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

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1,4-Dioxane-degrading bacterial consortia were enriched from forest soil (FS) and activated sludge (AS) using a defined medium containing 1,4-dioxane as the sole carbon source. These two enrichment cultures appeared to have inducible tetrahydrofuran/dioxane and propane degradation enzymes. According to qPCR results on the 16S rRNA and soluble di-iron monooxygenase genes, the relative abundances of 1,4-dioxane-degrading bacteria to total bacteria in FS and AS were 29.4% and 57.8%, respectively. For FS, the cell growth yields (Y), maximum specific degradation rate ($V_{\max}$), and half-saturation concentration (K_m) were 0.58 mg-protein/mg-dioxane, 0.037 mg-dioxane/mg-protein·h, and 93.9 mg/l, respectively. For AS, Y, $V_{\max}$, and K_m were 0.34 mg-protein/mg-dioxane, 0.078 mg-dioxane/mg-protein·h, and 181.3 mg/l, respectively. These kinetics data of FS and AS were similar to previously reported values. Based on bacterial community analysis on 16S rRNA gene sequences of the two enrichment cultures, the FS consortium was identified to contain 38.3% of *Mycobacterium* and 10.6% of *Afipia*, similar to previously reported literature. Meanwhile, 49.5% of the AS consortium belonged to the candidate division TM7, which has never been reported to be involved in 1,4-dioxane biodegradation. However, recent studies suggested that TM7 bacteria were associated with degradation of non-biodegradable and hazardous materials. Therefore, our results showed that previously unknown 1,4-dioxane-degrading bacteria might play an important role in enriched AS. Although the metabolic capability and ecophysiological significance of the predominant TM7 bacteria in AS enrichment culture remain unclear, our data reveal hidden characteristics of the TM7 phylum and provide a perspective for studying this previously uncultured phylotype.

Keywords: 1,4-Dioxane-degrading bacterial consortia, candidate division TM7, *Mycobacterium*, *Afipia*, kinetic parameters, soluble di-iron monooxygenase

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

cancer [19, 50]. The International Agency for Research on Cancer also categorizes 1,4-dioxane as Group 2B (possibly carcinogenic to humans) [19]. Consequently, the World Health Organization has established the guidelines for 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 (5×10^{-6} atm m³/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 *Pseudonocardia dioxanivorans* CB1190 [30, 37], *P. benzenivorans* B5 [31], *Pseudonocardia tetrahydrofuranoxydans* K1 [18], *Pseudonocardia* sp. ENV478 [45], *P. sp.* D17 [41], *Mycobacterium vaccae* JOB5 [3], *Mycobacterium* sp. PH-06 [19], *Mycobacterium* sp. D6 [41], *Mycobacterium* sp. D11 [41], *Rhodococcus ruber* 219 [3], *Rhodococcus* sp. ENV425 [45], and *Rhodococcus* sp. RR1 [31]. The biodegradative pathways of 1,4-dioxane have also been reported in a gram-negative bacterium, *Afipia* sp. D1 [41], and in the fungus *Cordyceps sinensis* [35]. In particular, 8 bacterial strains (*P. dioxanivorans* CB1190, *P. benzenivorans* B5, *Pseudonocardia* sp. D17, *Mycobacterium* PH-06, *Mycobacterium* sp. D6, *Mycobacterium* sp. D11, *R. ruber* 219, and *Afipia* sp. D1) and a fungal strain (*C. sinensis*) have been found to be capable of growing on 1,4-dioxane as the sole carbon and energy source. However, the growth and removal rates of these microorganisms by 1,4-dioxane are known to be very low [50, 51] because the biological processes in these strains require a long hydraulic retention time.

Most of the 1,4-dioxane-degrading microorganisms are unable to use 1,4-dioxane as the sole carbon source because these strains are unable to induce necessary genes and/or to mineralize 1,4-dioxane completely [45]. For growth and gene induction support, these strains require primary

growth substrates such as tetrahydrofuran (THF) [3, 21, 48, 50], propane [3, 31], toluene [3, 21, 31], methane [31], and ethanol [21]. It was also shown that co-metabolic dioxane transformation was enhanced by the growth-inducing substrate THF [45, 50]. However, many other studies showed that THF inhibited the dioxane transformation of pure and mixed microbial cultures growing on THF as the primary substrate [31, 40, 48]. Therefore, although it was not impossible, dioxane degradation remained very limited for these strains.

In the present study, bacterial enrichment cultures with a high capability in degrading 1,4-dioxane as the sole carbon and energy source were obtained from forest soil (FS) and activated sludge (AS), and the bacterial composition and kinetic parameters of 1,4-dioxane biodegradation were analyzed. In addition, the genus *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)

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

Target gene	Primer	Sequence (5' → 3')	Amplicon size (bp)	Annealing temp. (°C)	Method	Reference
Eubacterial 16S rRNA gene	27F	AGAGTTTGATCMTGGCTCAG	1,503	50	Gene library and standard for qPCR ^a	[23]
	1492R	GGYTACCTTGTTACGACTT				
	357F	CTACGGGAGGCAGCAG	194	55	qPCR	[34]
	517R	ATTACCGCGGCTGCTGG				
SDIMO gene	NVC65	CARATGYTNGAYGARGTNCGNCA	1,100	55	Standard for qPCR ^b	[5]
	NVC58	CGDATRTRCRTC DATNGTCCA				
	NVC57	CAGTCNGAYGARKCSCGNAYAT	420	55	Gene library and qPCR	
	NVC66	CCANCCNGGRTAYTTRTTYTCRAACCA				

^a 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^T.

^b 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 reference strains were aligned with the CLUSTALW program [44]. Phylogenetic trees were constructed using the MEGA6 program [43]. An evolutionary distance matrix was generated according to the Kimura 2-parameter model [20], and a phylogenetic tree was inferred using the neighbor-joining method [36]. The neighbor-joining tree topology was evaluated by the bootstrap analysis with 1,000 replications [9].

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 copies were prepared with purified plasmid DNA containing the target region of *E. coli* KCTC 2441^T 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 ($R^2 > 0.98$ 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_{600}) 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:

$$\mu = \mu_{\max} \frac{S}{K_s + S} \quad (1)$$

$$v = V_{\max} \frac{S}{K_m + S} \quad (2)$$

where μ is the specific growth rate (h^{-1}), $\mu_{\max}$ is the maximum specific growth rate (h^{-1}), μ and $\mu_{\max}$ 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), $V_{\max}$ 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 $V_{\max}$, $\mu_{\max}$ and K_m , the nonlinear regression method (SigmaPlot, USA) was used. The growth yield (Y) was calculated using Eq. (3):

$$Y = \frac{X_2 - X_1}{S_2 - S_1} \quad (3)$$

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),

Fig. 1. Long-term enrichment cultivation of forest soil (FS) and activated sludge (AS) for 1,4-dioxane degradation.

(A) Enrichment culture of FS in a shaken flask at 30°C. (B) Enrichment culture of AS in an aerated (0.2 l/min) cylindrical reactor at room temperature (23–25°C). TSS of a previous culture was centrifuged and resuspended in phosphate-buffered saline three times before adding into the fresh medium for the next round of enrichment. The arrows on 270 days indicate sampling points for the analyses of kinetic parameters and bacterial community, and qPCR.

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 $\mu_{\max}$ of 1,4-dioxane degraders was reported to be in the range of 0.003–0.10 h^{-1} . The $\mu_{\max}$ of enriched AS on 1,4-dioxane was 0.008 h^{-1} , which was similar to those of industrial activated sludges (0.005–

Fig. 2. Time course of 1,4-dioxane and cell growth during enrichment on Days 270-272.

The error bars represent the standard deviation of three replicates.

0.014 h⁻¹). 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* DH5 α , 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 of KT832611–KT832646, KT992721–KT992771, KU000902–KU000912, and KX197123–KX197172.

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

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

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].

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

	Phylum	Class (Order)	Family	Genus	FS	AS	
Composition (%)	Actinobacteria	Acidimicrobiia (Acidimicrobiales)	Acidimicrobiaceae	<i>Aciditerrimonas</i>	2.13	-	
		Actinobacteria (Actinomycetales)	Mycobacteriaceae	<i>Mycobacterium</i>	38.30	0.99	
			Nocardiaceae	<i>Rhodococcus</i>	-	0.99	
	Armatimonadetes	Fimbriimonadia (Fimbriimonadales)	Fimbriimonadaceae	<i>Fimbriimonas</i>	-	3.96	
	Bacteroidetes	Flavobacteria (Flavobacteriales)	Cryomorphaceae	<i>Wandonia</i>	-	7.92	
		Sphingobacteria (Sphingobacteriales)	Chitinophagaceae	<i>Chitinophaga</i>	-	0.99	
			<i>Flavisolibacter</i>	4.26	-		
	<i>Terrimonas</i>		2.13	3.96			
	Chloroflexi	Unclassified Chloroflexi bacterium			6.38	-	
	Proteobacteria	Alpha-proteobacteria (Rhizobiales and Rhodospirillales)	Bradyrhizobiaceae	<i>Afipia</i>	10.64	3.96	
				<i>Nitrobacter</i>	-	0.99	
			Acetobacteraceae	<i>Acidisphaera</i>	-	0.99	
		Beta-proteobacteria (Burkholderiales)		<i>Gluconacetobacter</i>	-	0.99	
				<i>Paracaligenes</i>	-	0.99	
		Burkholderiaceae	<i>Albidiferax</i>	-	2.97		
			<i>Burkholderia</i>	-	0.99		
			<i>Pandoraea</i>	8.51	-		
		Gamma-proteobacteria (Legionellales and Xanthomonadales)		Comamonadaceae	<i>Variovorax</i>	-	0.99
				Legionellaceae	<i>Legionella</i>	-	0.99
		Xanthomonadaceae	<i>Dokdonella</i>	19.15	4.95		
			<i>Dyella</i>	-	4.95		
			<i>Rhodanobacter</i>	2.13	0.99		
			<i>Rudaea</i>	6.38	2.97		
	Verrucomicrobia		Unclassified Verrucomicrobia	-	1.98		
	Candidate division TM7		-	49.50			
	Candidate division OP10		-	0.99			
	Unclassified bacterium		-	0.99			
Diversity	Number of clones		47	101			
	Number of OTUs		10	23			
	Shannon-Weaver index of diversity (H' , $H' = -\sum P_i \ln P_i$) ^a		2.69	3.05			
	Simpson's index of diversity (D , $D = \sum P_i^2$) ^b		0.21	0.26			

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

^bSimpson's index; a higher number represents lower dominance; P_i : proportional abundance of ecosystem i.

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

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-1 and other 17 FS-clones (1) were of the following accession numbers: KT832628, KT832635, KT832636, KT832638, KT832639, KT832641-KT832643, KT992726, KT992728-KT992730, KT992732, KT992733, KT992736, KT992737, and KU000911. Armatimonadetes of clone AS-55 and other 3 FS-clones (2) were given accession numbers KT992753, KX197160, KX197170, and KX197171. Bacteroidetes of clone AS-54 and other 7 AS-clones (3) were of KT992752, KT992770, KX197123, KX197127, KX197144, KX197146, KX197153, and KX197163; and AS-1 and other 2 AS-clones (4) were of KT832611, KT832622, and KT992768. TM7 of clone AS-3 and other 43 AS-clones (5) of the following accession numbers were used for construction of the phylogenetic tree: KT832612, KT832614, KT832617, KT832620, KT832621, KT832623, KT832625, KT832627, KT992749, KT992751, KT992755, KT992758-KT992760, KT992762, KT992764-KT992767, KX197124, KX197126, KX197128, KX197131, KX197136, KX197138, KX197139, KX197141-KX197143, KX197145, KX197149, KX197150, KX197152, KX197154, KX197155, KX197157, KX197159, KX197162, and KX197164-KX197169. Beta-proteobacteria of clone FS-55 and other 3 FS-clones (6) were given the accession numbers KT832630, KT992731, KT992740, and KU000912. Gamma-proteobacteria of clone AS-23 and 2 other AS-clones, FS-44 and 2 other FS-clones (7) were KU000906, KX197134, KX197158, KT992725, KT992734, and KT992743. Clone FS-72 and 8 other FS-clones (8) were used KT832629, KT832631, KT832633, KT832637, KT832645, KT992721, KT992722, KT992735, and KT992739. Clone AS-52 and 4 other AS-clones (9) of the following accession numbers were used for construction of the phylogenetic tree: KT832618, KT992746, KT992750, KT992756, and KX197156.

Fig. 4. Phylogenetic tree of TM7-like bacterial clones within candidate division TM7.

The 16S rRNA gene sequences of representative TM7 bacterium clones were retrieved from the GenBank database and incorporated into the tree. TM7-like clones from enriched AS are bold-faced. The Subdivision 1 and 2 are shown with blue and red branches, respectively. Subdivision 1 and 2 are based on the classification systems of Dinis *et al.* [8], Hanada *et al.* [16], and Winsley *et al.* [46], which consist mainly of clones from natural environments and wastewater and humans and animals, respectively. The tree was constructed by using the neighbor-joining method and the sequences of *E. coli* (X80725) as an out-group. The bootstrap values above 50% are shown on the internal nodes. TM7 of clone AS-3 and other 42 AS-clones (1) of the following accession numbers were used for construction of the phylogenetic tree: KT832612, KT832614, KT832617, KT832620, KT832621, KT832623, KT832625, KT832627, KT992749, KT992751, KT992755, KT992758–KT992760, KT992762, KT992764–KT992767, KX197124, KX197126, KX197128, KX197136, KX197138, KX197139, KX197141–KX197143, KX197145, KX197149, KX197150, KX197152, KX197154, KX197155, KX197157, KX197159, KX197162, and KX197164–KX197169. Clones AS-72, AS-85, and AS-244 (2) were KT992745, KU000910, and KX197161, respectively.

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

Fig. 5. Quantification of the eubacterial 16S rRNA and soluble di-iron monooxygenase (SDIMO) genes in enriched FS and AS.

The values of gene copies were the average of quintuplicate experiments. Error bars represent the standard deviation.

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 $1.52 (\pm 0.07) \times 10^9$ copies/mg TSS and $0.45 (\pm 0.07) \times 10^9$ copies/mg TSS, respectively. For enriched AS, 16S rRNA and SDIMO gene copy numbers were $1.85 (\pm 0.08) \times 10^9$ copies/mg TSS and $1.07 (\pm 0.06) \times 10^9$ 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.*

Fig. 6. Phylogenetic tree of the partial sequences of soluble di-iron monooxygenase genes cloned from enrichment cultures FS and AS.

The tree was constructed by using the neighbor-joining method, and bootstrap values above 50% are shown on the internal nodes.

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 *prm* 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

Laplanche *et al.* [24] suggested that two independent bacterial communities encountering similar environmental perturbations (*e.g.*, heavy metals and acidic pH) exhibited the same overall taxonomical 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

phylum 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.* [6] 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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