrogen radiofrequency field held off resonance. The DCPMAS difference spectra shown at the top were obtained by using a DCPMAS pulse sequence with the nitrogen radiofrequency field held alternatively off and on resonance. DCPMAS spectra displayed at the very top had a gain of 10 over those shown directly below them.

degraded. Only a small amount of AMPA (about 0.5 to 0.7 mM), needed to supply phosphorus for growth, is further metabolized by *Pseudomonas* sp. strain LBr.

Since glyphosate and AMPA are both negatively charged species at neutral pH, transport of these compounds across a lipid bilayer, such as the plasma membrane of a cell, is likely to be a highly regulated process. Pipke et al. (11) have recently shown that glyphosate uptake in *Arthrobacter* sp. strain GLP-1 is strongly inhibited by other phosphonates or phosphate and depends on a source of energy. The metabolism of relatively large amounts of glyphosate by *Pseudomonas* sp. strain LBr would therefore seem to indicate that this organism has solved the problem of transporting large amounts of glyphosate into and AMPA out of the cell. Alternatively, the problem of transport may have been circumvented altogether by external degradation of glyphosate, perhaps within the periplasmic space and with the involvement of a membrane-bound or periplasmic enzyme. AMPA generated in this way would be free to diffuse back into the growth medium, where it could continue to build up, whereas the other product of the cleavage of the carboxymethyl carbon-nitrogen bond (most likely glyoxylate) could be transported into the cell. This scheme for the initial steps of detoxification of glyphosate by *Pseudomonas* sp. strain LBr would explain the CPMAS ^{13}C NMR results showing appreciable uptake and cellular incorporation of label from the carboxymethyl moiety of glyphosate.

Glyphosate metabolism via glycine. An unexpected characteristic of glyphosate metabolism within *Pseudomonas* sp. strain LBr, revealed by our DCPMAS ^{13}C NMR experiments on cells grown on $[2\text{-}^{13}\text{C},^{15}\text{N}]$ glyphosate, is the breakdown of a small but significant amount of glyphosate to glycine. The amount is only about 5% of the overall label metabolized by cells, but the breakdown does signify that *Pseudomonas* sp. strain LBr expresses an enzyme capable of cleaving the phosphonomethyl carbon-nitrogen bond.

One explanation for this capability is that the phosphonate generated by the bacterium for breaking down small amounts of AMPA needed to supply phosphorus for growth is also capable, at least in this microorganism, of directly acting on glyphosate as a substrate for the phosphonate reaction. The product of this reaction would, as is the case with AMPA, yield direct phosphorus for growth, and therefore the amount of glyphosate degraded by this pathway could inevitably be limited by the phosphorus requirement of the cell. Based on the amount of AMPA that *Pseudomonas* sp. strain LBr is capable of clearing from the medium (containing AMPA as the sole phosphorus source), no more than about 0.5 to 0.7 mM glyphosate could be metabolized in this way, even assuming that no AMPA (from the breakdown of glyphosate to AMPA) was separately used as a source of phosphorus. Consequently, partitioning of glyphosate between the two degradative pathways would naturally favor breakdown to AMPA, which does not lead to any discernible inhibition of enzyme activity or down-regulation of the genes involved in the process. We did not attempt in these experiments to determine a limit to the amount of glyphosate that *Pseudomonas* sp. strain LBr is capable of eliminating from the growth medium.

As to the possibility that the secondary capability of *Pseudomonas* sp. strain LBr to break down glyphosate to glycine stems from a minor contamination of a glycine-generating bacterium, such as *Pseudomonas* sp. strain PG2982 or *Arthrobacter* sp. strain GLP-1, within the supposedly pure culture of *Pseudomonas* sp. strain LBr, severe phosphorus starvation was needed to routinely bring about glyphosate-degrading conditions with this culture. Prior phosphate starvation has never been required with any of the glycine generators previously studied in our laboratory. Moreover, growth experiments on *Pseudomonas* sp. strain LBr using individual colonies from agar plates gave similar glyphosate-degrading results. Electron micrographs of cells

taken from a number of experiments showed all of the cells to be short rods which, depending on growth conditions, contained variable amounts of the large inclusion bodies shown by CPMAS ^{13}C NMR (4) to be composed of poly(β -hydroxybutyrate). Neither of the glycine generators we previously worked with contains poly(β -hydroxybutyrate), consistent with the claim that a single poly(β -hydroxybutyrate)-producing LBr strain of *Pseudomonas* sp. is responsible for the formation of both AMPA and glycine.

Pathway regulation. Although *Pseudomonas* sp. strain LBr is capable of detoxifying larger amounts of glyphosate than can other microorganisms, its ability to degrade glyphosate is still tied to phosphate regulation of the degradative pathways, since no glyphosate can be degraded by *Pseudomonas* sp. strain LBr in the presence of phosphate. This would seem to imply that the genes coding for breakdown of glyphosate to AMPA and for breakdown of AMPA to its products are located on the same operon, which is regulated by phosphate (or a phosphate-derived corepressor molecule). In any event, it would be useful if glyphosate detoxification in *Pseudomonas* sp. strain LBr could be uncoupled from phosphorus regulation, since this would open up the possibility of selecting for a mutant strain of *Pseudomonas* sp. strain LBr that expresses far higher levels of the glyphosate-degrading enzyme than is observed with the wild-type strain. Selective pressure could involve use of glyphosate as the sole carbon or nitrogen source and might yield a strain that would be easier to handle in experiments aimed at identifying, and cloning, the gene (or genes) involved in detoxification of glyphosate. Balthazor and Hallas (1), working with a different AMPA-generating bacterium isolated from activated sludge, found that, if properly handled, cells could be made to continue breaking down glyphosate to AMPA even in the presence of added phosphate. Thus, at least for one other known organism, the breakdown of glyphosate can be uncoupled from phosphate regulation.

The ability of *Pseudomonas* sp. strain LBr to detoxify high levels of glyphosate suggests that it may be useful for the genetic engineering of glyphosate resistance in plants. Of course, this prospect will depend, ultimately, on the nature and properties of the glyphosate-degrading enzyme that is present in *Pseudomonas* sp. strain LBr.

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