Uptake of Glyphosate by an *Arthrobacter* sp.

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The uptake of glyphosate (*N*-[phosphonomethyl]glycine) by an *Arthrobacter* sp. which can utilize this herbicide as its sole source of phosphorus was investigated. Orthophosphate suppressed the expression of the uptake system for glyphosate and also competed with glyphosate for uptake. The K_m for glyphosate uptake was 125 μM, and the K_i for orthophosphate was 24 μM. Organophosphonates as well as organophosphates inhibited glyphosate uptake, but only organophosphates and orthophosphate suppressed the uptake system. Glyphosate uptake was energy dependent, had a pH optimum of 6 to 7, and was differentially affected by divalent cations.

Many bacteria are known which can utilize organophosphonates as their sole sources of phosphorus (4). Cleavage of the carbon-phosphorus bond generally requires uptake of the organophosphonate into the cell. Specific and inducible uptake systems for 2-amino-ethylphosphonate (2-AEP) have been described for *Bacillus cereus* (16) and *Pseudomonas aeruginosa* (9), while other phosphonates are taken up via transport systems of the corresponding carboxylic acids (5). In general, it appears that free aminophosphonates are taken up less readily than phosphonopeptides (8).

As will be published elsewhere (R. Pipke, N. Amrhein, G. S. Jacob, and G. M. Kishore, submitted for publication), the degradation of the organophosphonate herbicide glyphosate (*N*-[phosphonomethyl]glycine) in an *Arthrobacter* sp. (designated *Arthrobacter* sp. strain GLP-1) has been investigated. While other microorganisms are known which cleave the carbon-phosphorus bond of glyphosate (1, 11, 17, 20), details of the pathway of glyphosate degradation are known only for a *Pseudomonas* sp. (7) and the *Arthrobacter* sp. mentioned above. In both microorganisms, orthophosphate suppresses glyphosate utilization. In a *Flavobacterium* sp., orthophosphate does not affect the degradation of glyphosate to aminomethylphosphonate, but it suppresses the utilization of aminomethylphosphonate as a source of phosphorus (1).

Suppression of glyphosate utilization by orthophosphate may be effected at the level of glyphosate uptake or glyphosate degradation or both. Here we report that the uptake of glyphosate by *Arthrobacter* sp. strain GLP-1 is mediated by a transport system which is subject to inhibition, as well as suppression, by orthophosphate and organophosphates.

MATERIALS AND METHODS

Chemicals. Glyphosate (free acid, 99.7% pure) was provided by Monsanto Agricultural Products Co., St. Louis, Mo. [3-^{14}C]glyphosate (specific activity, 344.1 MBq/mmol) and $^{32}P_i$ (carrier-free) were obtained from Amersham-Buchler, Braunschweig, Federal Republic of Germany (FRG). Organophosphates and organophosphonates were supplied by Sigma, Munich, FRG, with the exceptions of

methyl- and ethylphosphonic acids, which were from Ventron, Karlsruhe, FRG, and (1-amino-2-phenylethyl) phosphonic acid, which was a gift from CIBA-GEIGY AG, Basel, Switzerland.

Culture of bacteria. The isolation of *Arthrobacter* sp. strain GLP-1 will be described elsewhere (Pipke et al., submitted). The bacteria were grown and maintained on the synthetic medium of Winkler and De Haan (26) with 1% glucose as a carbon source and 0.1 M Tris hydrochloride, pH 7.0, instead of phosphate buffer. The respective sources of phosphorus are indicated in the text. All media were autoclaved prior to the addition of the phosphorus sources, which were added following filter sterilization. All glassware was washed with 1 N HCl and thoroughly rinsed with deionized and glass-distilled water to remove contaminating phosphate ions. Bacteria were grown at 30°C on a rotary shaker at 220 rpm, and growth was followed by measuring the turbidity of the medium (A_{650}).

Uptake experiments. The uptake of labeled glyphosate and orthophosphate, respectively, was measured by following the protocol of Piperno and Oxender (14). Cells from the logarithmic growth phase (A_{650} = 0.8 to 1.0) were collected by centrifugation at 10,000 × *g* for 5 min, washed in medium deficient of a phosphorus source, and adjusted to 15 mg (wet weight) per ml of the same medium. The suspension was stored on ice for no longer than 60 min before uptake experiments were started. A sample (0.2 ml) of the suspension was added to 1.5-ml polyethylene vials containing 1 ml of 0.1 M Tris hydrochloride, pH 7.0, and the respective test compound as indicated in the text. After incubation at 37°C for the appropriate time, 1 ml of the suspension was rapidly filtered by suction through membrane filters (2.5-cm diameter; 0.45-μm pore size; Millipore Corp., Bedford, Mass.) and washed with 10 ml of 0.1 M Tris hydrochloride, pH 7.0. The wet filters were transferred to 10 ml of Quickszint 212 (Zinsser, Frankfurt, FRG) in scintillation vials, and the radioactivity was determined after 1 h by liquid scintillation counting.

Extraction and assay of phosphatases. Cells in the logarithmic growth phase (A_{650} = 1.0) were collected by centrifugation, and proteins were extracted as described previously (18). Phosphatase activities were determined with *p*-NO_2-phenylphosphate as substrate (21).
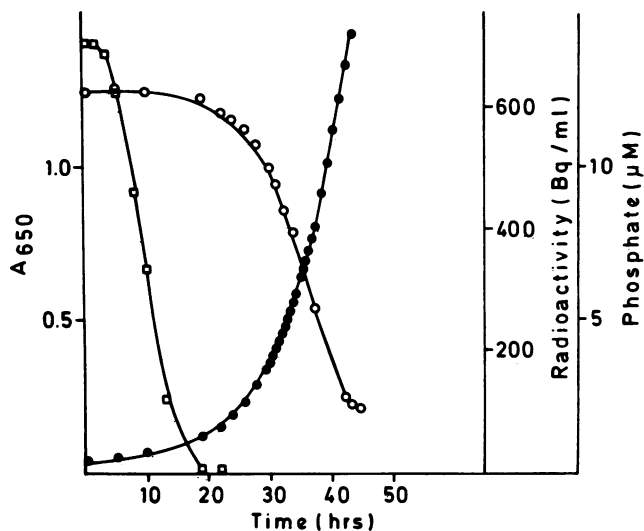
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FIG. 1. Growth of *Arthrobacter* sp. strain GLP-1 in the presence of both orthophosphate and glyphosate. Symbols: ●, A_{650}; □, concentration of orthophosphate in growth medium; O, radioactivity from [^{14}C]glyphosate in growth medium. The initial concentrations of orthophosphate and glyphosate were 14 and 250 μM, respectively. Even though in control experiments 14 μM orthophosphate alone supported growth of the bacteria to A_{650} of 0.8, no evidence for discontinuous (diauxotrophic) growth was found.

Assay of orthophosphate. Concentrations of orthophosphate were determined by the malachite green method of Lanzetta et al. (10).

RESULTS

Arthrobacter sp. strain GLP-1 is capable of utilizing a number of organophosphonates, including glyphosate, as a source of phosphorus (Pipke et al., submitted). When grown in the presence of both orthophosphate and glyphosate as sources of phosphorus, the bacteria completely removed the orthophosphate from the medium before they started to take up glyphosate (Fig. 1). This observation suggested that
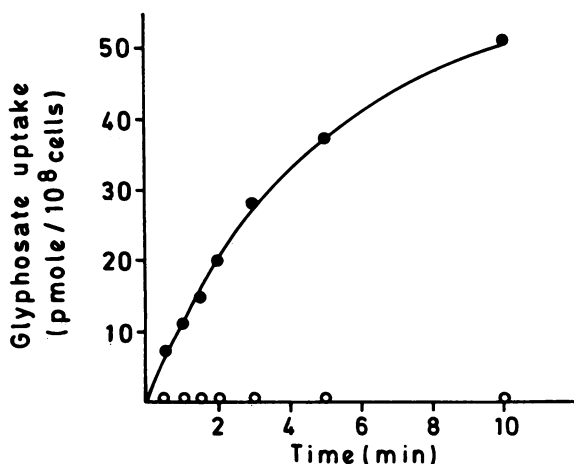

FIG. 2. Time course of uptake of glyphosate by *Arthrobacter* sp. strain GLP-1. Cells had been grown in minimal medium containing either 5 mM glyphosate (●) or 5 mM orthophosphate (O) before uptake of glyphosate was measured.

TABLE 1. Rates of glyphosate uptake by *Arthrobacter* sp. strain GLP-1 as a function of the phosphorus source in the growth medium before or after phosphorus starvation

Phosphorus source	Rate of uptake (pmol of glyphosate min^{-1} 10^{-8} cells^{-1})	
	Before[a]	After[a,b]
Glyphosate	11	84
Orthophosphate	0	59
Glucose 6-phosphate	0	30
Aminomethylphosphonate	12	80

[a] Cells in mid-log phase (A_{650} = 0.8 to 1.0) were used.

[b] The bacteria were grown in the presence of the indicated source of phosphorus and then sedimented and washed twice in phosphorus-deficient medium. After a subsequent incubation for 4 h in the absence of a phosphorus source, the uptake of glyphosate was determined.

orthophosphate inhibits or represses the uptake of glyphosate. Bacteria grown in glyphosate were capable of taking up the compound, while those grown in orthophosphate were not, even when washed twice in a phosphorus-deficient medium (Fig. 2). Thus, orthophosphate apparently represses the uptake system for glyphosate. Likewise, the uptake system was repressed by organophosphates, such as glucose 6-phosphate, but was functioning in cells grown in the presence of organophosphonates other than glyphosate, such as aminomethylphosphonate (Table 1). The rate of glyphosate uptake was, however, considerably enhanced when the cells had been deprived of any source of phosphorus prior to the uptake experiment (Table 1). Thus, phosphorus deprivation appears to derepress the system by which glyphosate is taken up. The nature of the phosphorus source also greatly affected the rate of transport of orthophosphate into *Arthrobacter* sp. strain GLP-1: cells grown in 5 mM orthophosphate accumulated it from a 50 μM solution at a rate of 0.09 nmol min^{-1} 10^{-8} cells, while the transport rate for cells grown in 5 mM glyphosate was 8.8 nmol min^{-1} 10^{-8} cells (1.11 × 10^8 cells have a wet weight of 1 mg).

As phosphatases are also known to be derepressed upon phosphorus deprivation of bacteria (6, 21, 24), we measured alkaline phosphohydrolase activity in *Arthrobacter* sp. strain GLP-1 growing in the absence or presence of orthophosphate and glyphosate. As expected, cells grown in the presence of orthophosphate had low phosphatase activity, while the activity of the enzyme was high in cells deprived of phosphorus or in cells growing in the presence of glyphosate (Table 2). It is also evident, however, that glyphosate had no effect on the level of phosphatase activity.

Therefore, phosphatase expression is regulated specifically by phosphate availability rather than generally by

TABLE 2. Extractable activity of alkaline phosphohydrolase in *Arthrobacter* sp. strain GLP-1 as a function of the phosphorus source in the growth medium[a]

Phosphorus source (1 mM)	Sp act (nkat mg of protein^{-1})
Orthophosphate	0.44
Glyphosate	29.1
Glyphosate[b]	35.4
No phosphorus source[b]	31.4

[a] Cells in mid-log phase (A_{650} = 0.8 to 1.0) were used.

[b] Cells were grown with 1 mM glyphosate as the phosphorus source and then incubated for a further 4.5 h in the presence or absence of glyphosate before phosphohydrolase activity was determined.
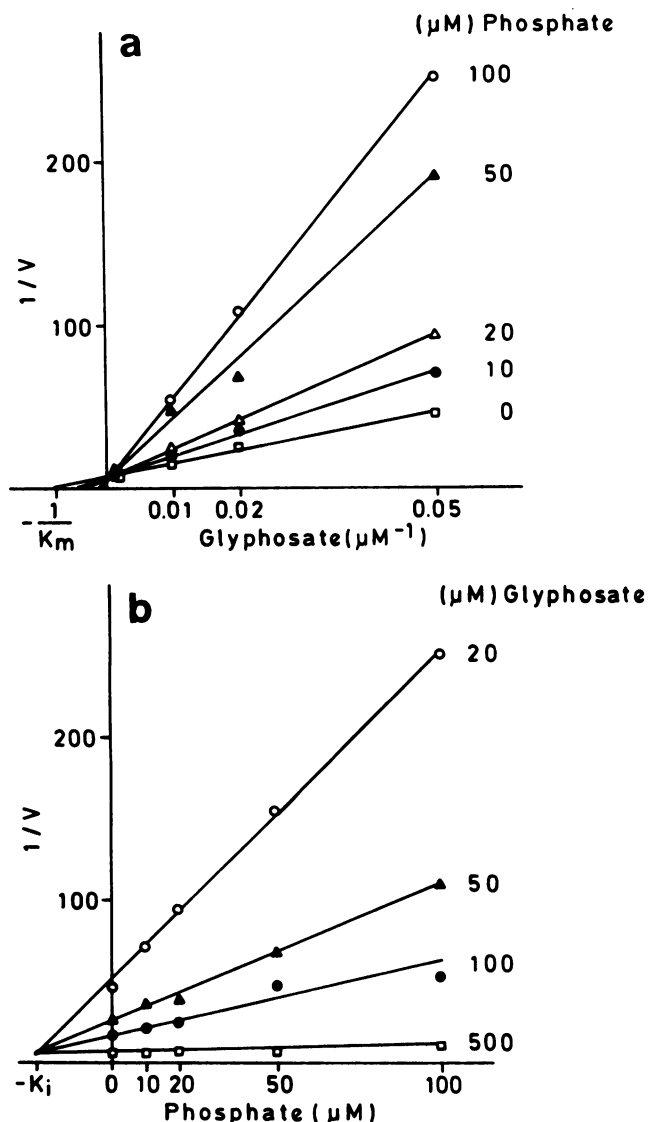
FIG. 3. Effect of orthophosphate on uptake of glyphosate by *Arthrobacter* sp. strain GLP-1. (a) Double reciprocal plot of the rates of glyphosate uptake as a function of glyphosate concentration, in the presence of increasing concentrations of orthophosphate. (b) Dixon plot of data shown in (a).

TABLE 3. Rate of uptake of glyphosate in *Arthrobacter* sp. strain GLP-1 in the presence of various compounds[a]

Compound (0.5 mM)	Rate of uptake (% of control)
None (control)	100[b]
Orthophosphate	13
Orthoarsenate	18
Glucose 6-phosphate	25
α-Glycerophosphate	17
β-Glycerophosphate	65
Erythrose 4-phosphate	44
Aminomethylphosphonate	35
1-Aminoethylphosphonate	37
2-AEP	34
(1-Amino-2-phenylethyl)phosphonate	52
Methylphosphonate	49
Ethylphosphonate	44
Fosfomycin	64
Sarcosine	98
Glycine	86
L-Alanine	95
L-Phenylalanine	96
L-Aspartate	99
Iminodiacetate	104

[a] Cells were grown in 5 mM glyphosate and used in mid-log phase (A_{650} = 0.8 to 1.0). Glyphosate concentration in uptake experiments was 50 μM.
[b] 100% = 21 pmol of glyphosate $min^{-1} 10^{-8} cells^{-1}$.

extent. Zn^{2+} was inhibitory (Table 4). No relationship with the stability constants of the respective metal glyphosate complexes (12) was evident.

DISCUSSION

The uptake of phosphate into bacteria such as *Escherichia coli* K-12 has been well investigated. *E. coli* K-12 possesses a constitutive low-affinity phosphate transport system, as well as a high-affinity phosphate-specific transport system which is efficiently repressed by orthophosphate (15, 19, 25). In *Arthrobacter* sp. strain GLP-1, cells grown in the pres-

phosphorus availability. When grown in the presence of 5 mM glyphosate as the sole source of phosphorus prior to the uptake experiments, the bacteria took up glyphosate with a K_m of 125 μM, and orthophosphate was a competitive inhibitor of glyphosate uptake with a K_i of 24 μM (Fig. 3a and b). Aminomethylphosphonate, likewise, inhibited glyphosate uptake competitively with a K_i of 50 μM (data not shown). Arsenate, organophosphates, and organophosphonates inhibited the uptake of glyphosate to varying degrees, while amino acids and the glyphosate analog iminodiacetate produced little, if any (in the case of glycine), inhibition (Table 3). The uptake of glyphosate was pH dependent, showing an optimum at pH 6 to 6.5 (Fig. 4), which indicates a preferential uptake of the glyphosate dianion (23). Of various divalent metal ions tested at 1 mM concentration, Ni^{2+} stimulated the rate of glyphosate uptake more than twofold, while Mn^{2+} and Ca^{2+} were stimulatory to a lesser
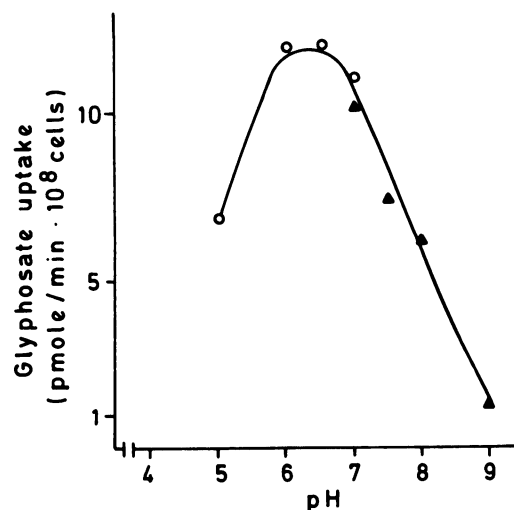

FIG. 4. Rate of glyphosate uptake by *Arthrobacter* sp. strain GLP-1 as a function of pH. Symbols: (O), 0.1 M Tris-maleate; (▲), 0.1 M Tris hydrochloride.

TABLE 4. Effect of divalent metal ions on glyphosate uptake by *Arthrobacter* sp. strain GLP-1[a]

Metal ion (1 mM)	Rate of uptake (% of control)
None (control)	100[b]
Ca^{2+}	141
Co^{2+}	83
Cu^{2+}	104
Mn^{2+}	168
Ni^{2+}	225
Zn^{2+}	40

[a] Cells in mid-log phase (A_{650} = 0.8 to 1.0) were used. Glyphosate concentration was 50 μM.
[b] 100% = 12.1 pmol of glyphosate min^{-1} 10^{-8} cells^{-1}.

ence of orthophosphate differ in orthophosphate uptake by nearly two orders of magnitude as compared with those grown in glyphosate. Glyphosate appears to be transported into the cells by a phosphate uptake system which is derepressed upon orthophosphate starvation, as it is not taken up by cells grown in the presence of orthophosphate or organophosphates (Fig 2, Table 1); furthermore, orthophosphate is an efficient competitive inhibitor of glyphosate uptake (Fig. 3). Phosphorus deprivation, rather than the presence of glyphosate, induces the system by which glyphosate is taken up (Table 1). In contrast, the transport system for 2-AEP in a strain of *B. cereus*, which utilized this phosphonate as its sole source of phosphorus, was inducible by 2-AEP as well as aminomethylphosphonate (16). While phosphate suppressed the induction of the transport system in *B. cereus*, it was ineffective in *P. aeruginosa*, in which 2-AEP also induced the system for its own uptake (9). In *P. aeruginosa*, phosphate had a much lower affinity for the uptake system than 2-AEP (K_m for 2-AEP = 6.2 μM; K_i for orthophosphate = 1 mM), while in *B. cereus* orthophosphate was found not to interfere with the uptake of 2-AEP at all (K_m for 2-AEP = 0.1 μM). These situations are clearly different from that observed with glyphosate uptake in *Arthrobacter* sp. strain GLP-1 (K_m for glyphosate = 125 μM; K_i for orthophosphate = 24 μM). This is the first report on the successful measurement of glyphosate uptake into a bacterium. Previous efforts to measure the uptake of glyphosate by *E. coli* and *Klebsiella pneumoniae*, organisms which are sensitive to inhibition of growth by glyphosate, had not been successful (B. Folwaczny and A. Schulz, unpublished observations). Likewise, the uptake of glyphosate by isolated plant cells was either extremely low or measurable only after prolonged incubation (2, 13). The accumulation and transport of glyphosate in the phloem, the assimilate-transporting tissue of higher plants, has been explained by the intermediate permeability mechanism (3). According to this mechanism, glyphosate diffuses preferentially, although passively, into the phloem, because a diffusion gradient is continually maintained by the mass flow toward metabolic sinks in the sieve tubes of the phloem.

It therefore appears that the glyphosate-degrading *Arthrobacter* sp. strain GLP-1 possesses a unique, hitherto undescribed transport system for glyphosate.

ACKNOWLEDGMENT

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