tfdA-Like Genes in 2,4-Dichlorophenoxyacetic Acid-Degrading Bacteria Belonging to the *Bradyrhizobium-Agromonas-Nitrobacter-Afipia* Cluster in α-*Proteobacteria*

Kazuhito Itoh,[1,2,3]* Rie Kanda,[1] Yoko Sumita,[4] Hongik Kim,[4] Yoichi Kamagata,[4] Kousuke Suyama,[1] Hiroki Yamamoto,[1] Robert P. Hausinger,[2,3] and James M. Tiedje[2,3]

Faculty of Life and Environmental Science, Shimane University, Matsue, Shimane 690-8504,[1] and Research Institute of Biological Resources, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8566,[4] Japan, and Department of Microbiology and Molecular Genetics[2] and Center for Microbial Ecology,[3] Michigan State University, East Lansing, Michigan 48824

The 2,4-dichlorophenoxyacetate (2,4-D)/α-ketoglutarate dioxygenase gene (*tfdA*) homolog designated *tfdAα* was cloned and characterized from 2,4-D-degrading bacterial strain RD5-C2. This Japanese upland soil isolate belongs to the *Bradyrhizobium-Agromonas-Nitrobacter-Afipia* cluster in the α subdivision of the class *Proteobacteria* on the basis of its 16S ribosomal DNA sequence. Sequence analysis showed 56 to 60% identity of *tfdAα* to representative *tfdA* genes. A MalE-TfdAα fusion protein expressed in *Escherichia coli* exhibited about 10 times greater activity for phenoxyacetate than 2,4-D in an α-ketoglutarate- and Fe(II)-dependent reaction. The deduced amino acid sequence of TfdAα revealed a conserved His-X-Asp-X_{146}-His-X_{14}-Arg motif characteristic of the active site of group II α-ketoglutarate-dependent dioxygenases. The *tfdAα* genes were also detected in 2,4-D-degrading α-*Proteobacteria* previously isolated from pristine environments in Hawaii and in Saskatchewan, Canada (Y. Kamagata, R. R. Fulthorpe, K. Tamura, H. Takami, L. J. Forney, and J. M. Tiedje, Appl. Environ. Microbiol. 63:2266–2272, 1997). These findings indicate that the *tfdA* genes in β- and γ-*Proteobacteria* and the *tfdAα* genes in α-*Proteobacteria* arose by divergent evolution from a common ancestor.

The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) has been used as a model compound to study evolution of bacterial catabolic genes for anthropogenic chemicals (10, 11, 16, 20, 21, 26, 35, 36, 37). A number of 2,4-D-degrading bacterial strains have been isolated and found to be distributed over many different phylogenetic groups (3, 5, 10, 12, 16, 20, 21, 25, 26, 35, 37). Most of the strains have been classified into two groups on the basis of the genetic and enzymatic properties of their 2,4-D-catabolizing traits. One group is composed of various genera in the β and γ subdivisions of the class *Proteobacteria*. They have 2,4-D-catabolizing genes that are homologous to *tfd* genes found in *Ralstonia eutropha* JMP134, a well-studied 2,4-D degrader (5, 26). The other group is composed of strains in the α subdivision of the class *Proteobacteria*. Their 2,4-D-catabolizing genes have shown less than 60% homology by hybridization to the *tfd* genes in the β- and γ-*Proteobacteria* (20, 21, 26, 37).

The *tfdA* gene encodes an α-ketoglutarate (α-KG)-dependent 2,4-D dioxygenase that converts 2,4-D into 2,4-dichlorophenol and glyoxylate while oxidizing α-KG to CO_2 and succinate as an initial step of 2,4-D mineralization (8, 9, 31, 32). The *tfdA* sequences fall into three distinct classes for members of the β- and γ-*Proteobacteria* (26). The class I *tfdA* genes, including that of *R. eutropha* JMP134, are usually located on broad-host-range, self-transmissible plasmids (5, 19, 36). The class II *tfdA* gene, which is only found in the chromosome of *Burkholderia* sp. strain RASC, is 76% identical to the class I

tfdA genes. The class III *tfdA* genes are 77 and 80% identical to the class I and class II *tfdA* genes, respectively. A *tfdA* phylogenetic tree with three distinct branches has been constructed, and interspecies gene transfer of the *tfdA* genes among the β- and γ-*Proteobacteria* was suggested by comparing the phylogenetic trees of the *tfdA* and 16S ribosomal (rDNA) genes (26).

An alignment of the *tfdA* genes was used to design PCR primer sets for amplifying *tfdA*-like genes from other sources (31, 37). In contrast to the 2,4-D-degrading β- and γ-*Proteobacteria*, none of 2,4-D-degraders belonging to the α-*Proteobacteria* has been found to yield *tfdA*-like PCR products (21, 26, 37). No hybridization was observed with *tfdA* even under low-stringency conditions, and α-KG-dependent 2,4-D dioxygenase activity was not detected. Therefore, the 2,4-D genes in this particular phylogenetic group were considered to be functionally and evolutionarily different from the *tfdA* genes in the former phylogenetic groups (21, 26, 37).

In order to understand the origin and evolutionary relationship of the 2,4-D catabolic genes, it is important to characterize the 2,4-D genes in the α-*Proteobacteria*. Recently we isolated the 2,4-D-degrading bacterial strain RD5-C2 from Japanese upland soil (18). RD5-C2 is a member of the *Bradyrhizobium-Agromonas-Nitrobacter-Afipia* cluster (14, 29) in the α-*Proteobacteria*, like the 2,4-D degraders previously isolated from the pristine environments in Hawaii and in Saskatchewan, Canada, and Chile (21). In this paper we report that RD5-C2 and other degraders isolated from the pristine environments possess *tfdA*-like genes that encode a protein belonging to the α-KG-dependent dioxygenase family (8, 9, 31, 32). Significantly, we show that the recombinant RD5-C2 protein exhibits

* Corresponding author. Mailing address: Faculty of Life and Environmental Science, Shimane University, Matsue, Shimane 690-8504, Japan. Phone: 81-852-32-6521. Fax: 81-852-32-6597. E-mail: itohkz@life.shimane-u.ac.jp.

greater activity for nonchlorinated phenoxyacetate over 2,4-D in contrast to the more uniform activities observed for the canonical TfdA.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. RD5-C2 and the other 2,4-D-degrading strains isolated from the pristine environments (21), HW13, HWK12, and BTH, were cultured on 2,4-D-containing basal medium (18) or HM medium (27). *Escherichia coli* HB101 and *E. coli* DH5α were used for the cloning and expression experiments, respectively. Luria-Bertani (LB) medium was used to grow *E. coli*. The *tfdA*-like gene from RD5-C2 (hereafter referred to as *tfdAα*) was cloned into pUC118 and then subcloned into pUC119. The plasmid vector pMAL-cRI (New England BioLabs) was used to construct the MalE-TfdAα fusion protein. The *tfdA*-like gene was fused to the *malE* gene, which encodes maltose-binding protein, and was expressed by using the *tac* promoter and translation initiation signals of maltose-binding protein.

Cloning and sequencing of *tfdAα*. To obtain whole DNA from the isolates, the cultures were grown on HM medium, washed with 1% NaCl, suspended in distilled water, frozen at −20°C, thawed at room temperature, and treated with proteinase K (10 µl of a 1-mg/ml concentration per 40 µl of sample) (Takara) in 40 mM Tris buffer solution (50 µl) containing 1% Tween 20, 0.5% Nonidet P-40 (Sigma), and 1 mM EDTA (pH 8.0). The mixture was incubated at 60°C for 20 min to digest proteins and then at 100°C for 5 min to inactivate the enzyme. After centrifugation (8,060 × *g*, 10 min) the supernatant was collected for gene amplification by PCR (15). A partial *tfdAα* fragment was amplified by using modified primers designed for the conserved regions of the *tfdA* genes from *R. eutropha* JMP134 and *Burkholderia* sp. strain RASC (3, 37): 5′-AC(C/G)GAGT TCTG(C/T)GA(C/T) ATG-3′ and 5′-(A/G)ACGCACGCG(G/A)TT(G/A)TCCC A-3′. The amplified *tfdAα* gene fragments were directly ligated into pT7Blue T-Vector (Novagen) and were transformed into *E. coli* HB101-competent cells according to the supplier's instructions. The inserted regions were amplified by PCR by using M13 primers M4 and RV (Takara) and were directly sequenced with an ABI 377 DNA sequencing system (Perkin-Elmer Japan) with the same primers.

The amplified *tfdAα* gene fragment was labeled with a DIG DNA Labeling Kit (Roche Molecular Biochemicals) and was shown to hybridize with a single ca. 4.0-kbp fragment of whole DNA *Sac*I digest (data not shown). DNA fragments of around 4.0 kbp were extracted from the gel, ligated into pUC118 to produce pTFD1-1, and transformed into *E. coli* DH5α-competent cells to create a genomic library. The DNA fragments were then screened by colony hybridization with the *tfdAα* gene fragment as a probe.

Plasmid DNA was purified from five positive clones, and the *Sac*I fragment was further digested with several restriction enzymes. The *Sal*I fragment was subcloned into pUC119, and the DNA sequence of the inserted fragment was determined as described previously. On the basis of the gene sequence, the following new primer set was constructed to amplify *tfdAα* from HW13, HWK12, and BTH: 5′-AC(C/G)GAGTTC(G/T)(C/G)CGACATGCG-3′ and 5′-GCGGT TGTCCCACATCAC-3′. The *tfdAα* genes were amplified, and the full sequences for HW13 and HWK12 and the partial sequence for BTH were determined by using a PCR in vitro cloning kit (Takara). All procedures described here were conducted according to the supplier's instructions.

Analysis of *tfdAα* sequence. The *tfdAα* sequences were compared with other available sequences by using the FASTA (28) and BLAST (2) algorithms. Sequences were aligned manually with the CLUSTALW program (34), and the sites where nucleotides were not resolved for all sequences were excluded from the alignments. Phylogenetic analysis was performed with three types of algorithms. CLUSTALW version 1.8 (34) was used as the software package for the neighbor-joining (NJ) analysis (30), and the bootstrap analysis based on 1,000 replicates was used to place confidence estimates on the tree. A maximum-likelihood (ML) analysis was carried out with the program package MOLPHY version 2.3b3 (1). An ML distance matrix was calculated by using NucML, and the NJ topology was reconstructed by using NJdist in MOLPHY as the starting tree for the ML method. An ML tree was obtained by using NucML with an R (local rearrangement search) option based on the HKY model (13). Local bootstrap probabilities were estimated by the resampling of estimated log-likelihood method (13, 24). A maximum-parsimony tree reconstruction was performed by using PAUP* software version 4.0b10 (33). A heuristic search was used with a random stepwise addition sequence of 100 replicates, tree-bisection-reconnection branch swapping, and the multrees option. A further analysis was run with 100 bootstrap replicates, each consisting of 30 additional random replicates. The Kishino-Hasegawa test (23) was carried out to survey the best

topology having the highest log-likelihood value among the most parsimonious trees. A consensus tree was obtained by using the strict method.

Construction of *malE-tfdAα* fusion plasmid. From pTFD1-1, plasmid pNK2 was constructed by subcloning the appropriate *Sal*I fragment into pUC119. *tfdAα* of RD5-C2 was amplified by PCR by using the primers 5′-GCTCTAGAATGA CGGTCCTGATCCGGCAG-3′ and 5′-CCCAAGCTTTGTTATTGTGCCCGC TACTCC-3′ to give *Xba*I and *Hin*dIII ends, respectively, and was inserted into the *Xba*I and *Hin*dIII cloning sites of the pMAL-cRI vector. The plasmid was transformed into *E. coli* DH5α-competent cells according to the supplier's instructions.

Preparation of cell extracts and enzyme assay. Unless stated otherwise, the experiments were done at 4°C. *E. coli* DH5α (pUC119-*tfdAα*) was grown overnight at 30°C in LB medium supplemented with 100 µg of ampicillin/ml. *E. coli* DH5α (pMAL-cRI-*tfdAα*) was grown to mid-log phase at 30°C in LB medium supplemented with 2 g of glucose/liter and 100 µg of ampicillin/ml, followed by induction with isopropyl-[β]-D-thiogalactopyranoside at 0.3 mM for 2 h. Cells were harvested by centrifugation (6,000 × *g*, 15 min) and suspended in a solution containing 10 mM Tris, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM leupeptin (pH 7.6). The suspended cells were passed through a French press at 120 MPa, and cell extracts were obtained by centrifugation (100,000 × *g*, 45 min). The cell extracts were examined by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were assayed for TfdA enzyme activity. Gels were stained with Coomassie brilliant blue, and phosphorylase b (M_r, 97,400), bovine serum albumin (M_r, 66,200), ovalbumin (M_r, 45,000), carbonic anhydrase (M_r, 31,000), soybean trypsin inhibitor (M_r, 21,500), and lysozyme (M_r, 14,400) (Bio-Rad) were used as standards. The MalE-TfdAα fusion protein was further purified by using an amylose resin column (New England BioLabs) according to the supplier's instructions. The fusion protein eluate was dialyzed against 10 mM Tris–0.1 mM EDTA (pH 7.4) prior to the enzyme assay. Protein concentration was determined by using a protein assay kit (Bio-Rad) with bovine serum albumin as the standard.

The enzyme activity was measured by the modified 4-aminoantipyrine method (8, 22). The typical assay mixture contained 1 mM α-KG, 50 µM ascorbate, 50 µM $(NH_4)_2Fe(SO_4)_2$, and 1 mM 2,4-D in 10 mM imidazole buffer (pH 6.75). The enzyme reaction was conducted at 30°C and was quenched by the addition of
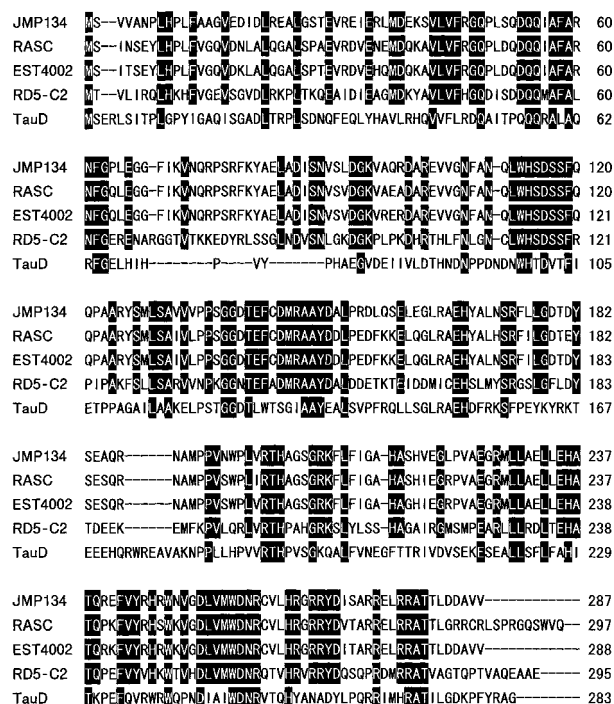

FIG. 1. Multiple alignment of the deduced amino acid sequences of RD5-C2 TfdAα and other α-KG-dependent dioxygenases. JMP134, RASC, and EST4002 are representative strains having class I, II, and III *tfdA* genes, respectively. TauD is taurine/α-KG dioxygenase of *E. coli*. Conserved residues are boxed.
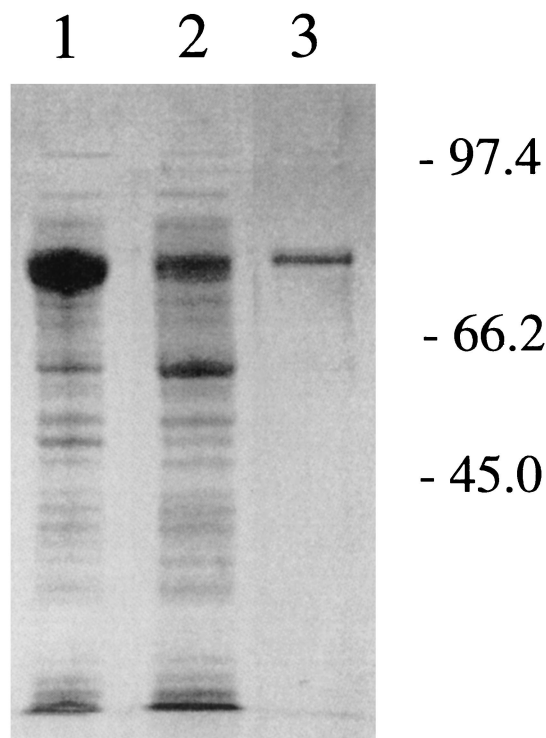
FIG. 2. Purification of MalE-TfdAα fusion protein from RD5-C2 (pMAL-cRI-tfdAα). Lane 1, whole-cell disruptant of RD5-C2 (pMAL-cRI-tfdAα); lane 2, supernatant of the disrupted cells; lane 3, purified MalE-TfdAα fusion protein eluted from an amylose resin column.

EDTA to give a final concentration of 5 mM. The concentration of released phenol derivative was measured as follows. One hundred microliters of a pH 10 buffer solution, 10 µl of 2% 4-aminoantipyrine, and 10 µl of 8% potassium ferricyanide were added to 1 ml of the reaction mixture. The absorbance at 510 nm was measured after 20 min. The assay was simultaneously conducted with purified *R. eutropha* JMP134 TfdA (9) for reference.

For the other phenoxyacetate derivatives, the corresponding phenol compounds were used as the standard in the assay. For the other α-ketoacids, phenoxyacetate was used as the substrate because this exhibited the highest activity among the phenoxyacetate derivatives used.

Nucleotide sequence accession number. The nucleotide sequences determined in this study have been deposited in the DDBJ/EMBL/GenBank database, and the accession numbers for *tfdAα* of RD5-C2, HWK12, HW13, and BTH are AB074490, AB074491, AB074492, and AB074493, respectively.

RESULTS

***tfdAa* gene and TfdAα amino acid sequences of RD5-C2.** The *tfdAα* sequence was found to most closely match that of *tfdA* of *R. eutropha* JMP134 and showed 56 to 60% identity to representative *tfdA* genes of JMP134, RASC, and *Achromobacter xylosoxidans* EST4002 (E. Vedler, V. Koiv, and A. Heinaru, Analysis of the 2,4-dichlorophenoxyacetic acid-degradative plasmid pEST4011 of *Achromobacter xylosoxidans* subsp. *denitrificans* strain EST4002; direct submission to GenBank [accession no. U32188], 1995), which were reported to have the class I, II, and III *tfdA* genes, respectively (26). The deduced amino acid sequence of RD5-C2 TfdAα was aligned with those of representative TfdA proteins and taurine/α-KG dioxygenase (TauD) from *E. coli*, which all belong to group II of the α-KG-dependent dioxygenase family (7, 8, 9, 17, 32) (Fig. 1). The

data indicated 80 to 93% identity of amino acids among the three classes of TfdA, whereas TfdAα showed lower identity (45 to 48%) to all classes of TfdA, as expected from the gene sequence analysis. TfdAα also showed lower identity to TauD (27%) than the other TfdA proteins (28%). Despite the divergence of TfdAα compared to the other sequences, all of these proteins possessed the two His and one Asp considered to be iron-binding ligands and an Arg suspected of forming a salt bridge with the C-5 carboxylate of α-KG (17). The four predicted active-site residues in TfdAα are His115, Asp117, His264, and Arg279.

TfdA activity of the MalE-TfdAα fusion protein. When *tfdAα* was overexpressed in *E. coli* by using a pUC119 derivative (pNK2), TfdAα protein was produced, as shown by a predominant band exhibiting an M_r of 36,000 following denaturing gel electrophoresis. However, most of the recombinant wild-type protein was insoluble and possessed no activity even when prepared from cells grown at 30 or 25°C (9) or when examined with cells cultured in 1 M sorbitol and 2.5 mM glycyl betaine (4). In contrast, the MalE-TfdAα fusion protein (M_r, 76,000) exhibited increased solubility, allowing its partial purification by amylose resin chromatography (Fig. 2). As shown in Table 1, the specific activity of purified MalE-TfdAα protein was greatly reduced in comparison to that of *R. eutropha* TfdA. Significantly, however, the MalE-TfdAα protein exhibited higher activity for phenoxyacetate derivatives that were less chlorinated than 2,4-D. In contrast, TfdA showed similar activity towards these phenoxyacetates, as reported previously (8). Besides the difference in specific activities between the substrates, MalE-TfdAα showed the same traits as TfdA (8) in terms of dependency on α-KG and ferrous iron (Table 2) and

TABLE 1. Specific activity of the MalE-TfdAα fusion protein[a]

Substrate	Sp act	
	MalE-TfdAα (pmol/min · µg of protein)	TfdA (nmol/min · µg of protein)
2,4-D	2.6	7.9
4-Chlorophenoxyacetate	6.5	8.6
Phenoxyacetate	21	8.7

[a] Experiments were performed at 30°C in 10 mM imidazole buffer (pH 6.75) containing 1 mM substrate, 1 mM α-KG, 50 µM ferrous iron, and 50 µM ascorbate.

TABLE 2. Requirement of α-KG and ferrous iron for activity of the MalE-TfdAα fusion protein

Conditions	Sp act (pmol/min · µg of protein)
Control[a]	25.1
−α-KG[b]	3.8
−Ascorbate[b]	21.3
−Ferrous iron[b]	3.7
−Enzyme[b]	2.9
−Phenoxyacetate[b]	2.1

[a] Experiments were performed at 30°C in 10 mM imidazole buffer (pH 6.75) containing 1 mM phenoxyacetate, 1 mM α-KG, 50 µM ferrous iron, and 50 µM ascorbate.
[b] Each component was removed from the reaction mixture.

TABLE 3. α-Ketoacid substrate specificity of the MalE-TfdAα fusion protein

Substrate[a]	Sp act (pmol/min · μg of protein)
α-KG	27.5
α-Ketoadipate	6.3
α-Ketovalerate	3.8
α-Ketobutyrate	2.5
α-Ketoisovalerate	1.3
α-Ketocaproate	0.6

[a] Experiments were performed at 30°C in 10 mM imidazole buffer (pH 6.75) containing 1 mM phenoxyacetate, 1 mM α-ketoacid, 50 μM ferrous iron, and 50 μM ascorbate.

in exhibiting the highest activity for α-KG among the α-keto-acids (Table 3).

Phylogenetic analysis of *tfdAα* genes from strain RD5-C2 and other 2,4-D-degrading α-Proteobacteria. Three 2,4-D-degrading strains belonging to α-Proteobacteria, previously isolated from pristine environments (21), were tested for the presence of a *tfdAα*-type gene by PCR amplification. The resulting *tfdAα* genes were sequenced and, along with RD5-C2 *tfdAα*, were subjected to phylogenetic analysis (Fig. 3). Since only four full-length open reading frames for *tfdA* are available

in the DDBJ/EMBL/GenBank database, partial sequences (307 bp) of *tfdA* and *tfdAα* were used for the analysis. The *tfdAα* genes of α-Proteobacteria comprise a distinct cluster compared to the three reported clusters associated with the representative *tfdA* genes. Gene sequence identity among the *tfdAα* representatives was 81 to 99%. Based on the full-length sequence comparisons, a similar phylogenetic tree was obtained. In addition, all three algorithms presented similar topologies in both the full-length and partial sequences (data not shown).

DISCUSSION

Previous studies of α-proteobacterial 2,4-D degraders indicated that the genes responsible for 2,4-D degradation were different from the canonical *tfd* genes and that these bacteria lack a TfdA-like enzyme, suggesting a different origin of the biodegradative pathway compared to the β- and γ-Proteobacteria (21, 26, 37). Our studies refute this interpretation; rather, strain RD5-C2 and other 2,4-D degraders within the *Bradyrhizobium-Agromonas-Nitrobacter-Afipia* cluster of α-Proteobacteria do harbor a *tfdA*-like gene (*tdfAα*), and the RD5-C2 TfdAα protein exhibits TfdA-like activity. This is the first unequivocal
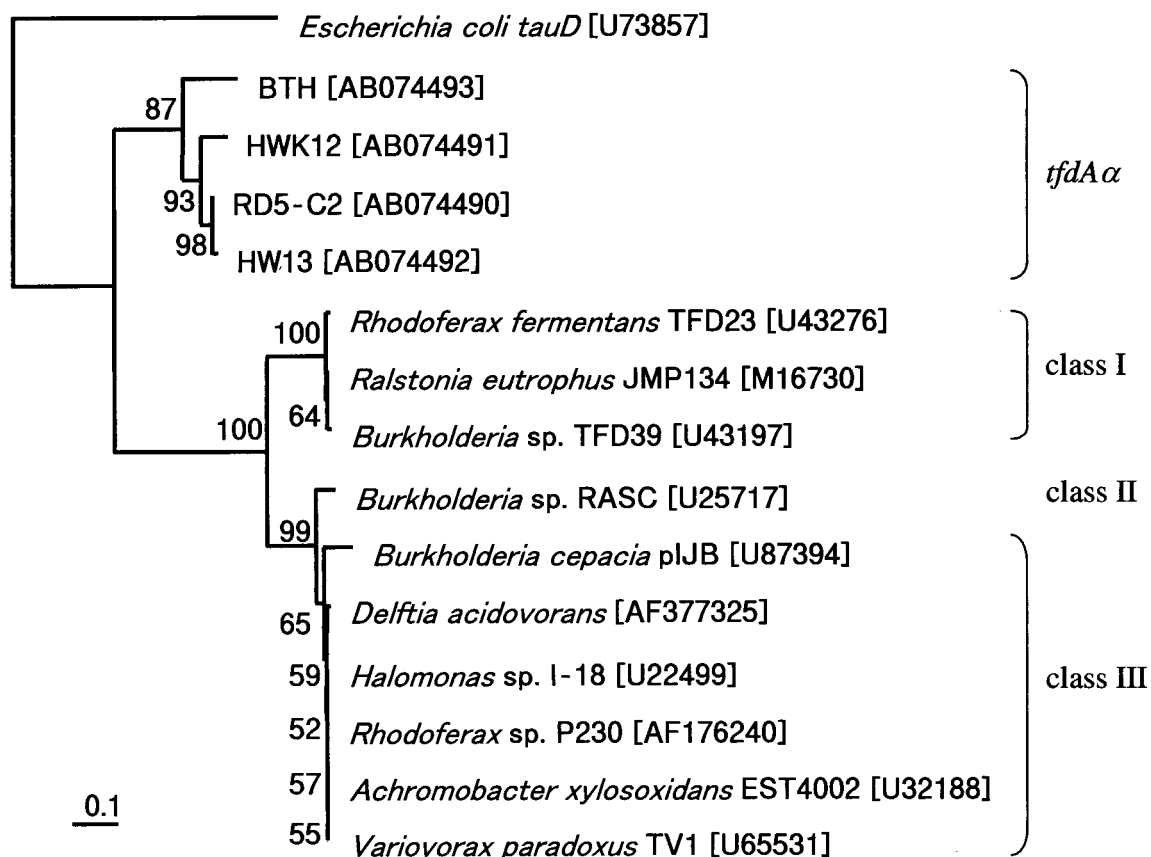

FIG. 3. Phylogenetic position of *tfdAα* among other genes of the α-KG-dependent dioxygenase family. The *tfdA* genes of JMP134, TFD39, and TFD23 are found in class I, the *tfdA* gene of RASC is in class II, and the other previously reported *tfdA* genes are class III representatives. *E. coli tauD* encodes taurine/α-KG dioxygenase. Designations in parentheses are DDBJ/EMBL/GenBank accession numbers. The phylogenetic tree was constructed on the basis of common partial sequences (307 bp) by using the NJ method. Bootstrap values above 50% are shown at nodes. The scale bar indicates substitutions per site.

evidence that *tfdA*-type genes and TfdA-like enzymes exist among α-, β-, and γ-proteobacterial 2,4-D degraders. Because the FASTA homology search with the DDBJ/EMBL/GenBank database indicated no *tfdAα*-like genes among microorganisms other than those presented in Fig. 3, it is difficult to envisage the original function of these genes. The low sequence identity (56 to 60%) of *tfdAα* genes with representative *tfdA* genes explains why they did not hybridize in prior studies, even under low-stringency conditions (21, 26, 37).

All α-*Proteobacteria* strains used in this study were isolated from pristine environments and Japanese arable soil that had had no history of chemical treatment, suggesting that *tfdAα* genes are distributed among soil strains independent of 2,4-D exposure. These *tfdAα* genes are likely to have some original function(s), and some strains with the appropriate *tfdAα* genes incidentally degrade 2,4-D.

On the basis of the 30% amino acid identity between TauD from *E. coli* and TfdA from *R. eutropha* JMP134, Eichhorn et al. (7) suggested that the two dioxygenases might have arisen by convergent evolution rather than from a common ancestor. In contrast, the *tfdA* and *tfdAα* genes are likely to have arisen by divergent evolution from a common ancestor, because they exhibit higher similarities and are separately distributed in β- and γ-*Proteobacteria* and α-*Proteobacteria*, respectively. Horizontal gene transfer is a well-known phenomenon, and interspecies gene transfer of *tfdA* genes was demonstrated by phylogenetic comparison of *tfdA* and 16S rDNA genes (26). On the other hand, phylogenetic trees of *tfdAα* and 16S rDNA genes are congruent (18), indicating evolution of the *tfdAα* genes without horizontal gene transfer occurring. On the basis of the findings that microorganisms isolated from pristine environments harbor *tfdAα* and that TfdAα has higher activity for nonchlorinated phenoxyacetate than 2,4-D, the *tfdAα* genes could be the origin of the *tfdA* genes. Horizontal transfer by transposons and/or plasmids and further adaptations to the anthropogenic environments would give rise to the well-studied *tfdA* representatives. Plasmid pJP4, carrying *tfdA*, was shown to be transferred to strains of α-, β- and γ-*Proteobacteria*; however, the 2,4-D-degrading phenotype could not be expressed in α-*Proteobacteria* such as *Rhizobium* sp. and *Agrobacterium tumefaciens* (5). Distribution of *tfdA*-like genes among non-2,4-D-degrading soil bacteria in many different phylogenetic groups was reported, and nucleotide sequence analyses of four isolates showed the highest homology with canonical *tfdA* (16). Sequence analysis of the *tfdA* genes from the other strains could reveal the evolutionary relationship between the *tfdA* and *tfdAα* genes.

TfdAα was experimentally found to be an α-KG-dependent dioxygenase, as previously observed for TfdA. The amino acid sequence of TfdAα clearly belongs to group II of the α-KG-dependent dioxygenase family, since it contains multiple conserved residues that are thought to function in catalysis (8, 17). The substrate for ancestral TfdA is unknown, but naturally occurring aryl-ether compounds released during degradation of lignin are a reasonable possibility (16). In addition, it is possible that the TfdA ancestor functioned in the metabolism of a cinnamic acid (6).

In the present study we showed that 2,4-D-degrading α-*Proteobacteria* possess *tfdAα* genes that arose from a common ancestor to the canonical *tfdA* genes, and it is considered that

ancestral *tfdAα* genes may serve as the origin of the *tfdA* genes on the basis of the following reasons. First, the 2,4-D-degrading α-*Proteobacteria* were isolated from pristine environments, widely distributed in Hawaii, and in Saskatchewan, Canada, and Chile and Japan, that had not been exposed to 2,4-D. Second, TfdAα is an α-KG- and Fe(II)-dependent dioxygenase that exhibits higher specific activity for nonchlorinated substrates. Thus, the chemistry of the TfdAα enzymes could be retained while modifying the substrate binding site to allow enhanced reactivity toward highly chlorinated 2,4-D to give rise to the TfdA activities. Further studies are needed to clarify the evolutionary relationships between *tfdAα* and the canonical *tfdA* genes and to understand the adaptation process of bacteria to chlorinated compounds.

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