Chlorophenol Hydroxylase Activity Encoded by TfdB from 2,4-Dichlorophenoxyacetic Acid (2,4-D)-Degrading Bradyrhizobium sp. Strain RD5-C2

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The tfdB gene encoding chlorophenol hydroxylase and its homolog were found in 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading strain RD5-C2, which belongs to Bradyrhizobium sp. of α-Proteobacteria. The nucleotide and deduced amino acid sequence identities of the two genes, designated tfdBa and tfdBb, were 60% and 57% respectively. Their nucleotide sequences most closely matched those of previously reported tfdB, which consisted of those from 2,4-D-degrading β- and γ-Proteobacteria and Sphingomonas sp. in α-Proteobacteria, with 61–67% identity. The TfdBa expressed in Escherichia coli showed the highest activity for 2,4-dichlorophenol but a narrower range of activity for the other chlorophenols than previously reported TfdBs. In the case of TfdBb, however, no observable activity for any chlorophenols or phenol was detected, although production of a protein with an appropriate molecular size was observed. Based on codon usage patterns and the GC content of the genes, it probable that the tfdB genes in the 2,4-D-degrading Bradyrhizobium sp. were obtained through horizontal gene transfer.

Key words: chlorophenol hydroxylase; tfdB; 2,4-dichlorophenoxyacetic acid (2,4-D); Bradyrhizobium sp.

The herbicide 2,4-D (2,4-dichlorophenoxyacetic acid) has been used worldwide since the 1940s and many 2,4-D-degrading bacterial strains have been isolated that belong to different phylogenetic groups.1–8) Based on the phylogenetic character and genes involved in the degradation of 2,4-D, they have been classified into the following three classes:5) The class I degraders are composed of various genera in the β- and γ-Proteobacteria. They have 2,4-D-catabolizing genes that are homologous to the tfd genes found in Cupriavidus necator (formerly Ralstonia eutropha) JMP134, a well studied 2,4-D degrader.9,10) The class II degraders are composed of Sphingomonas sp. in α-Proteobacteria. It is possible that cadAB genes are involved in the degradation of 2,4-D in this class of degraders.11,12) The class III degraders belong to Bradyrhizobium sp. in α-Proteobacteria. Most of them were isolated from pristine environments and possessed both a tfdA-like gene (tfdAα) and cadAB genes.5,11,13)

The first step in 2,4-D degradation is cleavage of the ether linkage by TfdA or CadAB to produce 2,4-dichlorophenol (2,4-DCP) and glyoxylate.12,14) Degradation of 2,4-DCP proceeds by a hydroxylation reaction to form 3,5-dichlorocatechol (3,5-DCC), followed by cleavage of the aromatic ring to 2,4-dichloromuconate.9) 2,4-DCP monoxygenase and 3,5-DCC dioxygenase, encoded by tfdB and tfdC respectively, mediated the above mentioned reactions successively.9,15)

Despite the difference in the enzymes that attack the ether linkage of 2,4-D, both class I and II degraders have been found to harbor tfdB. The genes were classified into six groups by PCR-RFLP analysis, and independent evolution of the tfdB and tfdC genes is probable.8) Studies of the TfdB enzyme have been limited to a few examples. The active component of TfdB from C. ne- cator JMP134 was found to be a homotetramer,16) and it had the highest activity for 2,4-DCP among several chlorophenol compounds.17) Recently, a second TfdB (TfdBII), from C. necator JMP134, was purified, and it exhibited properties in substrate specificity similar to the first one.18) 2,4-DCP hydroxylases from Burkholderia cepacia 2a (formerly Acinetobacter sp.) and Defluvi- bacter lusatiensis also showed the highest activity for 2,4-DCP, and all the hydroxylases examined were NADPH or NADH dependent.19–21) but there is no information about dichlorophenol hydroxylase and the genes that encode them among type III degraders. Since the strains of this class were isolated from pristine environments, it is important to clarify the genes and enzymes involved in the degradation of 2,4-DCP to understand the origin and evolutionary relationships of 2,4-D catabolic genes. The aim of this study was to
identify the \textit{tfdB} gene among class III 2,4-D-degraders, and to compare its phylogenetic and enzymatic properties with those of previously reported TfdBs.

### Materials and Methods

**Bacterial strains and media.** RD5-C2 and further 2,4-D-degrading \textit{Bradyrhizobium} strains, HW13, HWK12, and BTH, were cultured in 2,4-D-containing basal medium\(^{39}\) or HM medium.\(^{22}\) \textit{E. coli} JM109 and pT7Blue T-Vector (Novagen, Tokyo) were used for gene cloning, and \textit{E. coli} BL21 and pTV118N (Takara, Tokyo) were used in expression experiments. LB medium was used to grow \textit{E. coli}.

**PCR and sequencing of \textit{tfdB}**. Whole DNA samples were obtained from isolates according to the method described previously.\(^{1,23}\) Partial \textit{tfdB} fragments were amplified using primers originally constructed by Vallaeys et al.\(^{8}\) and modified based on the available \textit{tfdB} sequences, 5'-CSCACATCCACCCGCCACARCC-3' and 5'-CRGGCTGGAATSACCCARTA-3'. The amplified \textit{tfdB} gene fragments were directly ligated into pT7Blue T-Vector and transformed into \textit{E. coli} JM109 competent cells. The inserted regions were amplified by PCR using M13 primers, M4 and RV (Takara), and directly sequenced with an ABI 310 DNA sequencing system (Perkin-Elmer, Tokyo) using the same primers. The full sequences of \textit{tfdB} were determined in RD5-C2 using a PCR in vitro Cloning Kit (Takara). All procedures described here were conducted according to the suppliers’ instructions. Briefly, the DNA sample was digested by \textit{EcoRI}, \textit{HindIII}, \textit{PstI} or \textit{SalI}, and ligated with the corresponding cassette. DNA fragments containing \textit{tfdB} were amplified and sequenced by PCR using the cassette primer and primers constructed based on the known sequence in \textit{tfdB}.

**Analysis of \textit{tfdB} sequence.** The \textit{tfdB} sequences were compared with other available sequences using the FASTA\(^{24}\) and the BLAST\(^{25}\) algorithms. Sequences were aligned using the CLUSTALW program,\(^{26}\) and phylogenetic analysis was performed using CLUSTALW with neighbor-joining analysis.\(^{27}\)

**Comparison of codon usage frequency and GC content.** The codon usage patterns and GC contents of \textit{tfdB} examined were compared with those of the \textit{fdxA} and \textit{cadA} genes of these strains, and the nodulation (\textit{nod}), NifA-regulated, and housekeeping genes of \textit{Bradyrhizobium japonicum}\(^{28}\) to elucidate the origin of \textit{tfdB} in 2,4-D-degrading \textit{Bradyrhizobium} strains. Patterns of synonymous codon frequency, except for stop codons and codons for methionine and tryptophan, were grouped by cluster analysis (Ward linkage using squared Euclidean distance) using the SPSS for Windows software (ver. 11.0.1 J, SPSS Japan, Tokyo).

**Construction of clones for expression of \textit{TfdB}**. Two sets of whole \textit{tfdB} genes (\textit{tfdBa} and \textit{tfdBb}) of RD5-C2 were amplified by PCR using primers 5'-CATGCCATGGGAACACGGGAAAGGCAGTCG-3' and 5'-CGGAATTCTTTATCCGACCGAGGCGTTGC-3' for \textit{tfdBa}, and 5'-CATGCCATGGGAACACGGGAAAGGAGTCG-AG-3' and 5'-CGGAATTCTTCAACCGCCGGCGGCAG- GACTTCC-3' for \textit{tfdBb}, to give \textit{NcoI} and \textit{EcoRI} sites at the ends of the forward and reverse primer respectively, and inserted into the \textit{NcoI}-\textit{EcoRI} cloning site of the pTV118N vector after digestion by \textit{NcoI} followed by \textit{EcoRI}, which gave the proper position of the initial codon and the direction of the genes inserted. The plasmid was transformed into \textit{E. coli} BL21 competent cells according to the supplier’s instructions. Correct insertion and sequence were confirmed by PCR and sequencing using M13 primers, M4 and RV-N (Takara).

**TfdB enzyme assay.** Unless otherwise stated, the experiments were conducted at 4°C. \textit{E. coli} BL21 (pTV118N-\textit{tfdB}) was grown to mid-log phase at 30°C using LB medium supplemented with 50 \mu g/ml of ampicillin, followed by induction with IPTG at 0.4 mM for 3 h. The cells were harvested by centrifugation (12,000 \times g, 10 min), and suspended in 10 mM Tris and 1 mM EDTA (pH 8.0). The cell pellets collected by centrifugation were frozen at \(-80°C\). The frozen cells were further treated with liquid nitrogen and disrupted 3 times for 30 seconds by the freeze-fracturing method using Cryo-Press CP-100WP (MicroTech, Chiba, Japan). The powdered samples were suspended in 100 mM \textit{KH}_{2}PO_{4}/Na_{2}HPO_{4} buffer (pH 7.6),\(^{17}\) and crude cell extracts were obtained as supernatant after centrifugation (100,000 \times g, 40 min). The crude cell extracts were examined by denaturing SDS–polyacrylamide gel electrophoresis and assayed for TfdB enzyme activity. Gels were stained with GelCode Blue Stain Reagent (Takara), and Perfect Protein Markers, 10–225 kDa (Novagen) were used as standards and for estimation of protein concentration.

The typical enzyme assay mixture contained 0.2 mM NADPH and 0.1 mM each of chlorophenol or phenol in phosphate buffer.\(^{17}\) The chlorophenols used are listed in Table 2. The enzyme reaction was conducted at 25°C for 60 min. Degradation of the chlorophenol (or phenol) and production of the corresponding chlorocatechol (or catechol) were periodically monitored by HPLC (LC-10AT, Shimadzu, Kyoto, Japan), equipped with a Sumipax ODS-A212 column (Sumika Chemical Analysis Service, Osaka, Japan). The mobile phase was a mixture of acetonitrile–0.1% acetic acid (60:40) at a flow rate of 1.0 ml min\(^{-1}\). The chlorophenols and phenol were detected with a Shimadzu SP10-10A UV–visible light detector at the corresponding suitable wavelengths between 269 and 288 nm, and identified based on their specific retention times. Representative retention times for the substrates and their products were 5–10 min and 4–6 min respectively under the above conditions.
Southern hybridization. Each amplified tfdBa and tfdBb gene fragment (about 440 bp) was labeled with a DIG DNA Labeling Kit (Roche Molecular Biochemicals, Tokyo) and used as a probe for Southern hybridization analysis. The DNA samples were digested with SalI and blotted onto Hybond N nylon membranes (Amersham, Tokyo), then hybridized with a labeled probe under low-stringency conditions, as described previously. All procedures described were also conducted according to the supplier’s instructions.

Nucleotide sequence accession number. The nucleotide sequences determined in this study have been deposited in the DDBJ/EMBL/GenBank database, and the accession numbers run from AB262586 to AB262590.

Results

Gene and amino acid sequences and phylogenetic analysis of tfdB

The tfdB fragments (438 bp) were successfully amplified in all 2,4-D-degrading Bradyrhizobium strains, RD5-C2, HW13, HWK12 and BTH, used in this study, and two different tfdB-like genes were found in strain RD5-C2. The full sequence of the two tfdB-like genes of RD5-C2, tfdBa (1788 bp) and tfdBb (1791 bp), were determined. Their nucleotide and deduced amino acid sequence identities were 60% and 57%, respectively, and their nucleotide sequences most closely matched those of previously reported tfdB, at 63–67% and 61–67% respectively. The deduced amino acid sequence showed similar identity (62–66%) in tfdBa, but relatively lower identity (55–57%) in tfdBb as compared with those in the gene sequence. As shown in Fig. 1, tfdBs were phylogenetically separated into five clusters. Among these, two clusters consisted of tfdB from class I 2,4-D-degraders of which tfdBa and tfdBb of C. necator JMP134 are representatives. One cluster consisted of tfdB of class II Sphingomonas 2,4-D-degraders, and two clusters consisted of tfdB of class III 2,4-D-degraders in which tfdBa and tfdBb of Bradyrhizobium sp. RD5-C2 are represented. Based on full-length sequence comparisons, a similar phylogenetic tree was obtained (data not shown). The gene sequence identities on a full-length basis among the tfdB representatives were 61–67%.

Codon usage frequency and GC content

The genes of Bradyrhizobium sp. examined were separated into two groups based on their GC contents, as summarized in Table 1. Separation of the two groups by GC content and codon usage pattern were in good agreement (data not shown). The first group, which had a lower total GC content (55.7–57.8%), consisted of tfdBa, cadA of 2,4-D-degrading Bradyrhizobium sp., nod, and NifA-regulated genes of B. japonicium. The second group was made up of tfdBb, tfdAα, and housekeeping genes of B. japonicium, and showed higher total GC contents (64.4–67.0%). The difference between the two groups was distinctive when the GC-bias at the third codon position was considered.

Southern hybridization

As shown in Fig. 2, a positive signal from the tfdB probe of RD5-C2 was detected in all the Bradyrhizobium strains examined. In the case of the tfdB probe, it
hybridized with all the strains but HWK12 at different positions from that of the \textit{tfdBa} probe. The position of the signals were almost same between RD5-C2 and HW13, but different from those of HWK12 and BTH in \textit{tfdBa} and the \textit{tfdBb} probe. Weak signals were observed in hybridization with the \textit{tfdBb} probe at the same position as the signals detected with the \textit{tfdBa} probe. It is assumed that they are a cross hybridization of \textit{tfdBa} genes with the \textit{tfdBb} probe due to the low stringency conditions. Weak signals of cross hybridization by the \textit{tfdBa} probe might have been due to experimental conditions such as the specific activity of the probe, etc.

Substrate specificity of TfdBa and TfdBb of RD5-C2

When \textit{tfdBa} and \textit{tfdBb} were overexpressed in \textit{E. coli} BL21 using pTV118N, both TfdB proteins were produced at the expected size, about 65 kDa, by denaturing gel electrophoresis. The relative activities of the tested chlorophenols and phenol are shown in Table 2, accompanied by data on other TfdBs reported previously for \textit{C. necator} JMP134,17,18) \textit{B. cepacia} 2a,19) and \textit{D. lusatiensis} S1.21) In TfdBa crude extract, the highest activity was observed for 2,4-DCP (about 0.5 nmol/min/\textmu g of protein), as with previously characterized TfdB, but it showed a narrower range of activity for chlorophenols than the other TfdBs. The TfdB of \textit{D. lusatiensis} S1 showed activity for all the chlorophenols listed, whereas TfdBa was active only for 4-CP, 2,3-DCP, 2,4-DCP, 3,4-DCP, and 3,5-DCP. In contrast,

Table 1. GC Content of \textit{tfdB}-Like Genes of \textit{Bradyrhizobium} sp.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Number of genes</th>
<th>Total</th>
<th>1st codon position</th>
<th>2nd codon position</th>
<th>3rd codon position</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{tfdBa}</td>
<td>4</td>
<td>55.7</td>
<td>62.0</td>
<td>43.6</td>
<td>61.5</td>
</tr>
<tr>
<td>\textit{tfdBb}</td>
<td>1</td>
<td>67.0</td>
<td>65.3</td>
<td>46.3</td>
<td>89.6</td>
</tr>
<tr>
<td>\textit{tfdBb}</td>
<td>4</td>
<td>64.4</td>
<td>65.3</td>
<td>39.2</td>
<td>89.0</td>
</tr>
<tr>
<td>\textit{caddA}</td>
<td>4</td>
<td>55.7</td>
<td>59.1</td>
<td>43.8</td>
<td>64.5</td>
</tr>
<tr>
<td>\textit{nod} genes</td>
<td>12</td>
<td>57.8</td>
<td>na</td>
<td>na</td>
<td>64.4</td>
</tr>
<tr>
<td>\textit{NifA-regulated} genes</td>
<td>14</td>
<td>57.7</td>
<td>na</td>
<td>na</td>
<td>69.4</td>
</tr>
<tr>
<td>\textit{Housekeeping} genes</td>
<td>19</td>
<td>64.9</td>
<td>na</td>
<td>na</td>
<td>86.4</td>
</tr>
</tbody>
</table>

When \textit{tfdBa} and \textit{tfdBb} were overexpressed in \textit{E. coli} BL21 using pTV118N, both TfdB proteins were produced at the expected size, about 65 kDa, by denaturing gel electrophoresis. The relative activities of the tested chlorophenols and phenol are shown in Table 2, accompanied by data on other TfdBs reported previously for \textit{C. necator} JMP134,17,18) \textit{B. cepacia} 2a,19) and \textit{D. lusatiensis} S1.21) In TfdBa crude extract, the highest activity was observed for 2,4-DCP (about 0.5 nmol/min/\textmu g of protein), as with previously characterized TfdB, but it showed a narrower range of activity for chlorophenols than the other TfdBs. The TfdB of \textit{D. lusatiensis} S1 showed activity for all the chlorophenols listed, whereas TfdBa was active only for 4-CP, 2,3-DCP, 2,4-DCP, 3,4-DCP, and 3,5-DCP. In contrast,

Table 2. Relative Activity of TfdB for Chlorophenols and Phenol

<table>
<thead>
<tr>
<th>Substrate</th>
<th>RD5-C2</th>
<th>pJP4</th>
<th>Strain S1</th>
<th>Strain 2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>&lt; 1</td>
<td>0</td>
<td>3</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>2-CP</td>
<td>&lt; 1</td>
<td>0</td>
<td>11 (13)</td>
<td>31</td>
</tr>
<tr>
<td>3-CP</td>
<td>&lt; 1</td>
<td>0</td>
<td>3 (6)</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>4-CP</td>
<td>68</td>
<td>0</td>
<td>29 (30)</td>
<td>68</td>
</tr>
<tr>
<td>2,3-DCP</td>
<td>26</td>
<td>0</td>
<td>15</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>2,4-DCP</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2,5-DCP</td>
<td>&lt; 1</td>
<td>0</td>
<td>15</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>2,6-DCP</td>
<td>&lt; 1</td>
<td>0</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>3,4-DCP</td>
<td>30</td>
<td>0</td>
<td>&lt; 2</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>3,5-DCP</td>
<td>5</td>
<td>0</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>2,4,5-TCP</td>
<td>&lt; 1</td>
<td>0</td>
<td>&lt; 5 (13)</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>2,4,6-TCP</td>
<td>&lt; 1</td>
<td>0</td>
<td>&lt; 5</td>
<td>&lt; 2</td>
</tr>
</tbody>
</table>

Experiments were performed at 25°C in 100 mM KH\textsubscript{2}PO\textsubscript{4}/Na\textsubscript{2}HPO\textsubscript{4} buffer (pH 7.6) containing 0.1 mM substrate, 0.2 mM NADPH, 1 mM β-mercaptoethanol, 0.1 mM EDTA, and 2 μM FAD.

aLedger et al.18) Data from Liu and Chapman17) are presented in parentheses.
bFritsche et al.20), Makdessi and Lechner.21)
cBeadle and Smith.19) dNot determined.
TfdBb showed no observable activity towards any chlorophenols tested or towards phenol, although production of a protein with an appropriate molecular size was observed, as with TfdBa.

**Discussion**

Vallaey et al.\(^8\) identified the presence of tfdB genes among several genera of class I and class II 2,4-D degraders with the six RFLP patterns. They were separated phylogenetically into three clusters (Fig. 1). The tfdB genes of class I and class II degraders were separated on both levels, but the separation among the class I degraders was unclear. After study,\(^8\) 2,4-D degrading *Bradyrhizobium* strains in α-Proteobacteria, class III degraders, were found in pristine environments\(^5\) and in arable soil with no history of pesticides application.\(^3\) It appeared in this study that this new class of 2,4-D degraders also harbors a tfdB gene with 2,4-DCP hydroxylase activity. The unique environments where they were isolated suggest that TfdBs in this class of 2,4-D-degraders are likely to have original functions other than the degradation of 2,4-DCP. It is possible, however, that the substrates are naturally occurring chlorophenols.\(^29\)

In the case of 2,4-D-degrading β- and γ-Proteobacteria, class I degraders, horizontal gene transfer of tfd genes has been demonstrated experimentally\(^20,21\) and by the incongruent distribution of tfd genes among several genera.\(^6,22\) This suggested also in Fig. 1, in which one cluster of class I degrader consisted of *Burkholderia* sp., *Cupriavidus* sp., *Pseudomonas* sp., and *Ralstonia* sp., and another cluster of the same class degrader consisted of *Burkholderia* sp., *Cupriavidus* sp., *Delftia* sp., *Pseudomonas* sp., *Ralstonia* sp., *Rhodoferax* sp., and *Variovorax* sp. In contrast, phylogenetic clusters of tfdB in α-Proteobacteria were distinctly separated as between *Bradyrhizobium* sp., *Defluviobacter* sp., and *Sphingomonas* sp., indicating evolution of the tfdB genes in this group without recent horizontal gene transfer.

Two kinds of tfdB-like genes were found in the four 2,4-D-degrading *Bradyrhizobium* strains, except for one strain, HWK12. Perhaps they have different origins, based on differences in codon usage pattern and GC content (Table 1). A comparison with the nodulation and NifA-regulated genes in *B. japonicum*, which are considered to be acquired relatively late in evolution via lateral gene transfer from other microorganisms,\(^28\) suggests the possibility that tfdBa was obtained, since the GC contents of both groups were distinctively distinguished from those of housekeeping genes. No other homologous genes were detected in southern hybridization.

Regarding the requirement of NADPH or NADH for the activity (data not shown) and the highest activity for 2,4-DCP, the five TfdBs had similar characteristics although their phylogenetic separations were distinct (Fig. 1 and Table 2). The narrower substrate spectrum of TfdBa in RD5-C2 is unique. Since RD5-C2 was isolated from the uncontaminated environment without any pesticides, higher activity of TfdAa for less chlorinated phenoxyacetate is perhaps reasonable,\(^13\) but TfdBa showed weaker activity for 4-CP and no significant activity for 2-CP and 3-CP or non-chlorinated phenol. The presence of another TfdB homolog, TfdBb, is interesting. Two sets of tfdB genes have also been found on the plasmid pJP4 of *C. necator* JMP134, and they showed similar activities and specificity profiles in spite of the distinct separation in the phylogenetic tree.\(^16\) In the case of TfdBb, since no activity was observed for any phenol examined, no functional consideration is possible.

The codon usage pattern and the GC content of the 2,4-D-degradation-related genes in *Bradyrhizobium* sp. present interesting features in combination with their enzyme activities. *CadAB* and *tfdBa* genes are supposed to be obtained through gene transfer, and are responsible for the degradation of 2,4-D and 2,4-DCP.\(^12,13\) Both enzymes showed higher activity for chlorinated compounds than non-chlorinated ones.\(^12\) but it should be noted that *cadAB* and *tfdB* are likely to have evolved for natural compounds, in which case the degradation of 2,4-D and 2,4-DCP is coincidental, since the strains were isolated from pristine environments.\(^5\) The organization of the *cadAB* and *tfdB* genes has not yet been examined. The *tfdAa* and *tfdBb* genes are supposed to have different origins from the genes mentioned above. TfdBb showed no apparent activity for any phenols in this study. Its functions are unclear, but it is possible that the gene served as the origin of the tfdB genes of 2,4-D degraders. Further studies are needed to clarify the evolutionary relationships between the genes and their original functions as well as their organization to understand the adaptation process of bacteria to chlorinated compounds.

**References**


