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Enhanced Mineralization of [U-^{14}C]2,4-Dichlorophenoxyacetic Acid in Soil from the Rhizosphere of *Trifolium pratense*

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Enhanced biodegradation in the rhizosphere has been reported for many organic xenobiotic compounds, although the mechanisms are not fully understood. The purpose of this study was to discover whether rhizosphere-enhanced biodegradation is due to selective enrichment of degraders through growth on compounds produced by rhizodeposition. We monitored the mineralization of [U-^{14}C]2,4-dichlorophenoxyacetic acid (2,4-D) in rhizosphere soil with no history of herbicide application collected over a period of 0 to 116 days after sowing of *Lolium perenne* and *Trifolium pratense*. The relationships between the mineralization kinetics, the number of 2,4-D degraders, and the diversity of genes encoding 2,4-D/α-ketoglutarate dioxygenase (*tfdA*) were investigated. The rhizosphere effect on [^{14}C]2,4-D mineralization (50 μg g^{-1}) was shown to be plant species and plant age specific. In comparison with nonplanted soil, there were significant ($P < 0.05$) reductions in the lag phase and enhancements of the maximum mineralization rate for 25- and 60-day *T. pratense* soil but not for 116-day *T. pratense* rhizosphere soil or for *L. perenne* rhizosphere soil of any age. Numbers of 2,4-D degraders in planted and nonplanted soil were low (most probable number, <100 g^{-1}) and were not related to plant species or age. Single-strand conformational polymorphism analysis showed that plant species had no impact on the diversity of α-*Proteobacteria tfdA*-like genes, although an impact of 2,4-D application was recorded. Our results indicate that enhanced mineralization in *T. pratense* rhizosphere soil is not due to enrichment of 2,4-D-degrading microorganisms by rhizodeposits. We suggest an alternative mechanism in which one or more components of the rhizodeposits induce the 2,4-D pathway.

The rhizosphere is the zone of soil directly influenced by the presence of plant roots. It receives inputs of an array of low (e.g., sugars, organic and amino acids, phenolics, and other secondary metabolites)- and high (e.g., cellulose, lignin, mucilage, proteins)-molecular-mass compounds as a result of rhizodeposition and is a zone of complex plant-microbe interactions (56). Seminal work by Hsu and Bartha (23) demonstrated that mineralization of organophosphate pesticides in the rhizosphere is increased relative to that in nonplanted soils. Since this study, numerous reports of rhizosphere-enhanced biodegradation have been published for several classes of organic pollutants, including polyaromatic (2, 35, 48) and aliphatic (5, 38, 48) hydrocarbons, chlorophenols (3, 13), chlorophenoxyacetic acids (3), chlorobenzoates (46), *s*-triazines (32), and surfactants (29).

One suggested reason for rhizosphere-enhanced biodegradation is the possible presence, as a result of rhizodeposition, of high densities of active and diverse heterotrophic microorganisms on root surfaces, a situation which will facilitate complex degradative pathways through activities of consortia and conjugative horizontal transfer of catabolic genes carried on mobile genetic elements (6). Another suggested reason for rhizosphere-enhanced xenobiotic biodegradation is that components of the rhizodeposits (exudates, root materials, or their decomposition products) may be structural analogs for the pollutant in question (6, 20). It follows that the presence of a pollutant analog in the rhizosphere may select for pollutant-degrading microorganisms capable of directly using the analog for growth, act as a cooxidized substrate during cometabolism, or serve as an effector molecule in the induction of xenobiotic catabolic pathways (43). Furthermore, some investigators (45, 47, 57) hold the view that the rhizodeposition of compounds stimulating xenobiotic biodegradation may not be merely a passive process that fortuitously results in enhanced biodegradation. On the contrary, it is suggested that the plant, on sensing the presence of a xenobiotic in its rhizosphere, actively alters the composition of its rhizodeposits to stimulate xenobiotic degradation and that this response is an extension of the plant's defenses against natural allelopathic chemicals. However, despite much speculation regarding the causes, most experimental studies are descriptive and solely report the phenomenon of rhizosphere-enhanced biodegradation. Thus, empirical studies are required in order to dissect the mechanisms of enhanced biodegradation in the rhizosphere. Only if we gain a better understanding of plant-soil-microorganism-pollutant interactions can we rationally exploit the system for effective rhizoremediation of contaminated land.

One xenobiotic for which rhizosphere-enhanced biodegradation has been reported is 2,4-dichlorophenoxyacetic acid (2,4-D) (3), a herbicide widely used for the control of broad-leaved weeds in both agriculture and domestic applications. Many 2,4-D-degrading bacteria have been isolated both from 2,4-D-exposed (9, 26) and from nonexposed (pristine) (24, 27) soils. Of these, the best studied is the β-proteobacterium *Ralstonia eutropha* JMP134(pJP4), originally isolated from 2,4-D-exposed soil (9). The initial step in 2,4-D breakdown, as described for *R. eutropha* JMP134(pJP4), is mediated by an

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TABLE 1. Chemical properties of Sourhope soil[a]

Soil	pH[b]	Total organic carbon (mg g^{-1})[c]	Freundlich parameters for adsorption of 2,4-dichlorophenol[d]		
			K_f	n	K_{OC}
Nonplanted	4.03 (0.007)	24.61 (0.80)	5.75 (1.02)	0.83 (0.006)	233.6
L. perenne rhizosphere	4.02 (0.003)	21.03 (0.51)	5.60 (1.02)	0.82 (0.007)	266.3
T. pratense rhizosphere	4.04 (0.008)	24.52 (0.71)	5.47 (1.01)	0.83 (0.005)	223.1

[a] Soil was sampled from the rhizospheres of *L. perenne* and *T. pratense* after 60 days of plant growth. Values are means (standard errors), $n = 4$.
[b] Determined in $CaCl_2$ after removal of root debris.
[c] Determined by wet oxidation after removal of root debris.
[d] The concentration dependence of 2,4-dichlorophenol adsorption to *L. perenne, T. pratense* (with incorporated root debris), and nonplanted soil was investigated after 1 h (25°C) over triplicate initial aqueous concentrations of 0.2, 2, 10, 50, and 250 μg of [^{14}C]2,4-dichlorophenol ml^{-1}. Data are expressed as the linear form of the Freundlich equation ($r^2 > 0.99$ in all cases), $\log S = \log K_f + n \log A$, where S is the adsorbed concentration (in micrograms per gram), A is the aqueous concentration (in micrograms per milliliter), $\log K_f$ is the intercept, and n is the elasticity of sorption. K_{oc} is calculated as K_f/percent total organic carbon × 100.

α-ketoglutarate-dependent dioxygenanse, encoded by the *tfdA* gene, which cleaves the acetate side chain to produce 2,4-dichlorophenol (15, 16, 49). *tfdA* shows only low homology to one other previously characterized gene (*tauD* of *Escherichia coli* [53]). Other 2,4-D-degrading isolates are distributed among the α, β, and γ subdivisions of the class *Proteobacteria* (24, 27, 34). Isolates in the β- and γ-*Proteobacteria* possess *tfdA*-like genes with more than 76% sequence similarity to the canonical *tfdA* genes in *R. eutropha* JMP134(pJP4) (34). Until recently it was thought that isolates belonging to the α-*Proteobacteria* did not possess *tfdA*-like genes (27, 28, 34), and Kitagawa et al. (28) have characterized a novel gene cluster, *cadRABKC*, that is responsible for 2,4-D degradation in *Bradyrhizobium* sp. strain HW13 but shows no similarity to *tfdA* genes. *cadA* homologs have been detected in other bradyrhizobia and in α-proteobacterial degraders belonging to the genus *Sphingomonas* (28). However, more recent research has reported that slow-growing 2,4-D-degrading bacteria isolated from pristine soils and belonging to the *Bradyrhizobium-Agromonas-Nitrobacter-Afipia* (BANA) cluster within the α-*Proteobacteria* possess *tfdA*-like genes with approximately 60% sequence identity to the canonical *tfdA* gene (25).

The high diversity uncovered in *tfdA*-like genes suggests that the selection for genotypes may have been operating before the first use of 2,4-D in the early 1940s, and several workers have postulated the existence of an ancestral *tfdA* gene encoding a degradative enzyme with the highest affinity for a naturally occurring analog of 2,4-D (11, 17, 22, 25). Indeed, the protein encoded by the *tfdA*-like gene in BANA cluster isolates from pristine soil has a higher affinity for nonchlorinated phenoxyacids than for 2,4-D (25). The facts that pristine soils can mineralize 2,4-D (17) and that 2,4-D-degrading bacteria can be isolated from them (24, 27) suggest that *tfdA*-like genes are maintained for a function other than 2,4-D degradation. Dunning Hotopp and Hausinger (11) tested a range of alternative substrates for canonical TfdA and concluded that the original substrate may have been a plant-derived compound, such as a cinnamic acid derivative. Given the diversity of compounds produced in the rhizosphere, it is reasonable to hypothesize that other natural substrates for TfdA may be found in the rhizodeposits and that this may explain rhizosphere-enhanced mineralization of 2,4-D. In this study we monitored 2,4-D mineralization kinetics in pristine soils collected from the rhizospheres of *Lolium perenne* and *Trifolium pratense* and related them to the number of 2,4-D degraders and the diversity of *tfdA*-like genes. We present evidence to suggest that enhanced mineralization in the *T. pratense* rhizosphere is not due to selection of 2,4-D-degrading microorganisms in the rhizosphere by rhizodeposit analogs of 2,4-D but may be due to induction of the 2,4-D pathway by one or more components of the rhizodeposits.

MATERIALS AND METHODS

Soil. The brown forest soil used in this study was collected from the Macaulay Land Use Research Institute experimental site at Rigg Foot (Sourhope Research Station, Cheviots, Scotland). The Rigg Foot site is upland grassland, dominated by *Agrostis capillaris*, and has not been exposed to 2,4-D. Detailed vegetation and soil data can be obtained from the Natural Environmental Research Council (United Kingdom) Soil Biodiversity website (http://mwnta.nmw.ac.uk/soilbio/baseline98A.htm). Soil samples were collected from a depth of 10 to 40 cm, sealed in sterile polyethylene bags, and transported to the laboratory, where they were sieved (mesh size, <2.8 mm) and stored at 4°C until use. Some properties of the soil are shown in Table 1.

Experimental design. Plants were grown in boiling tubes containing field-moist (0.36 g of H_2O g of dry $soil^{-1}$; 62.3% maximum holding capacity) soil (equivalent dry weight, 12 g). Replicate tubes were planted with 20 seeds of either *L. perenne* or *T. pratense* (Herbiseed, Twyford, England) per tube. As controls, additional tubes were left nonplanted but were otherwise treated identically to the planted tubes. Tubes were closed with polyurethane foam bungs and incubated at 20°C with a light-dark cycle of 16 h of light (4,200 lx) and 8 h of dark. The water content was gravimetrically adjusted to the initial field value with sterile distilled water every 2 days. Toward the end of the time course, plants began to show signs of nutrient stress. Therefore, at 87 and 106 days after planting, tubes for each planting treatment received a solution of NH_4NO_3 and KH_2PO_4 as part of the 2-day watering to give 35, 25, and 30 μg of N, P and K, respectively, g of $soil^{-1}$. Additional tubes received no fertilizer. Four replicate tubes per plant treatment were destructively sampled 0, 25, and 60 days after planting, and three replicates were sampled for each plant × fertilizer treatment on day 116: shoots were excised and discarded, and the root was chopped finely with a sterile scalpel and homogenized with the soil (i.e., the entire below-soil-surface contents of the tube were defined as the rhizosphere). Plants within replicate treatments grew equally well (coefficient of variation for dry weight of shoots, <25%). Subsamples were taken for determination of 2,4-D mineralization potential and microbiological and molecular analysis.

Soil biochemical and microbiological analysis. Soil dehydrogenase activity was determined by the iodonitrotetrazolium chloride (INT; Sigma-Aldrich Co. Ltd., Gillingham, Dorset, United Kingdom) method (51, 55). An aqueous 0.2% (wt/vol) solution of INT was added to the soil sample (dry weight, 0.5 g). Following incubation in the dark (25°C, 48 h), the reaction was terminated by addition of 10 ml of *N,N*-dimethyl formamide–ethanol (1:1, vol/vol), soil was removed by centrifugation, and the absorbance of the supernatant was determined at 464 nm. The amount of INT formezan (INTF) produced was calculated by reference to an INTF calibration curve.

Soil subsamples (dry weight, 0.5 g) were used as the basis for a 10-fold dilution series for the determination of culturable bacterial numbers and most probable numbers of 2,4-D degraders ($MPN_{2,4\text{-}D}$). For culturable numbers, dilutions (50 μl) were spread onto nutrient agar (Oxoid), and plates were incubated at 25°C

and counted after 72 h. For $MPN_{2,4\text{-}D}$, ignition tubes (five replicates for each dilution) containing 1.8 ml of basal medium supplemented with L-[U-^{14}C]2,4-D (0.050 g liter^{-1}; 28.7 kBq liter^{-1}; Sigma-Aldrich Co., Ltd.) were inoculated with dilutions (200 μl). The basal medium (modified from reference 10) was composed of the following (measured in grams per liter unless otherwise stated): Na_2HPO_4 (2.78), KH_2PO_4 (1.0), $CaNO_3 \cdot 4H_2O$ (0.05), $(NH_4)_2SO_4$ (1.0), $MgSO_4 \cdot 7H_2O$ (0.2), Casamino Acids (0.005), and trace element solution (1 ml per liter) (27). To obtain 10^{-1} most-probable-number (MPN) dilutions, soil (0.2 g [dry weight basis]) was weighed directly into ignition tubes. After incubation (9 weeks, 20°C), the remaining radioactivity was quantified, and tubes containing ≤60% of that for the noninoculated controls were scored positive. $MPN_{2,4\text{-}D}$ estimates were derived by reference to MPN tables (1). A dilution series constructed from 25-day harvest soil that had previously been exposed to 2,4-D (50 μg g^{-1}) for 36 days was also used to inoculate MPN tubes.

[U-^{14}C]2,4-D and [U-^{14}C]2,4-dichlorophenol fate. [^{14}C]2,4-D mineralization was determined for soil harvested after 0, 25, 60, or 116 days of plant growth. [^{14}C]2,4-dichlorophenol mineralization was determined for the 25-day harvest sample only. In addition, [^{14}C]2,4-D mineralization kinetics were also recorded for nonplanted soil exposed previously to 2,4-D (50 μg g^{-1}; 36 days). Soil subsamples (4 g [dry weight basis]) in EPA vials (40 ml; gas-tight tetrafluoroethylene-silicone septa; Sigma-Aldrich Co., Ltd.) were amended with [U-^{14}C]2,4-D or [U-^{14}C]2,4-dichlorophenol (50 μg g^{-1}; 250 Bq g^{-1}) in a sufficient volume of sterile distilled water to bring the soil moisture content to 70% of the maximum moisture-holding capacity. A test tube (internal diameter, 75 by 9 mm) containing NaOH (1 ml; 1 M) was placed on the surface of the soil to trap the $^{14}CO_2$ released. Vials were incubated at 25°C. On sampling days, the NaOH solution was taken for quantification of radioactivity and replaced with fresh NaOH solution. At the end of the mineralization experiment (36 days), soil subsamples (0.5 g [wet weight]) were extracted with water (1 ml) by vortexing (30 s), the soil was removed by centrifugation (16,100 × *g*, 5 min), 800 μl of the supernatant was decanted, and 200 μl was used for ^{14}C determination. The water-extracted soil pellet was further extracted with methanol (1 ml, 30 s), 1 ml of the supernatant was decanted, and radioactivity in a 400-μl aliquot was determined. The remaining aqueous and methanolic supernatants were stored at −20°C for high-performance liquid chromatography (HPLC) analysis. The recovery efficiency of this sequential extraction method was tested by using soil freshly spiked with 2,4-D and was found to be 99.7% ± 0.5%.

The extracted soil pellet was dried (105°C), and levels of nonextractable residues were determined by CrO_3 oxidation as described by Dalal (7) by using NaOH (2 ml; 4 M) to trap the $^{14}CO_2$ evolved. In all cases, radioactivity was quantified by using a Beckman LS6000TA Liquid Scintillation system programmed to count each sample for 5 min. UltimaGold (Packard Bioscience, Groningen, The Netherlands) was used as the scintillant in a 1:4 (for aqueous extract and 1 M NaOH trap samples) or 1:10 (for methanolic samples) sample-to-cocktail ratio. HionicFluor (Packard) was used as the scintillant for 4 M NaOH traps (sample-to-cocktail ratio, 1:12).

HPLC analysis of extracts was performed as described previously (44).

DNA template isolation. DNA was extracted from soil exactly according to the method of Griffiths et al. (19) except that bead beating was performed using white quartz sand (0.2 g) mixed with glass beads (0.3 g; 425 to 600 U) (both from Sigma-Aldrich Co. Ltd.) as the matrix and a mini-beadbeater (Biospec Products) set to beat for 30 s at 2,500 rpm.

PCR amplification and cloning. Two primer sets were used: those designed by Vallaeys et al. (52) by sequence alignment of the *tfdA* genes from *R. eutropha* JMP134(pJP4) and *Burkholderia* sp. strain RASC and those (*tfd*Aα1 [5′-CCGG CGTCGATCTGCGCAAG-3′] and *tfd*Aα2 [5′-GTTGACGACGCGCGCCGA CA-3′]) designed to amplify a 359-bp region of the *tfdA*-like gene (encoding 2,4-D α-ketoglutarate-dependent dioxygenase) recently characterized in α-*Proteobacteria* (25) by alignment of complete *tfdA*-like sequences from strains RD5-C2 (GenBank accession number AB074490), HWK12 (AB074491), and HW13 (AB074492). Reaction mixtures contained 1× PCR buffer, 2.5 mM $MgCl_2$, 1× Q solution, 1 U of HotStarTaq DNA polymerase (QIAGEN, Crawley, United Kingdom), deoxynucleoside triphosphate solution (200 μM [each] dATP, dCTP, dGTP, and dTTP), forward and reverse primers (0.4 μM each), 0.5 μl of template DNA, and sterile distilled water up to 25 μl. Reaction mixtures were incubated at 95°C for 15 min to activate the HotStarTaq prior to the start of the temperature program. For the *tfd*Aα primer set, the temperature program was 40 cycles of denaturation (1 min at 94°C), annealing (45 s at 67°C), extension (1 min at 72°C), and final extension (10 min at 72°C). For the Vallaeys et al. (52) primer set, we used a touchdown program: 23 cycles of denaturation (1 min at 94°C), annealing (1 min at 65°C, decreasing by 1°C cycle^{-1} to 42°C), and extension (1.5 min at 72°C), followed by 17 cycles of denaturation (1 min at 94°C), annealing (1 min at 42°C), extension (1.5 min at 72°C), and final extension (10 min at 72°C). The ability of *tfd*Aα1 and -2 to amplify *tfdA*-like gene fragments from soil was confirmed by sequencing. Putative partial *tfdA*-like fragments obtained by PCR amplification of DNA extracted from nonplanted soil before and after 2,4-D treatment (Fig. 5) were ligated into the pGEM-T Easy vector (Promega, Southampton, United Kingdom) and transformed into *E. coli* JM109 competent cells (Promega), according to the manufacturer's instructions. White colonies were checked for inserts by PCR using primers *tfd*Aα1 and -2. Plasmid DNA extracted (QIAprep Spin Miniprep kit; QIAGEN) from six unique (as determined by single-strand conformational polymorphism [SSCP]) randomly selected clones was used as the basis for sequencing (Comfort Read; MWG Biotech, Ebersberg, Germany) using M13 primers.

Analysis of cloned *tfdA*-like sequences. Cloned *tfdA*-like sequences were compared with selected reference *tfdA*-like sequences in the GenBank database. Partial cloned sequences and full reference sequences were aligned by using ClustalX (version 1.81), and reference sequences were trimmed so that only contiguous nucleotides were used for subsequent analysis. A phylogenetic tree was constructed by using the neighbor-joining method and Jukes-Cantor distances (Phylip, version 3.6a3). Bootstrap analysis with 100 replicates was used to place confidence estimates on the tree. *E. coli tauD* (encoding taurine/α-ketoglutarate dioxygenase [53]) was used as an outgroup.

SSCP analysis. The SSCP analysis protocol was based on the method described by Schwieger and Tebbe (41). Prior to electrophoretic analysis, aliquots of PCR products were mixed with denaturing loading buffer (95% formamide, 10 mM NaOH, 0.25% [wt/vol] bromophenol blue, 0.25% [wt/vol] xylene cyanol [41]) in a 1:5 sample-to-buffer ratio. Samples were denatured (95°C for 3 min) and then immediately cooled on ice water. Denatured samples (4 μl) were loaded onto a 0.6X SequaGel MD (National Diagnostics, Hull, United Kingdom) gel (40 by 33 cm with 0.4-mm spacers) and run at 600 V and 21 ± 2°C for 20 h by using a Hoefer SQ3 sequencing tank and an EPS601 (Amersham Pharmacia Biotech) power pack. The gel was silver stained according to the Promega Silver Sequence DNA sequencing system (39). The SSCP gels were composed of sample lanes with several conformer bands per lane. Band positions were assigned and scored either 1, for present, or 0, for absent. The resulting binary matrix was used as the basis for cluster analysis using the matching binary similarity coefficient and single linkage method (Intercooled Stata 7 for Windows; Stata Corporation, College Station, Tex.).

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers AY193866 to AY193870.

RESULTS

Culturable numbers and dehydrogenase activity. In soil harvested after only 25 days of plant growth, plant treatment had a significant effect ($P = 0.006$ by analysis of variance [ANOVA]) on culturable numbers (Fig. 1A). The effect was most pronounced in the *T. pratense* rhizosphere (CFU were 4.0 × 10^7, 1.6 × 10^7, and 5.8 × 10^6 g^{-1} for *T. pratense*, *L. perenne*, and nonplanted soils, respectively). The *T. pratense* rhizosphere also showed significantly increased dehydrogenase activity on day 25 ($P = 0.017$ by ANOVA) and day 60 ($P = 0.013$ by ANOVA) (Fig. 1B). Results for both culturable number and dehydrogenase activity followed the same treatment order (from highest to lowest, *T. pratense*, *L. perenne*, and nonplanted soils).

$MPN_{2,4\text{-}D}$. Before the pristine soil was exposed to 2,4-D, $MPN_{2,4\text{-}D}$ were low (<100 g^{-1}; below detectable limits in some replicates) for material harvested either from nonplanted soil or from *L. perenne*- or *T. pratense*-planted soil (Fig. 2A). In contrast to culturable numbers or dehydrogenase activity (Fig. 1), $MPN_{2,4\text{-}D}$ were not related to planting treatment ($P = 0.063$ by the Kruskal-Wallis test) or harvest day ($P = 0.33$ by the Kruskal-Wallis test) (Fig. 2A). However, soil sampled from 25-day-old rhizospheres or nonplanted controls and incubated in the presence of 2,4-D (50 μg g^{-1}, 36 days) had significantly ($P < 0.001$ by the Kruskal-Wallis test) higher $MPN_{2,4\text{-}D}$ (Fig. 2B) than non-2,4-D-exposed soil (Fig. 2A). In 2,4-D-exposed

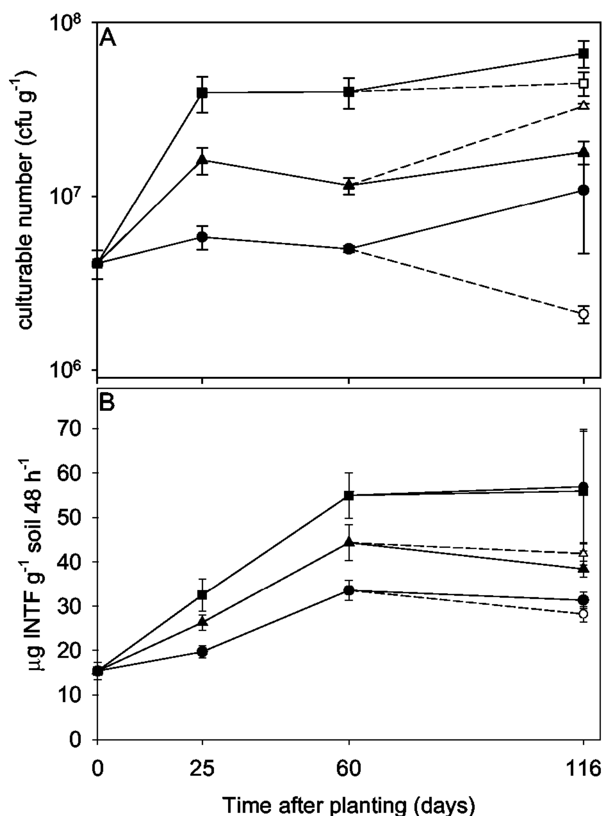

FIG. 1. Effects of plant species and plant age on the number of culturable bacteria (A) and dehydrogenase activity (B) in soil sampled from the rhizosphere of *L. perenne* (▲) or *T. pratense* (■) or in nonplanted controls (●). Soils were fertilized on days 87 and 106 with NH_4NO_3 and KH_2PO_4 to give 35 μg of N g^{-1}, 25 μg of P g^{-1}, and 30 μg of K g^{-1}. Open symbols, fertilized treatments. Error bars, standard errors.

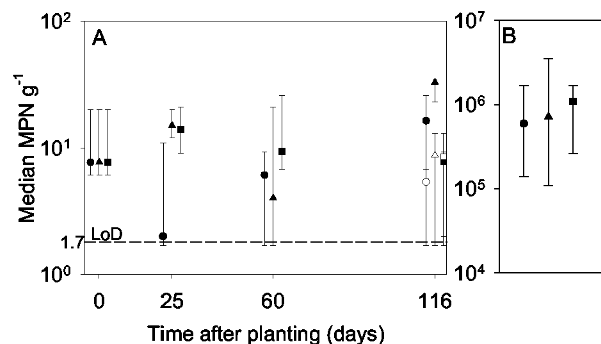

FIG. 2. (A) Effects of plant species and plant age on $MPN_{2,4\text{-}D}$ in pristine soil sampled from the rhizosphere of *L. perenne* (▲) or *T. pratense* (■) or from nonplanted controls (●). Soils were fertilized on days 87 and 106 with NH_4NO_3 and KH_2PO_4 to give 35 μg of N g^{-1}, 25 μg of P g^{-1}, and 30 μg of K g^{-1}. Open symbols, fertilized treatments. (B) $MPN_{2,4\text{-}D}$ in soil sampled from 25-day-old rhizospheres or nonplanted controls and incubated in the presence of 50 μg of 2,4-D g^{-1} for 36 days. Error bars, maximum and minimum values. LoD, limit of detection.

soil, median $MPN_{2,4\text{-}D}$ ranged from 6×10^5 g^{-1} (nonplanted) to 1×10^6 g^{-1} (*T. pratense*), although plant treatment did not have a significant effect ($P = 0.91$ by the Kruskal-Wallis test).

2,4-D and 2,4-dichlorophenol mineralization and 2,4-D ^{14}C mass balance. Cumulative $^{14}CO_2$ evolution from nonplanted, *L. perenne*-planted, or *T. pratense*-planted soil amended either with [U-^{14}C]2,4-D over the entire time course (0 to 116 days) (Fig. 3A to E) or with [U-^{14}C]2,4-dichlorophenol at the day-25 harvest (Fig. 3B) was determined. Mineralization of 2,4-D by nonplanted soil previously exposed to 2,4-D (50 μg g^{-1}, 36 days) is shown for comparison (Fig. 3A). To enable a quantitative comparison of mineralization kinetics, curves were fitted to the function $Y = a[1 + (t/t_0)^b]^{-1}$, and parameters (asymptote [a], time at which the maximum rate is reached [t_1], and maximum rate) were compared for each sampling time and treatment (Fig. 4). The t_1 and rate parameters were calculated from the function as described previously (42). Generally, cumulative mineralization curves were sigmoidal, except for that of 2,4-D in fertilized nonplanted soil (day 116 [Fig. 3E]), where mineralization was minimal (<2.5% mineralized in 36 days). For soils not previously exposed to 2,4-D, 2,4-D mineralization curves were characterized by a lag phase prior to the onset of exponential $^{14}CO_2$ evolution, during which time mineralization occurred at a low and linear rate (~0.2% day^{-1}). This is in contrast to results for soil preexposed to 2,4-D (containing an enriched 2,4-D-degrading population of 6×10^5 MPN g^{-1}) (Fig. 2B), where exponential $^{14}CO_2$ evolution was immediate (Fig. 3A). Nevertheless, independently of whether the soil had previously been exposed to 2,4-D, the percent $^{14}CO_2$ evolution always approached an asymptote of ~70 (Fig. 3 and 4A). The mineralization asymptote was also independent of planting treatment (Fig. 3 and 4A). However, there was a pronounced reduction in the length of the lag phase for *T. pratense*-planted versus *L. perenne*-planted or nonplanted soil, particularly on days 25 and 60 (Fig. 3B and C). The reduction in the lag phase length is reflected by the parameter t_1 (expressed in days). For example, t_1 is 22.0 ± 0.6 or 15.3 ± 0.7 days for day-60-harvested nonplanted or *T. pratense*-planted soil, respectively (Fig. 4B). The rhizosphere of *T. pratense* also had a significant ($P < 0.05$ by ANOVA) effect on the maximum rate of 2,4-D mineralization on harvest days 25 and 60 (e.g., for day 25, the maximum rate for the *T. pratense* rhizosphere was 10.0% day^{-1} while that for nonplanted soil was 7.6% day^{-1} [Fig. 4C]). By day 116, the *T. pratense* rhizosphere effect persisted only in fertilized treatments (Fig. 4B and C).

In contrast to that of 2,4-D, 2,4-dichlorophenol mineralization (Fig. 3B) proceeded after a shorter lag phase (t_1, 5.8 to 6.4 days [Fig. 4B]), but there was no significant effect ($P > 0.05$ by ANOVA) of either *L. perenne* or *T. pratense* planting on any of the parameters describing mineralization (Fig. 4D to F).

Quantification of the fate of the 2,4-D ^{14}C (as $^{14}CO_2$, water-extractable ^{14}C, methanol-extractable ^{14}C, and nonextractable residues released by wet oxidation) at the end of the experiment revealed, except for the fertilized nonplanted soil, no significant difference ($P > 0.1$) in the partitioning of the ^{14}C among the four fractions tested (data not shown). Less than 3% of the ^{14}C could be water or methanol extracted, and the remainder of the ^{14}C (15 to 27%) could be recovered only by wet oxidation. Thus, it is likely that the 2,4-D was completely degraded and that the remaining nonextractable ^{14}C fraction was associated with the soil organic matter and microbial biomass. For nonplanted fertilized soils (day 116), where mineralization was inhibited, the ^{14}C could be accounted for as

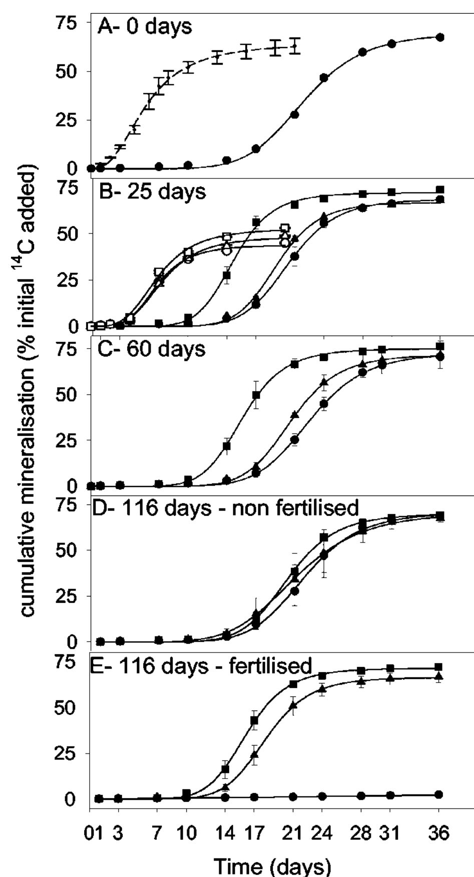

FIG. 3. Effects of plant species, plant age at harvest (0, 25, 60, or 116 days old), and NPK fertilization on mineralization of 2,4-D (solid symbols) or 2,4-dichlorophenol (open symbols) at 50 μg g^{-1} in soil sampled from the rhizosphere of *L. perenne* (▲), the *T. pratense* rhizosphere (■), or nonplanted controls (●). Dashed line in panel A shows mineralization of 2,4-D in nonplanted soil previously exposed to 50 μg of 2,4-D g^{-1} for 36 days. Soils were fertilized on days 87 and 106 with NH_4NO_3 and KH_2PO_4 to give 35 μg of N g^{-1}, 25 μg of P g^{-1}, and 30 μg of K g^{-1}. Error bars, standard errors.

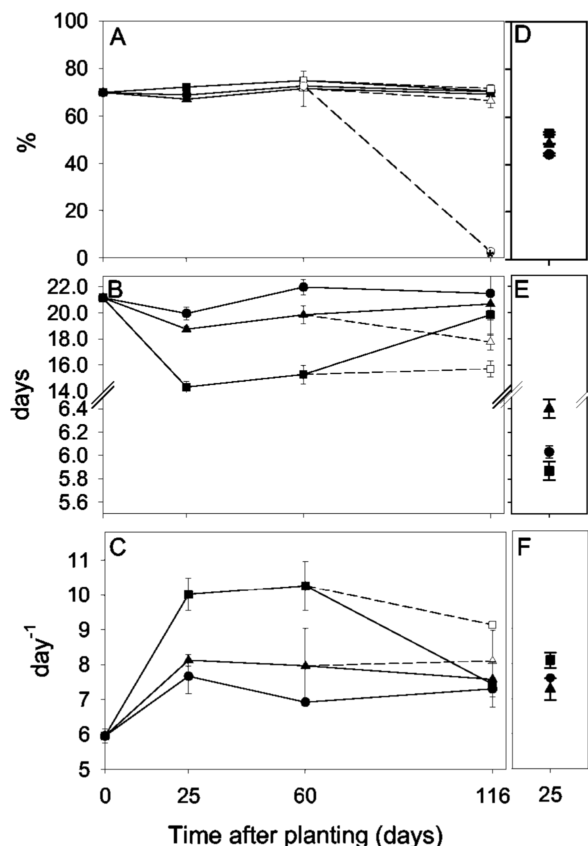

FIG. 4. Effects of plant species and plant age on 2,4-D (A to C) and 2,4-dichlorophenol (D to F) mineralization parameters in soil sampled from the rhizosphere of *L. perenne* (▲), *T. pratense* (■), or nonplanted controls (●). Effects are expressed as asymptotic percentage mineralized (A and D), time point at which the maximum mineralization rate is reached (t_1) (B and E), or calculated maximum rate (C and F). Soils were fertilized on days 87 and 106 with NH_4NO_3 and KH_2PO_4 to give 35 μg of N g^{-1}, 25 μg of P g^{-1}, and 30 μg of K g^{-1}. Open symbols represent fertilized treatments. Error bars, standard errors.

follows: water extractable, 36.7% ± 2%; methanol extractable, 20.2% ± 2%; nonextractable but released by wet oxidation, 50.2% ± 2%. Subsequent HPLC analysis revealed that the water- and methanol-extractable ^{14}C was 2,4-D associated. The reason for the inhibition of 2,4-D transformation and mineralization by NPK fertilization in nonplanted soil is not known, although we speculate that addition of relatively high concentrations of inorganic salts caused osmotic stress. In planted soil, NPK did not have the same detrimental effect, presumably because the plants took up a significant proportion of the fertilizer.

PCR-SSCP analysis of diversity of *tfdA*-like genes. For PCR of DNA extracted from soil, we initially used primers designed by sequence alignment of *tfdA* genes from the β-proteobacterial degraders *R. eutropha* JMP134(pJP4) and *Burkholderia* sp. strain RASC (52). However, we were not able to obtain any PCR products with these primers, even for soil samples that had previously been exposed to 2,4-D (50 μg g^{-1}, 36 days) and, according to the $MPN_{2,4\text{-}D}$ analysis, contained approximately 10^6 degraders g^{-1} (Fig. 2B). In preliminary experiments using template DNA extracted from soil inoculated with a 10-fold dilution series of *Burkholderia* sp. strain RASC (50), we determined that the detection limit for the PCR was approximately 10^4 *tfdA* copies g^{-1} of soil (data not shown). Therefore, if the 2,4-D-degrading population present in the soil possessed the β- and γ-proteobacterial *tfdA*-like gene, we would expect to be able to obtain a PCR product in the 2,4-D-exposed soil. Even when we used DNA extracted from positive $MPN_{2,4\text{-}D}$ tubes as a template, we did not achieve amplification. However, PCR of DNA extracted from soil using the primers (*tfd*Aα1 and -2) designed to target *tfdA*-like genes from α-*Proteobacteria* yielded faint PCR products of the expected size (359 bp) from soil not previously exposed to 2,4-D and more-distinct products from soil exposed to 2,4-D (50 μg g^{-1}) for 36 days (Fig. 5A). Furthermore, DNA was extracted from the highest-dilution (10^{-5} and 10^{-6}) positive $MPN_{2,4\text{-}D}$ tubes set up with 2,4-D-exposed soil sampled at the end of the mineralization experiments. This DNA was used as a basis for PCR with the *tfd*Aα primers; of the 26 samples tested, 22 yielded a product of the correct size, suggesting that the α-proteobacterial *tfdA*-like gene was numerically important in soil exposed to 2,4-D.

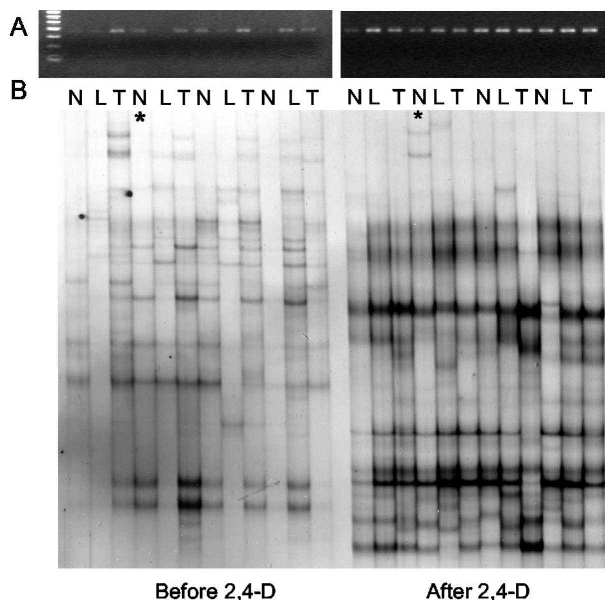

FIG. 5. PCR amplification using *tfd*Aα primers (A) and subsequent SSCP analysis (B) of *tfdA*-like genes in Sourhope soil before and after 2,4-D treatment. Each lane contains a PCR product obtained from DNA extracted from an independent, destructively sampled soil sample that either was not planted (N) or was planted with *L. perenne* (L) or *T. pratense* (T) and was harvested after 25 days. Asterisked lanes contain putative partial *tfdA*-like fragments that were cloned subsequently; clones were sequenced to check primer fidelity.

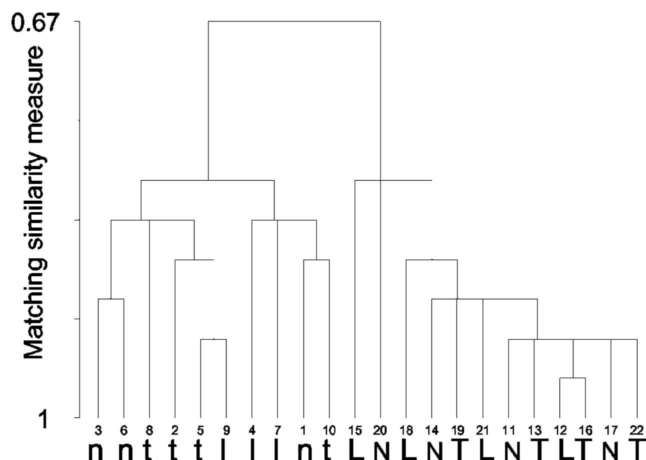

FIG. 7. Hierarchical cluster analysis (using matching binary similarity distances and the single linkage method) of the *tfdA*-like SSCP banding patterns shown in Fig. 5B. N, L, and T, replicate sample lanes from nonplanted, *L. perenne*-planted, and *T. pratense*-planted soils, respectively. Lowercase letters, non-2,4-D-exposed samples; capital letters, 2,4-D-exposed samples.

To check primer fidelity, putative soil *tfdA*-like PCR products were cloned and six clones were sequenced. Phylogenetic comparisons with reference strain *tfdA* genes (representatives of β- and α-proteobacterial degraders) placed the clones on the α-proteobacterial branch of the *tfdA* tree (Fig. 6). The partial *tfdA*-like DNA sequences cloned from soil had 83.2 to 99.4% identity to the common partial *tfdA*-like sequence of the α-proteobacterial strain RD5-C2 (25) (GenBank accession number AB074490).

SSCP analysis of the resulting PCR products revealed differences in the position and abundance of *tfdA*-like conformer bands between non-2,4-D-exposed and 2,4-D-exposed soils which were discernible by eye (Fig. 5B). Indeed, hierarchical cluster analysis of SSCP band patterns (Fig. 7) confirmed that the samples clustered according to 2,4-D exposure history but that plant treatment had no effect on the diversity of *tfdA*-like genes either in non-2,4-D-exposed or in 2,4-D-exposed soils.

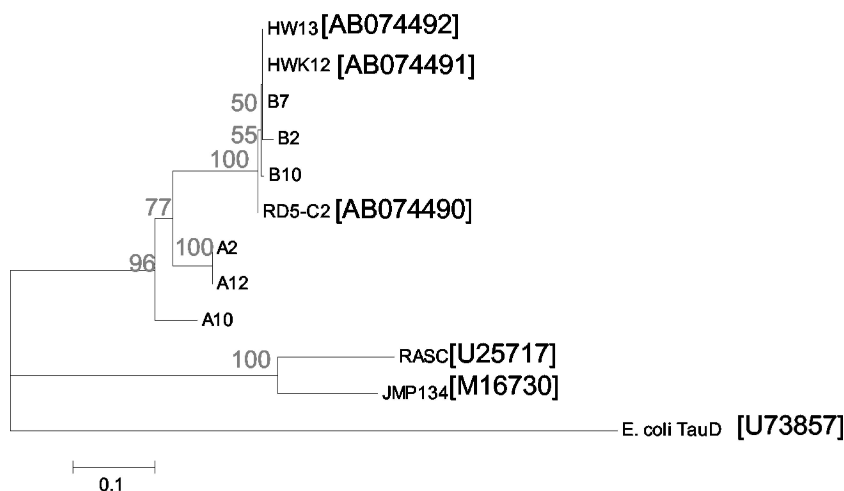

FIG. 6. Phylogenetic positions of *tfdA* sequences cloned from Sourhope soil among *tfdA* sequences from *R. eutropha* JMP134(pJP4), *Burkholderia* sp. strain RASC, and reference strains HW12, HW13, and RD2-C5. Strains HW12, HW13, and RD2-C5 are members of the BANA cluster of the α-*Proteobacteria* most closely related to *Bradyrhizobium* spp. *E. coli tauD* encodes taurine/α-ketoglutarate dioxygenase. The neighbor-joining dendrogram (Jukes-Cantor distances) was constructed from reference sequences and common partial sequences (357 bp) of *tfdA* PCR amplified by using primers *tfd*Aα1 and *tfd*Aα2. Soil clone designations begin with "B" (for "before 2,4-D application") or "A" (for "after 2,4-D application"). Bootstrap confidence limits (percentages) are given at each branch. Scale bar represents a Jukes-Cantor distance of 0.1. GenBank accession numbers are given in brackets after strain designations.

DISCUSSION

After only 25 days of plant growth, culturable microbial numbers and activity were increased in soil planted with *T. pratense* or *L. perenne*. This is known as the "rhizosphere effect," documented by many authors who report the 2- to 10-fold increases in microbial number and activity in planted soil over nonplanted soil seen in our present study (4, 43). The rhizosphere effect was more pronounced in the *T. pratense* than in the *L. perenne* rhizosphere despite the fact that *L. perenne* produced a root system that was more extensive and had greater biomass than that of *T. pratense* (data not shown). The reason for the difference between the microbial properties of *L. perenne*- and *T. pratense*-planted soils is unknown. We were not able to detect any effect of the rhizospheres on the organic carbon content of the soil (Table 1). However, it can be supposed that *T. pratense* produced exudates either in greater quantity (although this was not detected by the wet oxidation organic carbon method) or of superior quality to those of *L. perenne*, or both.

Aside from the rhizodeposit-mediated impact on gross microbial parameters, such as culturable heterotrophs or activity of a global respiratory enzyme (dehydrogenase), our main aim was to dissect the impact of plant roots on 2,4-D mineralization and numbers and diversity of 2,4-D degraders. Our hypothesis was that plant roots deposit compounds to the rhizosphere that are natural analogs of the 2,4-D pathway and enhance 2,4-D biodegradation by serving as either (i) a substrate for 2,4-D catabolic enzymes, (ii) a cooxidized substrate during cometabolism, or (iii) an effector molecule in the induction the pathway. The results of the mineralization assays show that there was indeed an effect of the rhizosphere on 2,4-D biodegradation but that the effect was dependent on plant species (*T. pratense*) and plant age (for nonfertilized soil, the effect was confined to younger [<60-day] plants).

If enhanced 2,4-D mineralization was due to the presence of rhizodeposit 2,4-D analogs that could be broken down by Tfd enzymes and used as a source of carbon for growth, then we would expect *T. pratense* to have an impact on the numbers and diversity of 2,4-D degraders. Although activities and culturable numbers increased in the rhizosphere, $MPN_{2,4\text{-}D}$ in the pristine soil were low (<100 g^{-1}) and were not affected by plant treatment. In contrast, soil previously exposed to 2,4-D (50 μg g^{-1}) gave $MPN_{2,4\text{-}D}$ estimates of $\sim 1 \times 10^6$ g^{-1}, showing that the $MPN_{2,4\text{-}D}$ method used was effective at detecting increases in 2,4-D degrader numbers in response to a selective pressure. Other workers (8) have also shown that MPN assays can sensitively detect the enrichment of 2,4-D degraders in aquifer sediments exposed to phenoxy acid herbicides. If rhizodeposition was selectively enriching the pristine soil, then the rhizosphere-to-nonrhizosphere ratio (R:S) for 2,4-D degraders would exceed that for the total heterotrophs. In an early study, Sandmann and Loos (40) used an MPN method to determine R:S values for 2,4-D degraders in the rhizospheres of sugarcane and African clover. They found that rhizosphere soil sampled from sugarcane, with no previous exposure to phenoxyacetic acid herbicides, had an enriched 2,4-D degrading population ($R{:}S_{2,4\text{-}D} = 105$; $R{:}S_{heterotroph} = 6$). Similarly, Fang et al. (12) reported an enrichment of phenanthrene degraders in soil planted with various grass species. The results of those studies are in contrast to our findings, since we calculate an $R{:}S_{2,4\text{-}D}$ of 4.27 and a $R{:}S_{heterotroph}$ of 7.44 based on day-25 and -60 mean (heterotroph) or median ($MPN_{2,4\text{-}D}$) values for *T. pratense*-planted and nonplanted soils.

The soil used for our experiments was collected from an upland grassland with no history of 2,4-D application. The field site was remote from areas used for arable agriculture and therefore could not have received 2,4-D inputs from short-range spray drift. Since 2,4-D is nonvolatile and hydrophilic, it is unlikely to undergo long-range atmospheric transport and global distillation such as has been reported for other more volatile and hydrophobic xenobiotics (58). Thus, the fact that that this pristine soil had intrinsic 2,4-D mineralization ability (an observation common to other pristine soils [17]) is consistent with the idea that naturally occurring analogs of 2,4-D do exist, can be used directly as substrates for growth (17), and are responsible for the maintenance of the low numbers of 2,4-D-mineralizing microorganisms recorded here. However, the MPN evidence suggests that the putative analog was not an exclusive property of rhizosphere soil and therefore did not result directly from rhizodeposition.

Evidence from PCR analysis of DNA extracted from soil and positive $MPN_{2,4\text{-}D}$ tubes with two different primer sets (that targeting the β- and γ-proteobacterial *tfdA* and that targeting α-proteobacterial *tfdA*) suggests that 2,4-D degraders possessing the α-proteobacterial *tfdA*-like genes were present and numerically important in Sourhope soil. As indicated earlier, *tfdA*-like genes have been characterized only recently (25) in oligotrophic α-proteobacterial 2,4-D degraders isolated from pristine soils of Hawaii, Saskatchewan, and Japan (25, 27). 2,4-D mineralizers have proved difficult to isolate from pristine soils (17, 27), probably because of their slow-growing habits and sensitivity to high concentrations of organic nutrients (27). Indeed, despite many attempts, we were not able to resolve 2,4-D mineralizers from active enrichment cultures. However, using culture-independent PCR and subsequent SSCP analysis of *tfdA*-like PCR products, we were able to demonstrate the presence and also the diversity of *tfdA*-like genes in a pristine Scottish soil, adding further weight to the claim of Kamagata et al. (27) that this new class of 2,4-D degraders is widespread in nature.

We used SSCP analysis to examine the effect of the rhizosphere on the sequence diversity of *tfdA*-like genes. In our analysis, we also included soil from the three plant treatments that had previously been exposed to 2,4-D. We found that prior 2,4-D exposure had a pronounced effect on the diversity of 2,4-D-degradative genes, thus demonstrating the ability of the method to detect the impact of a selective pressure on the 2,4-D-degrading population. However, no plant treatment effect on diversity was detectable either in nonexposed or in 2,4-D-exposed soils. Taken together, the $MPN_{2,4\text{-}D}$ and PCR-SSCP evidence suggests that the rhizosphere does not exert a selective pressure on 2,4-D-degrading microorganisms. Rather the 2,4-D degraders present in Sourhope soil form a near-constant proportion of the soil community (both in numbers and in composition) and, in the absence of 2,4-D, are oblivious to the presence of a plant root. When 2,4-D is added to the soil, $MPN_{2,4\text{-}D}$ and PCR-SSCP evidence suggests that a subpopulation of 2,4-D degraders initially present in soil utilizes 2,4-D for growth; however, the composition of this population

of 2,4-D degraders also is not affected by plant treatment. In other words, the same subpopulation is degrading 2,4-D in the three treatments, but it is able to mineralize with a shorter lag time and at a higher rate in soil previously planted with *T. pratense*.

If the plant root (at least in the plant-soil system used in the present study) does not exert a direct selective pressure as a growth substrate on either the number or the diversity of 2,4-D degraders, an alternative explanation for the enhanced mineralization of 2,4-D in soil harvested from the *T. pratense* rhizosphere is required. Alternative explanations (alluded to earlier) include the involvement of an analog as an inducer or cometabolite, rhizodeposit-mediated promotion of the growth of 2,4-D degraders, or the operation of less-specific mechanisms such as rhizosphere-enhanced horizontal transfer of 2,4-D catabolic genes or the action of phytosurfactants to increase the concentration and bioavailability of 2,4-D in the soil aqueous phase. We think that some of these explanations are unlikely. First, the exponential kinetics of 2,4-D mineralization and the recorded increase in $MPN_{2,4\text{-}D}$ point toward a growth-linked, not a cometabolic process. Second, mating experiments with α-proteobacterial 2,4-D-degrading strains (HW13, HWK12, HWK13, and BTH) have shown that the *tfdA*-like gene is not transmissible (27). Third, there was no evidence of a bioavailability effect, as we could detect no difference in the parameters describing the partitioning of 2,4-dichlorophenol between the soil solid and aqueous phases (Table 1).

Therefore, one explanation not discounted relates to rhizodeposit-mediated promotion of the growth of 2,4-D degraders. For example, since it has been shown that TfdA has affinity for nonchlorinated substrates (11, 25), it may be that, once induced by the presence of 2,4-D, TfdA (or other 2,4-D pathway enzymes) was able to accept *Trifolium* rhizodeposits as substrates, thereby enhancing the growth rate of 2,4-D degraders. However, the explanation we favor is that a component of the rhizodeposits is able to act as an inducer of the *tfd* pathway. The regulation of the pathway in the newly discovered *tfdA*-like-gene-possessing α-proteobacterial degraders has not yet been characterized. Until we know more, we can only discuss the possibility of a rhizodeposit inducer in the context of our knowledge of the canonical *tfd* pathway in β-*Proteobacteria*. We know this pathway to be regulated by a LysR-type transcriptional regulatory protein encoded by *tfdR* (and, in JMP134, the identical *tfdS* gene) (31, 33, 54). The inducing effector molecule of the pathway is thought to be the intermediate 2,4-dichloromuconate (14). Presumably, on initial exposure to 2,4-D, low-level constitutive expression of the *tfd* pathway results in intracellular accumulation of the effector molecule and full induction of the pathway. In the present study, the low linear rate of $^{14}CO_2$ evolution during the lag phase and prior to the onset of exponential mineralization (Fig. 3) would be consistent with this process. It is reasonable to hypothesize that muconate derivatives will be present in the rhizosphere as a result of microbial metabolism of aromatic rhizodeposits. Leigh et al. (30) have shown that a variety of phenolic compounds are produced in the rhizosphere, particularly during the decay of dead roots. *cis,cis*-Muconate is the product of catechol metabolism via the central *ortho*-cleavage pathway (37), and many substituted phenols and aromatic acids (produced by rhizodeposition) could be degraded through this route, thus producing nonchlorinated *cis,cis*-muconates (21). Ogawa et al. (36) have reported that the LysR-type regulator of chlorocatechol degradation in *R. eutropha* can be activated by both chlorinated and nonchlorinated muconate (36). Thus, we consider it likely that an analog of 2,4-dichloromuconate produced in the *T. pratense* rhizosphere may activate transcription of the *tfd* genes by interaction with the regulatory TfdR-like protein.

2,4-D mineralization kinetics were both plant species and plant age specific. Boyle and Shann (3) also reported a specific plant effect on 2,4-D biodegradation: mineralization was enhanced to a greater extent in soil collected from a monocotyledon (e.g., *L. perenne*) rhizosphere than in soil from a dicotyledon (e.g., *T. pratense*) rhizosphere. This is the inverse of the findings of our present study. Because Boyle and Shann (3) did not determine the numbers or diversity of 2,4-D degraders in their study, it is difficult to explain the discrepancy in the results. However, in agreement with our work, Boyle and Shann found that the rhizosphere effect was specific for the first (*tfdA*-encoded) step of 2,4-D degradation, as there was no effect of either the monocot or dicot rhizosphere on the mineralization of the first metabolite of 2,4-D breakdown, 2,4-dichlorophenol. The quality and quantity of rhizodeposition are known to differ between plant species and also to be dependent on the growth stage of the plant (18). Thus, the rhizosphere effect of *T. pratense* may be due not solely to root death, as suggested by Leigh et al. (30), but also to more actively extruded components of the rhizodeposits. In order to narrow down the identity of the active compound(s) in the *T. pratense* rhizodeposits, we are currently conducting experiments to identify in which fraction of the rhizodeposits it predominates (exudates versus root structural compounds) and to examine what edaphic factors (e.g., pH, nitrogen content) control its production.

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