Identification of in situ 2,4-dichlorophenoxyacetic acid-degrading soil microorganisms using DNA-stable isotope probing

Alison M. Cupplesa, Gerald K. Simsb

aDepartment of Civil and Environmental Engineering, Michigan State University, East Lansing, MI 48824, USA
bUS Department of Agriculture, Agricultural Research Service, USA

Received 26 May 2006; received in revised form 6 July 2006; accepted 13 July 2006
Available online 31 August 2006

Abstract

Stable isotope probing (SIP) was used to investigate the microorganisms responsible for degradation of the herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D) in soil samples. Soils were unamended or amended with either unlabeled 2,4-D or UL(ring)13C-2,4-D. Degradation of 2,4-D was complete after 17 days, whereas little removal (11 ± 3%) was observed in the sterile controls. Terminal restriction fragment length polymorphism (TRFLP) on soil DNA after 17 days indicated a consistent increase in the relative abundance of one fragment (217 bp in Hae III digests) in soils spiked with 2,4-D (both unlabeled and labeled samples) compared to the unamended soils. DNA extracts from labeled and unlabeled 2,4-D amended soils were subject to ultracentrifugation, fractionation of centrifuged samples, followed by TRFLP on each fraction. TRFLP profiles from ultracentrifugation fractions illustrated that the same fragment experienced an increase in buoyant density (BD) in samples spiked with 13C-labeled 2,4-D. This increase in DNA BD indicates the organisms represented by this fragment were responsible for uptake and degradation of the herbicide. 16S rRNA sequencing of the heavy, 13C-enriched fraction suggests the organisms belong to the β subdivision of Proteobacteria. Herein, SIP facilitated the identification of unique organisms degrading 2,4-D in soil without the need for isolation and provided more direct evidence for a functional role of these organisms than would have been possible with the molecular-based methods alone.

Keywords: 2,4-dichlorophenoxyacetic acid; Stable isotope probing; Herbicide biodegradation; In situ biodegradation

1. Introduction

Understanding herbicide persistence is important from both the standpoints of herbicide efficacy and off-site movement to sensitive environments. A thorough understanding of agrochemical fate and transformation processes in soils is required to explain geographical variation in herbicide persistence and thus address both efficacy and persistence problems. We know environmental factors, such as temperature and moisture, can affect transformation rates, however, as yet, the influence of the species of microbes present has received little attention because the techniques have not been available for such studies. To date, the majority of data on herbicide degrading microorganisms has originated from laboratory experiments, such as enrichments, where the samples are exposed to environmental conditions differing greatly from those typical of field conditions. Consequently, the identity of the organisms able to degrade herbicides in a typical soil environment is still a great unknown. The recent development of stable isotope probing (SIP) enables, for the first time, function to be linked with identity without the need to culture the bacteria involved (Radajewski et al., 2000). With SIP, the ecology of herbicide biodegradation can be studied in soil systems containing a highly complex community, thus the microorganisms responsible for in situ herbicide degradation may at last be identified.

Stable isotope probing facilitates the identification of organisms assimilating an isotopically labeled compound from a sample containing a mixed community of organisms. The method involves incubation with the labeled compound, followed by nucleic acid extraction and density gradient ultracentrifugation to separate the light from the
heavy, label incorporated nucleic acids. The recovered nucleic acid fractions are then characterized by a number of available molecular-based methods to identify the organisms responsible for label uptake. A significant advantage to SIP, unlike many other DNA-based methods, is that only active DNA is targeted. To date, the method has been applied to assimilation of a variety of compounds, including glucose, caffeine, (Padmanabhan et al., 2003), naphthalene (Padmanabhan et al., 2003; Yu and Chu, 2005), phenol (DeRito et al., 2005; Manefield et al., 2002; Padmanabhan et al., 2003), methanol (Lueders et al., 2004c), methane (Morris et al., 2002), propionate (Lueders et al., 2004a) methyl bromide, methyl chloride (Miller et al., 2004), pentachlorophenol (Mahmood et al., 2005) and ammonium (Cupples et al., 2006).

The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), introduced in the 1940s, is extensively used worldwide to control broadleaf weeds. It is applied principally to control weeds in cereal crops (wheat, maize, rice and sorghum) and in grassland and turf areas. The significant history and introduced in the 1940s, is extensively used worldwide to control broadleaf weeds. It is applied principally to control weeds in cereal crops (wheat, maize, rice and sorghum) and in grassland and turf areas. The significant history and

2.2. Soil incubations

Soil samples (Westola soil, coarse-loamy, mixed, super-active, calcareous, thermic Typic Ustifluvents) were obtained from an agricultural field in Oklahoma previously under winter wheat production. Thus, these soils likely had previous agricultural exposure to 2,4-D. The Westola soil had a sandy loam surface texture, a pH in water of 7.8, cation exchange capacity of 15 mEq 100 g⁻¹, and contained 0.4% organic carbon (characterization performed by A&L Great Lakes Laboratory, Fort Wayne, IN). Samples were sieved (4 mm screen) and stored at 4°C until use. Soil samples were unamended or amended twice (day 0 and 10) with either unlabelled or 13C labeled 2,4-D (20 μg g⁻¹). Sterile controls were obtained by autoclaving repeatedly (three times in 1 day). Herbicide solutions (dissolved in acetonitrile, 100 μg ml⁻¹ for both unlabelled and labeled), were added to empty teflon centrifuge tubes and the acetonitrile was allowed to evaporate overnight. Control samples (no 2,4-D) received only acetonitrile. The next day, an ammonium sulfate (0.25 mg N g⁻¹ soil) solution (to bring final moisture level to 50% of the soil water holding capacity) was added, followed by the soil sample (2 g dry weight), and the contents were mixed. Capped tubes were stored in the dark and opened every few days to permit oxygen diffusion. Soils (no 2,4-D controls, autoclaved controls and unlabelled and labeled 2,4-D samples) were destructively sampled on day 7 and 17.

2.3. 2,4-D extraction and HPLC analysis

On day 7 and day 17 (chosen based on preliminary experiments), soil samples (1 g dry weight for each replicate) were removed for DNA extraction (see below) and the remaining soil (1 g dry weight) was extracted with an acetonitrile and calcium chloride solution (0.01 M) (60:40) (1:1, liquid to soil) overnight on a horizontal shaker in teflon centrifuge tubes. The sample was centrifuged (5 min at 9148g) and 400–500 μl samples were filtered (PVDF, 0.45 μm, 13 mm, Whatman Inc., Clifton, NJ) prior to reverse-phase high-performance liquid chromatography (HPLC, Hewlett Packard Series 1050, San Fernando, CA) analyses. The samples were either analyzed immediately, or, if analyzed later (within 3 days), were stored (4°C) until analyses. 2,4-D extraction recovery from this soil (triplicates samples, five concentrations) was found to be 87.3 ± 14.7% (non-autoclaved samples only). HPLC conditions were: injector volume, 100 μl; mobile phase flow rate, 0.9 ml min⁻¹; UV detector wavelength 233 nm; reverse-phase C₁₈ column (ProntoSIL C₁₈-EPS) and an isocratic mobile phase (acetonitrile: water: acetic acid, 50: 48: 2).

2.4. DNA extraction and ultracentrifugation

DNA was extracted (day 7 and 17) from soil samples with the Powersoil kit (Mobio Laboratories, Carlsbad, CA) following the manufacturer’s instructions. Ultracentrifugation was performed in Quick-Seal polyallomer tubes (13 x 51 mm, 5.1 ml, Beckman Coulter) in an Optima LE-80 K Preparative Ultracentrifuge (Beckman Instruments) equipped with a VTi 65.2 vertical tube rotor for 48 h,
184,000 gav (20 °C). Buoyant densities (BD) were measured with a model AR200 digital hand-held refractometer (Leica Microsystems Inc.). Following centrifugation, water was introduced with a precision pump (model PHD 2000, Harvard Apparatus, Holliston, MA) into the top of the centrifuge tube and fractions (150 μl) were collected at the bottom as previously described (Cupples et al., 2006; Lueders et al., 2004b), the BD of each fraction was then determined, CsCl was removed (Cupples et al., 2006; Griffiths et al., 2000; Lueders et al., 2004b) and purified DNA fractions were stored at −20 °C.

2.3. TRFLP and sequencing

Both the total DNA extracts and ultracentrifugation fractions were analyzed by 16S rDNA terminal restriction fragment length polymorphism (TRFLP) following standard procedures (Liu et al., 1997). PCR primers (Operon Biotechnologies) utilized were 27F-FAM (5′-AGAGTTT-GATCMTGGCTCAG, 5′ end-labeled with carboxyfluoresceine) and 1492R (5′-GGTTACCTTGTTACGACTT). PCR mixtures (100 μl) included the TaKaRa Ex Taq mixture (Takara Bio), primers (45 pmol each), and 5 μl DNA sample. The PCR program was: 94 °C (5 min); 94 °C (30 s), 55 °C (30 s), 72 °C (1.5 min) (30 cycles); 72 °C (5 min). PCR products were purified with the QIAquick® PCR purification kit (Qiagen Inc.), following the manufacturer’s instructions. The purified PCR products from the total DNA and fractions were digested with Hae III according to the recommended protocol (New England Biolabs). Additionally, heavy fractions were digested with Hha I, Mse I and Rsa I (New England Biolabs) following the manufacturer’s guidelines. DNA fragments were separated by capillary electrophoresis (model 3730xl Genetic Analyzer, Applied Biosystems) at the W. M. Keck Center (UIUC). Data were analyzed with GeneMapper V3.7 software (Applied Biosystems). Percent abundance of each fragment was determined as previously described (Yu et al., 2005). Heavy fraction 13C-labeled DNA was amplified (as above) then cloned into Escherichia coli TOP10 using a TOPO TA cloning kit (Invitrogen Corporation, Carlsburg, CA). Plasmids were extracted from the cloned cells with a QIAprep miniprep system (Qiagen, Inc.), and the insertions were sequenced at the W. M. Keck Center. The BLASTN search tool (Altschul et al., 1990) was used to find sequence homology and to determine the most similar sequences in the GenBank database. The Ribosomal Database Project II analysis tool “classifier” (Center for Microbial Ecology, Michigan State University) was utilized to assign taxonomic identity.

3. Results

3.1. 2,4-D biodegradation

The degradation pattern for 2,4-D was similar for soils amended with either the labeled or unlabeled herbicide; approximately half of the herbicide remained after 1 week and almost complete degradation was observed after 17 days. In contrast, little degradation was observed in the sterile controls (Table 1).

3.2. TRFLP of DNA extracts

DNA extracts from unamended (controls) and amended (labeled and unlabeled) soil samples were subjected to terminal restriction fragment length polymorphism (TRFLP) and the relative abundance of Hae III fragments from amended soils and control soils were compared. Only one fragment (217 bp) experienced an increase in relative abundance in 2,4-D amended samples compared to the controls (Fig. 1). The increase in abundance of this fragment was slight at day 7, however, by day 17 a significant (P < 0.001) increase, compared to the controls, was seen in all replicate samples amended with 2,4-D. DNA extracts were probed with primers designed (Lee et al., 2005) for a 2,4-D degrading gene (Ralstonia eutropha JMP134 type-tdfA); the correct size fragment (~300 bp) was observed among the bands produced (data not shown).

3.3. TRFLP results for SIP

DNA extracts from labeled and unlabeled 2,4-D amended soil samples were subject to ultracentrifugation, fractionation of centrifuged samples, followed by TRFLP

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>7</th>
<th>17</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D (13C)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55 ± 27</td>
<td>5 ± 10</td>
</tr>
<tr>
<td>2,4-D (13C)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>56 ± 21</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Sterile controls&lt;sup&gt;c&lt;/sup&gt;</td>
<td>85 ± 3</td>
<td>89 ± 3</td>
</tr>
</tbody>
</table>

<sup>a</sup>Taking into account extraction efficiency of 87.3%.
<sup>b</sup>Four replicates.
<sup>c</sup>Three replicates.

Fig. 1. Comparison of the relative abundance of TRFLP fragment 217 bp in 2,4-D amended and unamended soils after 7 and 17 days. Error bars represent standard deviations from 6–8 soil samples.
on each fraction. From TRFLP data, the relative abundance of each fragment was determined. In general, fragments were distributed throughout the gradient profile for both treatments, however a clear trend for fragment 217 bp could be observed (Fig. 2). To illustrate the specificity of enrichment for fragment 217 bp, compared to other fragments throughout the buoyant density profile, the relative abundance of the three dominant peaks (for labeled and unlabeled DNA) in TRFLP profiles are illustrated in Fig. 3. Notably, although all three fragments were found in heavy fractions of either treatment, only one peak (217 bp) experienced an increased relative abundance at higher buoyant densities in samples amended with $^{13}$C 2,4-D compared to the $^{12}$C 2,4-D control (Fig. 3C). The relative abundance of the other 2 dominant peaks remained essentially constant over the BD profile, indicating no increase in DNA BD, thus no $^{13}$C incorporation by the microorganisms represented by these fragments (Fig. 3A and B).

TRFLP on $^{13}$C enriched heavy fractions using additional restriction enzymes (Hae III, Hha I, Mse I and Rsa I) resulted in a unique dominant peak for each enzyme (Fig. 4). These fragment lengths were then compared to those obtained from in silico digests of 16S rRNA sequence data from $^{13}$C heavy fractions to determine the clones they represented. Of the 14 clones sequenced, three clones illustrated restriction enzyme cut sites that matched TRFLP results (Table 2) (sequences were deposited to GenBank under the Accession Nos DQ398882–DQ398884). The slight difference (2–3 bases) between the measured fragment lengths and those predicted using sequence data has been noted elsewhere (Clement et al., 1998; Liu et al., 1997; Osborn et al., 2000) and has yet to be explained (Yu et al., 2005). Clones 1 and 2 were 99% similar to each other and each was 97% similar to clone 3. The closest relatives of these three clones were determined (Table 3).

4. Discussion

The time for 2,4-D degradation in the soil studied was consistent with previous reports of 2,4-D degradation (Boivin et al., 2005; Gonod et al., 2003; Shaw and Burns, 1998, 2005). The coupling of stable isotope probing with TRFLP enabled us to determine which organisms were responsible for the observed 2,4-D degradation. To our knowledge, this is the first research to identify specific organisms able to assimilate 2,4-D while living in soil. TRFLP on DNA extracts demonstrated the organisms represented by peak 217 bp increased in abundance following amendment with 2,4-D, but not in unamended controls. Interestingly, the trend was the same in DNA extracts for both $^{13}$C labeled and unlabeled 2,4-D amended samples. Traces of 217 bp were observed throughout the buoyant density profile with enrichment of fragment 217 bp noted in the labeled 2,4-D control (Fig. 3C).

Fig. 3. Comparison of relative abundance of the three dominant fragments over a range of buoyant density (BD) from DNA extracted (day 17) from soil amended with either labeled ($^{13}$C) or unlabeled ($^{12}$C) 2,4-D. Replicate soil TRFLP data from day 17 and 7 illustrated the same trend.

Fig. 2. Comparison of heavy fraction TRFLP profiles from unlabeled ($^{12}$C) and labeled ($^{13}$C) 2,4-D amended soils to illustrate the dominance of fragment 217 bp in labeled heavy fractions compared to the unlabeled heavy fractions control. Replicates illustrated the same trend.
soils, indicating no isotope effect. The increase in relative abundance of fragment 217 bp in amended soils compared to unamended soil indicates 2,4-D is positively affecting the populations of the organisms represented by this fragment. Previously we would not know the mechanism causing this, (2,4-D may simply have been suppressing the competition allowing these particular organisms to thrive) however, stable isotope probing allowed us to determine the in situ process responsible. SIP illustrated that the same fragment (217 bp) became dominant in heavy fractions of labeled DNA but not unlabeled DNA. The comparison of TRFLP profiles over a range of BD from both labeled and unlabeled samples was an effective control for false positive results; a necessary concern considering the sensitivity of PCR. This approach may be particularly useful when small DNA BD shifts are expected because of low levels of incorporation (low substrate concentration or recalcitrant substrate). Another important control to consider concerns 13C cross-feeding (Gallagher et al., 2005), although we feel confident that no transfer of 13C between species occurred by day 7, we encourage others, perhaps using SIP for more recalcitrant compounds and thus longer time periods, to analyze samples over time to ensure this is not a problem.

Based on the partial rDNA sequences (1391–1395 bp) obtained, the three clones all belong to the subdivision of Proteobacteria, further, it is most likely that the organisms belong to the family of Comamonadaceae. Previous research indicates that 2,4-D degrading bacteria fall into three groups based on evolutionary and physiological characteristics (Kamagata et al., 1997). One group includes bacteria belonging to the β and γ subdivisions of Proteobacteria, including genera such as Rhodoferax, Bulkeleya, Ralstonia, Alcaligenes, Halomonas, Variovorax and Pseudomonas (Cavalca et al., 1999; Fulthorpe et al., 1995; McGowan et al., 1998; Vallaeyns et al., 1999). The second and third groups contains organisms in the α subdivision of Proteobacteria; those closely related to a Bradyrhizobium sp. (Kamagata et al., 1997) and those related to a species of the genus Sphingomonas (Ka et al., 1994a–c; Kamagata et al., 1997; McGowan et al., 1998; Vallaeyns et al., 1999). Based on their partial 16S rRNA gene sequences, the 2,4-D degrading microorganisms identified here likely belong to the first group of 2,4-D degraders. It is thought that the ability of this group to catabolize 2,4-D results from independent tdf gene recruitment through horizontal gene transfer (Fulthorpe et al., 1995; McGowan et al., 1998; Top et al., 1995; Vallaeyns et al., 1999). The presence of a PCR product of correct size using tdf targeted primers (Lee et al., 2005) further suggests the organisms identified here indeed belong to this group.
The partial 16S rRNA sequences of 2,4-D degrading bacteria identified here belong to a group of well-studied 2,4-D degraders (β subdivision of Proteobacteria), however, within this group they represent novel sequences, previously not associated with 2,4-D degradation. SIP has similarly enabled others to identify novel, previously undiscovered bacteria able to assimilate target compounds in mixed communities, such as anaerobic benzene degraders (Kasai et al., 2006) or methyl-chloride utilizing bacteria (Borodina et al., 2005). To our knowledge, this is the first application of SIP to investigate 2,4-D degradation in situ. The approach has facilitated the identification of soil organisms metabolizing 2,4-D under conditions more typical of those found in the environment. It is hoped this approach will enable the investigation of 2,4-D degrader diversity between soils, and its effect on degradation rates. This, in combination with 2,4-D functional gene SIP, will result in a more complete picture of 2,4-D degradation in agricultural areas.

Acknowledgements

We thank Jonathon Holt, Lynn Connor and Joanne Chees-Sanford for their extensive technical advice. Also, thanks to Loretta Ortiz-Ribbing and Jim Gilchrist (Northwest Oklahoma State University) for providing the soil. This work was supported by the Agricultural Research Service, US Department of Agriculture, project number 3611-12220-006-00D. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

References


Ka, J.O., Holben, W.E., Tiedje, J.M., 1994b. Genetic and phenotypic diversity of 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading bacteria isolated from 2,4-D-treated field soils. Applied and Environmental Microbiology 60, 1106–1115.


length polymorphisms of genes encoding 16S rRNA. Applied and Environmental Microbiology 63, 4516–4522.


