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Glyphosate-degrading isolates from environmental samples: occurrence and pathways of degradation

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Abstract The metabolism of the organophosphonate herbicide glyphosate was investigated in 163 environmental bacterial strains, obtained by a variety of isolation strategies from sites with or without prior exposure to the compound. Isolates able to use glyphosate as sole phosphorus source were more common at a treated site, but much less abundant than those capable of using the glyphosate metabolite aminomethylphosphonic acid (AMPA). Nevertheless, all 26 strains found to metabolise the herbicide did so via an initial cleavage of its carbon-phosphorus bond to yield sarcosine; no evidence for its metabolism or co-metabolism to AMPA was obtained.

Introduction

The metabolism of the phosphonate herbicide glyphosate [*N*-(phosphonomethyl)-glycine] has been extensively investigated both in the environment and under laboratory conditions. Microbial action is responsible for the degradation of the herbicide in the soil (Sprankle et al. 1975) and aminomethylphosphonic acid (AMPA) is the major metabolite detected (Rueppel et al. 1977). AMPA has also been reported to accumulate in industrial reactors degrading glyphosate-containing wastes (Murthy et al. 1989). Here glyphosate is rapidly degraded by a variety of microbial groups in the mixed substrate environment. Microorganisms isolated from such sources were able to transform glyphosate to AMPA with phosphate present in the growth medium but did not mineralize the compound further in the presence of phosphate (Balthazor and Hallas 1986; Hallas et al. 1988; Jacob et al. 1988; McAuliffe et al. 1990).

Only two organisms in which AMPA has been demonstrated as an intermediate of glyphosate degradation have been isolated from sources other than industrial glyphosate-degrading effluents (Pipke and Amrhein 1988; Lerbs et al. 1990). In contrast, direct cleavage of the C–P bond has been reported as the primary step in glyphosate degradation in diverse environmental and laboratory strains (Kishore and Jacob 1987; Pipke et al. 1987; Weidhase et al. 1990; Liu et al. 1991; Dick 1991). In these microorganisms, and in contrast to those isolated from industrial wastes, phosphate was inhibitory to glyphosate breakdown.

The metabolism of glyphosate has been investigated in few environmental bacteria isolated under non-selective conditions. This study aimed to investigate the occurrence of glyphosate-degrading activity in environmental isolates from populations that had or had not been exposed previously to glyphosate or to other phosphonates. It was anticipated that the strategies used would result in the isolation of representative strains responsible for glyphosate degradation in the environment.

Materials and methods

Source of inocula

Glyphosate-treated and untreated soils were fresh samples from a site described previously (Quinn et al. 1988). Extracts were prepared by shaking 1 g soil in 10 ml 0.85% NaCl for 1 h followed by brief centrifugation to remove suspended particles. A sample from the river Rhine was taken at Boppard, Germany, and was stored at 4°C prior to use.

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Media and growth conditions

Phosphate was removed from glassware by soaking in Decon 90 (Decon Laboratories, Hove, England) followed by rinsing in double-distilled water.

The mineral salts medium was buffered with 50 mM TRIS/HCl (pH 7.0) and consisted of (l^{-1}) NH_4Cl 5.0 g, $MgSO_4 \cdot 7H_2O$ 0.16 g, $CaCl_2 \cdot 2H_2O$ 0.08 g, trace elements solution (Krieg 1981) 1.0 ml, ethylenediaminetetraacetic acid ferric sodium salt 0.001 g. The following were added as filter-sterilised solutions after the basal medium had been autoclaved: potassium gluconate 5.0 g, sodium pyruvate 5.0 g, vitamin solution (Difco manual, 9th edition, p 251) 1.0 ml, phosphate, glyphosate or AMPA (adjusted to pH 7.0), to the final concentration stated in individual experiments. All incubations were done in a rotary shaker (100 rpm) at 29°C.

Enrichment procedures

Non-enriched microbial populations were obtained by serial dilution of extracts from each soil, subsequently cultured on casein/peptone/starch (CPS) agar (Collins and Willoughby 1962) with a reduced glycerol concentration (0.2 ml l^{-1}). Enriched populations resulted from culture in mineral salts medium with either glyphosate (2 mM) or phosphate (2 mM) as the phosphorus source: 50 ml medium in a 250-ml conical flask was inoculated with 0.5 ml extract from either glyphosate-treated or untreated soil. After 5 days incubation, cultures were plated on CPS agar.

"Analogue enrichment": inocula from glyphosate-treated soil or from the river Rhine were added to carbon-free medium (l^{-1}: NH_4Cl 5.0 g, $MgSO_4 \cdot 7H_2O$ 0.16 g, $CaCl_2 \cdot 2H_2O$ 0.08 g, trace elements solution 1.0 ml) or nitrogen-free medium (as carbon-free medium with the omission of NH_4Cl and with the addition of 5.0 g potassium gluconate as carbon source. Five carbon-limited and four nitrogen-limited cultures were set up for each source of inoculum – the following analogues or putative glyphosate-degradation products were added to individual cultures as either C or N sources: acetate, glycolate and glyoxalate, sarcosine and glycine, dimethylglycine, betaine, and glyphosine (*N*,*N*-diphosphonomethylglycine). Each culture contained a total of 5 g l^{-1} analogue(s) in equimolar ratios. Cultures were incubated for 2 weeks prior to the isolation of individual colonies on CPS agar. In each case isolates were chosen as representative of each population from plates that contained 50–100 colonies. Isolated colonies were purified and maintained on CPS agar.

Chemicals

Glyphosate, technical grade, was a gift from ICI Agrochemicals Division, Bracknell, UK, and was purified by recrystallisation from water. Methylphosphonate was supplied by Ventron GmBH, Karlsruhe, Germany. [3-^{14}C] Glyphosate, specific activity 52 mCi $mmol^{-1}$, was purchased from Amersham International PLC., Bucks., UK; [^{14}C]methylamine, specific activity 8 mCi $mmol^{-1}$ was obtained from Sigma Chemical Co., Poole, UK. Other biochemicals were supplied by Sigma. All chemicals were analytical-grade (Analar) reagents and were from BDH, Poole, UK.

Chromatography

Separation of glyphosate and its degradation products was achieved using Sigmacell type 100 cellulose (250 µm thickness) thin-layer chromatography plates with the following solvent: ethanol/water/ammonium hydroxide (17 M)/trichloroacetic acid/acetic acid (15 M) (55:35:2.5:3.5:2, v/v/v/w/v) (Shinabarger and Braymer 1986).

Amine-containing products were visualised with ninhydrin; Amplify (Amersham) was used to enhance the autoradiographic detection of ^{14}C-labelled compounds. For HPLC analysis, amine residues were derivatised with 9-fluorenylmethoxycarbonyl chloride and were detected at 280 nm using a Hewlett Packard 1090 Chemistation liquid chromatograph.

Autoradiographic detection of bacterial colonies that accumulate radiolabelled glyphosate

Solidifed medium was prepared by the addition of 1.5% Bactoagar (Difco Laboratories Inc., Detroit, Mich., USA) to the mineral-salts base. Filter-sterilised carbon and vitamin sources were added, prewarmed, once the molten agar had cooled to 55°C. The solidified agar was partially dried by incubation of the inverted petri dishes at 45°C for 2–3 h. A 0.5-ml sample of a solution of 0.6 µCi ml^{-1} [3-^{14}C]glyphosate with added unlabelled glyphosate was spread over the surface of the agar and adsorbed giving an approximate total glyphosate concentration of 0.4 mM. The agar was overlaid with sterile nitrocellulose before being streaked with individual bacterial isolates. After colony development, the filter was removed, oven-dried and coated in plastic foil for autoradiography.

Metabolic studies with radiolabelled glyphosate

To confirm the pathway(s) of degradation, cultures growing in mineral salts medium with 2 mM glyphosate as sole source of phosphorus were incubated overnight (18 h) and harvested in early log phase. The cells were washed in fresh phosphate-free mineral salts medium and were resuspended to a cell density of (A_{550}) 1.0 in aliquots of medium containing 1.0 µCi ml^{-1} ($\approx$ 18 µM) [3-^{14}C]glyphosate along with one of the following putative intermediates of glyphosate degradation: sarcosine (0.5 mM), phosphate (0.2 mM), methylamine (0.5 mM), methylphosphonate (0.2 mM) or AMPA (0.2 mM). Cultures were incubated for 4 h after which time the supernatants were analysed by TLC/autoradiography.

Results

Comparison of soil samples from the glyphosate-treated and untreated sites revealed both qualitative and quantitative differences between the soil microbial populations. The treated soil contained an approximately ten-fold higher population (cfu ml^{-1}) compared with the soil from the untreated site. However, the treated soil had a lower diversity of colony types apparent at the highest dilutions. A total of 32 and 15 distinct colony types were isolated from approximately 100 colonies each from untreated and treated soil respectively (Table 1).

A strategy to screen isolates for the ability to degrade glyphosate initially relied on the autoradiographic detection (Fig. 1) of colonies grown on phosphorus-free mineral-salts/agar containing the radiolabelled herbicide (0.4 mM). However, many strains that accumulated the radiolabel were subsequently unable to use glyphosate in liquid culture, suggesting that these strains transported the herbicide but were unable to degrade it; conversely, a few glyphosate-utilising strains did not accumulate the label. The addition of 0.2 mM

Table 1 Occurrence of the ability to utilise glyphosate and aminomethylphosphonic acid (*AMPA*) in environmental bacterial strains

Source of inoculum	Criterion of enrichment	Total number of distinctive colony types isolated	Number of distinctive colony types able to grow in mineral salts medium with the following as sole source of phosphorus:		
			Phosphate	Glyphosate	AMPA
Untreated soil	Growth on CPS agar	32	24	0	6
	Use of phosphate as P source	16	13	0	3
	Use of glyphosate as P source	12	9	5	6
Glyphosate-treated soil	Growth on CPS agar	15	14	3[a]	6
	Use of phosphate as P source	7	5	3	4
	Use of glyphosate as P source	6	4	2	4
Glyphosate-treated soil	Use of glyphosate analogues as C source	21	20	7	11
	Use of glyphosate analogues as N source	13	10	0	1
Rhine river water	Use of glyphosate analogues as C source	17	14	5	5
	Use of glyphosate analogues as N source	24	20	1	9
Total		163	133	26	55

[a] Two isolates able to utilise glyphosate were unable to use AMPA (CPS casein/peptone/starch)

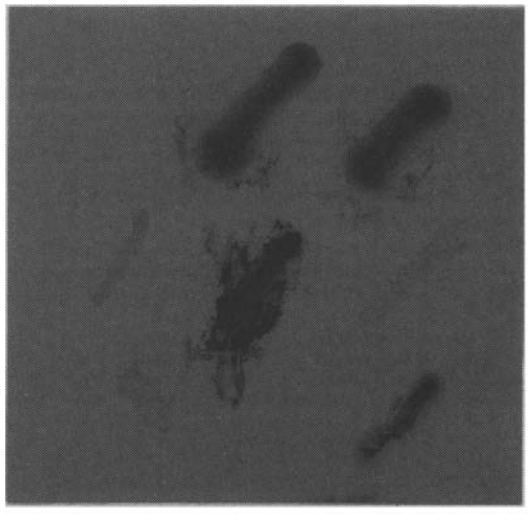

Fig. 1 Autoradiogram of bacterial colonies cultured on phosphate-free mineral-salts/agar in the presence of [3-^{14}C]glyphosate

phosphate to the agar resulted in the failure of all 27 organisms tested to accumulate the radiolabel.

Further investigation of the phosphonate-degrading properties of the isolates required culture in liquid mineral salts medium. Not all isolates grew in this medium with phosphate as P source; those that did were tested for growth with glyphosate or AMPA as sole source of phosphorus (Table 1). Of the 32 isolates from untreated soil, 24 grew readily in mineral salts medium replete with phosphate; of these, none subsequently grew with glyphosate as sole phosphorus source but 6 were able to use AMPA as sole phosphorus source. Of the 15 distinctive strains isolated from glyphosate-treated soil, 14 grew in phosphate-replete medium, and 3 and 6 respectively with glyphosate and AMPA as sources of phosphorus.

Enrichments with glyphosate as the sole source of phosphorus were readily established from both soil types, confirming the presence of herbicide-degrading bacteria in untreated soil. Organisms able to degrade AMPA were readily isolated from untreated soil without prior exposure to glyphosate; however, only enrichment with glyphosate as sole phosphorus source resulted in the isolation of individual strains able to degrade the herbicide. In contrast, glyphosate-degrading strains were amongst those isolated directly from glyphosate-treated soil (without enrichment) and following enrichment in phosphate-replete medium.

Each population resulting from analogue enrichment was dominated by up to 7 colony types. No growth was observed in enrichments with glyphosine as the sole source of carbon. There was no correlation between the analogue(s) used and the number of phosphonate-degrading strains subsequently isolated. Again, strains able to use AMPA as sole source of phosphorus were more frequent among isolates from both glyphosate-treated soil and Rhine water than strains able to grow with glyphosate as phosphorus source (Table 1).

Overall, the ability to cleave the C–P bond of AMPA and to use it as a phosphorus source was more widespread than the ability to use glyphosate: of 133 environmental isolates (Table 1) selected under a variety of conditions but all able to grow in mineral-salts medium containing P_i as sole phosphorus source, 31 were able to use only AMPA as the sole source of phosphorus, 24 used both AMPA and glyphosate and 2 used glyphosate as a phosphorus source but were unable to degrade AMPA.

The degradation pathways used by the glyphosate-degrading strains isolated were investigated. HPLC analysis of the supernatants of cultures growing with glyphosate as sole phosphorus source showed no metabolites attributable to glyphosate degradation. No degradation or modification of the herbicide was observed in the presence of phosphate. A parallel study (Dick 1991) with the previously isolated *Pseudomonas* sp. 4ASW (Quinn et al. 1989) and *Arthrobacter atrocyaneus* (Pipke and Amrhein 1988) showed that sarcosine and AMPA, their respective intermediates of glyphosate degradation, could be visualised by the incubation of glyphosate-grown log-phase cells with [3-^{14}C]glyphosate and an excess of the degradation product to "trap" the radiolabelled intermediates. This approach resulted here in the trapping of only [^{14}C]-sarcosine from cultures of all 26 isolates able to use glyphosate as sole P source. Typical results are shown in Fig. 2A. The identity of the degradation product as sarcosine was confirmed by digestion with sarcosine oxidase (Fig. 2B). Thus all 26 environmental isolates were capable of direct cleavage of the C–P bond of glyphosate.

Organisms unable to use glyphosate as sole P source were investigated for metabolism of glyphosate in complete medium in the presence of a limiting concentration of phosphate (0.2 mM). Neither potential intermediates, nor a reduction in the quantity of glyphosate present in the cultures was observed when the culture supernatants were subjected to HPLC analysis. In cultures containing [^{14}C]glyphosate, there was no evidence of degradation products or of changes in the intensity or R_F of the [^{14}C]-glyphosate spot.

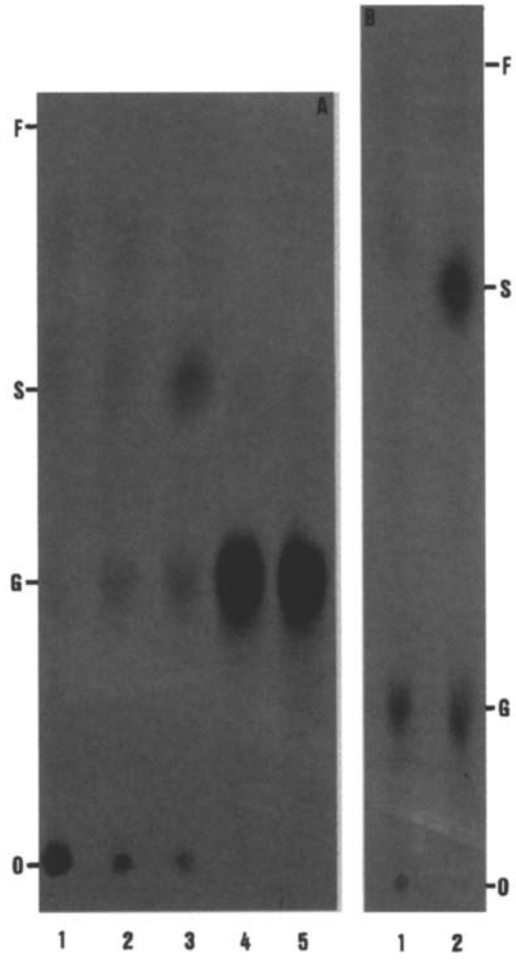

Fig. 2 **A** Typical TLC/autoradiogram showing the trapping of sarcosine resulting from glyphosate degradation by an environmental bacterial isolate. *Lanes 1–5* contained, as well as [3-^{14}C]glyphosate (18 μM), respectively no addition, methylamine (0.5 mM), sarcosine (0.5 mM), aminomethylphosphonic acid (0.2 mM), methylphosphonate (0.2 mM). *O* origin, *G* glyphosate, *S* sarcosine, *F* solvent front. **B** Confirmation of the identity of sarcosine by digestion with sarcosine oxidase. *Lane 1* typical culture supernatant treated with sarcosine oxidase. *Lane 2* untreated control. *O* origin, *G* glyphosate, *S* sarcosine, *F* solvent front

Discussion

Differences observed in the microbial populations isolated from soils sampled at glyphosate-treated and untreated sites were consistent with a proliferation of some microbial groups as a result of the repeated herbicidal treatment. Such differences were also observed by Quinn et al. (1988).

The enrichment of glyphosate-degrading isolates from both soil types confirmed the presence of glyphosate-degrading bacteria in untreated soil, although such strains appeared to be more frequent in glyphosate-treated soil. The higher frequency of AMPA-degrading isolates in both soil types suggests that the reported transient accumulation of AMPA in glyphosate-treated soils (Torstensson 1985) is unlikely to be due to the absence of a microflora able to degrade the compound.

The failure of some glyphosate-degrading strains to accumulate the radiolabelled herbicide when cultured on agar containing [3-^{14}C]glyphosate is consistent with the observed loss of a high proportion of the phosphonomethyl carbon as CO_2 following the cleavage of the C–P bond in *Pseudomonas* sp. PG 2982 (Kishore and Jacob 1987). Also, the failure of all 27 organisms tested to accumulate the radiolabel from agar in the presence of 0.2 mM phosphate highlights

the phenomenon of the inhibition of phosphonate transport by inorganic phosphate in environmental isolates in contrast to those from industrial wastes.

Many studies have suggested co-metabolism as a mechanism for the relatively slow rate of microbial degradation of glyphosate observed in the soil (Malik et al. 1989). Any investigation of co-metabolism precludes enrichment for the participating species as, by definition, there is no energetic or nutritional gain from the process (Horvath 1972). The use of structurally related analogues as an alternative enrichment strategy has been termed analogue enrichment and was tried following failure to obtain enrichments with glyphosate as sole carbon or carbon, nitrogen and phosphorus source (Quinn et al. 1988). The analogues used either were possible cleavage products of glyphosate provided as a carbon source, or provided a C–N bond similar to that in the herbicide molecule; organisms isolated on the basis of their ability to utilize such analogues might also be expected to utilize glyphosate.

In addition to the soils tested, a sample of the river Rhine was chosen for the likely exposure of its microflora to agricultural and industrial phosphonate detected as methylphosphonate in samples (Verweij et al. 1979). Despite attempts to enrich for organisms from both soils and Rhine water under conditions of analogue enrichment, where cleavage of the C–P bond was not a factor in the selection, no organisms were able to modify glyphosate in any way, at least under the conditions tested, that might contribute to the co-metabolism of the compound.

Glyphosate degradation in environmental organisms has been shown in this, and in many previous studies, to be inhibited by phosphate, yet in soil degradation must take place in the presence of phosphate. In axenic culture, this inhibition may be mediated by direct effects of phosphate on glyphosate transport or degradation and/or by the repression of these processes at the level of gene expression. Recent studies on phosphonate metabolism in *Escherichia coli* and *Enterobacter aerogenes* have shown that genes encoding both the transport and degradatory functions in phosphonate metabolism are subject to repression by inorganic phosphate as part of the PHO regulon (Wanner and Metcalf 1992; Lee et al. 1992). In the soil, microbial metabolism in the presence of phosphate but under conditions of nutritional stress may permit a low level of expression and operation of the phosphonate transport and degradatory proteins.

This study, to our knowledge, constitutes the largest investigation to date of glyphosate-degrading bacteria isolated from environmental sources, and underlines the differences in glyphosate degradation between environmental organisms and those from glyphosate-degrading industrial reactors.

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