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Natural selection for 2,4,5-trichlorophenoxyacetic acid mineralizing bacteria in agent orange contaminated soil

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Abstract

Agent Orange contaminated soils were utilized in direct enrichment culture studies to isolate 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and 2,4-dichlorophenoxyacetic acid (2,4-D) mineralizing bacteria. Two bacterial cultures able to grow at the expense of 2,4,5-T and/or 2,4-D were isolated. The 2,4,5-T degrading culture was a mixed culture containing two bacteria, *Burkholderia* species strain JR7B2 and *Burkholderia* species strain JR7B3. JR7B3 was able to metabolize 2,4,5-T as the sole source of carbon and energy, and demonstrated the ability to affect metabolism of 2,4-D to a lesser degree. Strain JR7B3 was able to mineralize 2,4,5-T in pure culture and utilized 2,4,5-T in the presence of 0.01% yeast extract. Subsequent characterization of the 2,4-D degrading culture showed that one bacterium, *Burkholderia* species strain JRB1, was able to utilize 2,4-D as a sole carbon and energy source in pure culture. Polymerase chain reaction (PCR) experiments utilizing known genetic sequences from other 2,4-D and 2,4,5-T degrading bacteria demonstrated that these organisms contain gene sequences similar to *tfdA*, *B*, *C*, *E*, and *R* (Strain JRB1) and the *tftA*, *C*, and *E* genes (Strain JR7B3). Expression analysis confirmed that *tftA*, *C*, and *E* and *tfdA*, *B*, and *C* were transcribed during 2,4,5-T and 2,4-D dependent growth, respectively. The results indicate a strong selective pressure for 2,4,5-T utilizing strains under field condition.

Introduction

Herbicide Orange, which is more commonly known as Agent Orange, was frequently used as a defoliant in the United States and was sprayed in large quantities in Southeast Asia during the Vietnam War. Agent Orange is composed of a mixture of 50% 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) and 50% 2,4-dichlorophenoxyacetic acid (2,4-D). The broadleaf herbicide 2,4-D has been found to be rapidly degraded in the environment, and the microorganisms capable of biodegrading 2,4-D are common in all geographical locations examined (Tiedje et al. 1997). The metabolic pathways involved in 2,4-D degradation have predominately been found in the α, β, and γ-*Proteobacteria*, and are located on large catabolic plasmids and/or within the chromosome. One of the best-studied bacterial systems for 2,4-D degradation is found in the β-*Proteobacteria*, *Wautersia* (formerly *Ralstonia*) (Vaneechoutte et al. 2004) eutropha JMP134 (Harker et al. 1989). *W. eutropha* JMP134 harbors a large ~80 kb catabolic plasmid designated, pJP4, containing the genes necessary for conversion of 2,4-D to β-ketoadipate which is subsequently metabolized

to succinate and acetyl-CoA by chromosomally encoded enzymes (Fulthorpe et al. 1995). This degradative pathway has been extensively studied by many laboratories around the world, and researchers are now beginning to understand the molecular mechanisms involved in degradation of 2,4-D in this organism.

Two bacteria have been demonstrated to grow on 2,4,5-T as a carbon and energy source. The first and best studied is the bacterial strain *Burkholderia cepacia* AC1100 (Daubaras et al. 1996; Kilbane et al. 1982), and the second is *Nocardiodes simplex* strain 3E which was isolated in 1990 by Golovleva et al. (1990). Both of these bacteria were isolated from enrichment cultures after many months of incubation under artificial laboratory selection conditions. *Burkholderia cepacia* AC1100, the postulated product of plasmid assisted molecular breeding (Kellogg et al. 1982) mediates complete mineralization of 2,4,5-T (Amy et al. 1985) in liquid culture and a complete 2,4,5-T metabolic pathway for *B. cepacia* AC1100 has now been elucidated (Gis & Xun 2003).

A fundamental goal of this research was to determine the potential for natural selection and bacterial catabolism of 2,4,5-T and 2,4-D associated with in Agent Orange contaminated soils. A scheme was devised consisting of two primary objectives: (1) to isolate and identify 2,4,5-T and 2,4-D degrading bacteria present in Agent Orange contaminated soils through isolation and subsequent phenotypic and genotypic analyses, and (2) to characterize 2,4,5-T and 2,4-D degradation by environmental bacteria isolated from Agent Orange contaminated soils. An understanding of the metabolic potential present in these contaminated soils will facilitate risk assessment and potential cleanup strategies at polluted sites containing mixtures of 2,4,5-T and 2,4-D.

In this paper, we report the rapid and direct isolation, identification, and characterization of a 2,4,5-T mineralizing bacterium, *Burkholderia* species strain JR7B3 and a 2,4-D degrading bacterium, *Burkholderia* species strain JRB1, from Agent Orange contaminated soils. Using primers specific for amplification of the *tfdA*, *B*, *C*, *E* and *tftA*, *C*, *E* genes, these bacteria were shown to contain genes similar to those previously characterized for 2,4-D degradation by *W. eutropha* JMP 134 and 2,4,5-T degradation in *B. cepacia* AC1100, respectively.

Materials and methods

Bacteria, plasmids, media, and chemicals

Escherichia coli strain DH5α (Invitrogen, San Diego, California) was grown in Luria Bertani broth (10 g/l tryptone, 10 g/l NaCl, 5 g/l yeast extract) and used in combination with the pCR 2.1 (Invitrogen, San Diego, California) plasmid vector for all cloning experiments as described by the manufacturer. *Wautersia eutropha* JMP 134 (formerly *Ralstonia eutropha* JMP134, Amy et al. 1985) and *B. cepacia* AC1100 (Kilbane et al. 1982) were grown in phosphate-buffered minimal salts medium (PAS), containing in grams per liter; 4.43 g K_2HPO_4, 1.72 g KH_2PO_4, 2.16 g NH_4Cl, 0.195 g $MgSO_4{\cdot}H_2O$, 0.01 g $FeSO_4{\cdot}2H_2O$, and 0.003 g $CaCl_2{\cdot}2H_2O$ (pH 7.2). Solidified PAS medium was prepared by addition of 15 g/l of Agarose (Difco) added prior to autoclaving. The large catabolic plasmid, pJP4 (Don & Pemberton 1985) was isolated from *W. eutropha* JMP 134 using the alkali lysis method as described by Sambrook et al. (1989). Quarter strength YEPG medium (0.25 g dextrose, 0.5 g polypeptone, 0.05 g yeast extract, 0.05 g NH_4NO_3 per liter; pH 7.0) was used for all heterotrophic colony counts. 2,4-dichlorophenol (2,4-DCP, 99%) and 2,4-D (99 + %) were purchased from Acros Orgainics (Pittsburgh, PA). 2,4,5-trichlorphenol (2,4,5-TCP, 99%) was purchased from Fluka Chemicals (Milwaukee, WI). 2,4,5-T (97%) was obtained from Aldrich chemical Company, Inc. (Milwaukee, WI). N,N-dimethylformamide (DMF, 99.9%), 2,4,5-T-ring-UL-^{14}C, and 2,4-D-ring-UL-^{14}C were purchased from Sigma Chemical Company (St. Louis, MO). Acetonitrile (HPLC grade) and Water (HPLC grade) were obtained form Fisher Scientific. All other chemicals were of analytical grade.

Soil characterization

Two soil samples contaminated with Agent Orange were received from Dr. James Cornette (Tyndall Air Force Base, Florida). These samples were obtained November 1996 and were stored at 4 °C prior to enrichment experiments which were initiated in July 1997. These soil samples are from a loading/unloading area at Hardstand 7, Eglin Air Force Base (Niceville, Florida). The

approximate depths of these samples were 0 in. (surface soil, SS) and 6 in. (core soil, CRS). A control soil (CS) was also obtained which was not contaminated with this herbicide. The 2,4,5-T, 2,4-D, 2,4,5-TCP, and 2,4-DCP concentrations were approximated by washing the soil samples using a hexane:acetone (1:1) solution and subsequent analysis of these extractions using HPLC as described below.

Enrichment cultures

Enrichment cultures were used to isolate 2,4,5-T and 2,4-D-degrading bacteria from Agent Orange contaminated soils. Briefly, 50 ml of PAS medium was combined with 5 g of soil from a surface contaminated soil for one set of cultures and contaminated soil from a 6 in. core sample for a second set of samples. To these soil slurries, 100 μl of a 500 mg/ml stock solution of 2,4,5-T or 2,4-D dissolved in DMF was added. These soil slurries were incubated at 28 °C and 150 rpm. After one week of incubation, aliquots of the soil slurries were serial diluted and plated on PAS media amended with the appropriate carbon source. These plates were incubated at 28 °C and monitored for colony formation. Additionally, all enrichment culture samples were plated on PAS media without 2,4,5-T or 2,4-D to control for false positives. Soil slurries were also sub-cultured into fresh PAS media containing the appropriate chemical and incubated for another week. Bacterial colonies that formed during the enrichment experiments, were re-inoculated into PAS liquid medium (without yeast extract) amended with the respective carbon source and grown to an approximate optical density (600 nm) of 0.25. Culture samples (1.0 ml) were routinely amended with 1.0 ml of 30% glycerol and frozen at −80 °C for long-term storage and further analyses.

Mineralization assays

About 0.5 ml samples from freezer stocks were washed two times with 1 × PAS medium and used to inoculate starter cultures containing 50 ml of PAS medium amended with 1 mg/ml of 2,4,5-T or 2,4-D. These starter cultures were incubated at 28 °C until mid-log growth phase was achieved (an optical density of 0.2 at 600 nm). From these starter cultures 0.5 ml was transferred to 250 ml Erlenmeyer flasks containing 50 ml of fresh PAS medium containing 1 mg/ml 2,4,5-T or 2,4-D. These cultures were allowed to incubate at 28 °C until mid-log phase was attained and then 5.0 ml was transferred to 40 ml EPA mineralization vials. An 8 ml glass vial, containing 0.5 ml of freshly prepared 0.5 N NaOH, was then inserted into the EPA mineralization vial, and these vials were sealed using open top screw caps fitted with Teflon-lined rubber septa. To each reaction vial of 2,4,5-T-ring-UL-^{14}C (89,074 CPM-JR7B and 106,287 CPM-JR7B3) or 2,4-D-ring-UL-^{14}C (93,223 CPM – JRB1) was then added. For each experiment, an abiotic control and a biotic control was prepared. Abiotic controls consisted of PAS media amended with 1 mg/ml 2,4,5-T or 2,4-D without any added cells. Biotic controls were prepared in the same manner as the test samples except that prior to addition of the 2,4,5-T-ring-UL-^{14}C or 2,4-D-ring-UL-^{14}C 200 μl of 2 N H_2SO_4 was added. All reaction vials were then incubated in the dark at 28 °C for 7–21 days. Test cultures were then amended with 200 μl of 2 N H_2SO_4 and incubated for an additional 2 h. ^{14}C concentrations associated with biomass, liquid media and evolved CO_2 was determined using a Beckman LS 5000 TD scintillation counter (Beckman, Fullerton, CA).

PCR analyses

PCR primers were developed using nucleic acid sequences obtained from the Genbank sequence database (National Center for Biotechnological Information, NCBI, http://www.ncbi.nlm.nih.gov). Specific primer sequences were chosen with the help of the Primer3 software algorithm (Whitehead Institute Center for Genome Research, http://www.genome.wi.mit.edu) and Blast 2.0 algorithm (NCBI). Primer sequences are listed in Table 1. All primers were synthesized using a Beckman oligo 100 DNA synthesizer (Beckman Instruments, Fullerton, CA) according to the manufacture's directions.

A modified touchdown PCR protocol (Hecker & Roux 1996) was used for amplification of 400–2300 bp nucleic acid sequences. PCR amplification was achieved using PCR beads (Amersham Pharmacia, Piscataway, New Jersey) according to manufacture's recommendations. Initial PCR analyses were conducted using whole bacterial cells from solid media. One small bacterial colony

Table 1. PCR primers used in this study

Target gene	Primers	Primer target region	Target accession number	Target size
tfdR - tfdA	L1 5′-cgt tcg tag tgc agg tgc-3′	52–69	M16730	*1610*
	R1 5′-ggt gcg tac atc ttg tgg-3′	1662–1647		
tfdA	*tfdA* L2 5′-cga aga cat cga cct tcg ag-3′	795–814	M16730	*589*
	tfdA R2 5′-gcc gat gaa gag aaa ctt gc-3′	1383–1364		
tfdB	*tfdB* L1 5′-tgt tag tca atg cgt ttc gtg-3′	4010–4030	M35097	*2218*
	tfdB R1 5′-cgt ctc acc tcg aat aga tcg-3′	6227–6207		
tfdC	*tfdC* L1 5′-caa gga tgt tgt cga tgc g-3′	351–369	M35097	*591*
	tfdC R1 5′-gcc ggg ata tga aac gtt gac c-3′	921–942		
tfdE	*tfdE* L1 5′-gtc gct ttg gtg cct acc t-3′	2328–2346	M35097	*655*
	tfdE R1 5′-tcc ttc aac att gca agc ag-3′	2982–2963		
tftA	*tftA* L1 5′-agc tcg att ctg gtg tac gg-3′	7–26	U80795	*798*
	tftA R1 5′-cgc att cag atc aag caa ga-3′	804–785		
tftC	*tftC* L1 5′-ggt cca gca gct caa gaa ag-3′	909–928	U83405	*522*
	tftC R1 5′-tat tcc gcg agc gat cta gt-3′	1430–1411		
tftE	*tftE* L1 5′-gtg acg tcg tgc ttt act cg-3′	71–90	U19883	1299
	tftE R1 5′-cta caa acg cga acc atc at-3′	1369–1350		
tftE	*tftE* L2 5′-gac aac cta tgc ggg tag tga-3′	642–662	U19883	617
	tftE R2 5′-cat gtc tgc agc gag ctc ta-3′	1257–1238		

was transferred to a sterile 200 μl thin wall PCR tube (Perkin Elmer, Foster City, CA) and combined with the following components: 1.5 μl Primer A (2.0 μM stock), 1.5 μl Primer B (2.0 μM stock), 23.5 μl ultrapure H_2O, and 1 PCR bead. Subsequent amplification of cloned PCR products was achieved using 1.5 μl Primer A (2.0 μM stock), 1.5 μl Primer B (2.0 μM stock), 22.5 μl ultrapure H_2O, 1.0 μl Template DNA (100 ng), and 1 PCR bead. All PCR products were stored at −20 °C prior to cloning, sequencing, and future analyses.

PCR products were cloned using Invitrogen's TA cloning kit (Invitrogen, San Diego, California) according to manufacturer's directions, and analyzed further. Inserts matching the molecular weight of the expected DNA band were sequenced by the Molecular Biology Resource Facility (The University of Tennessee, Knoxville) using either T7 or M13 based sequencing primers. Sequences obtained were analyzed using the Blast algorithm (NCBI) in order to determine similarities to published gene sequences.

RT-PCR analyses

To determine if the identified genetic sequences were expressed RT-PCR was utilized. Starter cultures containing 50 ml of PAS medium amended with 1 mg/ml of 2,4,5-T or 2,4-D were incubated at 28 °C until mid-log growth phase was achieved (an optical density of 0.2 at 600 nm). These starter cultures were the source of an inoculumn for test cultures. Briefly, 0.5 ml was transferred to 250 ml Erlenmeyer flasks containing 50 ml of PAS medium containing 1 mg/ml 2,4,5-T or 2,4-D. These cultures were allowed to incubate at 28 °C until mid-log phase was attained. RNA was then extracted using the Hot Phenol method, as previously described (Fleming et al. 1993). Rifampicin was used in some cases as an additional PCR control as previously described (Nagel et al. 1999). Reverse transcription reaction components were as follows: 200 μM dNTP, 5.0 mM DTT, 50 units of MMLV reverse transcriptase (Gibco BRL, Grand Island, New York), 1 × MMLV reaction buffer, 0.4 μM random primer (AAA GGA) and 1.0 μg of total RNA was used as the template. Reaction conditions for the reverse transcription were: an initial ramping down from 55 to 40 °C for primer annealing, reverse transcription at 37 °C for 60 min, and final 5.0 min incubation at 75 °C for enzyme inactivation. Reverse transcription reactions were stored at −20 °C prior to PCR. PCR beads (Amersham Pharmacia, Piscataway,

New Jersey) were used for subsequent PCR amplification. One PCR bead was combined with 21 μl HPLC grade H_2O, 1.5 μl of 2.0 μM primer L1, 1.5 μM of 2.0 μM primer R1, and 1.0 μl reverse transcription reaction. A modified touchdown PCR protocol (Hecker & Roux 1996) consisted of an initial denaturation at 92 °C for 4.0 min, 20 cycles of PCR including a denaturation at 92 °C for 15 s, annealing at (65–45 °C, 1 °C degrees/cycle) for 30 s, and primer extension at 72 °C for 30 s. Finally, a 7.0 min extension was followed by storage at 4 °C until gel electrophoresis.

Strain identification

Bacterial strains obtained from enrichment cultures were identified using 16S rRNA analysis. PCR was used to amplify 16S rDNA from total chromosomal DNA preparations using 1492r and 27f primers (Lane 1991; Weisburg et al. 1991). In some cases, PCR amplications were accomplished using whole bacterial cells obtained directly from plates with similar results. Briefly, 100 ng of genomic DNA was used as the template for PCR amplification using these primers (stock concentrations of 2 μM). PCR conditions consisted of 40 cycles of 95 °C (5 min), 50 °C (30 s), and 72 °C (2 min), followed by a final extension time of 7 min at 72 °C and storage at 4 °C. Subsequently, 10 μl of the PCR product was electrophoresed through a 1.5% agarose gel to verify the specificity of the PCR amplification, and samples with bands of the correct size were cloned and sequenced using primers 27f, 1492r, 907r, 926f, and 530f (Lane 1991).

RISA-RFLP analysis

Ribosomal intergenic spacer analysis (RISA) coupled with Restriction fragment length polymorphism (RFLP) was utilized to examine relatedness among members of the 2,4,5-T degrading bacterial consortia. For these studies a modified touchdown PCR protocol (Hecker & Roux 1996) was used for amplification of the intergenic region between the 23S and 16S rDNA. Specific primers for PCR amplification included 1055f (5′-ATG GCT GTC GTC AGC T-3′) and 23Sr (5′-CAA GGC ATC CAC CGT-3′). PCR was achieved using PCR beads (Amersham Pharmacia, Piscataway, New Jersey) according to manufacture's recommendations. PCR analyses were conducted using whole bacterial cells from solid media. One small bacterial colony was transferred to a sterile 200 μl thin wall PCR tube (Perkin Elmer, Foster City, CA) and combined with the following components: 1.5 μl Primer A (2.0 μM stock), 1.5 μl Primer B (2.0 μM stock), 23.5 μl ultrapure H_2O, and 1 PCR bead. The PCR conditions consisted of an initial denaturation at 92 °C for 4.0 min, 20 cycles of PCR including a denaturation at 92 °C for 15 s, annealing at (65–45 °C, 1 °C degrees/cycle) for 30 s, and primer extension at 72 °C for 30 s, followed by a final extension time of 7 min at 72 °C and storage at 4 °C. To the 25 μl PCR was added 3 μl of an appropriate 10X-reaction buffer and 1.0 μl each of either the *HHaI* and *RsaI* or *MspI* and *AluI* endonucleases. These digestions were incubated at 37 °C for 3 h, and analyzed using electrophoresis.

HPLC analyses

Concentrations of 2,4,5-T, 2,4-D, 2,4,5-TCP, and 2,4-DCP in culture supernatants were determined using HPLC. About 100 μl supernatant samples were taken from growing bacterial cultures and transferred to 1.5 ml microcentrifuge tubes. These were then centrifuged at approximately 16,100 $\times$ *g* to pellet bacterial cells, and the supernatant was frozen at −80 °C for HPLC studies. Immediately prior to analysis, samples were warmed at 37 °C for 5 min and 70 μl was transferred to an 1.8 ml amber autosampler vial containing 730 μl HPLC grade H_2O and 50 μg 4-bromobenzoic acid (4-BBA, internal standard) dissolved in 200 μl acetonitrile. Conditions for HPLC analysis were optimized for detection of the above compounds in 20% acetonitrile: 80% H_2O. Samples were eluted at a flow rate of 1.0 ml/min with 0.025% H_3PO_4-acetonitrile gradient, as follows: 0% acetonitrile (isocratic, 5.0 min), 0–50% acetonitrile (linear, 5.0 min), 50% acetonitrile (isocratic, 20 min), 50–0% acetonitrile (linear, 5.0 min). Absorbance of eluted compounds was monitored at 210 nm. Sample retention times were compared to known standards prepared and analyzed using identical protocols. Retention times (minutes) for compounds of interest were 4-BBA (16.2), 2,4-D (17.1), 2,4-DCP (18.0), 2,4,5-T (19.2), and 2,4,5-TCP (21.5).

Results

Soil characterization

HPLC analyses of Agent Orange contaminated soil samples approximated the 2,4,5-T and 2,4-D concentrations of these soils to be 73.8 mg/kg and 22.4 mg/kg, respectively, and very low concentrations of 2,4,5-TCP and 2,4-DCP were detected (<1.0 ppm). Total heterotrophic colony counts demonstrated that these soils contained $\sim 3.3 \times 10^7$ cfu/g for the CS, $\sim 5.2 \times 10^7$ cfu/g for the surface soil, and $\sim 2.8 \times 10^6$ cfu/g for the core soil.

Isolation of 2,4,5-T and 2,4-D degrading bacteria

Traditional enrichment culture experiments (<2 weeks), using these Agent Orange contaminated soils, readily resulted in the recovery of two bacterial cultures capable of using either 2,4,5-T or 2,4-D as a sole source of carbon and energy. These cultures were designated Agent Orange enrichment culture JR7B and JRB1, respectively. Transmission electron micrographs of JRB1 cultures revealed an organism of approximately $1.2\ \mu m \times 0.6\ \mu m$ in size. Similar electron micrographs of JR7B revealed several different sized bacterial cells. Further, subculturing of these colony types identified one bacterial strain, designated Strain JR7B3 which was able to mediate degradation of 2,4,5-T in the presence of additional nutrients (0.01% yeast extract). The other bacterial strain was designated JR7B2 and demonstrated no 2,4,5-T or 2,4,5-TCP transformation capacity. Transmission electron micrographs of Strain JR7B3 revealed a bacterial cell approximately $1.5\ \mu m \times 0.7\ \mu m$ in size. RISA coupled with RFLP analysis of JR7B3 and JR7B2 demonstrated that each one of these organisms had a unique banding pattern and therefore were genotypically distinct from each other (Figure 1). RISA-RFLP analysis of Strain JR7B3 and *B. cepacia* AC1100 revealed that these organisms might be closely related as evidenced by identical banding patterns (Figure 1).

16S phylogenetic analysis revealed that the 16S rRNA of the 2,4-D degrading bacterium (Strain JRB1, Table 2) was 97% similar to *Burkholderia glathei* (accession number U96935). Phylogenetic analyses of strain JR7B2 and strain JR7B3 revealed that they were 99% similar to *Burkholderia caribiensis* (accession number Y17009) and 97% similar to *Burkholderia graminis* (accession number U96941), respectively (Table 2).

Analysis of 2,4,5-T and 2,4-D degradation by bacteria from Agent Orange contaminated soil

Degradation of 2,4,5-T and 2,4-D by *Burkholderia* species strain JR7B3 and *Burkholderia* species strain JRB1, respectively, was examined using

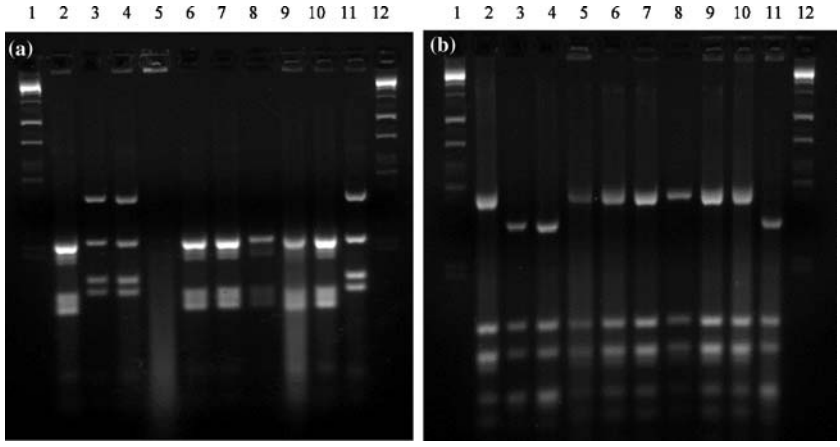

Figure 1. RISA-RFLP analysis of a 2,4,5-T degrading Agent Orange enrichment culture containing *Burkholderia* species strain JR7B2 and *Burkholderia* species strain JR7B3. PCR was used to amplify the 23S-16S intergenic region using primers 1055f and 23Sr. PCRs were then digested using *HhaI* + *RsaI* (a) or *MspI* + *AluI* (b) endonucleases. Incubation was 3 h at 37 °C. Lane designations are as follows: 1 kb ladder (1, 12), culture JR7B (2), strain JR7B3 (3), unidentified colony (4), strain JR7B2 (5), unidentified colony (6), colony G7 (7), colony N7 (8), colony 2 (9), colony 5 (10), *B. cepacia* AC1100 (11).

Table 2. Results from 16S sequence analysis of bacterial isolates obtained from Agent Orange soil enrichments (AOE)

Organism	Accession numbers for AOE sequences	Region of similarity	Similarity score	Name/accession number
AOE JRB1	AF439776	30–1447	1379/1420 (97%)	*Burkholderia glathei* U96935
AOE JR7B2	Not Submitted	525–999	475/476 (99%)	*Burkholderia caribiensis* Y17009
AOE JR7B3	AF439777	35–1430	1370/1398 (97%)	*Burkholderia graminis* U96941

PCR primers 27f, 1492r, 907r, 926f, and 530f were used to amplify 16S segments from strains *Burkholderia* species strain JRB1, *Burkholderia* species strain JR7B2, and *Burkholderia* species strain JR7B3.

mineralization and growing cell assays. Degradation studies utilizing *Burkholderia* species strain JRB1 demonstrated that 2,4-D was metabolized as a sole carbon and energy source (Figure 2). Mineralization assays confirmed strain JRB1 specific conversion of 2,4-D-ring-UL-^{14}C to CO_2 (~65.8%, Table 3). Similar degradation experiments demonstrated that the Agent Orange enrichment culture JR7B (bacterial mixed culture containing strains JR7B2 and JR7B3) metabolized 2,4,5-T as a sole carbon and energy source (Figure 3). Minearalization experiments confirmed culture JR7B specific conversion of 2,4,5-T-ring-UL-^{14}C to CO_2 (~79.2%, Table 4). Unexpectedly, the JR7B enrichment culture also mediated mineralization of 2,4-D-ring-UL-^{14}C (32.1%, Table 3). Mineralization experiments using only strain JR7B3 demonstrated conversion of 49.2% of 2,4,5-T-ring-UL-^{14}C to CO_2 during growth in the presence of 2,4,5-T and 0.01% yeast extract (Table 4). During the course of the mineralization experiments, a portion of the radiolabeled 2,4,5-T and 2,4-D substrates was unrecovered (predominantly 2,4,5-T). Specifically, this was due to assay difficulties resulting from the acidification procedure in which unmetabolized substrate precipitated. This also increased the experimental variability associated with these experiments.

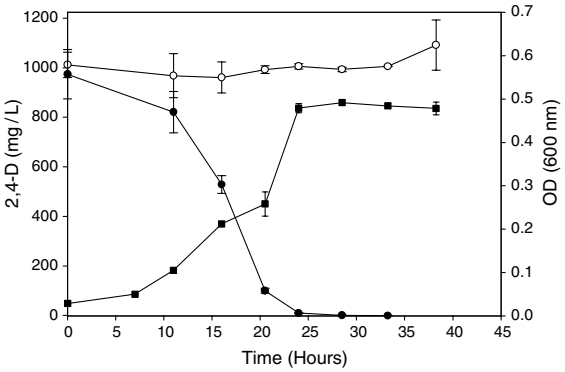

Figure 2. Burkholderia species strain JRB1 2,4-D degradation and growth curves. Bacterial cells were grown in PAS media supplemented with 1000 mg/l 2,4-D. 2,4-D concentrations for the bacterial culture (•) and an abiotic control (○) were quantified using HPLC analysis. Bacterial biomass (■) was determined by measuring the optical density of the culture (600 nm) using a DU-70 Spectrophotometer. All data points represent the average of three separate experiments. Error bars represent the standard deviation between these data.

Characterization of 2,4-D and 2,4,5-T degradative genes

PCR analyses of Strain JRB1 revealed that the *tfdA*, *B*, *C*, and *E* genes were present in this organism (Data Not Shown). Sequence analysis and subsequent bioinformatic studies of these PCR products revealed that these gene sequences were highly similar to 2,4-D degradative genes previously identified for strain *W. eutropha* strain JMP 134 (Table 5). The *tfdA* fragment sequence was 96% similar to the previous published *W. eutropha* sequence (accession number M16730). The PCR amplification of this gene was designed to target the intergenic region between the *tfdR* and the *tfdA*, and the size of the expected sequence was identical to that predicted from JMP 134. From this fragment, a 129 bp sequence of the *tfdR* was found to be 100% homologous to the JMP 134 sequence. PCR products were identified which were 99, 98, and 98% similar to previously published *tfdB*, *tfdC*, and *tfdE* gene sequences (accession numbers M35097, M35097, and M35097, respectively).

Examinations of strain JR7B3 by PCR demonstrated that gene sequences similar to the *tftA, C,* and *E* genes were present in this organism (Data Not Shown). None of the *tft* genes identified in

Table 3. 2,4-Dichlorophenoxyacetic acid mineralization assays for bacteria from Agent Orange contaminated soil

	CO_2	Biomass/solids	Supernatant	Total recovery
JRB1 culture	65.76 ± 7.7	14.41 ± 2.52	15.3 ± 3.04	95.48 ± 11.42
JRB1 culture + H_2SO_4	2.49 ± 0.27	5.64 ± 6.78	65.93 ± 8.28	72.25 ± 15.89
Abiotic control	1.45 ± 0.21	4.25 ± 7.36	69.49 ± 22.63	74.1 ± 16.15
JR7B culture	32.08 ± 3.26	6.94 ± 6.01	39.45 ± 0.67	75.07 ± 10.24
JR7B culture + H_2SO_4	3.77 ± 0.45	3.96 ± 5.56	76.44 ± 8.00	83.16 ± 15.08
Abiotic control	3.12 ± 0.88	23.24 ± 2.88	48.71 ± 6.68	75.07 ± 10.24

Values represent percentage of ^{14}C detected (CPM) as evolved CO_2, cell biomass/solids, or culture supernatants. Each data point represents the average of three individual experiments.

strain JR7B3 were present in strain JR7B2 (Data Not Shown). Identified genes were found to be similar to those previously described for *B. cepacia* AC1100 (Table 5). PCR amplification products from these studies corresponded with expected band sizes associated with control amplifications using *B. cepacia* AC1100 (Data Not Shown). Subsequent cloning, sequencing, and computer analysis revealed gene sequences which were 97% similar to the 104–508 bp region of the *tftA* gene, 96% similar to the 909–1331 region of the *tftC* gene, and 98% similar to the 1010–1349 region of the *tftE* gene (Table 5).

Messenger RNA analysis for 2,4,5-T and 2,4-D degrading bacteria

PCR primers designed to examine degradative genes in *Burkholderia* species strain JRB1 and *Burkholderia* species strain JR7B3 were used to examine mRNA expression levels for the *tfdA, B, C* and the *tftA, C, E* genes to determine if these gene sequences were expressed during growth on 2,4,5-T and 2,4D, respectively. Expression analysis of *tfdA*, *tfdB*, and *tfdC* demonstrated that the these genes were transcribed during growth on 2,4-D (Figure 4a, b, and c) and that the size of the observed PCR product corresponded with amplification of control DNA (pJP4). *tfdA* and *tfdC* mRNA transcripts were not detected during growth on succinate alone (Figure 4a and b), although *tfdB* mRNA transcripts were observed (Figure 4c). Analogous experiments using the 2,4,5-T degrading consortia JR7B demonstrated that the *tftA*, *C*, and *E* genes were expressed during growth on 2,4,5-T as a sole carbon and energy source (Figure 5a, c, and b). These experiments also demonstrated that the *tftA*, *C*, and *E* genes were not expressed during growth on succinate as the sole carbon and energy source (Figure 5a, c,

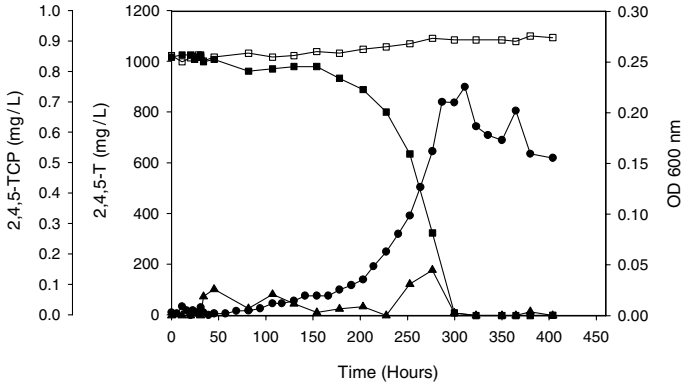

Figure 3. 2,4,5-T degradation and growth curves for the Agent Orange enrichment culture JR7B. The enrichment culture was grown in PAS media supplemented with 1000 mg/l 2,4,5-T. HPLC was used to quantify 2,4,5-TCP (▲) and 2,4,5-T for both the bacterial culture (■) and an abiotic control (□). The culture's optical density (●) was determined spectrophotometrically (600 nm).

Table 4. 2,4,5-Trichlorophenoxyacetic acid mineralization assays for bacteria from Agent Orange contaminated soil

	CO_2	Biomass/solids	Supernatant	Total Recovery
JR7B culture	79.18 ± 4.32	7.31 ± 1.28	5.83 ± 2.97	88.22 ± 5.56
JR7B culture + H_2SO_4	0.27 ± 0.005	9.29 ± 1.8	46.67 ± 7.55	53.72 ± 6.24
Abiotic control	2.68 ± 0.01	6.04 ± 0.77	31.71 ± 6.06	36.33 ± 6.47
JR7B3 culture	49.24 ± 4.09	4.84 ± 0.16	2.94 ± 0.13	57.02 ± 4.38
JR7B3 culture + H_2SO_4	0.23 ± 0.01	48.21 ± 1.03	47.13 ± 1.25	95.57 ± 2.28
Abiotic control	0.11 ± 0.03	50.41 ± 1.30	50.21 ± 0.85	100.74 ± 2.17

Values represent percentage of ^{14}C detected as evolved CO_2, cell biomass/solids, or culture supernatants. Each data point represents the average of three individual experiments.

and b). Note that in Figure 5d 16S control amplifications are similar for 2,4,5-T, 2,4,5-T + rifampicin, and succinate cultures. Decreases in 16S amplification product for succinate + rifampicin cultures probably indicate RNA degradation associated with this sample.

Discussion

This study describes the isolation and characterization of two *Burkholderia* species capable of mediating mineralization of 2,4,5-T and 2,4-D. One mixed culture (Agent Orange enrichment culture JB) was identified which was capable of mineralizing both 2,4-D (32.1%) and 2,4,5-T (79.2%). Strain JR7B3 was isolated from this mixed culture and was capable of mineralizing 2,4,5-T (49.2%) during nutrient dependent growth in the presence of 2,4,5-T. A second apparently pure bacterial culture containing *Burkholderia* species strain JRB1 was also identified which was capable of mineralizing 2,4-D. The 16S sequence of strain JR7B3 was found to be 97% similar to *B. graminis* and contained genes similar to those involved in 2,4,5-T metabolism by *B. cepacia* AC1100. The 16S sequence of the 2,4-D degrading bacterium, strain JRB1, is 97% similar to *B. glathei*. Strain JRB1 was shown to completely degrade 2,4-D during growth on this compound as a sole carbon and energy source. Strain JR7B3 was able to mineralize 2,4,5-T in the presence of additional growth nutrients or in association with a mixed culture containing strain JR7B2. Transmission electron micrographs of these bacterial strains demonstrated similar cell morphologies as those observed for *B. cepacia* AC1100. One notable difference was the presence of exopolysaccharide in association with *B. cepacia* AC1100 cells during growth on PAS containing 1000 mg/l 2,4,5-T.

Agent Orange contaminated soil samples were the source of inoculation for these bacterial

Table 5. Summary of important bioinformatic information obtained from analysis of sequenced PCR products

Clone designation	Size of fragment analyzed*(bp)	Accession number for Strain B1 and 7B3 sequences	Similarity score	Gene	Accession number for known genes
B1A(2)	129	AF439768	129/129 (100%)	*tfdR (477–605)*	S80112
B1A(2)	404	AF439769	359/373 (96%)	*tfdA (1293–1611)*	M16730
B1B(5)	310	AF439770	143/144 (99%)	*tfdB (6052–6194)*	M35097
B1C(6)	590	AF439771	583/592 (98%)	*tfdC* (351–942)	M35097
B1E(8)	655	AF439772	648/655 (98%)	*tfdE (2328–2982)*	M35097
WA(13)	513	AF439773	400/411 (97%)	*tftA (104–508)*	U11420
7C	433	AF439774	417/433 (96%)	*tftC (909–1331)*	U83405
7E	350	AF439775	338/343 (98%)	*tftE (1010–1349)*	U19883

*Sequences include the gene and any untranslated regions outside gene boundaries which were amplified during PCR.

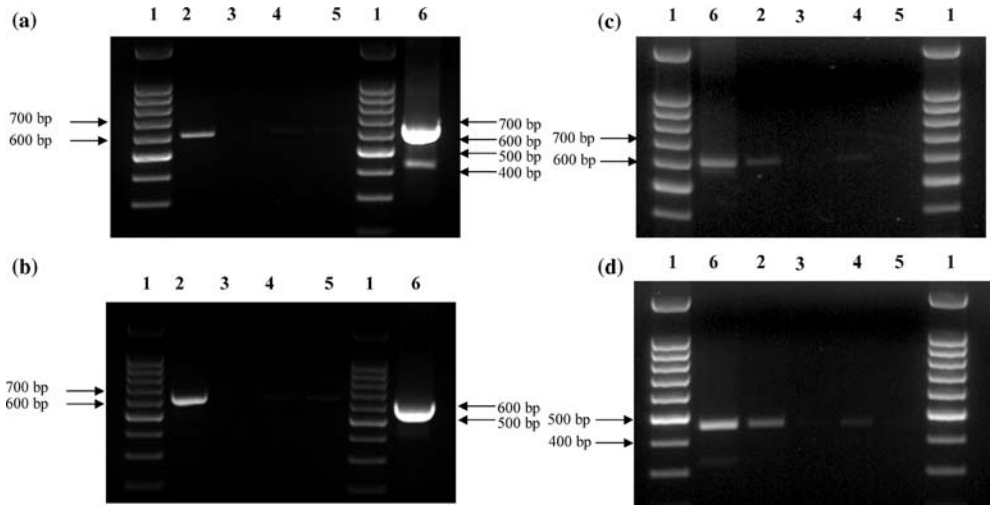

Figure 4. RT-PCR analysis of *tfdA*, *tfdB*, and *tfdC* in *Burkholderia* species strain JRB1. Figure illustrates agarose gel electrophoresis of RT-PCR using specific primers for (a) *tfdA*, (b) *tfdC,* (c) *tfdB*, (d) 16S rRNA. For these studies total RNA was isolated from cultures containing: (2) PAS + 2,4-D, (3) PAS + 2,4-D (Rif), (4) PAS + succinate, and (5) PAS + succinate (Rif). Control DNA for each primer was included (6), and 100 bp DNA ladders (1) were used for size references. The abbreviation ''Rif'' denotes cultures that were treated with rifampicin for 64 min prior to total RNA extraction.

strains. These soils originated from a loading/unloading area and were historically heavily contaminated with Herbicide Orange. 2,4,5-T and 2,4-D concentrations were approximately 73.8 and 22.4 mg/kg of soil, and it is therefore possible that much of the chlorophenoxy-herbicide remaining in these soils is recalcitrant to bacterial degradation due to sorption to the soil organic

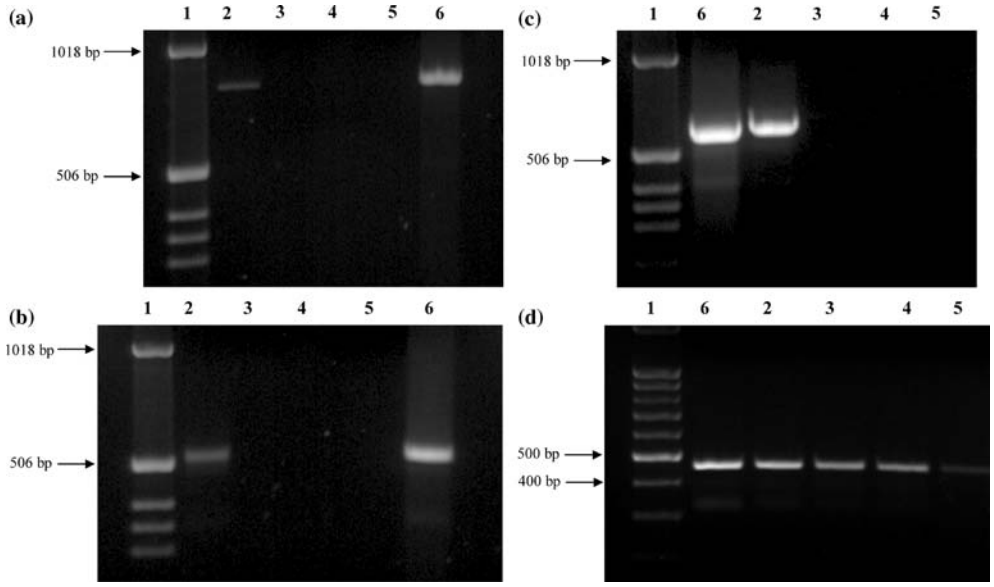

Figure 5. RT-PCR analysis of *tftA*, *tftC*, and *tftE* in Agent Orange enrichment culture JR7B. Figure illustrates gel electrophoresis of RT-PCR using specific primers for (a) *tftA*, (b) *tftC,* (c) *tftE*, (d) 16S rRNA. For these studies total RNA was isolated from cultures containing: (2) PAS + 2,4,5-T, (3) PAS + 2,4,5-T (Rif), (4) PAS + succinate, and (5) PAS + succinate (Rif). Control DNA for each primer was included (6), and 100 bp DNA ladders were used for size references. The abbreviation ''Rif'' denotes cultures that were treated with rifampicin for 64 minutes prior to total RNA extraction.

matter. Isolation of 2,4-D and 2,4,5-T degrading bacterial cultures from these soils required very little enrichment culturing, and growth was apparent after the first week for 2,4-D degrading cultures and after 3 weeks for 2,4,5-T degrading cultures. Growth of 2,4-D degrading bacteria (JRB1) was fairly rapid on PAS minimal media plates containing 1000 mg/l 2,4-D with visible colonies appearing within 2–3 days. Growth of 2,4,5-T degrading bacteria (JR7B3) was slower with visible colonies appearing after 1–1½ weeks. Additionally, the Agent Orange enrichment culture JR7B was able to form minute colonies on 2,4-D plates after 2–2½ weeks.

Genetic analysis of these bacterial strains using PCR identified 2,4,5-T and 2,4-D degradative genes. Strain JRB1 was shown to contain genes that were similar to previously published 2,4-D degradative genes. Gene sequences identified in this strain had regions of high similarity to the *tfdA* (96%), *tfdB* (99%), *tfdC* (98%), *tfdE* (98%), and *tfdR* (100%). Genetic elements similar to published sequences were also found in the 2,4,5-T degrading Strain JR7B3. Specifically, these included genic regions are similar to the *tftA* (97%), *tftC (96%)*, and *tftE (98%)* genes. Furthermore, expression analysis confirmed 2,4,5-T and 2,4-D specific transcription for all genes, respectively, except for *tfdB*. The observed expression of *tfdB* during succinate specific growth of JRB1 suggests either a basal level of expression or activation of the *tfdB* gene under these growth conditions.

This study is the first to demonstrate that 2,4,5-T and 2,4-D degrading bacteria can co-exist in Agent Orange contaminated soils. Previous studies have relied on prolonged selective conditions within the laboratory, such as plasmid assisted molecular breeding (Kilbane et al. 1982), to allow bacteria to adapt to growth on 2,4,5-T as a sole carbon and energy source. It has been postulated that microbial consortia may be more widely spread in association with 2,4-D breakdown in the environment than has previously been thought (Tiedje et al. 1997). Based on these experiments, this certainly seems to be likely for 2,4,5-T degradation as well.

In the original 'plasmid-assisted molecular breeding' experiments giving rise to *B. cepacia* AC1100 (Kilbane et al. 1982) unspecified microbial cultures from undefined waste sites, including Love Canal and Eglin Air Force Base, along with strains harboring catabolic plasmids were cultivated via chemostats selection (Kellogg et al. 1981). After 8 months of cultivation with 500 mg/l 2,4,5-T a degradative mixed culture was achieved. Given the results reported from this present study and the fact that both studies apparently used Agent Orange contaminated soils from Eglin Air Force Base, it is not clear that strain AC1100 necessarily evolved through the laboratory molecular breeding approach. Possibly, such cultivation conditions may have selected for resident organisms such as strain JR7B3 which based on this current study appears to have evolved naturally in 2,4,5-T contaminated soil. Given more than 20 year time differential between the two investigations it could also be argued that natural evolutionary processes generating strain JR7B3 accomplished the same as molecular breeding, however this is indeterminate. It is interesting that most of the important metabolic potential necessary for 2,4,5-T transformation were found in a single bacterium. Further analysis of these bacterial strains will allow the potential dynamic interaction of 2,4,5-T and 2,4-D degradation pathways to be studied. Results from such studies have the potential for facilitating bioremediation and bioaugmentation efforts at sites contaminated with 2,4,5-T and 2,4-D mixtures.

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