Organophosphonates, characterized by the presence of a stable, covalent carbon-to-phosphorus (C—P) bond, are of widespread occurrence in the environment. Natural and synthetic organophosphonates are of importance, with the latter being utilized extensively in the chemical industry (26). By far the most important use of synthetic organophosphonates, however, is as herbicides, with glyphosate (16), the world’s leading agrochemical, worth in excess of $1 billion per year to its manufacturer, Monsanto Company, St. Louis, Mo.

The organophosphate C—P bond may be cleaved by a range of enzymes, including C—P hyase (27) and various hydrolases (24, 26). Additionally, the C—P bond of phosphonopyruvate can be intramolecularly rearranged to form a phosphate ester, phosphoenolpyruvate, by the action of the enzyme phosphoenolpyruvate phosphomutase (17). However, C—P bond cleavage is not the only route by which organophosphate biodegradation may proceed with both transaminases (22) and oxidoreductases (4), acting on parts of organophosphonate molecules other than the C—P bond; indeed, microorganisms that degrade organophosphonates without C—P bond cleavage have been described in recent years (15, 23).

Organophosphate metabolism has traditionally been studied in greatest detail within soil and soil microorganisms, largely due to scientific interest in the environmental fate of the herbicide glyphosate. Few attempts have been made to investigate the biodiversity of microorganisms capable of degrading organophosphonates (26), and studies have concentrated mainly on gram-negative, mesophilic bacteria, although recent work has attempted to redress this imbalance (9, 14, 19). While the isolation, biochemical characterization, and taxonomic description of thermophilic microbial strains have proceeded apace in recent years (18), biodegradation studies with such microorganisms are relatively scarce, and studies with organophosphonates are nonexistent. Here, we report for the first time the ability of a thermophilic bacterium to cleave the C—P bond of a number of organophosphonates and demonstrate a thermotolerant glyphosate oxidoreductase activity in cell extracts of the same.

The thermus isolation medium of Atlas (2) was prepared and solidified with 1.5% purified agar (Oxoid, Basingstoke, United Kingdom). Samples of domestic central heating system water could utilize a number of organophosphonates as the sole phosphorus source for growth at 60°C. During growth on glyphosate, aminophosphate release to the medium was observed, and in cell extracts, a glyphosate oxidoreductase-type activity, producing stoichiometric amounts of aminophosphate and glyoxylate from glyphosate, was detectable.

A strain of *Geobacillus caldoxylosilyticus* from central heating system water could utilize a number of organophosphonates as the sole phosphorus source for growth at 60°C. During growth on glyphosate, aminophosphate release to the medium was observed, and in cell extracts, a glyphosate oxidoreductase-type activity, producing stoichiometric amounts of aminophosphate and glyoxylate from glyphosate, was detectable.

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TABLE 1. Range of organophosphonate substrates utilized by G. caldoxylosilyticus T20 as the sole phosphorus or nitrogen source

<table>
<thead>
<tr>
<th>Organophosphonate substrate</th>
<th>Growth (µg of protein ml⁻¹) on substrate a⁻¹</th>
<th>Sole P source (1 mM)</th>
<th>Sole N source (5 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>315</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td>30</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Methylphosphonate</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethylphosphonate</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphonophenylate</td>
<td>45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aminomethylphosphonate</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Aminoethylphosphonate</td>
<td>45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Amino-3-phosphono propionate</td>
<td>75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphonycin</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphono formate</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphono acetate</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-[Phosphonomethyl]-glycine</td>
<td>310</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphonomethyl-iminodiacetate</td>
<td>320</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Phosphonopropionate</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Phosphonopropionate</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Amino-4-phosphono butyrate</td>
<td>55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Phosphono butyrate</td>
<td>110</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Phosphono butyrate</td>
<td>40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Results were scored negative if the protein yield, as measured by the method of Ahmad et al. (1), was less than 20% of that of the positive control containing 1 mM inorganic phosphate. Results are the mean of duplicates, which on no occasion varied by more than 5%.

that the microorganism was utilizing some 0.4 mM glyphosate-derived-phosphorus for growth under these conditions. The production of AMPA was confirmed by spiking high-performance liquid chromatography (HPLC) samples with authentic AMPA (Sigma-Aldrich Chemical Co., Poole, United Kingdom) and also by carrying out ¹H- and ³¹P-nuclear magnetic resonance analyses of culture supernatant samples concentrated 20-fold as previously described (24). Spectra were recorded at room temperature in D₂O on a Bruker DRX spectrometer (Karlsruhe, Germany) operating at 300.13 MHz for ¹H and 121.50 MHz for ³¹P. No AMPA production was observed in uninoculated control flasks, nor did decomposition of the organophosphonates used occur at 60°C, as measured by HPLC and inorganic phosphate determination.

Organophosphonate biodegradation by a thermophilic bacterium has not previously been reported. The present study proves that an obligately thermophilic microorganism can cleave C—P bonds and utilize a number of organophosphonates for growth. Notably, the strain did not utilize either 2-aminoethylphosphonate or 2-amino-3-phosphonopropionate, two natural organophosphonates, the biodegradation of which is facilitated by a large majority of environmental isolates (26). Because G. caldoxylosilyticus T20 is a thermophile, it is unsurprising that the range of organophosphonates utilized is different from those of previously studied mesophilic microorganisms. As previously reported for many isolates (26), however, phosphate starvation was required for organophosphonate biodegradation. G. caldoxylosilyticus T20 was unable to utilize AMPA as the sole P source, suggesting that while AMPA produced intercellularly from glyphosate may be metabolized, the strain is incapable of transporting and utilizing exogenously supplied AMPA. This observation may be explained if organophosphonate biodegradation in this microbe is controlled at the level of the transporter (12), rather than by the specificity of C—P bond cleavage enzymes.

G. caldoxylosilyticus T20 was grown on glyphosate as the sole P source, cell extracts were prepared by sonication, and when assayed for glyphosate oxidoreductase (GOX) by the method of Barry and Kishore (4) at 50°C, the level of release of dinitrophenylhydrazine (DNPH)-reactive material (corresponding to an activity of 0.6 nmol min⁻¹ mg⁻¹) was above that in control experiments lacking glyphosate substrate or cell extract. This DNPH-reactive material was confirmed as glyoxylate by the HPLC method of Qureshi et al. (21) following extraction with ethyl acetate. AMPA production and glyoxylate release were stoichiometric and linear with time up to 60 min (Fig. 2). Unlike the GOX activity described by Barry and Kishore (4), which occurred at 30°C, no activity was detectable in T20 cell extracts at less than 50°C, which is unsurprising, because the source organism does not grow below this temperature. However, the level of activity in isolate T20 is comparable to that reportedly obtained in cell lysates of a number of microorganisms obtained from a glyphosate waste treatment plant described in U.S. patent no. 5776760 (4).

In order to assess the similarity of G. caldoxylosilyticus GOX to published sequences, specific oligonucleotide primers targeting an internal region of the sequence of the wild-type GOX gene (4) from isolate LBAA (sequence ID no. 3) were designed with the aid of the Oligo Primer Analysis Software (Oligo version 5; NBI). The designations and sequences of the

FIG. 1. Growth of G. caldoxylosilyticus T20 on glyphosate (1.0 mM) as the sole phosphorus source in defined thermophile medium with an (NH₄)₂SO₄ nitrogen source (2.6 g liter⁻¹) and a glucose-glycerol-sucrose carbon source (3 g liter⁻¹ each), o, A₆₅₀; □, glyphosate; □, 2-aminoethylphosphonate. PMG, glyphosate.
The biodegradation of the herbicide glyphosate via the AMPA pathway by a thermophilic microorganism has not been reported before now. While conversion of glyphosate to AMPA is the accepted mechanism for detoxification of this herbicide in soil (15), no microorganism that conclusively exhibits this capability has been isolated (6). To date, our understanding of this phenomenon is based almost exclusively upon work carried out by Monsanto on microbes within a glyphosate waste treatment plant, which also metabolize the herbicide via this pathway (3, 4, 5, 8, 10, 13). The present study shows for the first time the conclusive production of AMPA from glyphosate both in vivo and in vitro by a microorganism not obtained from an industrial source.

It has previously been shown that *Arthrobacter atrocyaneus* ATCC 13752 could degrade glyphosate via an AMPA intermediate (20), despite this microorganism being deposited in culture collection prior to the invention of glyphosate. This suggests that the enzyme or enzymes responsible for glyphosate biodegradation via the AMPA pathway have a different, natural substrate rather than having evolved to facilitate glyphosate biodegradation since the introduction of the chemical and its widespread use. As with *A. atrocyaneus* ATCC 13752, isolate T20 was isolated from a source unlikely to have been exposed to glyphosate. It would appear therefore that the ability to degrade glyphosate to AMPA is present in a range of genetically diverse bacteria. Future studies will examine whether this is the result of one or many different enzymes.

**REFERENCES**