Diversity of 2,4-Dichlorophenoxyacetic Acid (2,4-D) and 2,4,5-Trichlorophenoxyacetic Acid (2,4,5-T)-Degrading Bacteria in Vietnamese Soils

NGUYEN L. HUONG1, KAZUHITO ITOH*, and KOUSuke SUYAMA1

1 Faculty of Life and Environmental Science, Shimane University, Matsue, Shimane 690–8504, Japan

(Received March 30, 2007—Accepted May 23, 2007)

Diverse 2,4-D and 2,4,5-T-degrading bacteria were isolated country-wide from ten Vietnamese soils, with or without a history of exposure to Agent Orange. The 353 degraders were phylogenetically grouped into three major categories; *Burkholderia* spp. (43.3% of all degraders), *Sphingomonas* spp. (40.2%), and *Ralstonia* spp. (15.3%) and two minor ones; *Bradyrhizobium* sp. (0.8%) and *Nocardioides* sp. (0.3%). The 2,4,5-T degraders, 65% of all degraders, were isolated from all soil samples and their 16S rRNA genes were the most homologous with that of *Sphingomonas* spp., *Burkholderia* spp. or *Bradyrhizobium* sp. The following four degradative genes were found by PCR: *tfdA* (*tfdAα*) in the *Burkholderia* spp., *Ralstonia* spp., *Bradyrhizobium* sp. and *Nocardiooides* sp.; *tfdB* in all degraders; *tftA* (*cadA*) in the *Sphingomonas* spp., *Burkholderia* sp. and *Bradyrhizobium* sp.; *tftC* only in the *Burkholderia* sp. The degraders among *Burkholderia* spp. were isolated only from the central and southern sites, while those among *Ralstonia* spp. were found only at the north sites with one exception. The *Sphingomonas* spp. were isolated country-wide, but four phylogenetically different groups were found at one site, while only one group was found at the other five sites. At least three different plasmids that carried the *tfd* genes were found in the *Burkholderia* spp. and *Ralstonia* spp. without relation to the sites and the phylogenetic groups. These results suggest that the 2,4-D- and 2,4,5-T-degrading microbial consortia have spread countrywide and are diverse on a genetic as well as geographic basis.

Key words: 2,4-D, 2,4,5-T, degrading bacteria, degradative genes, plasmid

The phenoxyherbicides 2,4-D and 2,4,5-T have been widely used for the control of broad-leaf weeds since the 1940s. Many 2,4-D-degrading microorganisms have been isolated worldwide from a variety of environments such as agricultural soils, sediments, waste treatment facilities, and pristine soils1,6,7,12,15,17,21,27,29. The bacteria capable of biodegrading 2,4-D are categorized into three groups based on their degradation enzymes and physiological properties17. The first group is the copiotrophic and fast-growing bacteria in β- and γ-Proteobacteria such as *Achromobacter*, *Burkholderia*, *Delftia*, *Halomonas*, *Pseudomonas*, *Ralstonia*, *Rhodococcus*, and *Variovorax* isolated from human-disturbed sites. These bacteria contain *tfd* genes which are often encoded on transmissible plasmids6,7,23,28. The second group is the copiotrophic and fast-growing strains in α-Proteobacteria belonging to the genus *Sphingomonas*. They have been isolated from environments that have encountered chlorinated chemicals15,30. A CadA homolog with 54–58% deduced amino acid identity with TftA of the 2,4,5-T-degrading *Burkholderia cepacia* AC1100 was found, but its function has not been confirmed yet14,19. The TfdB and TfdC of this genus show 55–63% and 65–68% identity with counterparts of the first group, respectively30. The third group consists of the oligotrophic bacteria belonging to the genus *Bradyrhizobium* in α-Proteobacteria. They have been isolated from pristine soils in Hawaii, Canada, and Chile17 and subsequently from an arable soil in Japan12. They are characterized by CadAB with 54–59% deduced amino acid identity with TftAB, and TfdAα with 43–46% identity with TfdA of the first group13,14.

In contrast to the diversity of 2,4-D-degrading bacteria,
only a few 2,4,5-T-degrading bacteria have been found. Three strains have been demonstrated to grow on 2,4,5-T as a carbon and energy source. The first is *B. cepacia* AC1100, created by the technique of plasmid-assisted molecular breeding\(^{18}\). The biochemistry, enzymology, and genetics of the 2,4,5-T-degrading pathway in AC1100 have been studied in detail. 2,4,5-T oxygenase encoded by *tftAB* converts 2,4,5-T to 2,4,5-tricholorophenol (2,4,5-TCP)\(^{4,36}\), which is further oxidized by chlorophenol 4-monoxygenase encoded by *tftCD*\(^{8,11,35}\). The second is *Nocardioides simplex* strain 3E isolated in 1990 from soils treated long-term with 2,4,5-T\(^{9}\). No information on the genes involved in the degradation by 3E has been published up to now. After more than 20 years, a third 2,4,5-T-degrading bacterium, *Burkholderia* sp. JR7B3, was isolated from soil contaminated with Agent Orange (a mixture of 50% 2,4-D and 50% 2,4,5-T) in Florida\(^{25}\). The strain was shown to contain degradative genes, and the deduced amino acid sequences were almost identical with TftA (92%), TftC (92%), and TftE (99%) of *B. cepacia* AC1100\(^{25}\).

Beside many reports on the isolation and characterization of biodegrading strains, there have been a few studies on the diverse 2,4-degrading bacteria in environments. Ka *et al.*\(^{15}\) studied the diversity of 2,4-D-degrading bacteria isolated from small agricultural plots treated with 2,4-D at different concentrations. Twenty-seven of 47 isolated 2,4-D-degrading bacteria were identified as *Sphingomonas*, *Pseudomonas*, and *Alcaligenes* by conducting a fatty acid methyl ester analysis, and characterized based on hybridization patterns obtained with *tfd* gene probes. Vallaeys *et al.*\(^{32}\) examined the diversity of 68 isolates of 2,4-D degraders in agricultural soil by performing a PCR restriction fragment length polymorphism (PCR-RFLP) analysis of the 16S rRNA and *tfd* genes, and showed the involvement of diverse bacterial species in the degradation of 2,4-D in the soil. Macur *et al.*\(^{22}\) had examined the diversity of dominant 2,4-D-degrading bacteria in agriculture soil under increasing concentrations of 2,4-D using PCR DGGE of 16S rRNA genes coupled with a traditional isolation method. They showed that the dominant 2,4-D-degrading isolates were *Variovorax*, *Achromobacter*, *Mesorhizobium*, and *Bradyrhizobium* at 10 ppm of 2,4-D, and *Burkholderia* at 500 ppm. In the Florida soil contaminated with Agent Orange, only two bacteria, *Burkholderia* sp. JR7B3 and JRB1, were isolated as 2,4,5-T- and 2,4-D-degraders, respectively\(^{25}\). The degrading gene sequences were highly similar to the previously reported *tft* and *tfd* genes. Besides this, there is little information on 2,4-D- and 2,4,5-T-degrading bacteria in soils contaminated heavily with Agent Orange long term.

In the Vietnam War, Agent Orange was used extensively between 1962 and 1971 as a defoliant. An estimated more than 45 million kg of this herbicide was used in South Vietnam\(^{26}\). In this paper, we focus on studying indigenous 2,4-D- and 2,4,5-T-degrading bacteria isolated from Agent Orange-contaminated and uncontaminated soils in the northern, central and southern regions of Vietnam. A variety of 2,4-D- and 2,4,5-T-degrading bacteria were found, such as *Sphingomonas*, *Ralstonia*, *Burkholderia*, *Bradyrhizobium*, and *Nocardioides*, and the genes and plasmids involved in the degradation were investigated in relation to the location isolated as well as phylogenetic classification.

**Materials and Methods**

**Soil samples**

Soil samples were collected at northern (VN1 and VN2), central (VN3 and VN4), and southern (VN5 to VN10) sites in Vietnam during September 2004 (Fig. 1). The samples...
The experiment was conducted in duplicate for each soil sample. The herbicides were about 70 mg L\(^{-1}\) of 2,4-D and 2,4,5-T. The sample was centrifuged (1,880 \(\times\) g, 20 min) to remove soil particles, and the amounts of 2,4-D and 2,4,5-T in the supernatant were determined by HPLC. When 2,4-D or 2,4,5-T disappeared from the soil-water suspension, 5 ml of a 0.25% aquatic solution of 2,4-D or 2,4,5-T sodium salt (pH 7.0) was added to obtain the initial concentration (0.1 g L\(^{-1}\)) of the herbicides. The experiment was conducted in duplicate for each soil sample.

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

When the first and second disappearance of 2,4-D and 2,4,5-T were observed, a one milliliter aliquot of each soil-water suspension was taken and diluted with sterilized distilled water to prepare a series of dilutions from 10\(^{-1}\) to 10\(^{-8}\). One hundred-microliter portions of the dilutions from 10\(^{-5}\) to 10\(^{-8}\) were plated onto 2,4-D- or 2,4,5-T-containing basal agar medium (g L\(^{-1}\)) [2,4-D or 2,4,5-T (0.1), K\(_2\)HPO\(_4\) (0.5), KH\(_2\)PO\(_4\) (0.5), NH\(_4\)NO\(_3\) (0.25), MgCl\(_2\)-6H\(_2\)O (0.2), CaCl\(_2\)-2H\(_2\)O (0.02), Na\(_2\)MoO\(_4\)-2H\(_2\)O (0.002), FeSO\(_4\)-7H\(_2\)O (0.001), MnSO\(_4\)-7H\(_2\)O (0.001), and agar (15)]. After one to two weeks of incubation at 25°C, a plate with an adequate number of colonies was selected.

The ability of all the isolates to degrade 2,4-D and 2,4,5-T was determined using the basal liquid medium containing 2,4-D or 2,4,5-T at 100 mg L\(^{-1}\). After two weeks of incubation with shaking at 25°C, a one-milliliter aliquot was centrifuged (21,880 \(\times\) g, 10 min), and the supernatant was subjected to quantification of the remaining 2,4-D or 2,4,5-T by HPLC.

**HPLC of 2,4-D and 2,4,5-T**

The analysis of 2,4-D and 2,4,5-T was conducted with a high-performance liquid chromatograph (LC-10AT, Shimadzu, Kyoto, Japan), equipped with a SUMIPAX ODS-A212 column (id., 6 mm; length, 150 mm) (Sumika Chemical Analysis Service, Osaka, Japan). The mobile phase was a mixture of acetonitrile-0.1% acetic acid (60:40) with a flow rate of 1.0 ml min\(^{-1}\). 2,4-D and 2,4,5-T were detected using a Shimadzu SP10-10A UV-visible light detector at 284 nm and identified based on their retention times.

**DNA extraction, PCR amplification, and sequence analysis**

To obtain whole DNA from isolates, the cultures were

---

<table>
<thead>
<tr>
<th>Soil name</th>
<th>Sites</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Soil type</th>
<th>Texture</th>
<th>MWHC</th>
<th>pH (H(_2)O)</th>
<th>Total C (g kg(^{-1}))</th>
<th>Total N (g kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>VN1</td>
<td>Cau Dien</td>
<td>21°02'26&quot;N</td>
<td>105°45'43&quot;E</td>
<td>Fluvisol</td>
<td>Sandy loam</td>
<td>465</td>
<td>8.1</td>
<td>20.8</td>
<td>1.6</td>
</tr>
<tr>
<td>VN2</td>
<td>Van noi</td>
<td>21°08'50&quot;N</td>
<td>105°48'51&quot;E</td>
<td>Acrisol</td>
<td>Sandy loam</td>
<td>434</td>
<td>7.8</td>
<td>7.9</td>
<td>0.8</td>
</tr>
<tr>
<td>VN3</td>
<td>Quang tri</td>
<td>16°44'39&quot;N</td>
<td>107°06'52&quot;E</td>
<td>Fluvisol</td>
<td>Sandy loam</td>
<td>287</td>
<td>5.4</td>
<td>4.7</td>
<td>0.5</td>
</tr>
<tr>
<td>VN4</td>
<td>Hue</td>
<td>16°17'25&quot;N</td>
<td>107°24'43&quot;E</td>
<td>Ferralsol</td>
<td>Sandy loam</td>
<td>366</td>
<td>5.2</td>
<td>6.5</td>
<td>0.6</td>
</tr>
<tr>
<td>VN5</td>
<td>Khanh hoa</td>
<td>12°39'13&quot;N</td>
<td>109°00'54&quot;E</td>
<td>Acrisol</td>
<td>Sandy loam</td>
<td>289</td>
<td>5.0</td>
<td>6.0</td>
<td>0.5</td>
</tr>
<tr>
<td>VN6</td>
<td>Binh my</td>
<td>11°09'25&quot;N</td>
<td>106°45'03&quot;E</td>
<td>Acrisol</td>
<td>Sandy loam</td>
<td>270</td>
<td>4.5</td>
<td>7.3</td>
<td>0.5</td>
</tr>
<tr>
<td>VN7</td>
<td>Chanh my</td>
<td>10°59'27&quot;N</td>
<td>106°37'43&quot;E</td>
<td>Fluvisol</td>
<td>Clay</td>
<td>688</td>
<td>4.0</td>
<td>56.3</td>
<td>3.6</td>
</tr>
<tr>
<td>VN8</td>
<td>Tam hiep</td>
<td>10°57'48&quot;N</td>
<td>106°52'01&quot;E</td>
<td>Fluvisol</td>
<td>Clay loam</td>
<td>454</td>
<td>5.3</td>
<td>12.3</td>
<td>1.3</td>
</tr>
<tr>
<td>VN9</td>
<td>Phu hua</td>
<td>10°59'06&quot;N</td>
<td>106°38'12&quot;E</td>
<td>Acrisol</td>
<td>Loamy sand</td>
<td>245</td>
<td>6.0</td>
<td>6.0</td>
<td>0.5</td>
</tr>
<tr>
<td>VN10</td>
<td>Bien hoa</td>
<td>10°58'32&quot;N</td>
<td>106°49'32&quot;E</td>
<td>Acrisol</td>
<td>Sandy loam</td>
<td>256</td>
<td>5.7</td>
<td>8.2</td>
<td>0.6</td>
</tr>
</tbody>
</table>

---

\(^a\) FAO classification\(^{15}\).

\(^b\) Maximum water-holding capacity (g kg\(^{-1}\)).

Concentrations of 2,4-D and 2,4,5-T were less than 1.0 mg kg\(^{-1}\) in VN1-9, and 144 and 194 mg kg\(^{-1}\), respectively, in VN10.
grown on a 2,4-D- and 2,4,5-T-containing basal liquid 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⁻¹ solution per 40 µl of sample) (Takara, Tokyo, Japan) in a 40 mM Tris buffer solution (50 µl) containing 1% Tween 20, 0.5% Nonidet P-40 (Sigma, Tokyo, Japan), and 1 mM EDTA (pH 8.0). The mixture was incubated at 60°C for 20 min to digest proteins and then at 95°C for 5 min to inactivate the enzyme. After centrifugation (21,880×g, 10 min), the supernatant was collected for gene amplification by PCR.

PCR amplification of the 16S rRNA, tfdA (tfdAα), tfdB, tfbA (cadA), and tfC genes of each isolate was performed using primers (Table 2) with a Mycycler™-thermal cycler (BioRad, Tokyo, Japan). The reactions were performed for 2 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C, and then a final 2 min at 72°C. The reaction mixtures contained 2 µl of 10× reaction buffer, 5 mM of each dNTP, 10 pmol of each primer, 0.5 U of Taq DNA Polymerase (Bioneer, Seoul, Korea), 0.8 µl of template DNA, and enough sterilized distilled water to make up 20 µl. PCR products were examined by 1% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV transillumination. PCR products were purified using a QuickStep 2 PCR Purification kit (Edge Biosystems, Gaithersburg, USA).

Cycle sequencing of the PCR products was performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Tokyo, Japan) with 30–100 ng of template DNA, 3.2 pmol of the respective primer, 1 µl of premix (2.5×), and 3.5 µl of buffer (5×) in a total volume of 20 µl. PCR was performed for 1 min at 96°C, and then 25 cycles of 10 sec at 96°C, 5 sec at 50°C, and 4 min at 60°C. The products were purified with Performa DTR Gel Filtration Cartridges (Edge Biosystems), precipitated with 70% ethanol, dissolved in 15 µl of Hidi-Formamide (Applied Biosystems), and then denaturated at 96°C for 2 min and chilled on ice. The sequencing analysis was carried out on an ABI Prism, 3100-Avant Genetic Analyzer (Hitachi, Tokyo, Japan).

The gene sequences were compared with those in the DNA Data Bank of Japan using the BLAST algorithm. Sequences were aligned, a phylogenetic analysis was performed with the CLUSTAL W (1.8) program using the neighbor-joining method, and a bootstrap analysis based on 1000 replicates was used to place confidence estimates on the tree.

**Plasmid preparation**

The extraction of plasmid was performed according to the procedures described by Birnboim and Doly3. Isolates were grown in 4 ml of PTYG liquid medium (g L⁻¹) [peptone (0.25), tryptone (0.25), yeast extract (0.5), glucose (0.5), MgSO₄·7H₂O (0.03), and CaCl₂·2H₂O (0.003)] with shaking at 25°C for 4 days. The cells were harvested by centrifugation (21,880×g, 10 min) and thoroughly suspended in 100 µl of TE. The suspension was mixed thoroughly with 200 µl of SDS-alkaline solution (0.2N NaOH and 1% SDS)

<table>
<thead>
<tr>
<th>Table 2. Primers used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Applying</strong></td>
</tr>
<tr>
<td>16S rRNA</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>tfdA</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>tfdAα</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>tfdB</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>tfC</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>cadA</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>tfC</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
by inverting the tube and kept on ice for 5 min, and then 150 µl of potassium acetate (3M, pH 5.2) was added. The contents of the tube were gently mixed by inversion and kept on ice for 5 min, then centrifuged at 21,880×g for 10 min. The supernatant was transferred into a new tube and treated with RNase (10 mg ml⁻¹) at 37°C for 30 min. The plasmid preparation was washed with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated by adding 1/10 of sodium acetate (3M, pH 5.2) and 2 volumes of ethanol. The pellet was dissolved in 20 µl of TE.

Gel electrophoresis of the plasmid was performed using 0.75% SeaPlaque GTG agarose (Cambrex Bio Science Rockland, Rockland, USA) at 100 V for 1 hour in 0.8×TAE (32 mM Tris, 16 mM acetate, and 0.8 mM EDTA, pH 8.0). After electrophoresis, gels were stained with ethidium bromide and photographed under UV illumination.

Hybridization

The strains were grown on PTYG liquid medium at 25°C with shaking for 4–7 days. Cells were collected by centrifugation at 6,000×g for 10 min. The cells were resuspended and lysed with 4 ml of lysozyme solution (0.5 mg ml⁻¹) at 38°C for 10 min, then 5 ml of a Proteinase K-SDS mixture (each 0.2 mg ml⁻¹) was added and the incubation was continued at 38°C for 1 hour. The crude DNA preparation was washed with phenol-chloroform-isoamyl alcohol (25:24:1), and treated with RNase (10 mg ml⁻¹) at 38°C for 1 hour in TE buffer (pH 8.0). The DNA samples after complete digestion with Sall (Toyobo, Tokyo, Japan), and the plasmids subjected to agarose gel electrophoresis were transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech, Tokyo, Japan). The amplified ffdA, ffdB, tfcA, and tfcC fragments of Burkholderia sp. M38-VN3-2W and cadA fragment of Sphingomonas sp. 52-VN6-1W were labeled by a DIG DNA labeling kit (Roche Applied Science, Tokyo, Japan) and used as probes for hybridization analysis. The hybridizations with each probe were performed under the low-stringency conditions described previously. The detection of a hybridized probe was realized using the DIG Luminescent Detection kit (Roche Applied Science) according to the proposed protocol.

Nucleotide sequence accession number

The nucleotide sequences determined in this study have been deposited in the DDBJ, EMBL, and GenBank databases under accession numbers AB299569 to AB299603 for the 16S rRNA gene, AB299604 to AB299627 for ffdA and tfdA, AB299628 to AB299662 for tfdB, AB299663 to AB299675 for tfcA and cadA, and AB299676 to AB299678 for tfcC.

Results

Degradation of 2,4-D and 2,4,5-T in soil-water suspension

At the start of incubation, 2,4-D and 2,4,5-T concentrations in soil-water suspensions were about 100 mg L⁻¹. The degradation of 2,4-D was found in all soil-water suspensions within 1 week with one exception in which 2,4-D was degraded in the third week in one VN5. Degradation of 2,4,5-T was observed in VN2, VN3, VN8, and VN10 within 1 or 2 weeks, while it took 2-5 weeks for the degradation to occur in VN4, VN5, VN6, VN7, and VN9. 2,4,5-T disappeared within 1 or 2 weeks after the lag period. In VN1 and one of VN4 and VN5, 2,4,5-T gradually decreased during 7–10 weeks of incubation (Table 3). The subsequent degradations took only one week for 2,4-D and 2,4,5-T to be degraded in most cases.

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

Samples were taken after 1 and 2 weeks of incubation for all soil-water suspensions, and at 4 or 5 weeks for VN4, VN5, VN6, VN7, and VN9, and at 10 weeks for VN1, VN4, and VN5. More than seven hundred colonies were picked up, and three hundred and fifty three bacteria with 2,4-D- and/or 2,4,5-T-degrading ability were isolated (Table 3). Proportions of the degraders among the isolates varied from 12% in VN7 to 90% in VN2. They all degraded 2,4-D, and 65% of them showed the ability to degrade 2,4,5-T. Phylogenetic analyses based on the partial 16S rRNA gene sequences (ca. 500 nucleotides) of the isolates indicated that they appeared to be clustered into five groups: Burkholderia, Sphingomonas, Ralstonia, Bradyrhizobium, and Nocardioïdes. Their numbers and distributions in each soil are shown in Table 3. Dominant genera were Burkholderia, Sphingomonas, and Ralstonia. Distributions of the major degraders differed among the sites: Sphingomonas and Ralstonia from VN1 and VN2, Sphingomonas and Burkholderia from VN4, VN5, VN6, and VN10, and only Burkholderia from VN3, VN7, VN8, and VN9 (one Ralstonia strain in VN9). Both minor degraders, Bradyrhizobium and Nocardioïdes, were isolated from VN10.

Thirty-five representative strains were selected as a different phylogenetic group from each soil, and a phylogenetic tree was constructed based on almost complete 16S rRNA gene sequences (ca. 1400 nucleotides) as shown in Fig. 2.
The genus *Sphingomonas* were isolated from VN1, VN2, VN4, VN5, VN6, and VN10. All isolates belong to this group could degrade both 2,4-D and 2,4,5-T (Table 4). The *Sphingomonas* degraders were tentatively classified at the species level into five groups based on 16S rRNA gene sequences (Fig. 2). All isolates of group S(I) from VN1, VN2, VN4, VN5, and VN6 showed identical 16S rRNA gene sequences and were most homologous with that of *S. chungbukensis* (97%). In contrast, groups S(II), S(III), S(IV), and S(V) were isolated from VN10, and their most homologous sequences were those of *S. agrestic* (98%), *S. herbicidovorans* (98%), *S. paucimobilis* (100%), and *S. yabuuchiae* (94%), respectively.

Various groups of *Burkholderia* were found in the central and south Vietnamese soils, but not in the northern soils. The *Burkholderia* degraders were tentatively classified at the species level into five groups based on 16S rRNA gene sequences (Fig. 2). Distribution patterns of these groups among the locations and dominance among the groups differed as shown in Table 3. Groups B(II) and B(V), which were most homologous with *B. cepacia* AC1100 (99%) and *B. sacchari* JS150 (100%), respectively, were able to degrade 2,4-D and 2,4,5-T, whereas the other groups could degrade only 2,4-D (Table 4). The 16S rRNA gene nucleotide sequences of all group B(II) isolates from VN3, VN8, and VN9 were identical, and those of group B(V) isolated from VN3, VN6, VN7, VN8, and VN10 were also identical.

Members of the genus *Ralstonia* were isolated from VN1, VN2, and VN9, and could degrade 2,4-D but not 2,4,5-T (Table 4). They were tentatively classified at the species level into two groups based on 16S rRNA gene sequences (Fig. 2). Groups R(I) and R(II), which were most homologous with *R. taiwanenesis* (98–99%) and *R. eutropha* (99%), respectively, were found in VN1, but only group R(I) was found in VN2.

The *Bradyrhizobium* strains were isolated only from
2,4-D and 2,4,5-T Degraders in Soils of Vietnam

VN10, and were most homologous with \textit{B. elkanii} (99%). They showed 2,4-D- and 2,4,5-T-degrading ability. The \textit{Nocardioides} strain was also found only in VN10 and was most homologous with \textit{N. dubius} (95%). The strain degraded only 2,4-D.

Distribution and phylogenetic analysis of 2,4-D- and 2,4,5-T-degradative genes

The distribution of 2,4-D- and 2,4,5-T-degradation-related genes among the degraders is summarized in Table
Almost all *Burkholderia*, *Ralstonia*, *Bradyrhizobium*, and *Nocardioides* strains examined showed the amplification of *tfdA* (or *tfdAα*) and *tfdB* gene fragments by PCR. A few exceptions were group B(II) strains isolated from VN8 and VN9. They showed no PCR product for *tfdA*. The absence of *tfdA*-like was confirmed by Southern hybridization by using the *tfdA* gene fragment of the *Burkholderia* strain as a probe (data not shown). In *Sphingomonas* strains, *tfdB* gene fragments were amplified in all strains and the absence of a *tfdA*-like gene was confirmed by the Southern hybridization.

Based on the nucleotide sequences (ca. 310 bp), *tfdA* (*tfdAα*) genes were phylogenetically divided into three groups (Fig. 3 and Table 4). The major group (group I) consisted of *Ralstonia*, *Nocardioides*, and *Burkholderia* strains that differed from each other by 5 nucleotides at most (data not shown). The nucleotide (deduced amino acid) sequences of *tfdA* in this group were 99–100% (98–100%) identical with the previously reported *tfdA* (TfdA) of *R. eutropha* JMP134. The sites where they were isolated included all locations, but the isolates from VN2 and VN3 were distinguished from the other strains with the same difference of one nucleotide. The *Burkholderia* strains from VN10 showed the presence of *tfdA* with 86–87% nucleotide (86–89% in deduced amino acid) identity to the first group (group II). The phylogenetic position was between *tfdA* of *R. eutropha* JMP134 and *Burkholderia* sp. RASC (Fig. 3). The nucleotide (deduced amino acid) sequence of *tfdAα* amplified from the *Bradyrhizobium* strain was 92% (91%) and 53% (44%) identical to that from 2,4-D-degrading *Bradyrhizobium* sp. RD5-C2 and *R. eutropha* JMP134, respectively (group III).

The *tfdB* nucleotide sequences (ca. 490 bp) were phylogenetically separated into three groups. The first group consists of *tfdB* from the *Ralstonia*, *Nocardioides*, and *Burkholderia* strains, which were all 98–100% identical (97–100% in deduced amino acid) to that of *R. eutropha* JMP134 (group I). In group I, group B(V) strains formed one cluster, and group B(II) strains and the strains isolated from VN2 and VN3 formed another cluster. The second group was *Sphingomonas* *tfdB*, which were all 89–91% identical (94–98% in deduced amino acid) to that of *tfdB* of *S. herbicidivorans* MH (group II). The nucleotide (deduced amino acid) sequences of the *tfdB* in this group were 63–
64% (62–66%) identical with the tfdB (TfdB) of *R. eutropha* JMP134. The tfdB nucleotide sequences of the strains isolated from VN2, VN4, VN5, and VN6 belonging to group S(I) were identical. The third group (group III) was *Bradyrhizobium* tfdB with 59% homology (64% in deduced amino acid) to tfdB of *Bradyrhizobium* sp. RD5-C2 (Fig. 4 and Table 4). The nucleotide (deduced amino acid) sequence of the tfdB of this strain was 64% (62%) identical with the tfdB (TfdB) of *R. eutropha* JMP134.

The tfA (cadA) gene fragments (ca. 490 bp) were amplified by PCR only in the 2,4,5-T-degrading strains in contrast to tfdA which was distributed among both the 2,4-D- and 2,4,5-T-degrading bacteria. As an exception, group B(V) 2,4,5-T-degrading bacteria showed an absence of the tfA (cadA) gene by PCR and Southern hybridization using the tfA gene fragment of group B(II) strains as a probe (data not shown). The phylogenetic analysis clearly demonstrated that the genes were divided into three groups: 99% (99% in deduced amino acid) identical to that of *B. cepacia* AC1100 (group I), 91% (96–97%) to cadA of *Sphingomonas* sp. B6–10 (group II), and 92% (97%) to a cadA-like gene of *B.
elkanii USDA94 which has no ability to degrade 2,4-D (group III). The nucleotide (deduced amino acid) sequence identity of the last group with that of 2,4-D-degrading Bradyrhizobium sp. RD5-C2 was 61% (58%) (Fig. 5 and Table 4). The nucleotide (deduced amino acid) sequences of group II and group III were 31% (45%) and 62% (48%) identical with tftA (TftA) of B. cepacia AC1100, respectively. The tftA nucleotide sequences of all the Vietnamese group B(II) isolates were identical, and the nucleotide sequences of cadA of group S(I) degraders were also identical.

The tftC gene fragments were amplified only in group B(II) strains, and no obvious signal was observed in the other strains by Southern hybridization analysis (data not shown). The nucleotide (deduced amino acid) sequence identity was 94% (94%) and 93% (92%) with those previ-
2,4-D and 2,4,5-T Degraders in Soils of Vietnam

Petersen et al. (1998) previously reported in B. cepacia AC1100 and Burkholderia sp. JR7B3, respectively. Differences among the \textit{tftC} sequences of the Vietnamese group B(II) degraders were one nucleotide in the 456 bp fragment (data not shown).

\textbf{Plasmid}

To elucidate the locations of the degrading genes and diversity of the plasmids on which the genes reside, the plasmids were compared by agarose gel electrophoresis and hybridized with the genes for some strains. A representative result of the electrophoresis and hybridization with the \textit{tfdA} probe is shown in Fig. 6. The result of hybridization with the \textit{tfdB} probe was the same as in Fig. 6 (data not shown). The presence of plasmids was observed in most of the isolates except for \textit{Sphingomonas} spp., \textit{Bradyrhizobium} sp., \textit{Nocardioides} sp. and group B(V) degraders. The \textit{tfdA} and \textit{tfdB} genes were detected in most of the plasmids, whereas neither \textit{tftA} nor \textit{tftC} was hybridized with the plasmids of group B(II) degraders. The size and distribution of the plasmids varied as follows. Group B(III) degraders from VN8 and VN9, group B(IV) degraders from VN9, and group B(I) degraders from VN4 had plasmids of the same size which hybridized with the \textit{tfdA} and \textit{tfdB} probes. Group R(I) degraders from VN1 and VN9 had three plasmids each of the same size with the \textit{tfdA} and \textit{tfdB} genes detected in the largest plasmid. Whereas plasmids of the same size were...
generally distributed in each genus among the isolates from different sites, different plasmids existed in isolates from the same site such as groups R(I) and R(II) degraders from VN1. In addition, different plasmids were also present in the same species in the case of group B(III) degraders from VN3 and VN8. Various plasmids were harbored by group B(II) degraders, but no probes hybridized with any of them.

Discussion

The degradation of 2,4-D started in all soil-water suspensions after 1 week and 2,4-D-degrading bacteria were successfully isolated from all samples after the first degradation of 2,4-D. The isolated 2,4-D-degrading bacteria were phylogenetically the same as those that have been previously reported. Major degraders belonged to the genera *Burkholderia*, *Sphingomonas*, and *Ralstonia* as in other environments examined (6,12,13,17). In almost all the *Burkholderia* spp. and *Ralstonia* spp., both *cadA* and *tfdB* were found with more than 99% identity with previously reported sequences in this group of 2,4-D-degraders, indicating the ubiquitous nature of the 2,4-D-degrading genus and genes involved. In *Sphingomonas* spp., both *cadA* and *tfdB* were found with more than 90% identity with those in the *Sphingomonas* 2,4-D-degraders. To date, the 2,4-D-degrading *Sphingomonas* sp. has been believed to have no *tfdA* gene based on the negative results of PCR amplification and hybridization (15,23,31). As minor 2,4-D-degrading bacteria, *Bradyrhizobium*, which can degrade both 2,4-D and 2,4,5-T, was found in VN10. This class of 2,4-D-degraders have been isolated from soils without a history of 2,4-D application such as pristine environments (37). Isolation of this class of 2,4-D-degraders in contaminated soil and their ability to degrade 2,4-D as well as 2,4,5-T suggest adaptation to contaminated environments for proliferation. The *tfdA* and *cadA* gene sequences of the Vietnamese *Bradyrhizobium* sp. were 92% and 91% identical to those of 2,4-D-degrading *Bradyrhizobium* sp. isolated from pristine environments, in contrast, the identity was 59% in the case of *tfdB*, suggesting different origins of these genes. The other minor isolate was the *Nocardiooides* strain, found to have the same 2,4-D-degrading genes as the *Burkholderia* spp. and *Ralstonia* spp. Although identified previously as 2,4,5-T-degrading bacteria, it is the first in this class of bacteria to harbor *tfdA* and *tfdB* which would be obtained through horizontal gene transfer.

The 2,4,5-T-degrading bacteria were isolated from all soils within 5 weeks, and within 2 weeks from VN2, VN3, VN8, and VN10. It is interesting to note that the 2,4,5-T-degrading bacterium *Burkholderia* sp. JR7B3 was isolated after 3 weeks in an enrichment culture (25), it took 8 to 10 months for *B. cepacia* AC1100 (8), and it took 21 months for *Nocardiooides* sp. (9). More abundant and diverse 2,4,5-T-degrading bacteria were isolated from the Vietnamese soils. The highly contaminated environments may have enhanced the adaptation and proliferation of the 2,4,5-T-degrading bacteria. The 2,4,5-T degrading bacteria were found not only in group B(II) degraders, the most homologous with *B. cepacia* AC1100, but also in group B(V) degraders, *Sphingomonas* spp. and *Bradyrhizobium* sp. In previous studies, *Burkholderia* sp. JR7B3 was isolated from Agent Orange-contaminated soil in Florida and contained *tfdA* and *tftC* genes which had 97% and 96% identity to those involved in 2,4,5-T’s degradation by *Burkholderia* sp. AC1100 (25). In our experiment, the same genes with more than 99% and 92% identity to the previously reported *tfdA* and *tftC* were also identified in group B(II) strains, respectively, in addition to *tfdA* and *tfdB*. As *tfdA* and *tfdB* are almost identical among the Vietnamese isolates, it is supposed that *tfdA* and *tftC* are essential for the degradation of 2,4,5-T. *Burkholderia* sp. AC1100 is a laboratory-bred 2,4,5-T-degrading strain that emerged by mixing several catabolic plasmids and sediments for 8 to 10 months (31), and *Burkholderia* sp. JR7B3 was the first 2,4,5-T-degrading strain to be isolated from a natural environment. Our results show the additional example that a similar genetic adaptive process occurred naturally in the 2,4,5-T-contaminated environments. Group B(V) strains, found in VN3, VN6, VN7, VN8, and VN10, could degrade 2,4-D and 2,4,5-T, however, neither *tfdA* nor *tftC* was found, suggesting that the degraders have different enzyme systems for the degradation of 2,4,5-T.

The genus *Sphingomonas* is becoming important in environmental microbiology because various xenobiotic-degrading organisms belong to this group (31). Previously, a *S. herbicidovarans* MH has been described that degrades phe-noxalkanoic acid herbicides such as meprop, dichlorprop, 2,4-D, 4-chloro-2-methylphenoxyacetic acid (MCPA), and 2,4-dichlorophenoxybutanoic acid (2,4-DB) (20,24), although this strain could not degrade 2,4,5-T (20). In this study, group S(III) strains, most homologous with *S. herbicidovarans*, were isolated as 2,4,5-T-degraders. In addition, phylogenetically different 2,4,5-T-degrading *Sphingomonas* strains were isolated, suggesting that the members of this genus have the ability to adapt for degradation of 2,4,5-T in highly contaminated soil environments.

The geographic distribution of the 2,4-D- and 2,4,5-T-degrading bacteria showed some characteristics based on their phylogeny, degradation-related genes, and plasmids.
Major degraders were different in each soil sample (Table 3). The Burkholderia strains were isolated only in the central and southern areas, and there was one phylogeny in VN5 and VN10, but a few phylogenies at the other sites. Based on the tfdA and tfdB gene sequences, the isolates from VN2 and VN3 were grouped into one cluster. In spite of the similarity of the degradation genes between VN2 and VN3, the plasmids in which the genes reside differed in size (data not shown). Most of the tfdA and tfdB gene sequences from the other locations were identical and some plasmids were the same size, suggesting a country-wide distribution of the genes by plasmid-mediated horizontal gene transfer. However, some plasmids of different sizes were found in Burkholderia spp. as well as Ralstonia spp. In the case of Ralstonia spp., they were isolated from the same soil. These observations suggest that the degradative genes have been distributed on diverse plasmids among several species of the genera. In contrast, group B(II) degraders have almost identical 16R rRNA, tfdB, cadA, and tfdC genes among the isolates from VN3, VN8, and VN9. Group B(V) degraders also have almost identical 16R rRNA and tfdB genes among the isolates from VN3, VN6, VN7, VN8, and VN10. Both are degraders of 2,4-D and 2,4,5-T, and each strain may have spread to central and southern areas in Vietnam.

In contrast to Burkholderia spp., the Ralstonia strains were only isolated from the north with one exception in VN9, and the major phylogeny differed between the sites. The geographic separation of the Burkholderia and Ralstonia strains might be related to the area’s history: with and without heavy and long-term contamination by Agent Orange. The degradative genes and plasmids were so diverse because the genes were different between group R(I) degraders from VN1 and VN2, and the plasmids were different between group R(I) and R(II) degraders both isolated from VN1.

In Sphingomonas spp., only group S(I) degraders were found in VN1, VN2, VN4, VN5, and VN6, while four phylogenetically different groups were found in VN10. The former degraders have almost identical 16R rRNA, cadA and tfdB genes, which are different from those of Sphingomonas spp. isolated from VN10. It is suggested that the same strain of degrader may be present around the country, while diverse Sphingomonas spp. exist in VN10 that has been heavily contaminated by Agent Orange for a long time.

From the standpoint of sampling sites, major degraders were Sphingomonas and Ralstonia in VN1 and VN2, Sphingomonas and Burkholderia in VN4, VN5, VN6, and VN10, and only Burkholderia in VN3, VN7, VN8, and VN9. What determines distribution and dominant degraders in relation to contamination with herbicides is a challenging question because several factors including the microbial community and physico-chemical properties of soils may affect proliferation of the degraders. No relationship between dominant degraders and soil characteristics was observed in this study.

In addition, it is clear that the enrichment-cultivating conditions may have selected for 2,4-D- and 2,4,5-T-degrading bacteria in the soil samples. The enrichment process may result in a different microbial community structure from the original one. Therefore, this should be taken into account when considering a microbial community based on cultured microorganisms.

To the best of our knowledge, this is the first study that has demonstrated the diverse 2,4-D- and 2,4,5-T-degrading bacteria in Vietnamese soils which have been contaminated with Agent Orange for a long time. Microbial consortia may be more diverse and widely spread in association with the adaptation to high concentrations of 2,4-D and 2,4,5-T in the environment than has been found in this study. Further analysis of these bacteria will allow for elucidating how they adapt to xenobiotic chemicals in the environment.

References

8) Gisi, M.R., and L. Xun. 2003. Characterization of chlorophenol 4-monoxygenase (TfD) and NADH: flavin adenine dinucle-


