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# Pristine Environments Harbor a New Group of Oligotrophic 2,4-Dichlorophenoxyacetic Acid-Degrading Bacteria

YOICHI KAMAGATA,[1,2]* ROBERTA R. FULTHORPE,[1]† KATSUNORI TAMURA,[1,2]‡ HIDETO TAKAMI,[1,2]§ LARRY J. FORNEY,[1] AND JAMES M. TIEDJE[1]

*NSF Center for Microbial Ecology, Michigan State University, East Lansing, Michigan 48824,[1] and Research and Development Corporation of Japan, Chiyoda-ku, Tokyo 100, Japan[2]*

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**2,4-Dichlorophenoxyacetic acid (2,4-D)-degrading bacteria were isolated from pristine environments which had no history of 2,4-D exposure. By using 2,4-D dye indicator medium or $^{14}$C-labeled 2,4-D medium, six strains were isolated from eight enrichment cultures capable of degrading 2,4-D. Phylogenetic analyses based on 16S ribosomal DNA (rDNA) sequencing and physiological properties revealed that one isolate from Hawaiian volcanic soil could be classified in the genus *Variovorax* (a member of the β subdivision of the class *Proteobacteria*) and that the other five isolates from Hawaiian volcanic soils, Saskatchewan forest soil, and Chilean forest soil have 16S rDNAs with high degrees of similarity to those of the *Bradyrhizobium* group (a member of the α subdivision of the class *Proteobacteria*). All the isolates grow slowly on either nutrient media (0.1× Bacto Peptone-tryptone-yeast extract-glucose [PTYG] or 0.1× Luria broth [LB] medium) or 2,4-D medium, with mean generation times of 16 to 30 h, which are significantly slower than previously known 2,4-D degraders. Nutrient-rich media such as full-strength PTYG and LB medium did not allow their growth. PCR amplification using internal consensus sequences of *tfdA* (a gene encoding an enzyme for the first step of 2,4-D mineralization, found in pJP4 of *Alcaligenes eutrophus* JMP134 and some other 2,4-D-degrading bacteria) as primers and Southern hybridization with pJP4-*tfdA* as a probe revealed that the isolate belonging to the genus *Variovorax* carried the *tfdA* gene. This gene was transmissible to *A. eutrophus* JMP228 carrying a plasmid with a mutant *tfdA* gene. The other five isolates did not appear to carry *tfdA*, and 2,4-D-specific α-ketoglutarate-dependent dioxygenase activity could not be detected in cell lysates. These results indicate that 2,4-D-degrading bacteria in pristine environments are slow-growing bacteria and that most of their phylogenies and catabolic genes differ from those of 2,4-D degraders typically isolated from agricultural soils or contaminated environments.**

2,4-Dichlorophenoxyacetic acid (2,4-D) is an anthropogenic chemical that is used as a broad-leaf herbicide. Since no compounds analogous to 2,4-D have been found so far in nature, the origin of the initial genes in this pathway is unknown. A number of 2,4-D degraders have been isolated (4, 9, 12, 17, 22, 23, 25), and most of the strains that have been well characterized fall into two groups. One group is composed of various genera in the β and γ subdivisions of the class *Proteobacteria* that contain 2,4-D-degrading genes similar to the *tfd* genes found in the well-studied strain *Alcaligenes eutrophus* JMP134 (5, 6, 10, 14, 20, 21, 24, 26, 27, 30). The *tfd* genes are often encoded on transmissible plasmids, and they appear to have been horizontally spread at least among these two phylogenetic subdivisions (16, 31). The other group is composed of strains in the α subdivision of the class *Proteobacteria*, to date mostly in the genus *Sphingomonas* (17–19). These strains do not hybridize to *tfdA*, *tfdB*, or *tfdC* genes, nor do they contain the α-ketoglutarate-dependent 2,4-D dioxygenase enzyme (TfdA) found in the other group. Hence, the pathway and genes involved in 2,4-D degradation in this group are unknown. Strains from both of these groups have been isolated from environments that have encountered chlorinated chemicals, primarily agricultural soils, sediments, and waste treatment facilities. These strains are reasonably easily isolated and grow readily in culture.

2,4-D degraders have yet to be isolated from uncontaminated environments with no prior history of 2,4-D exposure, however. If such 2,4-D-degrading isolates are obtained, we may be able to gain insight into the evolutionary origin of the genes. In a recent study, we evaluated 2,4-D and 3-chlorobenzoate (3CBA) degradation in 668 pristine soil samples from six regions of the world (13). These soils had not been disturbed by human activity and had no evidence of exposure to pesticides or other xenobiotics. Both chemicals were degraded in these soils, although 2,4-D degradation typically occurred after a longer lag period and occurred in fewer samples (59%) than did 3CBA degradation. More surprising, however, was the fact that only five 2,4-D degraders could be isolated, even after several approaches were used, while 610 3CBA degraders could be isolated. Hence, the organisms responsible for 2,4-D degradation in these pristine sites appear to be different from those in the two above-mentioned groups from disturbed sites. The failure to isolate 2,4-D degraders from the pristine soils showing 2,4-D mineralization could be because the organisms have special nutritional or other cultural requirements, are sensitive to moderate concentrations of 2,4-D, or are members of consortia or because the degradation is by cometabolism. In this paper, we report a new group of slow-growing, oligotrophic, 2,4-D-degrading bacteria that were isolated from these and other pristine soils.

* Corresponding author. Present address: National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Tsukuba, Ibaraki 305, Japan. Phone: 81-298-54-6026. Fax: 81-298-54-6005. E-mail: kamagata@nibh.go.jp.

† Present address: Division of Physical Sciences, University of Toronto at Scarborough, Scarborough, Ontario M1C 1A4, Canada.

‡ Present address: Institute of Molecular and Cellular Biosciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan.

§ Present address: Deep-Sea Microorganisms Research Group, Japan Marine Science and Technology Center (JAMSTEC), Kanagawa, Yokosuka 237, Japan.

## MATERIALS AND METHODS

**Soil samples.** Eight soil samples were chosen from the global soil sample collection studied by Fulthorpe et al. (13); four showed 2,4-D mineralization in the previous study, and four did not. These samples were collected from six different geographic regions: central California, southwestern Australia, southwestern Africa, central Chile, northern Saskatchewan (Canada), and northwestern Russia. Sites were chosen in nature preserves, remote areas, wilderness parks, or areas for which there was no documentation of exposure to chloroaromatic pesticides and no history of cultivation, excavation, or transportation activity. The sampling protocol and properties of each soil sample, including pH, moisture content, carbon content, and texture, are described elsewhere (13). Twenty-two soil samples were from the isolated, young land mass of Hawaii. Seventeen samples were from the chronosequence sites studied by Crews et al. (2). These are all volcanic (basalt) ash-derived soils that support montane rain forests of native vegetation, 1,200 m in elevation, with a relatively constant mean temperature (16°C) and rainfall distribution (2,500 mm/year). The sites and their soil ages (i.e., the amount of time since they were formed by volcanic activity) are as follows: Thurston, 200 years; Laupahoehoe, 20,000 years; Kohala, 150,000 years; and Kokee, 4 million years.

The other five Hawaiian samples were taken from a semiarid region in Volcano National Park (Kipuka Keana Bihopa) from two soils buried by lava flows which occurred prior to human contact. These specimens were obtained by digging horizontally into the soil under the lava rock caps from a vertical cliff face (Hilina pali) that had formed by the fracture of the lower slope, exposing the edges of these buried soils. These soils are isolated from surface leaching and new plant carbon by 2 to 5 m of solid lava rock that overlays each soil layer (8). The ages of the soil carbon in the two soil layers sampled, as determined by radiocarbon studies, are 4,800 and 10,700 years (8). The former layer is 7 to 8 m below the soil surface and is covered by three lava rock layers, and the latter is 11 to 12 m deep and is covered by four rock layers. Water flow is primarily vertical through rock fractures. The sampled soil remains moist due to high humidity resulting from the absence of evaporation and transpiration. The buried soils were low in organic matter (1.2% for the 4,800-year-old site) compared to the surface soil. The surface vegetation is seasonal submontane woodland which has been invaded by African grasses.

All soil samples were collected from 5 to 20 cm below the soil surface, transferred into aseptic bags, and stored at −4°C until use. Intersample contamination was carefully avoided by ethanol and flame sterilizing the trowels and corers between samples.

**Media and reagents.** The preparation of the basal medium (2,4-D medium) used for enrichment, isolation, and cultivation of isolates throughout the experiments was described previously (13, 29). This medium contained (in grams per liter, unless otherwise noted) 2,4-D (0.025 to 0.125), $KH_2PO_4$ (1.36), $Na_2HPO_4$ (1.41), $(NH_4)_2SO_4$ (0.3), $MgSO_4 \cdot 7H_2O$ (0.05), $CaCl_2 \cdot H_2O$ (0.0058), trace metal solution (5 ml/liter), and agar (for plates only, 20). The trace metal solution contained (in grams per liter) $FeSO_4 \cdot 7H_2O$ (0.55), $ZnSO_4 \cdot 7H_2O$ (0.23), $MnSO_4 \cdot 7H_2O$ (0.34), $Co(NO_3)_2 \cdot 6H_2O$ (0.075), $CuSO_4 \cdot 5H_2O$ (0.047), and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.025).

[$^{14}$C]2,4-D-amended agar medium contained 0.5 μCi of $^{14}$C-ring-labeled 2,4-D per 25 ml of the 2,4-D medium described above. The dye indicator agar medium (2,4-D-BTB medium) for isolation of 2,4-D degraders contained (in grams per liter, unless otherwise noted) 2,4-D (0.125), Casamino Acids (CA; 0.025), yeast extract (YE; 0.025), $KH_2PO_4$ (0.136), $Na_2HPO_4$ (0.141), $(NH_4)_2SO_4$ (0.12), $MgSO_4 \cdot 7H_2O$ (0.02), $CaCl_2 \cdot H_2O$ (0.0023), bromothymol blue (BTB; 0.05), trace metal solution (1 ml/liter), and agar (20 g/liter). BTB is a pH indicator with a dark blue-green color at pH 7; it turns yellow as a result of proton formation when 2,4-D in the medium is mineralized.

Luria broth (LB), peptone-YE-tryptone-glucose (PTYG), 0.1× PTYG, 0.1× LB, and Trypticase soy broth (Difco) and R2A (Difco) media were also used for the culture of isolates and recipient strains for mating experiments.

Enrichment was performed in Falcon tubes (15 ml), and the other cultures (for growth rate measurement and harvesting of cells for enzyme assays) were grown in 125-ml flasks with 25 ml of medium or in 1-liter flasks with 300 ml of medium on a rotary shaker.

**Enrichment.** Approximately 1 g of soil was transferred into 5 ml of 2,4-D medium containing (per liter) 25 mg of 2,4-D, 100 mg of CA, and 100 mg of YE in a 15-ml Falcon tube and incubated at 30°C on a rotary shaker. The tube rack was tilted to ensure aeration. Aliquots were removed at 2- or 3-day intervals and analyzed with a Hewlett-Packard 1050 series high-performance liquid chromatograph equipped with a Lichrosorb RP-18 column (Merck), with 60% methanol–40% acidic water as the mobile phase. The enrichment products which showed complete mineralization of 2,4-D were transferred to fresh 2,4-D medium with a 5 to 20% inoculum.

**Isolation.** Isolation of 2,4-D degraders was performed in two ways, using either 2,4-D-BTB medium or $^{14}$C-ring-labeled 2,4-D medium. The colonies that turned the 2,4-D-BTB medium yellow were picked and purified on the same medium. Alternatively, enrichment cultures were plated out onto $^{14}$C-ring-labeled 2,4-D medium. After a 1- to 2-week incubation, colonies which accumulated [$^{14}$C]2,4-D were detected by autoradiography (13). The colonies which corresponded to the radioactivity were picked from the master plates and purified on 2,4-D-BTB medium. 2,4-D and $^{14}$C-ring-labeled 2,4-D were obtained from Sigma Chemical Company.

**Molecular methods.** Whole DNA was extracted from the isolates in accordance with protocols described previously (1). Repetitive extragenic palindromic (REP)-PCR patterns were obtained using Rep-1 and Rep-2 as primers and extracted DNA as the template (3, 33). Amplification products were separated by electrophoresis on a 1% agarose gel to visualize the patterns. All isolates were analyzed at the same time using the same batch of amplification mixtures and electrophoresis gel.

A partial sequence of the *tfdA* gene was amplified by PCR with two primers which we previously designed based on the nucleotide sequences of the *tfdA* genes in *A. eutrophus* JMP134 and *Burkholderia* sp. strain RASC (32): 5′-AAC GCAGCG(G/A)TT(G/A)TCCCA-3′ and 5′-ACGGAGTTCTG(C/T)GA(C/T) ATG-3′. The primers were expected to amplify 362 bp of nucleotides within the *tfdA* sequence. The amplified product was purified by 1.5% agarose gel electrophoresis and sequenced with the PCR primers as the sequencing primers. Sequencing was carried out at the Michigan State University sequencing facility with an automated fluorescence sequencer (model 373A; Applied Biosystems).

Southern hybridization and detection were performed in accordance with the protocol of the Genius system (Boehringer Mannheim, Indianapolis, Ind.) under high-stringency conditions (50% formamide and 5% blocking agent in the hybridization solution). Digoxigenin-labeled *tfdA*, *tfdB*, and *tfdC* probes were made as previously described (12).

The entire 16S ribosomal DNA (rDNA) gene of each isolate was amplified from genomic DNA by using fD1 and rD1 as the primers (34). The amplified product was purified by electrophoresis on a 1% agarose gel, cloned by using a TA cloning kit and *Escherichia coli* JM109 (Novagen), and sequenced using 5′-GGTTACCTTGTTACGACTT-3′ (*E. coli* positions 1510 to 1492; reverse), 5′-ACGGGCGGTGTGTACAAG-3′ (*E. coli* positions 1406 to 1389; reverse), 5′-TTGCGCTCGTTGCGGGACT-3′ (*E. coli* positions 1111 to 1093; reverse), 5′-CATCGTTTACGGCGTGGAC-3′ (*E. coli* positions 821 to 803; reverse), 5′-GTATTACCGCGGCTGCTGG-3′ (*E. coli* positions 536 to 518; reverse), and 5′-TCTGGTTGATCCTGCCAGAG-3′ (*E. coli* positions 8 to 27; forward), all designed by Hiraishi (15), as primers.

**2,4-D dioxygenase assay.** Cells grown on 2,4-D medium were disrupted with a French press or by sonication and centrifuged (8,000 × *g*) in 20 mM Tris-HCl buffer with 0.5 mM EDTA (pH 7). The supernatant was assayed for α-ketoglutarate-dependent 2,4-D dioxygenase activity as described previously (10, 11).

**Mating.** 2,4-D-degrading isolate strains HW1, HW13, HWK12, and BTH were used as donors for the mating experiment. The recipients used were *A. eutrophus* JMP228 carrying pBH501aE and the same strain lacking this plasmid (31). pBH501aE is a pJP4 derivative obtained via a site-specific deletion of the 566-bp *Nru*I fragment of the *tfdA* gene into which an *Npt*II (kanamycin resistance) cassette had been inserted (31). *A. eutrophus* JMP228 is a rifampin-resistant mutant derived from *A. eutrophus* JMP134 which has been cured of pJP4. Mating between the isolate and the recipient strain was performed in accordance with the method of Top et al. for plasmid capture via conjugation (31). Transconjugants capable of mineralizing 2,4-D were selected on 2,4-D-BTB medium containing kanamycin. In order to assess background growth, the series of diluted suspension was plated on LB medium containing the antibiotic(s) or on 2,4-D medium. Growth of the donor could be differentiated from growth of the recipient because the recipient grew fast on LB-antibiotic medium whereas the donor did not and, also, the donor grew on 2,4-D medium but the recipient did not.

## RESULTS

**Enrichment and isolation of 2,4-D degraders from pristine sites.** The strategy used to attempt to culture 2,4-D degraders was to supply additional nutrients in the form of CA and YE, to use lower concentrations of 2,4-D than those used before (13), to gradually increase the 2,4-D/CA-YE ratio, and to screen for activity by determining acid production and $^{14}$C uptake from [$^{14}$C]2,4-D so that activity due to consortia, cometabolism, or fastidious strains could still be detected. Three of the four continental soils that degraded 2,4-D in the previous study (13) yielded active enrichments under these conditions (Table 1), and the four previously inactive soils were also inactive in this assay. Five of the 22 Hawaiian soils also degraded 2,4-D (Table 1).

We attempted to isolate 2,4-D degraders from primary or secondary enrichments, but all attempts were unsuccessful, probably because there were still so many microorganisms which were not related to 2,4-D degradation. Thus, these cultures were repeatedly transferred (>10 times) to fresh 2,4-D medium. In the early stage of enrichments, we used 25 ppm of 2,4-D and 100 ppm each of YE and CA. After three to five

TABLE 1. Characteristics of the 2,4-D-degrading enrichments from which the 2,4-D-degrading isolates were obtained

| Strain | Origin of isolate[a] | Soil classification[b] | [2,4D]/[CA-YE] (ppm/ppm)[c] | Serial transfers[d] | Days required for complete degradation |
|---|---|---|---|---|---|
| HW1 | Hawaii, Thurston (200) | Hydric Dystrandept | 125:0 | 15 | 7–10 |
| HW13 | Hawaii, volcanic cliff | —[f] | 125:0 | 15 | 7–10 |
| HWK11 | Hawaii, Laupahoehoe (20,000) | Typic Hydrandept | 125:0 | 14 | 7–10 |
| HWK12 | Hawaii, Laupahoehoe (20,000) | Typic Hydrandept | 125:0 | 15 | 7–10 |
| HWK24[e] | Hawaii, Kohala (150,000) | Typic Placandept | 25:100 | 10 | >14 |
| BTH | Saskatchewan, Bittern | Albic luvisols | 25:0 | 10 | >10 |
| CHK[e] | California, Chabot | Chromic luvisols | 25:25 | 12 | >14 |
| RCO | Chile, Rio Clarillo | Chromic luvisols | 125:0 | 12 | 7–10 |

[a] The numbers in parentheses indicate the number of years since the soil was deposited by volcanic eruption.
[b] Hawaiian soils, U.S. Soil Survey classification, in reference 2; non-Hawaiian soils, FAO classification, in reference 12.
[c] Concentrations of 2,4-D, CA, and YE after the fifth transfer.
[d] Number of times transferred prior to isolation attempt.
[e] Pure cultures could not be obtained from these enrichments (see the Results section).
[f] —, sample was from a soil profile formed prior to burial by a lava flow 4,800 years ago.

transfers to fresh medium, we increased the 2,4-D concentration from 25 to 125 ppm and decreased the concentrations of YE and CA (Table 1) in order to increase the fraction of microorganisms involved in 2,4-D degradation. We were not able to remove the organic nutrients for 2,4-D degradation in enrichments HWK24 (Hawaiian volcanic soil) and CHK (Californian soil), and the time required for complete mineralization of 2,4-D fluctuated in these two cases throughout the enrichment. Therefore, the six remaining enrichment cultures were the focus for isolation of 2,4-D degraders by using 2,4-D-BTB medium or $^{14}$C-ring-labeled 2,4-D medium. The enrichment from Chile was particularly difficult to resolve. 2,4-D degradation was readily maintained in the enrichment culture, which consisted of two morphotypes. One morphotype was easily isolated, fast growing, and had no activity on 2,4-D. The other morphotype (strain RCO) grew poorly on 0.1× PTYG but eventually produced acid from 2,4-D. 2,4-D degradation by this strain was confirmed in liquid culture, but the activity occurred at a much slower rate than in the original consortium.

Single-colony isolation was repeated at least three times on 2,4-D-BTB medium. The purity was checked by plating the cells on 0.1× PTYG medium and examining the colonies under a microscope. Six 2,4-D-degrading isolates, HW1, HW13, HWK11, HWK12, BTH, and RCO, were obtained by the above-mentioned methods and used for further study.

**Morphological and growth properties of the isolates.** Table 2 lists the isolates and their morphological and growth properties. Most of the isolates were long bent rods that were 2 to 3 μm long and 0.6 to 0.8 μm in diameter (Fig. 1). Cells of strain HW1, however, were short rods that were 1.2 μm long, had a diameter of 0.8 μm, and sometimes occurred in pairs (Fig. 1). Colonies of HW1 were very small, less than 1 mm in diameter after 1 week of incubation on 2,4-D-BTB medium. The colonies on 0.1× PTYG were comparatively bigger than those on 2,4-D-BTB medium and their surfaces were glossy and yellowish. Strains HW13, HWK11, HWK12, BTH, and RCO produced slimy white colonies on both 2,4-D BTB and 0.1× PTYG media, with diameters of over 2 to 3 mm.

One of most outstanding features of these isolates was that all of them are slow-growing bacteria compared to previously described 2,4-D degraders (17). Figure 2 shows typical growth curves of strain HW13 on 2,4-D medium and 0.1× PTYG medium. Strain HW13 had a doubling time of approximately 20 h on 2,4-D medium, and it took 7 days to mineralize 125 mg of 2,4-D per liter. No intermediate products were detected by high-performance liquid chromatography analysis. Similar results were obtained with strains HWK11, HWK12, BTH, and RCO. Strain HW1 also required more than 7 days to mineralize 2,4-D, and the cells formed clumps more than 1 mm in diameter.

TABLE 2. Physiological and genetic properties of 2,4-D-degrading isolates

| Isolate | Morphology | Growth on[a]: | | | | $T_d$ (h)[b] | REP-PCR type | Presence of *tfdA* gene as determined by: | | TfdA dioxygenase activity[c] | Transmissibility of 2,4-D gene[d] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1× PTYG | 0.1× PTYG | TSB | R2A | | | PCR | Southern hybridization | | |
| HW1 | Short rod | + | + | + | +[e] | 20 | I | + | + | + | + |
| HW13 | Long bent rod | − | + | − | +[e] | 16 | II | − | − | − | − |
| HWK11 | Long bent rod | − | + | − | +[e] | 20 | III | − | − | − | − |
| HWK12 | Long bent rod | − | + | − | +[e] | 20 | III | − | − | − | − |
| BTH | Long bent rod | − | + | − | NT[f] | 30 | IV | − | − | − | − |
| RCO | Long bent rod | − | ±[g] | − | NT | >30 | V | − | − | NT | NT |

[a] Growth on indicated agar media; −, no growth after a 1-month incubation. TSB, Trypticase soy broth; R2A, Difco.
[b] Doubling time on 0.1× PTYG medium; average of duplicates.
[c] α-Ketoglutarate-dependent dioxygenase activity present (+) or absent (−).
[d] 2,4-D gene is (+) or is not (−) transmissible to a *tfdA* mutant recipient, *A. eutrophus* JMP228(pBH501aE).
[e] Very slow growth.
[f] NT, not tested.
[g] ±, much slower growth.

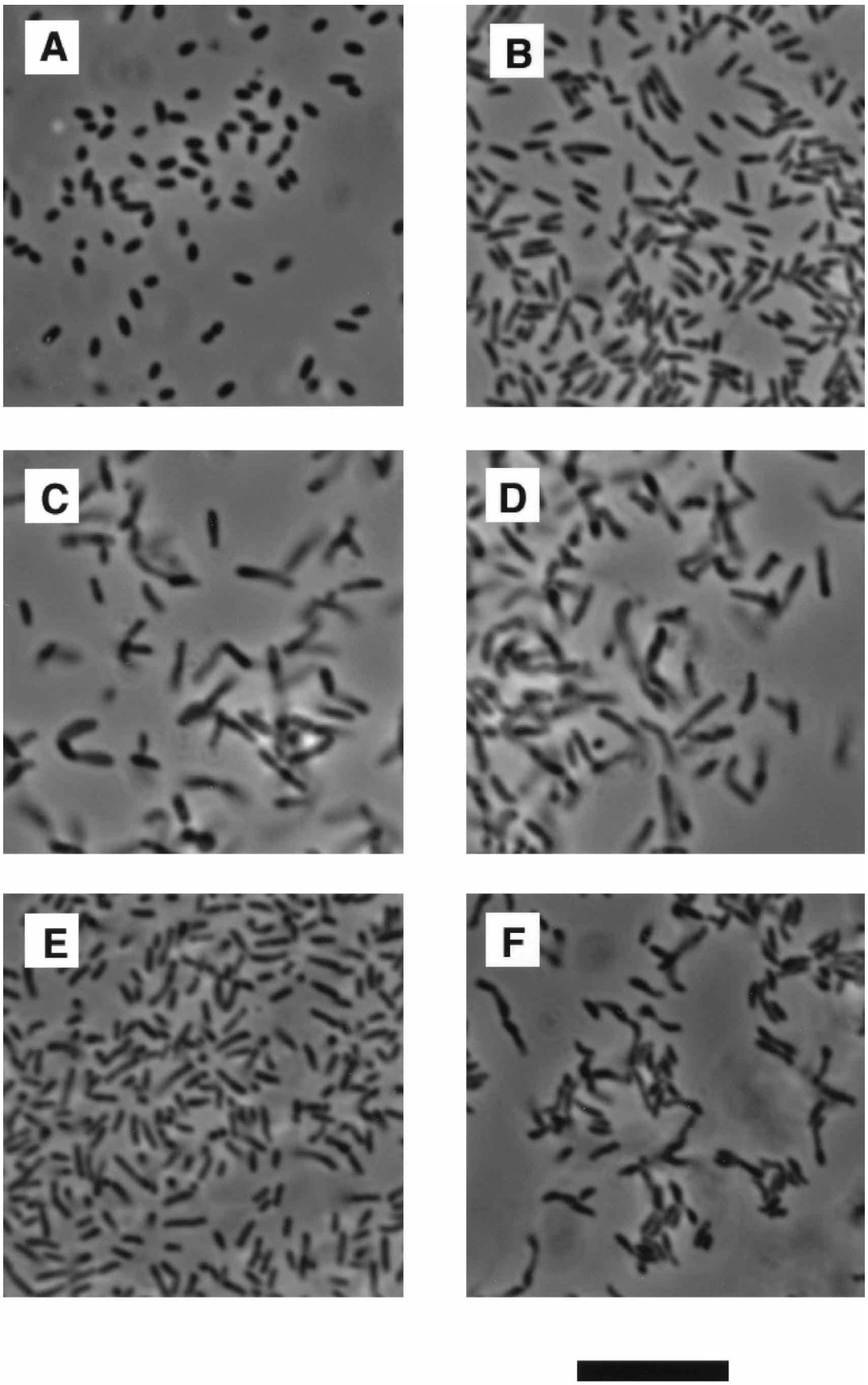


FIG. 1. Photomicrographs of 2,4-D-degrading isolates from pristine soil samples. (A) Strain HW1 from Hawaii. (B) Strain HW13 from Hawaii. (C) Strain HWK11 from Hawaii. (D) Strain HWK12 from Hawaii. (E) Strain BTH from Saskatchewan. (F) Strain RCO from Chile. Bar, 10 μm.

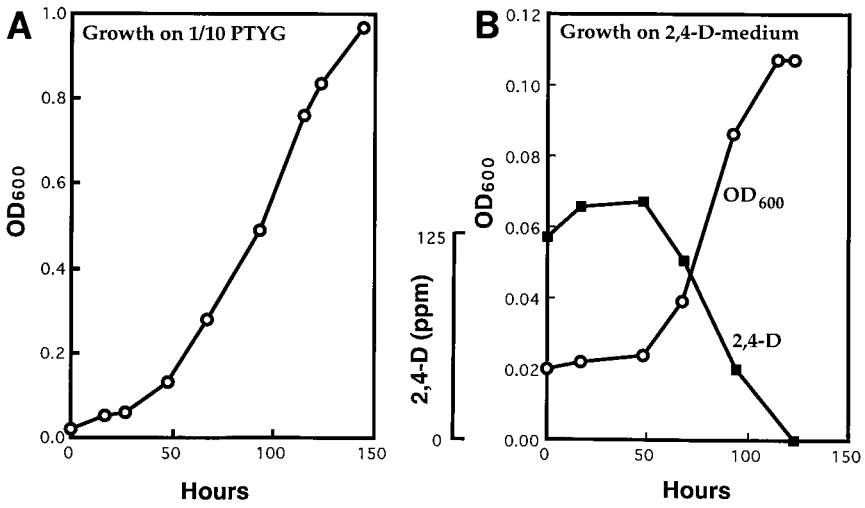


FIG. 2. Typical growth curves of and 2,4-D mineralization by strain HW13. (A) Growth on 0.1× PTYG medium. (B) Growth (○) and 2,4-D mineralization (■) on 2,4-D medium.

**Phylogenetic and physiological properties of the isolates.** All of the isolates were fingerprinted by REP-PCR (data not shown). Although HWK12 and HWK11 had identical patterns, the others gave different patterns (Table 2), suggesting that five genotypically different strains were obtained from the pristine samples. The REP patterns were compared to those of the other known 2,4-D and 3CBA degraders in our laboratory collection (12), but no identical or even similar patterns were found. This indicates that the isolates from pristine sites are genetically novel 2,4-D-degrading strains.

We sequenced the entire 16S rDNA genes of the isolates and compared these sequences to the GenBank 16S rDNA database. The 16S rDNA gene sequence of strain HW1 (GenBank accession no. D89026) most closely matched (98%) that of members of the genus *Variovorax*. Colonies of strain HW1 were glossy and of a yellowish color, indicative of carotenoid production. Strains HW13, HWK11, HWK12, BTH, and RCO were found to have sequences similar to those of members of the genera *Bradyrhizobium*, *Nitrobacter*, *Blastobacter*, *Afipia*, *Rhodopseudomonas*, and *Agromonas* (Table 3). All of these genera are members of the α subdivision of the class *Proteobacteria* and are phylogenetically closely clustered. Members of the genus *Bradyrhizobium*, especially the species *B. elkanii*, had sequences most similar to all of the isolates.

**2,4-D degradation properties of the isolates.** PCR amplification of a *tfdA* internal sequence was attempted for every isolate (Table 2). Of the six isolates, only strain HW1 produced an amplification product of the expected size. Nucleotide sequencing revealed that the amplified product has 97% sequence similarity to *tfdA* from pJP4 (GenBank accession no. AB001107). A *tfdA*-related gene did not appear to be present in the other strains, since Southern blots of *Eco*RI digests of genomic DNA of each strain showed that the *tfdA* probe hybridized only to the DNA of strain HW1. No hybridization to the DNA of any strain occurred when *tfdB* and *tfdC* were used as probes (data not shown). α-Ketoglutarate-dependent 2,4-D dioxygenase activity was detected in strain HW1 cells, whereas no activity could be detected in the remaining strains.

**Transfer of the strain HW1 *tfdA* gene to *A. eutrophus* JMP228 (pBH501aE), a *tfdA* mutant strain.** Mating experiments were performed to determine whether the 2,4-D degradation gene(s) could be transferred to other microorganisms (Table 2). Of the isolates tested, the 2,4-D degradation phenotype was transferred only from HW1 to the *tfdA* mutant strain [*A. eutrophus* JMP228(pBH501aE)]; it occurred at a frequency of $4 \times 10^{-8}$. The 2,4-D degradation phenotype was not transferred into *A. eutrophus* JMP228 cells lacking pBH501aE. Genes conferring growth on 2,4-D were not transferable by conjugation between the other isolates and the two recipients.

## DISCUSSION

We succeeded in isolating 2,4-D-metabolizing organisms from both the continental and Hawaiian soils. The strategy of using progressively less organic supplement and higher concentrations of 2,4-D in each of the enrichment steps seems to have worked, although it should be noted that it did not work in all cases. One of the most interesting features of these isolates is the fact that all of them are slow-growing microorganisms which, except for strain HW1, are sensitive to high concentrations of nutrients. The canonical, well-known 2,4-D degrader *A. eutrophus* JMP134(pJP4) grows well on nutrient-rich media and doubles every 1.5 h on 0.1× PTYG medium (data not shown). In contrast to this, for example, strain BTH, which was found in pristine spruce forest soil in northern Saskatchewan, had a 30-h doubling time on 0.1× PTYG medium and could not grow on standard (rich) medium such as 1× PTYG or LB. The isolate did not grow well on 2,4-D medium in the absence of an organic supplement, either, and we were not able to obtain a reproducible specific growth rate under these conditions. This indicates that the isolate may require other nutrients for growth and for mineralization of 2,4-D. Ka et al. also found that there are slow-growing 2,4-D-degrading populations as well as fast-growing degraders in agricultural soils (17, 19). However, the growth rates of their slow-growing bacterial group are still higher (doubling times, 4 to 9 h) than the rates of the isolates which we obtained. It appears that when undiluted environmental samples are enriched in broth cultures containing a high 2,4-D concentration, only fast-growing degraders outgrow slow growers in the cultures (17). Since only slow growers were obtained from our enrichment cultures without diluting soil samples, there are apparently no fast-growing 2,4-D-degrading microorganisms in these soils.

TABLE 3. Percent sequence similary values for 16S rDNA sequences of our isolates belonging to the α subdivision of the class *Proteobacteria* versus those of other members of the same group

| Strain[a] | % Sequence similarity vs | | | |
|---|---|---|---|---|
| | HW13 | HWK12 | BTH | RCO |
| HW13 (D89027) | | 98 | 97 | 95 |
| HWK12[b] (D89028) | 98 | | 98 | 96 |
| BTH (D89029) | 97 | 98 | | 96 |
| RCO (D89030) | 95 | 96 | 96 | |
| *Bradyrhizobium elkanii* (BEU35000) | 98 | 98 | 97 | 94 |
| *Bradyrhizobium* sp. LMG 9980 (X70404) | 99 | 99 | 97 | 96 |
| *Bradyrhizobium lupini* (X87273) | 97 | 97 | 96 | 95 |
| *Bradyrhizobium japonicum* LMG 6138T (S46916) | 97 | 97 | 97 | 95 |
| *Blastobacter denitrificans* (X66025) | 96 | 96 | 96 | 94 |
| *Nitrobacter winogradskyi* ATCC 25381 (L35506) | 96 | 96 | 96 | 94 |
| *Agromonas oligotrophica* JCM1494 (D78366) | 96 | 97 | 96 | 95 |
| *Rhodopseudomonas palustris* ATCC 17001 (D25312) | 96 | 96 | 96 | 94 |
| *Afipia felis* (M65248) | 95 | 95 | 94 | 95 |
| *Beijerinckia indica* ATCC 9039 (M59060) | 89 | 90 | 90 | 89 |
| *Rhizobium huakuii* IFO15243 (D13431) | 88 | 89 | 90 | 88 |
| *Sphingomonas paucimobilis* IFO13935 (D13725) | 83 | 83 | 84 | 82 |

[a] The first four strains are our isolates. The nonparenthetic designations following the bacterial names are the names of the strains or culture collection numbers. Designations in parentheses are GenBank accession numbers.

[b] The 16S rDNA of strain HWK11 is not shown here, but it is genotypically identical to that of strain HWK12 based on the REP-PCR pattern (see Table 2).

All of our new isolates might be described as oligotrophs because of their inability to grow on rich media, but the evolutionary reasons for this are unclear except in the case of HW13. The oligotrophic nature of the Hawaiian isolate HW13 is consistent with its habitat of origin. This isolate came from soil that had been sealed from new sources of carbon by a lava flow 4,800 years ago and thus must have depended on its oligotrophic capabilities for survival. This soil also appears to have been isolated from both immigrant microorganisms and synthetic chemicals by the solid lava cap. Hence, these 2,4-D degraders likely existed in this geographically isolated site before the soil was covered by lava as a result of volcanic activity.

The HW1 strain isolated from Hawaiian volcanic soils was identified as a member of the genus *Variovorax* based on its 16S rDNA sequence and the characteristic yellow colonies formed on 2,4-D and nutrient media. Many strains in the genus *Variovorax* formerly belonged to the genus *Alcaligenes* (35), which previously contained many 2,4-D degraders. Dunbar et al. recently found that a large number of *Variovorax* strains dominated the 2,4-D-degrading population in an agricultural 2,4-D-amended soil (7). These strains were genetically very diverse as determined by genomic REP-PCR fingerprinting. REP-PCR patterns of these isolates were compared with those of our isolate, but none of the patterns matched, suggesting that our isolate is genotypically different.

Of six isolates, five strains are phylogenetically related to members of the genus *Bradyrhizobium* and its relatives, a group not previously known to contain 2,4-D degraders. Recently, Saitou et al. isolated a number of oligotrophic, slow-growing bacteria that appeared to be dominant members of the soil community (28). They describe them as being members of the *Bradyrhizobium-Agromyces-Nitrobacter-Afipia* (BANA) cluster. Our isolates also appear to be members of this cluster, but their further taxonomic placement should await analysis of the new soil BANA members.

Most of 2,4-D-degrading bacteria that were previously described are members of the β subdivision of the class *Proteobacteria*, including *Burkholderia* spp., *Alcaligenes* spp., *Rhodoferax fermentans*, and *Comamonas testosteroni*. It is particularly interesting that to date only 2,4-D degraders belonging to the β and γ subdivisions of the class *Proteobacteria* carry a gene with 60% or more sequence similarity to the canonical *tfdA* of pJP4 in *A. eutrophus* JPM134. We found that only one of our isolates, strain HW1, is a member of the β subdivision, and it carries a *tfdA* gene that is highly similar to *tfdA* from pJP4.

In contrast to these findings, PCR amplification and Southern hybridization studies revealed that isolates HW13, HWK11, HWK12, BTH, and RCO did not seem to carry genes similar to *tfdA*. We attempted to identify the 2,4-D-degrading enzyme by using an assay for α-ketoglutarate-dependent 2,4-D dioxygenase developed with *A. eutrophus* JMP134 with cells disrupted through a French press or by sonication, but all attempts were unsuccessful. Similar results were also observed with 2,4-D degraders belonging to the genus *Sphingomonas*, another member of the α subdivision of the class *Proteobacteria* (data not shown). These strains did not contain a *tfdA*-type gene but grew rapidly on media with 500 to 1,000 ppm of 2,4-D. Attempts were made to detect 2,4-D-degrading enzyme activity by modifying the assay conditions and replacing cosubstrates with other compounds, but activity could not be detected. The reason why no activity was detected still remains to be clarified, but it is very likely that the enzyme involved in the first step of 2,4-D mineralization in these bacteria is quite different from the α-ketoglutarate-dependent 2,4-D dioxygenases found in the members of β and γ subdivisions of the class *Proteobacteria*.

Of six isolates, only strain HW1 carries the *tfdA* gene, and that gene was transmissible to another species (non-2,4-D degrading) although the frequency was low. In other studies, we found that the transconjugant retained the plasmid pBH501aE and that the *tfdA* gene of strain HW1 was inserted in the area where the disrupted *tfdA* gene was located (unpublished data). We also used the same strain of *A. eutrophus* from which the plasmid was cured for the mating experiment, but no 2,4-D-degrading transconjugant was obtained. These results suggest that homologous recombination occurred in the recipient after the gene was transferred from the host to the recipient. We also carried out matings involving HW13, HWK12, and BTH as donors and *A. eutrophus* JMP228 with and without pBH501aE as the recipient; however no 2,4-D-degrading transconjugant was observed.

Our research indicates several important points. First, as described in our previous paper (13), 2,4-D-degrading microorganisms are widespread in pristine environments but are very difficult to culture. Second, most of the 2,4-D degraders found in pristine soils are slow-growing microorganisms and are sensitive to high concentrations of organic nutrients. This suggests that a new class of 2,4-D degraders should be added to the previous two (Table 4). Our five isolates define this new class III. This class may be more frequently represented in nature than is now recognized. Third, 2,4-D degraders which are members of the α subdivision of the class *Proteobacteria* do not carry the canonical *tfdA* gene, although microorganisms which are members of the β and γ subdivisions do. Furthermore, the former do not appear to have transmissible 2,4-D-degrading properties while the latter do (Table 4). Fourth, if the transmissible gene is the origin of the canonical gene, the origin of the fast-growing 2,4-D degraders that are widespread in agricultural soils and other human-impacted environments in which 2,4-D is being applied could be explained as being the

TABLE 4. Three classes of 2,4-D-degrading isolates

| Class | Subdivision(s) of *Proteobacteria* | Nutrition | Growth pace | 2,4-D gene | Transmissible 2,4-D degradation trait |
|---|---|---|---|---|---|
| I | β and γ | Copiotrophic | Fast | *tfdA* | Yes |
| II | α (*Sphingomonas*) | Copiotrophic | Fast | Non-*tfd* | No |
| III | α (BANA group) | Oligotrophic | Slow | Non-*tfd* | No |

result of horizontal gene transfer from slow growers, like strain HW1. Since HW1 comes from an isolated, non-2,4-D-selected environment, it may represent a strain more typical of the origin of the 2,4-D degradation trait. Finding a second group of 2,4-D degraders that apparently does not have the *tfd* genes or the Tfd dioxygenases suggests that more emphasis needs to be placed on understanding the unknown but apparently common non-*tfd* pathways.

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