

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

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## 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) <sup>13</sup>C-2,4-D. Degradation of 2,4-D was complete after 17 days, whereas little removal ( $11 \pm 3\%$ ) 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 <sup>13</sup>C-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, <sup>13</sup>C-enriched fraction suggests the organisms belong to the  $\beta$  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.

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## 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

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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 extensive use pattern of 2,4-D perhaps explains why it is one of the most studied herbicides. Many 2,4-D degrading bacteria have been isolated and characterized from a range of environmental conditions (Cavalca et al., 1999; Fulthorpe et al., 1996, 1995; Itoh et al., 2004, 2002; Kamagata et al., 1997; Kitagawa et al., 2002; Maltseva et al., 1996; McGowan et al., 1998; Suwa et al., 1996; Tonso et al., 1995; Top et al., 1995; Vallaeyes et al., 1997). However, until recently, we could only speculate on which bacteria were actively responsible for 2,4-D degradation in the soil environment. The ability to examine the microbial ecology of herbicide degradation with confidence is important for explaining variations in herbicide efficacy and persistence in agricultural soils. Here, we investigate 2,4-D degradation in soil and identify the organisms responsible for this *in situ* degradation using  $^{13}\text{C}$ -DNA targeted SIP. The herbicide was chosen for reasons expressed above and its effectiveness as a carbon source, as well as its polar nature, which minimizes concerns over bioavailability in a soil matrix (Sims and Cupples, 1999).

## 2. Materials and methods

### 2.1. Chemicals

Unlabeled 2,4-D (99%) and labeled 2,4-D (UL(ring)  $^{13}\text{C}$ -2,4-D, 99%,  $100\ \mu\text{g ml}^{-1}$  in acetonitrile) were purchased from Chem Service (West Chester, PA) and Cambridge Isotope Laboratories, Inc (Andover, MA), respectively. Organic solvents and water were Optima grade (Fisher Scientific, Pittsburg, PA).

### 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  $15\ \text{mEq } 100\ \text{g}^{-1}$ , 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  $4\ ^\circ\text{C}$  until use. Soil samples were unamended or amended twice (day 0 and 10) with either unlabelled or  $^{13}\text{C}$  labeled 2,4-D ( $20\ \mu\text{g g}^{-1}$ ). Sterile controls were obtained by autoclaving repeatedly (three times in 1 day). Herbicide solutions (dissolved in acetonitrile,  $100\ \mu\text{g ml}^{-1}$  for both unlabeled 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 ( $0.25\ \text{mg N g}^{-1}$  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 unlabeled 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  $400\text{--}500\ \mu\text{l}$  samples were filtered (PVDF,  $0.45\ \mu\text{m}$ , 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 ( $4\ ^\circ\text{C}$ ) until analyses. 2,4-D extraction recovery from this soil (triplicates samples, five concentrations) was found to be  $87.3 \pm 14.7\%$  (non-autoclaved samples only). HPLC conditions were: injector volume,  $100\ \mu\text{l}$ ; mobile phase flow rate,  $0.9\ \text{ml min}^{-1}$ ; UV detector wavelength 233 nm; reverse-phase  $\text{C}_{18}$  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 ( $13 \times 51\ \text{mm}$ , 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  $g_{av}$  (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  $\mu$ l) 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.

### 2.5. 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  $\mu$ l) included the TaKaRa Ex Taq mixture (Takara Bio), primers (45 pmol each), and 5  $\mu$ l 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<sup>®</sup> 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 <sup>13</sup>C-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 eutrophia* 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 1  
Percent 2,4-D remaining in soil samples over the experimental period<sup>a</sup>

Time (days)	7	17
2,4-D ( <sup>12</sup> C) <sup>b</sup>	55 ± 27	5 ± 10
2,4-D ( <sup>13</sup> C) <sup>b</sup>	56 ± 21	0 ± 0
Sterile controls <sup>c</sup>	85 ± 3	89 ± 3

<sup>a</sup>Taking into account extraction efficiency of 87.3%.

<sup>b</sup>Four replicates.

<sup>c</sup>Three replicates.

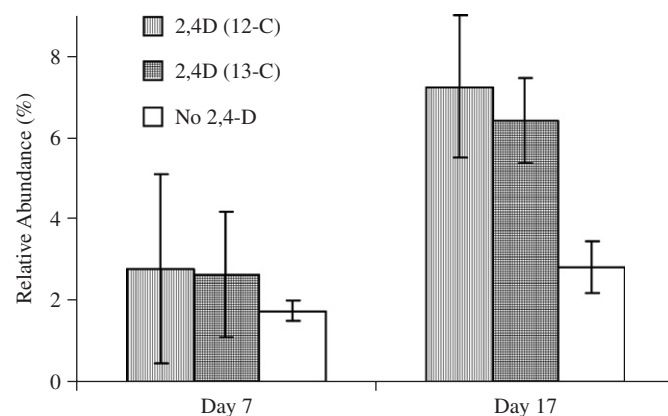


Fig. 1. Comparison of the relative abundance of TRFLP fragment 217 bp in 2,4-D amended and unamended soils after 7 and 17 days. Error bars represent standard deviations from 6–8 soil samples.

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  $^{13}\text{C}$  2,4-D compared to the  $^{12}\text{C}$  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  $^{13}\text{C}$  incorporation by the microorganisms represented by these fragments (Fig. 3A and B).

TRFLP on  $^{13}\text{C}$  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  $^{13}\text{C}$  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  $^{13}\text{C}$  labeled and unlabeled 2,4-D amended

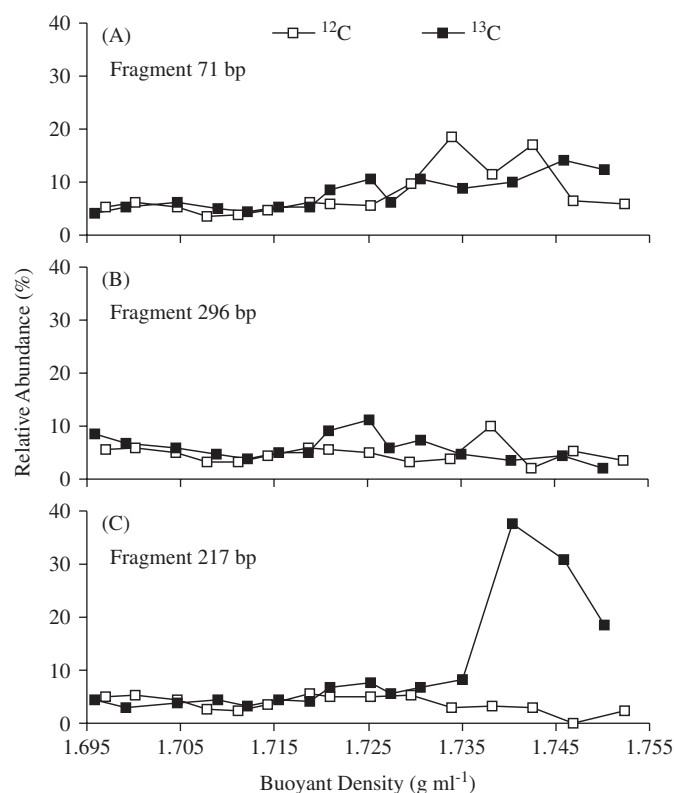


Fig. 3. Comparison of relative abundance of the three dominant fragments over a range of buoyant density (BD) from DNA extracted (day 17) from soil amended with either labeled ( $^{13}\text{C}$ ) or unlabeled ( $^{12}\text{C}$ ) 2,4-D. Replicate soil TRFLP data from day 17 and 7 illustrated the same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

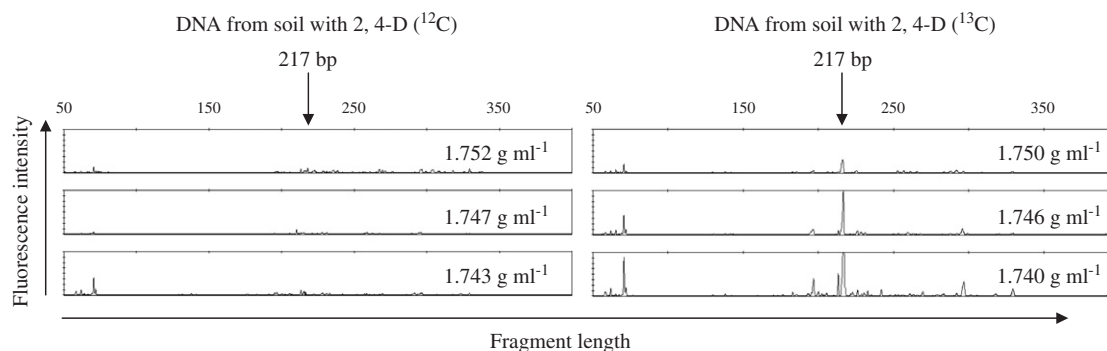


Fig. 2. Comparison of heavy fraction TRFLP profiles from unlabeled ( $^{12}\text{C}$ ) and labeled ( $^{13}\text{C}$ ) 2,4-D amended soils to illustrate the dominance of fragment 217 bp in labeled heavy fractions compared to the unlabeled heavy fractions control. Replicates illustrated the same trend.

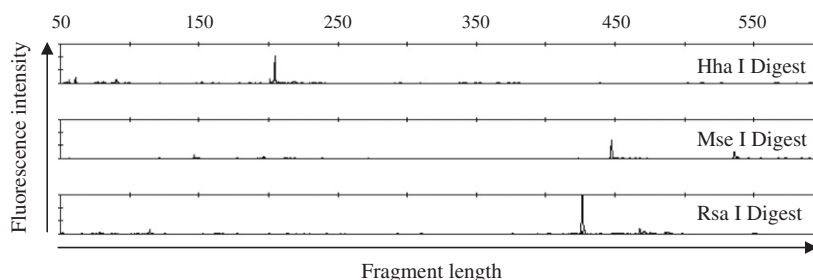


Fig. 4. TRFLP profiles from heavy fractions (1.740–1.746 g ml<sup>-1</sup>) of labeled 2,4-D amended soil with three different restriction enzymes.

Table 2

Comparison of dominant fragments in heavy fraction TRFLP to clone restriction enzyme cut sites predicted from sequence analyses

Restriction enzyme	TRFLP	Clones <sup>a</sup>
Hae III	217	219
Hha I	205	207
Mse I	447	450
Rsa I	426	429

<sup>a</sup>Cut site was the same in all three clones.

Table 3

Closest matches between 16S rRNA gene sequences from organisms able to uptake 2,4-D in these investigations and sequences from the GenBank database

Clone	Percent similarity <sup>a</sup>	Closest relative in Genbank <sup>b</sup>	Genbank accession no.	Reference
1 and 2	96%	<i>Comamonadaceae</i> bacterium MPsc	AY651926	Wang, Y and J.-D Gu, unpublished
	96%	Uncultured bacterium, clone HDBW-WB06	AB237669	Shimizu, S., M. Akiyama, T. Naganuma and Y. Ishijima, unpublished
	96%	Uncultured bacterium	DQ248266	Brinkman, C. K., F. S. Colwell and R. L. Crawford, unpublished
3	99%	<i>Ramlibacter</i> sp. HTCC332	AY429716	Connon et al., 2005
	97%	Uncultured bacterium	AY725253	Chee-Sanford, J. C., L. M. Connor, T. J. Holman and G. K. Sims, unpublished
	97%	Uncultured bacterium	AY725246	Chee-Sanford, J. C., L. M. Connor, T. J. Holman and G. K. Sims, unpublished

<sup>a</sup>Percent similarity between the sequenced clone and the sequence from GenBank.

<sup>b</sup>Identities are based on the three closest NCBI sequences.

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 <sup>13</sup>C cross-feeding (Gallagher et al., 2005), although we feel confident that no transfer of <sup>13</sup>C 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  $\beta$  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  $\beta$  and  $\gamma$  subdivisions of *Proteobacteria*, including genera such as *Rhodospirillum rubrum*, *Bulkholderia*, *Ralstonia*, *Alcaligenes*, *Halomonas*, *Variovarax* and *Pseudomonas* (Cavalca et al., 1999; Fulthorpe et al., 1995; McGowan et al., 1998; Vallaey et al., 1999). The second and third groups contains organisms in the  $\alpha$  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; Vallaey 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; Vallaey 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 ( $\beta$  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.

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### References

- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215, 403–410.
- Boivin, A., Amellal, S., Schiavon, M., van Genuchten, M.T., 2005. 2,4-dichlorophenoxyacetic acid (2,4-D) sorption and degradation dynamics in three agricultural soils. *Environmental Pollution* 138, 92–99.
- Borodina, E., Cox, M.J., McDonald, I.R., Murrell, J.C., 2005. Use of DNA-stable isotope probing and functional gene probes to investigate the diversity of methyl chloride-utilizing bacteria in soil. *Environmental Microbiology* 7, 1318–1328.
- Cavalca, L., Hartmann, A., Rouard, N., Soulas, G., 1999. Diversity of *tfdC* genes: distribution and polymorphism among 2,4-dichlorophenoxyacetic acid degrading soil bacteria. *FEMS Microbiology Ecology* 29, 45–58.
- Clement, B.G., Kehl, L.E., DeBord, K.L., Kitts, C.L., 1998. Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities. *Journal of Microbiological Methods* 31, 135–142.
- Connon, S.A., Tovanabootr, A., Dolan, M., Vergin, K., Giovannoni, S.J., Semprini, L., 2005. Bacterial community composition determined by culture-independent and -dependent methods during propane-stimulated bioremediation in trichloroethene-contaminated groundwater. *Environmental Microbiology* 7, 165–178.
- Cupples, A.M., Shaffer, E.A., Chee-Sanford, J.C., Sims, G.K., 2006. DNA buoyant density shifts during  $^{15}\text{N}$  DNA stable isotope probing. *Microbiological Research*, in press, doi:10.1016/j.micres.2006.01.016.
- DeRito, C.M., Pumphrey, G.M., Madsen, E.L., 2005. Use of field-based stable isotope probing to identify adapted populations and track carbon flow through a phenol-degrading soil microbial community. *Applied and Environmental Microbiology* 71, 7858–7865.
- Fulthorpe, R.R., McGowan, C., Maltseva, O.V., Holben, W.E., Tiedje, J.M., 1995. 2,4-dichlorophenoxyacetic acid-degrading bacteria contain mosaics of catabolic genes. *Applied and Environmental Microbiology* 61, 3274–3281.
- Fulthorpe, R.R., Rhodes, A.N., Tiedje, J.M., 1996. Pristine soils mineralize 3-chlorobenzoate and 2,4-dichlorophenoxyacetate via different microbial populations. *Applied and Environmental Microbiology* 62, 1159–1166.
- Gallagher, E., McGuinness, L., Phelps, C., Young, L.Y., Kerkhof, L.J., 2005.  $^{13}\text{C}$ -carrier DNA shortens the incubation time needed to detect benzoate-utilizing denitrifying bacteria by stable-isotope probing. *Applied and Environmental Microbiology* 71, 5192–5196.
- Gonod, L.V., Chenu, C., Soulas, G., 2003. Spatial variability of 2,4-dichlorophenoxyacetic acid (2,4-D) mineralisation potential at a millimetre scale in soil. *Soil Biology & Biochemistry* 35, 373–382.
- Griffiths, R.I., Whiteley, A.S., O'Donnell, A.G., Bailey, M.J., 2000. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Applied and Environmental Microbiology* 66, 5488–5491.
- Itoh, K., Kanda, R., Sumita, Y., Kim, H., Kamagata, Y., Suyama, K., Yamamoto, H., Hausinger, R.P., Tiedje, J.M., 2002. *tfdA*-like genes in 2,4-dichlorophenoxyacetic acid-degrading bacteria belonging to the *Bradyrhizobium-Agromonas-Nitrobacter-Afipia* cluster in *alpha-Proteobacteria*. *Applied and Environmental Microbiology* 68, 3449–3454.
- Itoh, K., Tashiro, Y., Uobe, K., Kamagata, Y., Suyama, K., Yamamoto, H., 2004. Root nodule *Bradyrhizobium* spp. harbor *tfdA* alpha and *cad4*, homologous with genes encoding 2,4-dichlorophenoxyacetic acid-degrading proteins. *Applied and Environmental Microbiology* 70, 2110–2118.
- Ka, J.O., Holben, W.E., Tiedje, J.M., 1994a. Analysis of competition in soil among 2,4-dichlorophenoxyacetic acid-degrading bacteria. *Applied and Environmental Microbiology* 60, 1121–1128.
- 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.
- Ka, J.O., Holben, W.E., Tiedje, J.M., 1994c. Use of gene probes to aid in recovery and identification of functionally dominant 2,4-dichlorophenoxyacetic acid-degrading populations in soil. *Applied and Environmental Microbiology* 60, 1116–1120.
- Kamagata, Y., Fulthorpe, R.R., Tamura, K., Takami, H., Forney, L.J., Tiedje, J.M., 1997. Pristine environments harbor a new group of oligotrophic 2,4-dichlorophenoxyacetic acid-degrading bacteria. *Applied and Environmental Microbiology* 63, 2266–2272.
- Kasai, Y., Takahata, Y., Manefield, M., Watanabe, K., 2006. RNA-based stable isotope probing and isolation of anaerobic benzene-degrading bacteria from gasoline-contaminated groundwater. *Applied and Environmental Microbiology* 72, 3586–3592.
- Kitagawa, W., Takami, S., Miyauchi, K., Masai, E., Kamagata, Y., Tiedje, J.M., Fukuda, M., 2002. Novel 2,4-dichlorophenoxyacetic acid degradation genes from oligotrophic *Bradyrhizobium* sp. strain HW13 isolated from a pristine environment. *Journal of Bacteriology* 184, 509–518.
- Lee, T.H., Kurata, S., Nakatsu, C.H., Kamagata, Y., 2005. Molecular analysis of bacterial community based on 16S rDNA and functional genes in activated sludge enriched with 2,4-dichlorophenoxyacetic acid (2,4-D) under different cultural conditions. *Microbial Ecology* 49, 151–162.
- Liu, W.T., Marsh, T.L., Cheng, H., Forney, L.J., 1997. Characterization of microbial diversity by determining terminal restriction fragment

- length polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology* 63, 4516–4522.
- Lueders, T., Pommerenke, B., Friedrich, M.W., 2004a. Stable-isotope probing of microorganisms thriving at thermodynamic limits: syntrophic propionate oxidation in flooded soil. *Applied and Environmental Microbiology* 70, 5778–5786.
- Lueders, T., Manefield, M., Friedrich, M.W., 2004b. Enhanced sensitivity of DNA and rRNA based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environmental Microbiology* 6, 73–78.
- Lueders, T., Wagner, B., Claus, P., Friedrich, M.W., 2004c. Stable isotope probing of rRNA and DNA reveals a dynamic methylotroph community and trophic interactions with fungi and protozoa in oxic rice field soil. *Environmental Microbiology* 6, 60–72.
- Mahmood, S., Paton, G.I., Prosser, J.I., 2005. Cultivation-independent *in situ* molecular analysis of bacteria involved in degradation of pentachlorophenol in soil. *Environmental Microbiology* 7, 1349–1360.
- Maltseva, O., McGowan, C., Fulthorpe, R., Oriol, P., 1996. Degradation of 2,4-dichlorophenoxyacetic acid by haloalkaliphilic bacteria. *Microbiology—UK* 142, 1115–1122.
- Manefield, M., Whiteley, A.S., Griffiths, R.I., Bailey, M.J., 2002. RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. *Applied and Environmental Microbiology* 68, 5367–5373.
- McGowan, C., Fulthorpe, R., Wright, A., Tiedje, J.M., 1998. Evidence for interspecies gene transfer in the evolution of 2,4-dichlorophenoxyacetic acid degraders. *Applied and Environmental Microbiology* 64, 4089–4092.
- Miller, L.G., Warner, K.L., Baesman, S.M., Oremland, R.S., McDonald, I.R., Radajewski, S., Murrell, J.C., 2004. Degradation of methyl bromide and methyl chloride in soil microcosms: use of stable C isotope fractionation and stable isotope probing to identify reactions and the responsible microorganisms. *Geochimica Et Cosmochimica Acta* 68, 3271–3283.
- Morris, S.A., Radajewski, S., Willison, T.W., Murrell, J.C., 2002. Identification of the functionally active methanotroph population in a peat soil microcosm by stable-isotope probing. *Applied and Environmental Microbiology* 68, 1446–1453.
- Osborn, A.M., Moore, E.R.B., Timmis, K.N., 2000. An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environmental Microbiology* 2, 39–50.
- Padmanabhan, P., Padmanabhan, S., DeRito, C., Gray, A., Gannon, D., Snape, J.R., Tsai, C.S., Park, W., Jeon, C., Madsen, E.L., 2003. Respiration of  $^{13}\text{C}$ -labeled substrates added to soil in the field and subsequent 16S rRNA gene analysis of  $^{13}\text{C}$ -labeled soil DNA. *Applied and Environmental Microbiology* 69, 1614–1622.
- Radajewski, S., Ineson, P., Parekh, N.R., Murrell, J.C., 2000. Stable-isotope probing as a tool in microbial ecology. *Nature* 403, 646–649.
- Shaw, L.J., Burns, R.G., 1998. Biodegradation of 2,4-D in a noncontaminated grassland soil profile. *Journal of Environmental Quality* 27, 1464–1471.
- Shaw, L.J., Burns, R.G., 2005. Rhizodeposition and the enhanced mineralization of 2,4-dichlorophenoxyacetic acid in soil from the *Trifolium pratense* rhizosphere. *Environmental Microbiology* 7, 191–202.
- Sims, G.K., Cupples, A.M., 1999. Factors controlling degradation of pesticides in soil. *Pesticide Science* 55, 598–601.
- Suwa, Y., Wright, A.D., Fukimori, F., Nummy, K.A., Hausinger, R.P., Holben, W.E., Forney, L.J., 1996. Characterization of a chromosomally encoded 2,4-dichlorophenoxyacetic acid alpha-ketoglutarate dioxygenase from *Burkholderia* sp. strain RASC. *Applied and Environmental Microbiology* 62, 2464–2469.
- Tonso, N.L., Matheson, V.G., Holben, W.E., 1995. Polyphasic characterization of a suite of bacterial isolates capable of degrading 2,4-D. *Microbial Ecology* 30, 3–24.
- Top, E.M., Holben, W.E., Forney, L.J., 1995. Characterization of diverse 2,4-Dichlorophenoxyacetic acid-degradative plasmids isolated from soil by complementation. *Applied and Environmental Microbiology* 61, 1691–1698.
- Vallaey, T., PerselloCartieaux, F., Rouard, N., Lors, C., Laguerre, G., Soulas, G., 1997. PCR-RFLP analysis of 16S rRNA, *tfdA* and *tfdB* genes reveals a diversity of 2,4-D degraders in soil aggregates. *FEMS Microbiology Ecology* 24, 269–278.
- Vallaey, T., Courde, L., McGowan, C., Wright, A.D., Fulthorpe, R.R., 1999. Phylogenetic analyses indicate independent recruitment of diverse gene cassettes during assemblage of the 2,4-D catabolic pathway. *FEMS Microbiology Ecology* 28, 373–382.
- Yu, C.P., Chu, K.H., 2005. A quantitative assay for linking microbial community function and structure of a naphthalene-degrading microbial consortium. *Environmental Science & Technology* 39, 9611–9619.
- Yu, C.P., Ahuja, R., Sayler, G., Chu, K.H., 2005. Quantitative molecular assay for fingerprinting microbial communities of wastewater and estrogen-degrading consortia. *Applied and Environmental Microbiology* 71, 1433–1444.