Structural and Kinetic Characteristics of 1,4-Dioxane-Degrading Bacterial Consortia Containing the Phylum TM7

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Introduction

1,4-Dioxane is a colorless and flammable liquid with a faintly sweet odor. It has been widely used as a stabilizer for chlorinated solvents such as 1,1,1-trichloroethane, trichloroethene (TCE), and tetrachloroethene [31, 32, 40, 50]. 1,4-Dioxane is also used as a solvent in the manufacturing processes of ethylene glycol and ethylene oxide [38].

Owing to its extensive use, 1,4-dioxane has frequently been detected as a persistent contaminant in surface water and groundwater [19, 40, 50]. 1,4-Dioxane is recognized as a serious pollutant in aquatic environments because of its acute and chronic toxicity as well as its suspected carcinogenicity [4, 42]. The United States Environmental Protection Agency classifies 1,4-dioxane as one of the Class 2B carcinogens with a high possibility of causing human...
1,4-dioxane in drinking water at 50 μg/l since 2006. In Korea, the Ministry of Environment (MOE) has set the quality limit of 1,4-dioxane in drinking water at 50 μg/l since 2012. Furthermore, the MOE has regulated the concentration of 1,4-dioxane in industrial wastewater effluents at below 4 mg/l since 2013.

Since 1,4-dioxane has a high miscibility with water, low Henry’s constant \( (5 \times 10^5 \text{ atm m}^3/\text{mol at 20°C}) \), and high hydrophilic nature (log \( K_{ow} \) = -0.27) \([27, 31, 33, 50]\), it can persist for a long time, especially in aquatic environments \([40]\). Among traditional physicochemical technologies, advanced oxidation processes (AOPs) using ozone, hydrogen peroxide, UV light, and their combinations have been found to be effective in removing 1,4-dioxane \([41]\). However, AOPs require high energy consumption and operational costs.

On the other hand, microbial degradation of 1,4-dioxane is considered as a cost-effective and efficient treatment technology \([3, 15, 39, 51]\). Therefore, several attempts have been made to isolate and characterize 1,4-dioxane-degrading bacteria. To date, dioxane metabolism has been reported in 12 gram-positive bacterial strains, including P. dioxanivorans \([41]\), Rhodococcus \( \text{sp. ENV478} \) \([45]\), Pseudonocardia \( \text{tetrahydrofuranoxydans} \) K1 \([18]\), Pseudonocardia \( \text{sp. ENV478} \) \([45]\), P. benzenivorans \( B5 \) \([31]\), Pseudonocardia \( \text{tetrahydrofuranoxydans} \) K1 \([18]\), Pseudonocardia \( \text{sp. ENV478} \) \([45]\), P. benzenivorans \( B5 \) \([31]\), and Mycobacterium \( \text{vaccae} \) \([18]\). In particular, the genus \( \text{Mycobacterium} \) and candidate division TM7 capable of growing on 1,4-dioxane were reported in this study.

### Materials and Methods

#### Enrichment Culture of 1,4-Dioxane-Degrading Bacterial Consortia

For enrichment, 10 g of FS collected from a forest near the Yongin Campus of Myongji University and of AS obtained from a domestic wastewater treatment plant in Yongin, Korea were washed three times with 200 ml of ammonium mineral salts (AMS) \([37]\) medium. Then pre-washed FS was added into a 500 ml Erlenmeyer flask containing 200 ml of AMS medium so that the concentration of total suspended solids (TSS) was 1,200 mg/l. This flask was incubated aerobically at 30°C on a shaker rotating at 120 rpm. The pre-washed AS was cultured in a 5 L cylindrical reactor (ID 170 mm x H 260 mm) containing 2 L of AMS medium and 1,200 mg/l of TSS. The reactor was stirred at 200 rpm and was supplied with air at 0.2 l/min using a flowmeter (Dwyer Instruments, USA). The reactor was kept at room temperature (22–25°C). For both enrichment cultures, AMS medium was supplemented with about 200–250 mg/l of 1,4-dioxane as the sole carbon and energy source. After about 3 weeks of enrichment culture (5–6 successive transfers), the concentration of 1,4-dioxane was lowered to about 100 mg/l. Subsequently, enrichment cultivation was continued for 300 days by transferring TSS into fresh AMS every week in early stages and every 2–3 days in later stages. TSS of the previous culture was centrifuged and washed three times with phosphate-buffered saline (pH 7.4) before transfer to remove mineral salt precipitates.

#### Gene Library Construction and Phylogenetic Analysis

The genomic DNA of the enriched culture was extracted using the FastDNA SPIN kit for soil (MP Biomedicals, USA) according to the manufacturer’s instructions. For the extracted genomic DNA, the 16S rRNA and soluble di-iron monooxygenase (SDIMO)
Dioxane-Degrading Bacterial Communities and Kinetics

Table 1. Primer sets for the 16S rRNA and SDIMO genes used in this study.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer</th>
<th>Sequence (5' → 3')</th>
<th>Amplicon size (bp)</th>
<th>Annealing temp. (°C)</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eubacterial 16S rRNA gene</td>
<td>27F</td>
<td>AGAGTTTGATCMTGGCTCAG</td>
<td>1,503</td>
<td>50</td>
<td>Gene library and standard for qPCR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[23]</td>
</tr>
<tr>
<td></td>
<td>1492R</td>
<td>GGYTACCCTTGTACGACTT</td>
<td>1,503</td>
<td>50</td>
<td>qPCR</td>
<td>[34]</td>
</tr>
<tr>
<td></td>
<td>357F</td>
<td>CTACGGGAGGCAGCAG</td>
<td>194</td>
<td>55</td>
<td>qPCR</td>
<td>[5]</td>
</tr>
<tr>
<td></td>
<td>517R</td>
<td>ATTACCGCGGCTGCTGG</td>
<td>1,100</td>
<td>55</td>
<td>Standard for qPCR&lt;sup&gt;b&lt;/sup&gt;</td>
<td>[23]</td>
</tr>
<tr>
<td>SDIMO gene</td>
<td>NVC65</td>
<td>CARATGYTGAYGARGTNCGNCA</td>
<td>420</td>
<td>55</td>
<td>Gene library and qPCR</td>
<td>[23]</td>
</tr>
<tr>
<td></td>
<td>NVC57</td>
<td>CCANCCNGGRTAYTTITYTICTAAA</td>
<td>420</td>
<td>55</td>
<td>qPCR</td>
<td>[23]</td>
</tr>
</tbody>
</table>

<sup>a</sup> A plasmid standard of the 16S rRNA gene containing the target region was generated for primer 27F/1492R using DNA extracted from Escherichia coli KCTC 2441<sup>T</sup>.

<sup>b</sup> A plasmid standard of the SDIMO gene containing the target region was generated for primer NVC65/NVC58 using DNA extracted from the enrichment culture FS.

genes were amplified by PCR using the 27F/1492R and NVC57/NVC66 primer sets, respectively (Table 1). PCRs were carried out using Ex Taq polymerase (Takara Bio, Japan) with the following thermal cycles: initial denaturation at 95°C for 3 min; 30 cycles consisting of 95°C for 30 sec, 50–55°C for 30 sec, and 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR products were electrophoresed on a 1% (w/v) agarose gel and purified with the QIAquick PCR Purification Kit (Qiagen, Germany). PCR products of the 16S rRNA gene were ligated into the T-Blunt PCR Cloning Kit (Solgent, Korea) and cloned into Escherichia coli DH5α according to the manufacturer’s instructions. The nucleotide sequences of the cloned 16S rRNA and SDIMO genes were determined using the ABI 3730XL DNA Analyzer (Applied Biosystems, USA) at Bionics (Korea). The sequences were identified by BLASTN and the Classifier Services of the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov). Chimeric sequences were examined by the Find Chimeras tool of DECIPHER (http://decipher.cee.wisc.edu/FindChimeras.html). The 16S rRNA and SDIMO gene sequences of the enrichment and FS standard for qPCR<sup>a</sup>

For determining the abundance of total bacteria (16S rRNA gene) and 1,4-dioxane-degrading bacteria (SDIMO gene), 16S rRNA and SDIMO genes were amplified by quantitative PCR (qPCR) using the 357F/517R and NVC57/NVC66 primer sets, respectively (Table 1). The standards for quantifying the 16S rRNA and SDIMO gene sequences were generated using quintuplicate 10-fold dilutions of plasmid DNA. Target copy numbers for each reaction were calculated from the standard curves by assuming that the average molecular mass of a double-stranded DNA molecule was 660 g/mol. For all qPCR assays, there was a linear relationship between the log of target copy number and the calculated threshold cycle value across the specified concentration range (R² > 0.98 in all cases). The copy number of 1,4-dioxane-degrading bacteria in the total bacterial population was estimated by assuming an average of three ribosomal operons per bacterial cell and one SDIMO operon per 1,4-dioxane-degrading bacterial cell [11].

Quantitative Real-Time PCR

For determining the abundance of total bacteria (16S rRNA gene) and 1,4-dioxane-degrading bacteria (SDIMO gene), 16S rRNA and SDIMO genes were amplified by quantitative PCR (qPCR) using the 357F/517R and NVC57/NVC66 primer sets, respectively (Table 1). The standards for quantifying the 16S rRNA and SDIMO gene sequences were prepared with purified plasmid DNA containing the target region of E. coli KCTC 2441<sup>T</sup> and FS enrichment culture, respectively. The PCR mixture contained 10 µl of iQ SYBR Green SuperMix (Bio-Rad, USA), 10 ng of salmon sperm DNA as an internal amplification control (Sigma-Aldrich, USA), 0.3 µM of each primer, 2 µl of DNA template, and DNA-free water, yielding a total volume of 20 µl. qPCR was carried out with the Rotor-Gene Q (Qiagen) using the following cycling parameters: initial denaturing at 95°C for 15 min, followed by 45 cycles of 95°C denaturation for 15 sec, 55°C of annealing for 20 sec, and 72°C of extension for 25 sec. The fluorescence signal was measured at 55°C during each cycle. Melting curve analysis was performed to confirm the specificity of the qPCR results. Each test included triplicate reactions per DNA sample and the appropriate sets of standards.

A plasmid standard containing the target region was generated for each primer set using DNA extracted from the appropriate positive control sample (Table 1). The amplified products were run on a 1% agarose gel to confirm the specificity of the amplification and the identified standard DNA sequence within plasmids. DNA concentrations were determined by the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, USA). Standard curves were generated using quintuplicate 10-fold dilutions of plasmid DNA. Target copy numbers for each reaction were calculated from the standard curves by assuming that the average molecular mass of a double-stranded DNA molecule was 660 g/mol. For all qPCR assays, there was a linear relationship between the log of the plasmid DNA copy number and the calculated threshold cycle value across the specified concentration range (R² > 0.98 in all cases). The copy number of 1,4-dioxane-degrading bacteria in the total bacterial population was estimated by assuming an average of three ribosomal operons per bacterial cell and one SDIMO operon per 1,4-dioxane-degrading bacterial cell [11].

Estimation of Kinetic Parameters

For kinetics analysis, enriched FS and AS were cultivated in AMS medium containing 100 mg/l of 1,4-dioxane as the sole carbon and energy source. Cell density was monitored by measuring the optical density at 600 nm (OD<sub>600</sub>) or by measuring TSS (mg/l)
filtered with 0.2 μm membranes (Advantec, Japan) and dried at 105°C for 12 h. 1,4-Dioxane (soluble organic carbon) in the culture medium was measured as non-purgeable organic carbon (NPOC) using a TOC analyzer (TOC-VCPH, Shimadzu, Japan). In comparing the calculated kinetic parameters with previously reported values, the TSS concentration was converted to cellular protein concentration by using the conversion factor of 1 g TSS = 0.4 g protein [31]. For the degradation kinetics studies, the Monod (Eq. (1)) and Michaelis-Menten (Eq. (2)) equations were adopted as follows:

\[
\mu = \frac{\mu_{\text{max}} S}{K_s + S}
\]

\[
v = \frac{V_{\text{max}} S}{K_m + S}
\]

where \( \mu \) is the specific growth rate (h\(^{-1}\)), \( \mu_{\text{max}} \) is the maximum specific growth rate (h\(^{-1}\)), \( \mu \) and \( \mu_{\text{max}} \) are calculated using OD\(_{600}\) data, \( S \) is the concentration of 1,4-dioxane in mg/l, \( v \) is the specific degradation rate for 1,4-dioxane (mg 1,4-dioxane/mg protein·h), \( V_{\text{max}} \) is the maximum substrate degradation rate (mg 1,4-dioxane/mg protein·h), and \( K_m \) is the half-saturation constant for 1,4-dioxane in mg/l. In order to obtain \( V_{\text{max}} \), \( \mu_{\text{max}} \), and \( K_m \), the nonlinear regression method (SigmaPlot, USA) was used. The growth yield (\( Y \)) was calculated using Eq. (3):

\[
Y = \frac{X}{X_0 - X/2 - S/2 - S}
\]

where \( X \) is the biomass (protein) concentration (mg/l), and \( S \) is the concentration (mg/l) of the substrate (1,4-dioxane).

**Results**

**Enrichment Culture and Kinetics**

During the early stages of enrichment, the initial 1,4-dioxane concentration in the fresh medium was 100–280 mg NPOC/l and the concentration was changed to 100–140 mg NPOC/l in the later stages. The TSS of the culture was transferred to the fresh medium when concentrations of NPOC decreased below about 40 mg/l. As shown in Fig. 1, the degradation rate of 1,4-dioxane stabilized after 150–180 days. Subsequently, 1,4-dioxane was rapidly consumed in 2–3 days after transfer to fresh medium. In order to determine the kinetic parameters of enriched FS and AS, the concentrations of TSS and 1,4-dioxane were measured between Days 270 and 272. As shown in Fig. 2, the concentration of dioxane decreased from 110 to 7.2 mg NPOC/l (93.5% removal), while the TSS concentration of FS increased from 400 to 550 mg/l in 48 h. In the case of enriched AS, the concentration of 1,4-dioxane decreased from 140 to 10.1 mg NPOC/l (92.8% removal), and the concentration of TSS increased from 430 to 540 mg/l. These batch data were processed to obtain specific growth rates and specific 1,4-dioxane degradation rates for time intervals, and fitted into the Monod and Michaelis-Menten equations.

The determined kinetic parameters of enriched FS, AS, and previously reported 1,4-dioxane degraders are summarized in Table 2. The \( \mu_{\text{max}} \) of 1,4-dioxane degraders was reported to be in the range of 0.003–0.10 h\(^{-1}\). The \( \mu_{\text{max}} \) of enriched AS on 1,4-dioxane was 0.008 h\(^{-1}\), which was similar to those of industrial activated sludges (0.005–
The cell growth yields of enriched FS and AS were 0.58 and 0.34 mg protein/mg dioxane, respectively. These yields were in the range of previously reported values (0.03–0.50 mg protein/mg dioxane).

The V_{max} and K_{m} of enriched FS were 0.037 mg dioxane/mg protein·h and 93.9 mg/l, respectively. In the case of enriched AS, the V_{max} and K_{m} values were 0.078 mg dioxane/mg protein·h and 181.3 mg/l, respectively. The K_{m} values of FS and AS were in the range of other previously reported 1,4-dioxane-degrading bacteria (1.65–330 mg/l), and the K_{m} value of AS was similar to that of an industrial activated sludge (181.9 mg/l) [39] and P. dioxanivorans CB1190 (160 mg/l) [31]. The V_{max} value of FS was relatively lower than those of previous studies, probably because the enrichment culture obtained in this study contained non-dioxane degraders. Low V_{max} was also reported for Pseudonocardia sp. ENV478 (0.053 mg dioxane/mg protein·h) [43], Mycobacterium sp. D11 (0.052 mg dioxane/mg protein·h) [41], and an enrichment culture (0.05 mg dioxane/mg protein·h) [47].

Bacterial Community Analysis on 16S rRNA Gene Sequences

In order to identify the bacterial populations contained in enrichment cultures, 16S rRNA genes of enriched FS and AS (collected on Day 270) were PCR-amplified and cloned into E. coli DH5α, and 47 (FS) and 101 (AS) clones were analyzed. The obtained sequences of the cloned 16S rRNA gene were deposited in the GenBank/EMBL DNA database under the accession numbers KT832611–KT832646, KT992721–KT992771, KU000902–KU000912, and KX197123–KX197172.

**Table 2.** Kinetic parameters of 1,4-dioxane biodegradation by different cultures.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Temp. (°C)</th>
<th>H_{max} (h⁻¹)</th>
<th>Y (mg protein/mg dioxane)</th>
<th>V_{max} (mg dioxane/mg protein·h)</th>
<th>K_{m} (mg/l)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudonocardia dioxanivorans</em> CB1190</td>
<td>30</td>
<td>0.10</td>
<td>0.09</td>
<td>1.1 ± 0.008 at 1,000 ppm</td>
<td>160 ± 44</td>
<td>[31]</td>
</tr>
<tr>
<td><em>Pseudonocardia benzenivorans</em> B5</td>
<td>30</td>
<td>0.003</td>
<td>0.03</td>
<td>0.1 ± 0.006 at 1,000 ppm</td>
<td>330 ± 82</td>
<td>[31]</td>
</tr>
<tr>
<td><em>Pseudonocardia</em> sp. ENV478</td>
<td>30</td>
<td>-</td>
<td>-</td>
<td>0.053 at 25 ppm</td>
<td>-</td>
<td>[45]</td>
</tr>
<tr>
<td><em>Afipia</em> sp. D1</td>
<td>28</td>
<td>-</td>
<td>0.19</td>
<td>0.263 at 500 ppm</td>
<td>25.8</td>
<td>[41]</td>
</tr>
<tr>
<td><em>Mycobacterium</em> sp. D6</td>
<td>28</td>
<td>-</td>
<td>0.19</td>
<td>0.139 at 500 ppm</td>
<td>20.6</td>
<td>[41]</td>
</tr>
<tr>
<td><em>Mycobacterium</em> sp. D11</td>
<td>28</td>
<td>-</td>
<td>0.18</td>
<td>0.052 at 500 ppm</td>
<td>69.8</td>
<td>[41]</td>
</tr>
<tr>
<td><em>Pseudonocardia</em> sp. D17</td>
<td>28</td>
<td>-</td>
<td>0.22</td>
<td>0.096 at 500 ppm</td>
<td>59.7</td>
<td>[41]</td>
</tr>
<tr>
<td>Industrial activated sludge</td>
<td>-</td>
<td>0.062</td>
<td>0.344</td>
<td>-</td>
<td>1.65</td>
<td>[49]</td>
</tr>
<tr>
<td>Industrial activated sludge</td>
<td>-</td>
<td>0.005</td>
<td>-</td>
<td>-</td>
<td>181.9</td>
<td>[39]</td>
</tr>
<tr>
<td>Industrial activated sludge</td>
<td>25–35</td>
<td>0.010–0.043</td>
<td>0.18–0.50</td>
<td>0.12 at 100 ppm, 30°C</td>
<td>9.9</td>
<td>[12]</td>
</tr>
<tr>
<td>Enrichment culture</td>
<td>35</td>
<td>0.083</td>
<td>0.30</td>
<td>0.05 ± 0.003 at 200 ppm</td>
<td>12.6 ± 7.6</td>
<td>[48]</td>
</tr>
<tr>
<td>Enrichment culture-FS</td>
<td>30</td>
<td>0.0004</td>
<td>0.58</td>
<td>0.037 at 110 ppm</td>
<td>93.9</td>
<td>This study</td>
</tr>
<tr>
<td>Enrichment culture-AS</td>
<td>22–25</td>
<td>0.008</td>
<td>0.34</td>
<td>0.078 at 140 ppm</td>
<td>181.3</td>
<td>This study</td>
</tr>
</tbody>
</table>

For consistency of dimensions, a protein was assumed to constitute 40% of TSS, 43% of COD, and 50% of VSS [31]. Value converted from mg COD/l to mg 1,4-dioxane/l based on 1.82 mg COD/mg 1,4-dioxane [49].
16S rRNA gene sequences obtained from FS enrichment culture were assigned to four different phyla, including Proteobacteria (46.8%), Actinobacteria (40.4%), Bacteroidetes (6.4%), and Chloroflexi (6.4%) (Table 3). At the genus level, clones that belonged to Mycobacterium (38.3%), Dokdonella (19.2%), and Afipia (10.6%) were identified as members of large clusters. Aerobic 1,4-dioxane-degrading bacteria are usually gram-positive, and most of them belong to the Actinomycetales in the genera Mycobacterium, Pseudonocardia, and Rhodococcus [30, 41]. Afipia strains are known to be aerobic 1,4-dioxane-degrading gram-negative bacteria [41]. Mycobacterium and Afipia (ca. 48.9%) made up the largest population in enriched FS. The members of genus Mycobacterium were especially predominant in enriched FS, and the 18 clones (1 group) that showed 96–100% similarity with Mycobacterium were closely related to Mycobacterium sp. PH-06 according to the phylogenetic analysis (Fig. 3). Mycobacterium sp. PH-06 is well known to degrade 1,4-dioxane as the sole carbon and energy source [19]. The members of genus Afipia were also dominant in enriched

### Table 3. Bacterial compositions and diversity estimates of the 16S rRNA gene clone library.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class (Order)</th>
<th>Family</th>
<th>Genus</th>
<th>FS (%)</th>
<th>AS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Composition (%)</td>
<td>Actinobacteria (Actinomycetales)</td>
<td>Acidimicrobiaceae</td>
<td>Aciditerrimonas</td>
<td>2.13</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Actinobacteria (Actinomycetales)</td>
<td>Mycobacteriaceae</td>
<td>Mycobacterium</td>
<td>38.30</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>Actinobacteria (Actinomycetales)</td>
<td>Rhodococcaceae</td>
<td>Rhodococcus</td>
<td>-</td>
<td>0.99</td>
</tr>
<tr>
<td>Armatimonadetes</td>
<td>Fimbriimonadaceae (Fimbriimonadales)</td>
<td>Fimbriimonadaceae</td>
<td>Fimbriimonas</td>
<td>-</td>
<td>3.96</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Flavobacteriaceae (Flavobacteriales)</td>
<td>Cryomorphaceae</td>
<td>Wandonia</td>
<td>-</td>
<td>7.92</td>
</tr>
<tr>
<td>Sphingobacteria</td>
<td>Sphingobacteriaceae (Sphingobacteriales)</td>
<td>Chitinophagaceae</td>
<td>Chitinophaga</td>
<td>-</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Flavisolibacter</td>
<td></td>
<td>4.26</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Terrimonas</td>
<td></td>
<td>2.13</td>
<td>3.96</td>
</tr>
<tr>
<td>Chloroflexi</td>
<td>Unclassified Chloroflexi bacterium</td>
<td></td>
<td></td>
<td>6.38</td>
<td>-</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Alpha-proteobacteria (Rhizobiales and Rhodospirillales)</td>
<td>Bradyrhizobiaceae</td>
<td>Afipia</td>
<td>10.64</td>
<td>3.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nitrobacteraceae</td>
<td>Nitrobacter</td>
<td>-</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acetobacteriaceae</td>
<td>Acidisphaera</td>
<td>-</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gluconacetobacter</td>
<td></td>
<td>-</td>
<td>0.99</td>
</tr>
<tr>
<td>Beta-proteobacteria (Burkholderiales)</td>
<td>Alcaligenaceae</td>
<td>Paralcaligenes</td>
<td>-</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Burkholderiaceae</td>
<td>Albidiferax</td>
<td>-</td>
<td>2.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Burkholderiaceae</td>
<td>Burkholderia</td>
<td>-</td>
<td>0.99</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>Unclassified Verrucomicrobia</td>
<td></td>
<td></td>
<td>-</td>
<td>1.98</td>
</tr>
<tr>
<td>Candidate division TM7</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>49.50</td>
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<tr>
<td>Candidate division OP10</td>
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<td>-</td>
<td>0.99</td>
</tr>
<tr>
<td>Unclassified bacterium</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Diversity

- Number of clones: 47
- Number of OTUs: 10
- Shannon-Weaver index of diversity ($H'$, $H' = -\sum P_i \ln P_i$)$^a$: 2.69
- Simpson’s index of diversity ($D$, $D = \sum P_i^2$)$^b$: 0.21

$^a$Shannon-Weaver index; a higher number represents higher diversity; $P_i$: proportional abundance of ecosystem i.

$^b$Simpson’s index; a higher number represents lower dominance; $P_i$: proportional abundance of ecosystem i.
Dioxane-Degrading Bacterial Communities and Kinetics

Fig. 3. Phylogenetic tree based on the partial sequences of the 16S rRNA gene obtained from enrichment cultures FS and AS. The tree was constructed by using the neighbor-joining method and the sequences of Aquifex pyrophilus (M83548) as an out-group. The bootstrap values above 50% are shown at the internal nodes. Actinobacteria (1,4-dioxane-degrading bacteria) of clone FS-5 and other 17 FS-clones (1) were of the following accession numbers: KT832628, KT832635, KT832636, KT832638, KT832641-KT832643, KT992723, KT992726, KT992728-KT992730, KT992732, KT992733, KT992736, KT992737, and KU000911. Armamitomades of clone AS-55 and other 3 FS-clones (2) were given accession numbers KT992753, KX197160, KX197164, and KX197169. Beta-proteobacteria of clone FS-5 and other 17 FS-clones (3) were of KT832622, KT992768, and KT992769. TM7 of clone AS-5 and other 3 FS-clones (4) were given the accession numbers KT992753, KT992755, KT992757, and KT992759. Alpha rRNA of clone AS-2 and other 17 FS-clones (5) were of KT832622, KT992768, and KT992769. Members of clone AS-2 and other 17 FS-clones (6) were of KT832622, KT992768, and KT992769. Gamma-proteobacteria of clone FS-5 and other 3 FS-clones (6) were of KT832622, KT992768, and KT992769. Epsilonproteobacteria of clone AS-2 and other 17 FS-clones (7) were of KT832622, KT992768, and KT992769.
FS, and the five clones were related to *Afipia* sp. D1 through phylogenetic analysis. *Afipia* sp. D1 is also well known to degrade 1,4-dioxane as the sole carbon and energy source [41]. Many other genera, such as *Dokdonella* (19.2%) and *Pandoraea* (8.5%), have never been reported to be involved in 1,4-dioxane degradation. However, the bacterial strains
of genus *Dokdonella* were reportedly observed in enrichment culture with polycyclic aromatic hydrocarbons [1, 7]. Strains of the genus *Pandoraea* were also observed in polychlorobiphenyl-contaminated soil [13, 28].

16S rRNA gene sequences identified from AS enrichment culture showed relatively low similarity values (83–99%) with entries in the GenBank databases, and they were distributed over the phylogenetic tree away from the well-known 1,4-dioxane-degrading bacteria (Fig. 3). Major phyla in the enriched AS were candidatus Saccharibacteria (formerly candidate division TM7, 49.5%), Proteobacteria (27.7%), and Bacteroidetes (12.9%) (Table 3). At the genus level, members in enriched AS were more diverse than in enriched FS. The TM7 members were particularly predominant in enriched AS, as they occupied 49.5% of the clone libraries (Table 3, Fig. 3). Strains in the genera *Wandonia* (7.9%), *Dokdonella* (5.0%), and *Dyella* (5.0%) were also found. Table 3 also shows the diversity estimates of both libraries obtained by the Shannon–Weaver and Simpson’s indices. The diversities of enriched AS were a little higher than those of enriched FS.

Within the candidate phylum TM7, Dinis et al. [8] reported that Subdivision 1 consisted almost exclusively of OTUs obtained from natural and wastewater samples (environmental associated), whereas Subdivision 2 was mostly OTUs of human and animal sources (host associated). However, some clones from wastewater, soil, and contaminated sites were classified into the host-associated clade [16, 46]. In accordance with this classification, the 50 clones (3 groups) that showed low similarity (83–99%) with TM7 bacteria were closely related to Subdivision 1 (Fig. 4). This result indicated that the TM7-like bacterial group in enriched AS was important for 1,4-dioxane degradation in unknown ways.

### Copy Numbers of 16S rRNA and SDIMO Genes

SDIMOs were reportedly involved in metabolic and co-metabolic 1,4-dioxane degradation [11, 31]. SDIMOs are key multicomponent enzymes catalyzing the initial oxidation stage of hydrocarbons in phylogenetically and physiologically diverse bacteria; these enzymes could be categorized into five groups according to substrate specificity, sequence similarity, and component arrangement [5, 25]. Among them, Group 5 SDIMOs (THF/dioxane and propane monooxygenases) play an essential role in the initial oxidation stage of the cyclic ethers THF and 1,4-dioxane. Hence, it would be of interest to predict the dioxane degradation potential by assessing the SDIMO gene as a biomarker. The 16S rRNA and SDIMO gene copy numbers per 1 mg TSS are shown in Fig. 5. The abundance of 16S rRNA and SDIMO genes in the enriched FS sampled at Day 270 were 1.52 (±0.07) × 10^9 copies/mg TSS and 0.45 (±0.07) × 10^9 copies/mg TSS, respectively. For enriched AS, 16S rRNA and SDIMO gene copy numbers were 1.85 (± 0.08) × 10^9 copies/mg TSS and 1.07 (±0.06) × 10^9 copies/mg TSS, respectively. The average abundance relative to total bacteria (average SDIMO/16S rRNA gene copies) in enriched FS and AS were 29.4% and 57.8%, respectively. These results suggested that quantitative measurement of the SDIMO gene might be useful for monitoring 1,4-dioxane-degrading bacteria and probably other closely related pollutant-degrading bacteria.

In order to further verify that the amplicons obtained from enrichment cultures were fragments of the targeted SDIMO genes, 31 clone libraries were constructed with genomic DNA extracted from enriched FS and AS (collected on Day 270) by sequencing and alignment. Through SDIMO gene library construction and sequencing, it was confirmed that OTUs of enriched AS and FS contained the QSDE(S/A)RHI motif and belonged to Group 5 SDIMOs (data not shown). Phylogenetic analyses allocated all SDIMO clones of both enrichment cultures to the Group 5 SDIMOs with 97–98% similarity to *Rhodococcus* sp. YYL, *P.*
dioxanivorans CB1190, and Pseudonocardia sp. TY-7 (Fig. 6). In particular, the THF/dioxane monooxygenase of Rhodococcus sp. YYL was predominant, as it occupied 84.6% in FS and 77.8% in AS. Other clones were clustered into one group; specifically, the propane monooxygenase (prm) gene of P. dioxanivorans CB1190 or Pseudonocardia sp. TY-7. THF/dioxane monooxygenase is known to play a crucial role in dioxane biodegradation [26]. THF monooxygenase (thm) genes of Rhodococcus sp. YYL, Pseudonocardia sp. K1, and Pseudonocardia sp. ENV478 were highly homologous to the putative dioxane monooxygenase gene (dxm) cluster, based on estimates on the nucleotide sequence identity of each gene (above 97%) as well as generic component arrangement [26]. Although propane monooxygenases bear a close evolutionary relationship with THF/dioxane monooxygenases and are in the same subdivision (Group 5), they do not share the same substrate range. However, our studies with enrichment cultures suggested that the presence of genes associated with THF/dioxane monooxygenase and propane monooxygenase are promising indicators of 1,4-dioxane degradation, since enrichment cultures in this study were obtained using 1,4-dioxane as the sole carbon and energy source. As in our study, co-metabolism of 1,4-dioxane with bacteria expressing propane monooxygenases was observed in another study [26]. Gedalanga et al. [11] also reported that the prm gene was increasingly expressed in P. dioxanivorans CB1190 when over 50% of the 1,4-dioxane had been removed. Moreover, successful removal
of dioxane in microcosms inoculated with propanotrophs was achieved when the initial concentration of dioxane was as high as 10 mg/l [10]. Therefore, biodegradation of 1,4-dioxane might be correlated with the prn gene, as found in the enrichment culture of FS and AS. Sequences of the cloned Group 5 SDIMO gene were deposited in the GenBank/EMBL DNA database under the accession numbers KT832580–KT832610.

Discussion

Laplante et al. [24] suggested that two independent bacterial communities encountering similar environmental perturbations (e.g., heavy metals and acidic pH) exhibited the same overall taxonomic membership (i.e., members in both communities belong to the same high taxonomic rank). Concretely, they argued that community differentiation at the taxonomic diversity level reflected the presence and absence of a perturbation, whereas the community structure level accurately reflected the intensity of the perturbation. In the present study, microorganisms contained in FS and AS were enriched for 300 days using 1,4-dioxane as the sole source of carbon. Long-term exposure to an intense environmental perturbation (1,4-dioxane as a single substrate) significantly changed the microbial community and reduced biodiversity, and the activation of 1,4-dioxane degradation became evident regardless of microbial sources and cultivation conditions (Figs. 1 and 3, Table 3). However, bacterial communities of enriched FS and AS exhibited substantial differences (Fig. 2, Table 3). In enriched FS, previously reported 1,4-dioxane-degrading bacteria were so abundant that Mycobacterium spp. and Afipia spp. occupied approximately half of the enrichment FS. On the contrary, 1,4-dioxane-degrading bacteria were detected as minor populations among the 16S rRNA gene libraries constructed from enriched AS (5.94%). Surprisingly, the TM7-like bacteria were predominant, as they occupied 49.5% of the total clones of enriched AS.

Grady et al. [12] reported that the growth of a 1,4-dioxane degrader was highly sensitive to cultivation temperature (environmental stress). Previous studies discovered that the optimum temperature range for microbial degradation of 1,4-dioxane by Actinomycetales (genera Mycobacterium, Pseudonocardia, and Rhodococcus) was 28–35°C [2, 3, 19, 21, 30, 31, 35, 41, 45]. At a low temperature (below 20°C), 1,4-dioxane degraders showed slow growth and removal rates [12, 33, 48, 51]. Conversely, Zenker et al. [50] reported that a high temperature (35°C) was suboptimal for 1,4-dioxane biodegradation. Environmental TM7 bacteria were found mostly at ambient temperatures (16–25°C) [6, 16, 29, 47]. Therefore, it was thought that the 1,4-dioxane-degrading bacteria that belonged to Actinomycetales were absent in enriched AS because the enrichment culture of AS was carried out at room temperature (22–25°C). According to Bergey’s Manual of Systematic Bacteriology [14, 22], Afipia, Dokdonella, and Terrimonas grow well at 25–30°C; thus, these bacteria could commonly be enriched in FS and AS. It was concluded that the differences in the bacterial community were created by the different cultivation temperature, even though the main selective pressure of enrichment cultivation was identical (1,4-dioxane as the sole carbon source). Moreover, it was suspected that the presence of 1,4-dioxane and low temperature might stimulate growth of TM7-like bacteria.

Similarly to bacterial composition, enriched FS and AS showed differences in 1,4-dioxane degradation kinetics. The $K_m$ value of the enriched FS was confirmed at an intermediate level in previously reported $K_m$ values for 1,4-dioxane degradation, and was similar to that of Mycobacterium sp. D11 (Table 2). On the other hand, the $K_m$ of enriched AS was much higher than that of others, which could be advantageous for degrading high concentrations of 1,4-dioxane. Previous studies also emphasized that 1,4-dioxane-degrading bacteria with high $K_m$ could play a significant role in a 1,4-dioxane treatment of above 1 g/l [31, 41].

Interestingly, it was previously reported that 1,4-dioxane-degrading bacteria were not found in the 16S rRNA gene library of enriched AS, and its 1,4-dioxane degradation kinetic parameters were different from those of enriched FS. Another unique feature of enriched AS was that TM7-like bacteria were the most dominant group in the clones (Table 3). This finding suggested that TM7-like bacteria might degrade 1,4-dioxane by directly using it as a carbon and energy source or by metabolizing the by-products from 1,4-dioxane oxidation reaction in other microorganisms. The phylum candidate division TM7 has been observed with two monophyletic subdivisions at class-level clades [8]. Subdivision 1 is characterized by phylotypes mostly from environmental sources (soil, rhizosphere, marine, freshwater, wastewater, and contaminated sites), whereas Subdivision 2 includes phylotypes from animals, humans, and some environmental sources. TM7-like bacteria are of particular interest because they have been found in a variety of habitats; a stable culture of any species in this phylum has never been made, and habitats in which TM7 bacteria constitute the major population have so far not been reported [8, 17, 29, 46]. The relative abundance of this
phyllum within the samples ranged from 0.05% to 3.02% of the microbial community [8, 16, 46]. Our results showed that TM7-like bacteria occupied 49.5% of the clone libraries constructed from enriched AS, and most of the OTUs belonged to Subdivision 1 (environmental-associated clade). Furthermore, enriched AS actively removed 1,4-dioxane. Similar results were reported by Hanada et al. [16], who showed that members of the candidate phylum TM7 of the 16S rRNA gene clone library were prevalent (33%) in fully acclimated acidophilic nitrifying sequencing-batch reactors, but they could not clearly explain why TM7 bacteria were abundant. Connon et al. [14] reported that TM7 bacteria (385 bp of LH-PCR fragment) were predominant (31.5% of the total fragments) in the TCE-contaminated TM7 bacteria (385 bp of LH-PCR fragment) were abundant. Connon et al. [14] reported that TM7 bacteria (385 bp of LH-PCR fragment) were predominant (31.5% of the total fragments) in the TCE-contaminated groundwater treatment process using propane sparging, TCE, and cis-DCE (dichloroethene); propane removal rates were correlated with the increase in the 385 bp fragment proportion. Luo et al. [29] and Xie et al. [47] also reported on the candidate phylum TM7, where the dominant bacterium responsible for carbon uptake from toluene (11.3–62.1% of total T-RFLP fragments) and benzene (84.1% of the total T-RFLP fragments) in agricultural soil was identified by DNA stable isotope probing. In addition, a putative dihydroxybiphenyl dioxygenase (GenBank Acc. No. AKM80657) gene was suggested in TM7 metagenomic sequences (GenBank Acc. No. CP011211). However, genes encoding the degradation of toluene, benzene, and 1,4-dioxane have not been reported in TM7 bacteria. Although the ecophysiological significance and enzyme systems of the TM7-like bacteria remain unclear, these results suggested that candidate phylum TM7 might play an important role in 1,4-dioxane degradation. To our best knowledge, this is the first report on TM7 bacteria utilizing 1,4-dioxane as the sole carbon and energy source during biodegradation. Therefore, further studies are necessary to isolate microorganisms in TM7 and related taxa and to reveal the metagenomic characteristics of axenic cultures.

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References


