Biotransformation of Polychlorinated Dioxins and Microbial Community Dynamics in Sediment Microcosms at Different Contamination Levels

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Semi-anaerobic microcosms containing different levels of polychlorinated dibenzo-p-dioxins/dibenzo furans (PCDD/Fs) were constructed by seeding with different mass ratios of lake sediment and dioxin-contaminated soil and incubating with organic medium for 1 year. In all microcosms, PCDD/Fs were reduced as a first-order reaction with similar removal rate coefficients, and only trace amounts of less chlorinated congeners were produced as the intermediate and end products. This apparent complete dechlorination of PCDD/Fs seemed to be due to a combination of reductive dechlorination of PCDD/Fs and oxidative degradation of the dechlorinated products. Total cell counting, 16S rRNA gene clone library analyses and quinone profiling showed that the microcosms contained relatively constant total populations with members of the phyla Bacteroidetes, Firmicutes and Proteobacteria (especially “Deltaproteobacteria”) as the major constituents, independent of pollution levels. Quantitative real-time PCR with a specific primer set showed that the population density of “Dehalococcoides” and its phylogenetic relatives was highly correlated with the concentration of PCDD/Fs present. Some “Dehalococcoides” strains were isolated from the microcosms by repeated enrichment with chloroaromatics as the terminal electron acceptor. However, these isolates did not match with the major “Dehalococcoides”-related clones directly PCR-amplified. The results of this study suggest that PCDD/Fs in natural environments under given conditions are transformed with similar half-reduction rates independent of their concentrations, and a wide variety of “Dehalococcoides”-related bacteria play the primary role in this process.

Key words: polychlorinated dioxins, reductive dechlorination, “Dehalococcoides”, dehalorespiration, microcosm

Anthropogenic toxic compounds such as those of the so-called dioxin group, polychlorinated dibenzo-p-dioxins/dibenzo furans (PCDD/Fs) and coplanar polychlorinated biphenyls (co-PCBs), have been well documented to be widespread in different environmental compartments including aquatic sediment and soil. These chloroaromatics possibly undergo microbial breakdown as well as physicochemical degradation in the environment, as a number of studies have demonstrated the existence of a wide variety of dioxin-transforming microorganisms2,4,5. However, information about the effects of microbial activity on the fate and dynamics of dioxins in contaminated areas is still scanty.

In recent years, microbial reductive dechlorination of PCDD/Fs and PCBs has received intensive study, because this biological activity is not only possibly the initial step in the biotransformation of organohalogenes in the environment but also has important implications for engineered bioremediation2,4,5,8,9,10. Microbial dechlorination of PCDD/Fs has been demonstrated in studies of anaerobic microcosms using contaminated sediment and soil1,2,7,9,12,20,31,49,52. Recent research has also shown that the chlorobenzene-dechlorinating bacterium “Dehalococcoides” sp. strain CBDB123 and the chloroethene-dechlorinator “Dehalococcoides ethenogenes” strain 19524 have the ability to dechlorinate selected PCDD congeners11,18. Probably, there exists a large group of dehalorespiring bacteria within the phylum Chloroflexi, including “Dehalococcoides” species and their phylogenetic relatives reported to date as uncultured dechlorinating bacteria15,40,57,58,60. This assembly of dehalo-
respiring bacteria, termed here the "Dehalococci" group, may be omnipresent in a wide range of contaminated environments and contribute to the natural attenuation of PCDD/Fs and other chlorinated contaminants. However, little is known about the potential capacity of natural microbial communities to transform PCDD/Fs and the relationship between this biological process and the population dynamics of the "Dehalococci" group of bacteria. One of the reasons for this is that it is difficult to obtain axenic cultures of PCDD/F-dechlorinating bacteria and to grow them enough for full taxonomic characterization.

Previously, we studied the biotransformation of PCDD/Fs using a laboratory-scale semi-anaerobic microcosm with highly contaminated river sediment. In this microcosm, reductive dechlorination of PCDD/Fs and oxidative degradation of dechlorinated products might take place simultaneously, resulting in the apparent complete dechlorination of dioxins. A quantitative real-time PCR analysis with specific primer sets showed that the population levels of "Dehalococci" species and their phylogenetic relatives in the microcosms were in the order of 10^6 cells g^(-1) wet weight of sediment under steady-state conditions.

The present study was designed to obtain more definitive information about the potential capacity of sediment populations to transform PCDD/Fs. For this, we constructed semi-anaerobic sediment microcosms that contained different levels of PCDD/Fs and monitored the concentrations of PCDD/Fs and microbial community dynamics during a long-term period of incubation. The main purposes of this work were to envisage kinetic patterns of PCDD/F transformation in the microcosms and to elucidate the relationship between the level of contamination by PCDD/Fs and the population density of "Dehalococci" and phylogenetic relatives thereof as potent dechlorinators. We also made attempts to obtain pure cultures of "Dehalococci" from the microcosms by repeated enrichment with chlorinated aromatics as the terminal electron acceptor for dehalorespiration.

Materials and Methods

Microcosm construction

Mud samples taken from a core of sediment (depth 0–12 cm) of Lake Suwa, Japan, were used as the seed for constructing microcosms. The samples were used immediately upon return to the laboratory. A soil sample contaminated with a high concentration of PCDD/Fs (3.1 nmol g^(-1) dry wt) which was taken from a farmland area in Osaka Prefecture, Japan, was also used. The soil was dried by heating at 110°C for 48 h and then stored for 2 years at 10°C prior to use. Four microcosms that had different concentrations of PCDD/Fs, designated microcosms LS1, LS2, LS3, and LS4, were constructed in 230-ml screw-capped glass bottles. The sediment mud sample was mixed completely with an equal volume of phosphate-buffered saline (PBS; 10 mM potassium phosphate and 130 mM sodium chloride, pH 7.0) and pelleted by centrifugation at 5,000 × g for 10 min. The soil sample was also suspended in an equal volume of PBS and mixed completely. Then, the mud pellet and the soil slurry were introduced into screw-capped glass bottles at different mass ratios in a total of 100 g wet wt (Table 1). Each of the bottles with different levels of dioxins was prepared in triplicate. All of the bottles were completely filled with filter-sterilized OAM-2 medium, which is a modification of OAM-1 medium. The medium contained (per liter): 0.5 g of NH₄Cl, 0.5 g of KH₂PO₄, 0.2 g of NaCl, 0.2 g of MgCl₂·6H₂O, 0.05 g of CaCl₂·2H₂O, 1 ml of trace element solution SL8, 1 ml of vitamin mixture PV1, and 1 mM each of acetate, butyrate, formate, fumarate, pyruvate, and cysteine. For the control test, the bottles were filled with OAM-2 medium supplemented with 1% paraformaldehyde. The headspace of the bottles was not replaced with anoxic

<table>
<thead>
<tr>
<th>Microcosm</th>
<th>Mass ratio (g wet wt) mixed</th>
<th>PCDD/F concentration (Cₜₚₜ)</th>
<th>Total bacterial count (×10⁸ g⁻¹ dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sediment mud</td>
<td>Soil slurry</td>
<td>pmol g⁻¹ dry wt</td>
</tr>
<tr>
<td>LS1</td>
<td>100</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>LS2</td>
<td>99</td>
<td>1</td>
<td>52</td>
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<tr>
<td>LS3</td>
<td>91</td>
<td>9</td>
<td>310</td>
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<tr>
<td>LS4</td>
<td>10</td>
<td>90</td>
<td>2,900</td>
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¹ Measured by EtBr staining.
² Measured with a LIVE/DEAD BacLight Bacterial Viability kit.
gas, because this treatment had no stimulatory effect on the removal of PCDD/Fs as described previously\textsuperscript{40}. All the bottles were tightly capped and settled in a dark room at 25°C for 1 year. Every one month of incubation, the supernatant from the microcosms was removed and an aliquot of the sediment was sampled. Then, the bottles were filled up with fresh medium, mixed completely and incubated further. The sediment slurry and supernatant samples collected were stored separately at −20°C until the analysis.

Analysis of dioxins and related compounds

Dioxins were extracted from microcosm samples by the Soxhlet method and analyzed by high-resolution gas chromatography-mass spectrometry (GC/MS) to determine the total concentration of PCDD/Fs (C\textsubscript{tot}) and their congener profiles as described previously\textsuperscript{25}. The toxicity equivalent (TEQ) concentration was calculated based on the toxicity equivalent factors (TEFs) for different congeners redefined by van der Berg \textit{et al.}\textsuperscript{50}. In this study, mono-, di-, tri-, tetra-, penta-, hexa, hepta-, and octa-chlorinated congeners of dibenzo-p-dioxin (DD) and dibenzo-furan (DF) were abbreviated as CDD/Fs, TrCDD/Fs, TbCDD/Fs, HxCDD/Fs, HeCDD/Fs and OCDD/F, respectively. The terms PCDD/Fs and 1-3CDD/Fs were used to indicate the fractions of tetra- to octa-chlorinated congeners and of mono- to tri-chlorinated congeners, respectively. Non-chlorinated dioxins, catechol and salicylic acid from sediment and supernatant samples were extracted with ethyl acetate and measured by reverse-phase HPLC and photodiode array detection as reported previously\textsuperscript{27,60}. Hthalide (4,5,6,7-tetrachloro-1,3-dihydrobenzo[c]furan-2-one) and chlorobenzene were analyzed using a Hewlett-Packard 6890 series gas-liquid chromatograph and mass selective detector system as described previously\textsuperscript{58}.

Quinone analysis

Quinones from sediment samples were extracted with an organic solvent mixture and fractionated into menaquinone (MK-\textit{n}) (plus phylloquinone [K\textsubscript{1}]) and ubiquinone (Q-\textit{n}) fractions using Sep-Pak Vac 3 cc silica gel cartridges (Waters Corp., Milford, USA). Quinone components of each fraction were separated and identified by reverse-phase HPLC and photodiode array detection with external quinone standards. HPLC and mass spectroscopic detection with atomic pressure chemical ionization were also performed for the identification of plastoquinone (PQ-\textit{n}) and partially hydrogenated menaquinone species, MK-\textit{n}(H\textsubscript{\textit{n}}). Detailed information about the analytical procedure has been given previously\textsuperscript{24,30}.

Fluorescence microscopy

Total bacterial counts were measured by epifluorescence microscopy with ethidium bromide (EtBr) staining as described previously\textsuperscript{42,60}. Total viable counts were measured using a LIVE/DEAD BacLight\textsuperscript{TM} Bacterial kit (Molecular Probes, Eugene, USA) according to the manufacturer’s instructions and as reported previously\textsuperscript{40}. Fluorescence in situ hybridization (FISH) targeting 16S rRNA was performed with the domain-specific probes, EUB338 mix and ARC915, as described earlier\textsuperscript{60}. Stained specimens were observed under an Olympus BX-50 epifluorescence microscope equipped with a Flovel FD-120M digital charge-coupled device camera (Flovel Co., Tokyo, Japan). The number of stained cells was counted using the image analysis program WINROOF (Flovel).

DNA extraction and purification

Bulk DNA was extracted from the samples of sediment according to the protocol of Yoshida \textit{et al.}\textsuperscript{60} with minor modifications as previously described\textsuperscript{26}. The crude DNA extracted was further purified using a standard method including deproteinization with chloroform-isoamylalcohol and RNase treatment\textsuperscript{35}. The DNA solution thus obtained was diluted in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) as needed and used for PCR experiments.

Construction of 16S rRNA gene clone library

16S rRNA gene fragments from the DNA purified from microcosms were PCR-amplified with a pair of bacterial universal primers, fD1 (27F) and rP1 (1492r)\textsuperscript{55}, as described previously\textsuperscript{60}. The reaction profile consisted of pre-heating at 95°C for 2 min and 20 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1.5 min; the final step was followed by post-extension for 5 min. PCR products were separated by agarose gel electrophoresis, cut from the gel and then purified using a GENECLEAN Spin kit (Bio 101, Vista, USA). Purified PCR fragments were subcloned using a pTBlue Perfectly Blunt cloning kit (Novagen, Madison, USA). Transformation of \textit{Escherichia coli} competent cells was carried out according to a standard manual of molecular cloning\textsuperscript{60}. Plasmid DNA was isolated and purified by using the Wizard Plus Minipreps DNA Purification System (Promega Inc., Madison, USA) according to the manufacturer’s instructions.

Sequencing and phylogenetic analysis

Cloned 16S rRNA genes were sequenced using a Dye
Terminator Cycle Sequencing kit and an ABI PRISM®
3100-Avant Genetic Analyzer (Applied Biosystems, Foster
City, USA) according to the manufacturer’s instructions.
Sequence data were compiled by the GENETYX-MAC pro-
gram (Software Development Co., Tokyo, Japan), analyzed
for chimera with the CHIMERA_CHECK program version
2.714, and compared with those deposited in databases using
the BLAST search system5). The multiple alignment of se-
quences and calculation of the nucleotide substitution rate
(K_{sub}) with Kimura’s two-parameter model12) were
performed using the CLUSTAL W program50). Distance matrix
trees were constructed by the neighbor-joining method45),
and the topology of the trees was evaluated by bootstrapping
with 1,000 resamplings47). Alignment positions with gaps
were excluded from the calculations. A group of uncul-
tured clones and organisms having a sequence similarity
of 97% and more to one another was regarded as a single
operational taxonomic unit (OTU).

Real-time PCR
Real-time PCR assays for measurement of the population
density of the “Dehalococcoides” group (P_{ab}) were per-
formed by the nested PCR method as described
previously26,60). First, total bacterial 16S rRNA genes from
the microcosm DNA were amplified with the bacterial universal
primers fD1 and rP1 and purified as described above.
Then, nested real-time PCR was performed with the puri-
fied PCR product as the template and the “Dehalococ-
coides”-specific primers DHC793f and DHC946r and bac-
terial universal primers 357f and 517r. The reaction was
performed using a LightCycler FastStart DNA Master
SYBR GREEN I kit (Roche Molecular Biochemicals, India-
napolis, USA) and a LightCycler system (Roche Diagnos-
tics, Mannheim, Germany) according to the manufacturer’s
instructions. The copy number of the amplicons was cal-
culated using LightCycler software version 3.5 (Roche Diagnos-
tics, Mannheim, Germany).

Subcloning and sequencing of “Dehalococcoides”
clon es
Real-time PCR products amplified with primers
DHC793f and DHC946r were separated by agarose gel
electrophoresis, purified using a GENE CLEAN Spin kit,
and subcloned with a pTBlue Perfectly Blunt cloning kit
(Novagen, Madison, USA) as described for constructing the
16S rRNA gene clone library. Cloned 16S rRNA genes
were sequenced with an ABI cycle sequencing kit and an
ABI automated DNA sequencer as described above.

Enrichment, isolation and identification of “Dehalococcoides”

Based on collective information on the anaerobic cultiva-
tion of dechlorinating bacteria11,28, a chemically defined
anoxic medium (designated DHE1) was prepared under a
N_{2}/CO_{2} (80:20, [vol]/[vol]) atmosphere and used in 30-ml
anaerobic culture tubes and 60-ml serum bottles with a cul-
ture volume of 10 and 20 ml, respectively. The medium
consisted of (per liter): 2 mM acetate, 2 mM butyrate, 1 mM
cysteine, 0.5 g of NH_{4}Cl, 0.5 g of KH_{2}PO_{4}, 2.5 g of
NaHCO_{3} (filter-sterilized), 0.2 g of NaCl, 0.2 g of
MgCl_{2}·6H_{2}O, 0.05 g of CaCl_{2}·2H_{2}O, 1 ml of trace element
solution SL8, 1 ml of Se/W solution (0.5 g of NaOH, 3 mg
of NaSeO_{3}·5H_{2}O and 4 mg of Na_{2}WO_{4}·2H_{2}O per liter), 1 ml
of vitamin mixture PV1, 5 ml of 2% Na_{2}S·9H_{2}O solution
(filter-sterilized), 5 ml of 10% titanium (III) citrate solution
and 0.5 mg of resazurin. Specific inhibitors, bromoethansul-
fonate (BES, 5 mM) and molybdate (2 mM), were added as
needed. The pH of the medium was 6.9. The medium
was supplemented with 0.6% gelan gum (designated
DHE1G medium) when used as a solidified medium.
Pre-
liminarily, we made attempts to enrich “Dehalococcoides”
by use of 1,2,3,4-TCDD as the terminal electron acceptor
but were unsuccessful for unknown reasons. Therefore, the
enrichment medium was supplemented alternatively with
50 μM thiophene or 1,2,4-trichlorobenzene (as an acetone
solution) as a terminal electron acceptor; the solvent was
evaporated by using the N_{2}/CO_{2} gas mixture. The slurry
samples (1 ml) taken from the microcosms were introduced
into test tubes or bottles in which the headspace was re-
placed with a filter-sterilized gas mixture of CO_{2} and H_{2}
(90:10 [vol]/[vol]). The cultures were incubated on a recip-
rocral shaker at 30°C for 2 months and subjected to a GC/MS
analysis of dechlorinated products. The cultures capable
of possible dehalorespiration were transferred to fresh medi-
um (10% inoculum) and subcultured every 2 months. After
5 transfers, the enrichment cultures were diluted with lique-
fied DHE1G medium and incubated at 30°C for 4 to 6
weeks to produce single colonies. Very tiny colonies thus
obtained were picked from the tubes and stored in DHE1
medium.

The phylogenetice identity of the cultures was determined by
FISH as described above and denatured gel gradient
electrophoresis (DGGE) of PCR-amplified 16S rRNA
genes43). PCR-DGGE was performed by targeting the V3 re-
gion of 16S rRNA genes and using the Bio-Rad Dcode sys-
tem (Bio-Rad Laboratories, Hercules, USA) as reported
previously60). Major DGGE fragments were cut from the
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gel, purified using a GENECLEAN Spin kit, subcloned with a pTBlue Perfectly Blunt cloning kit, and then sequenced as described above.

Database accession numbers for sequences
The 16S rRNA gene sequences determined in this study were deposited under DDBJ accession numbers AB233385 to AB233404 and AB234235 to AB234287.

Results

General characteristics of microcosms
The 4 microcosms, LS1 to LS4, contained 18–2,900 pmol of PCDD/Fs and 10⁶ cells of bacteria per g (dry wt) at the start of incubation (Table 1). Also, the direct viable counts accounted for ca. 60% of the total at the beginning. The initial viable count of the soil itself, which had been dried and stored at 10°C for 2 years, was less than 1% of the total count (i.e., <10⁶ cells g⁻¹). Therefore, almost all of the viable populations in the microcosms might originate from the lake sediment, although one could not rule out the possibility that indigenous bacteria surviving in the soil grew during the incubation of the microcosms.

During 2 months of start-up incubation, all of the microcosms turned black with strong odor of sulfide, indicating vigorous activity by sulfate-reducing bacteria. Thereafter, the black coloring disappeared gradually with time. The pH of the microcosms ranged from 5.0 to 6.5 during this period and became stable at a neutral level after that. The total count in the microcosms increased to be in the order of 10⁰ cells g⁻¹ (dry wt) during the first 50 days of incubation, and thereafter steadied in a range of 8.0×10⁵ to 1.1×10⁶ cells g⁻¹ (dry wt). No marked differences were noted in appearance, pH and bacterial count among the 4 microcosms.

Kinetics of dioxin transformation
After a lag of 30–40 days, the concentration of PCDD/Fs ($C_{\text{den}}$) in the 4 microcosms decreased during the overall period of incubation and finally dropped to 50–54% of the initial concentrations on a molar basis (Fig. 1). This was also the case for the TEQ level of PCDD/Fs (data not shown). The control microcosm with paraformaldehyde exhibited only a slight reduction in the concentration of PCDD/Fs (3% decrease) during the overall period of incubation. The decrease in $C_{\text{den}}$ was relatively fast during start-up and became slower gradually with time in all cases. Thus, apart from the lag-time, the decrease in $C_{\text{den}}$ with time seemed to follow first-order kinetics as shown in Fig. 1. That is, $C_{\text{den}}$ remaining in the microcosms is given by:

$$C_t = C_0 e^{-kt}$$  

(1)

where $C_t$ and $C_0$ indicate $C_{\text{den}}$ on day $t$ and 0, respectively, and $k$ denotes a removal rate coefficient. The exponential transformation of equation (1) produces equation (2) as follows:

- $y = 18 e^{-0.020t}$
  $r^2 = 0.9766$

- $y = 52 e^{-0.0017t}$
  $r^2 = 0.9682$

- $y = 310 e^{-0.0017t}$
  $r^2 = 0.9655$

- $y = 2900 e^{-0.016t}$
  $r^2 = 0.9196$

Fig. 1. Changes in the concentrations of PCDD/Fs and 1-3CDD/Fs in microcosms LS1 (a), LS2 (b), LS3 (c) and LS4 (d). Regression equations approximating changes in PCDD/Fs and correlation coefficients ($r^2$) are shown. For the calculation, the lag time was ignored, because it was very short in the overall period of decay and thus had only a small effect on the results.
where $\ln(C_0/C_t)$ corresponds to the PCDD/F removal rate. On the basis of equation (2), all data on the removal rate obtained with the 4 microcosms were plotted against incubation time. As shown in Fig. 2, the removal rate could be expressed as a linear equation against time with an average $k$ value of 0.0018 (range, 0.0016–0.0020). From equation (2), the half-reduction time ($t_{1/2}$) of PCDD/Fs is given by:

$$t_{1/2}=\ln 2/k$$

On the basis of equation (3), the average half-reduction time of PCDD/Fs in the microcosms was estimated to be 13 months (range, 12–14 months).

A previous study showed that reductive transformation of dieldrin in anaerobic sediment cultures took place according to first-order kinetics(13).

**Dynamics of dioxin congeners and related compounds**

During the overall period of the experiment, all PCDD/F congeners were totally reduced, and only small amounts of 1-3CDD/Fs were accumulated as the intermediate or end products of dechlorination (Fig. 1 and Table 2). The concentrations of non-chlorinated dioxin congeners, DD and DF, were slightly increased during 2–3 months of the start-up period, and thereafter remained relatively unchanged or slightly decreased. Thus, although there remains the possibility that the dechlorinated products partly volatilized at each sampling time, a complete dechlorination of PCDD/Fs seemingly took place in the microcosms. The supernatant samples periodically removed from the microcosms did not contain chlorinated dioxins in detectable amounts but had significant amounts of catechol and salicylic acid (data not shown), which are the oxidatively degraded products of DD and DF, respectively(13,30). For example, the cumulative amounts of catechol and salicylic acid produced in microcosm LS4 corresponded to 2.9 and 0.5% of the total amount of PCDD/Fs removed during 1 year on a molar basis, respectively. Thus, the apparent complete dechlorination of PCDD/Fs seemed to be due to a combination of reductive dechlorination of PCDD/Fs and oxidative degradation of the dechlorinated products.

As shown in Table 2, there were marked differences in congener patterns as well as in concentrations of PCDD/Fs between microcosms LS1 and LS4. However, since the two microcosms gave similar removal constants for PCDD/Fs, the congener composition of PCDD/Fs seemed to have little or no effects on the dioxin removal rate.
16S rRNA gene clone analysis

To elucidate the microbial community structure of the dioxin-transforming microcosms, 16S rRNA gene clone libraries were constructed from two microcosms, LS1 and LS4, which contained the lowest and highest concentrations of PCDD/Fs among the test microcosms, respectively. Approximately 80 clone sequences (>1,400 bp each) were obtained from each of the clone libraries and checked for chimeras and ambiguous nucleotide positions. Of these clones, 67 from microcosm LS1 and 68 from microcosm LS4 could be identified and used for phylogenetic analysis. As shown in Fig. 3, almost all of the uncultured clones could be affiliated with either of 12 phyla including the WS1 division. The clone libraries from the two microcosms were similar to each other in phylogenetic composition at the phylum level, as members of the phyla Bacteroidetes, Firmicutes and Proteobacteria (especially the class “Deltaproteobacteria”) were the major constituents in both the microcosms. Also, the phylogenetic groups with which more than 2 clones from both the microcosms were affiliated were the phyla Chlorobi, Chloroflexi and Verrucomicrobia as well as the classes “Alpha-”, “Beta-” and “Gamma proteobacteria”. However, the 2 microcosms shared only 15% of clones identified as the common OTUs (>97% similarity). This suggests that a population shift took place in part from microcosm LS1 to microcosm LS4 due to the mixing with a large proportion of soil and/or the resultant extensive contamination with PCDD/Fs.

The neighbor-joining phylogenetic trees for the uncultured clones obtained from microcosm LS4 (i.e., those with LS4 in their name) are shown in Figs. 4, 5 and 6. Most of the LS4 clones grouped with the previously reported uncultured bacteria as single OTUs or their nearest neighbors. Interestingly, 51% of the LS4 clones, including those belonging to Acidobacteria, Bacteroidetes, Chlorobi, Firmicutes, Nitrospira, Proteobacteria and the WS1 division, were most closely related to the uncultured bacteria that have so far been detected in a PCDD/F-contaminated microcosm and other organochlorine-contaminated environments. Some Chloroflexi clones were detected but were located far from the “Dehalococcoides” group. These phylogenetic data indicate that members of the “Dehalococcoides” group were not major constituents of the microbial communities in the microcosms.

Quinone profiles of microcosms

The 4 microcosms under steady-state conditions produced both ubiquinones and menaquinones at a total concentration of 15 to 32 nmol g\(^{-1}\) (dry wt). A previous report has shown that 1 nmol of all the respiratory quinones corresponds to 2.5×10\(^8\) cells of respiratory bacteria\(^{26}\). Therefore, one could assume that the microcosm contained 3.8–8.0×10\(^8\) cells of respiratory bacteria per g of dry weight. This range of respiratory bacterial populations corresponded to 38–73% of the total population as measured by epifluo-

![Fig. 3. Phylogenetic composition of 16S rRNA gene clone libraries constructed from microcosms LS1 (a) and LS4 (b).](image-url)
Fig. 4. Neighbor-joining phylogenetic tree for the uncultured clones from microcosm LS4 and related organisms affiliated with the phyla of gram-negative bacteria other than Proteobacteria. *Aquifex pyrophila* is used as the outgroup to root the tree. Scale bar=2% substitution rate (Ksub).

Detailed information on the quinone profiles in microcosms LS1 and LS4 is given in Fig. 7. There were no marked differences in quinone patterns between the two microcosms. The major components detected were MK-6 to MK-8, which seemed to be derived from members of Bacteroidetes, Firmicutes, and “Delataproteobacteria”, when compared with the results of the clone library analysis. Ubiquinones accounted for 19–23 mol% of the total quinone content with Q-8, Q-9 and Q-10 as the main components. Apparently, these ubiquinone species were derived from members of “Alpha-”, “Beta-” and “Gammeproteobacteria”. Significant amounts of partially hydrogenated menaquinones (10–17 mol%), most of which might have originated from members of the phylum Actinobacteria, were also detected.
The finding as to partially saturated menaquinones did not agree with the results of the clone library analysis, which showed a small proportion (<5%) of Actinobacteria relative to the total community. The difference in efficiency of detection of Actinobacteria between the clone library and quinone analyses might result from biases with the former method (e.g., DNA extraction and PCR biases). Therefore, a polyphasic approach using multiple techniques is essential to characterize complex microbial communities.

Population dynamics of the “Dehalococcoides” group

Because of the possible role of the “Dehalococcoides” group in the dechlorination of PCDD/Fs in the microcosms, $P_{\text{obs}}$ was estimated by quantitative real-time PCR targeting 16S rRNA genes. The proportion of the “Dehalococcoides” rRNA gene to the total bacterial rRNA gene content remained relatively constant in each of the microcosms under steady-state conditions, a small percentage (in the order of $10^{-1}$ to $10^{-2}$) of the total content depending upon the microcosms (Table 3). By assuming the copy number of the
Fig. 6. Neighbor-joining phylogenetic tree for the uncultured clones from microcosm LS4 and related organisms categorized into the phyla of gram-positive bacteria (Actinobacteria and Firmicutes). *Actinobacteria* is used as the outgroup to root the tree. Scale bar=2% substitution rate (K$_{sub}$). Nodes showing a bootstrap percentage of more than 85% and of 50–84% (1,000 resamplings) are indicated by open circles and solid circles, respectively. For other explanations, see the legends to Fig. 4.

rRNA operon per genome in “Dehalococcoides” to be $3^1,47$ against an average copy number in bacteria of 3.8$^{19}$, the $P_{dhe}$ in the microcosms was presumed to be in the order of $10^4$ to $10^5$ cells g$^{-1}$ (dry wt) of sediment. The $P_{dhe}$ level as well as the total bacterial count in each microcosm was relatively constant during the overall period of incubation, except for a rapid increase during the start-up period ($\leq 50$ days from the beginning; data not shown). Our accompanying report has shown that the $P_{dhe}$ levels in the sediment and soil used as the seed were in the order of $10^4$ and $10^5$ cells g$^{-1}$ dry wt, respectively$^{26}$.

The results indicate that the $P_{dhe}$ changed depending upon the level of PCDD/Fs. To confirm this, logarithmic values of $P_{dhe}$ were plotted against those of $C_{dhe}$ based on the present and previous data (Fig. 8). There was a strong positive correlation between the $P_{dhe}$ and $C_{dhe}$ values which was statistically significant ($P<0.01$), thereby demonstrating the $C_{dhe}$-dependent proliferation of the “Dehalococcoides” group.

A clone library was constructed using the DNA fragments amplified by real-time PCR from microcosms LS1 and LS4 to confirm whether these were actually from the “Dehalococcoides” group. The phylogenetic tree shown in Fig. 9 revealed that all of the clones detected (i.e., those
with LS1-DHC and LS4-DHC in their names) were positioned within the "Dehalococcoides" group. However, only one clone, LS4-DHC18, clustered with the assembly of known "Dehalococcoides" strains, whereas the nearest neighbors of all other clones were the uncultured bacteria designated DF-140,57,58, OTU-15,41 and RFLP1729) and those previously detected in a PCDD/F-dechlorinating microcosm60).

**Enrichment and isolation of "Dehalococcoides"**

The enrichment of "Dehalococcoides" from the samples of sediment was performed by repeated subculturing with fthalide or 1,2,4-TCB as the terminal electron acceptor, and positive results were obtained with the samples from microcosms LS3 and LS4 when BES and molybdate, potent inhibitors for methanogens and sulfate reducers, respectively, were present in the enrichment cultures. The reductive dechlorination of fthalide and 1,2,4-TCB by enrichment cultures was confirmed by GC/MS-based detection of an unknown dechlorinated metabolite and monochlorobenzene as the end products, respectively. Although it was difficult to confirm cell growth in these dechlorinating cultures with the naked eye, FISH revealed that the cultures contained EUB338-positive bacteria with small cocoid cells (0.3–0.5 μm in diameter) occurring exclusively. Thus, these cultures were subjected to colony isolation in DHE1G medium. As a result, 4 strains, designated TUT1902, TUT1903, TUT1951 and TUT1952, could be isolated.

Upon the PCR-DGGE analysis (Fig. 10), all these cultures gave a single main band as indicated by an arrow whose sequences were identical with that of "Dehalococcoides" sp. strain CBDB1 (DDBJ/EMBL/GenBank accession number, AF230641), thereby confirming the cultural purity and phylogenetic identity of the 4 strains.

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**Table 3. Population levels of total bacteria and the "Dehalococcoides" group in different microcosms on day 360**

<table>
<thead>
<tr>
<th>Microcosm/sample</th>
<th>Total population (g⁻¹ dry wt)</th>
<th>% of &quot;Dehalococcoides&quot; 16S rRNA gene*</th>
<th>Presumed &quot;Dehalococcoides&quot; population (g⁻¹ dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS1</td>
<td>1.0±0.3×10¹¹</td>
<td>3.1±1.9×10⁻³</td>
<td>1.2±0.8×10⁵</td>
</tr>
<tr>
<td>LS2</td>
<td>9.2±1.9×10¹⁰</td>
<td>2.6±0.6×10⁻⁴</td>
<td>9.1±1.4×10⁵</td>
</tr>
<tr>
<td>LS3</td>
<td>8.6±1.5×10¹⁰</td>
<td>3.0±1.7×10⁻³</td>
<td>1.0±0.8×10⁵</td>
</tr>
<tr>
<td>LS4</td>
<td>9.1±2.1×10¹⁰</td>
<td>1.3±0.9×10⁻¹</td>
<td>4.6±1.1×10⁸</td>
</tr>
</tbody>
</table>

* Percentage of "Dehalococcoides" 16S rRNA gene to the total bacterial rRNA gene content.
Discussion

The results of the present study have confirmed our previous observations\(^{60}\) of PCDD/F-contaminated semi-anaerobic microcosms, and expanded our knowledge of the potential capacity of complex microbial communities to transform PCDD/Fs. In this study, the 4 test microcosms, containing a wide range of PCDD/F concentrations, exhibited similar reducing patterns of PCDD/Fs that could be approximated by a first-order reaction. This finding indicates that the population density and activity of dioxin-transforming microorganisms in each microcosm were constant during the overall period of incubation, apart from the initial short lag time. Thus, the 4 microcosms exhibited similar rate coefficients (\(k\)) ranging from 0.0016 to 0.0020 with an average of 0.0018. Previously, we found a half-reduction time for PCDD/Fs of 14 months in a river sediment microcosm, corresponding to a \(k\) value of 0.0017\(^{60}\). The range of values for the transformation rate may be a reflection of the potential capacity of natural microbial communities to degrade dioxins under the given conditions. To our knowledge, this is the first report to clearly define the half-life of PCDD/Fs under a given set of conditions by studying kinetic information. The half-life of PCDD/Fs found in our microcosms is much shorter than that recorded in soil\(^{49}\) and in lake sediment\(^{88}\).

Consistent with results reported previously\(^{60}\), the present study showed that all PCDD/F congeners in the 4 microcosms were totally reduced without the accumulation of significant amounts of 1-3CDD/Fs as intermediate or end products. Although there remains the possibility that some abiotic reactions\(^{35,36}\) were involved in the transformation, the apparent complete dechlorination of PCDD/Fs can be explained by assuming that the reductive dechlorination of PCDD/Fs and the oxidative degradation of the dechlorinated products took place simultaneously. Since our microcosms were a semi-anaerobic system made by filling up the vessels with medium without sparging anoxic gas, there might be some chance of aerobic dioxin-degrading bacteria growing using a small amount of dissolved oxygen. In fact, the oxidatively degraded products of DD and DF, catechol and salicylate, respectively, were accumulated in the microcosms. The detection of significant amounts of ubiquinones and the PCR clones possibly derived from ubiquinone-containing Proteobacteria (i.e., "Alpha-","Beta-", and "Gamma-proteobacteria") demonstrates that the microcosms allowed the growth of aerobic bacteria. From the microcosms as well as from the soil used as the seed\(^{21}\), we have already isolated a number of DF-degrading aerobic bacteria belonging to the phyla Actinobacteria, Firmicutes and Proteobacteria (Hiraishi et al., manuscript in preparation). It is also necessary to clarify whether anaerobic oxidation of DD and DF takes place in a semi-anaerobic microcosm such as that used in this study.

The 16S rRNA gene clone library analyses and quinone profiling revealed that members of the phyla Bacteroidetes, Firmicutes and Proteobacteria (especially "Delta-proteobacteria") were the major constituents of the total population in the microcosms under steady-state conditions. Interestingly, the majority of the clones detected in this study were most similar to those previously found in chlorinated solvent-contaminated aquifer\(^{49}\) and in microbial communities capable of reductive dechlorination of PCDD/F\(^s\), TCDD\(^s\), PCBs\(^{29,30}\), chlorobenzenes\(^{4,57}\) and 1,2-dichloropropane\(^{49}\). In view of the results of these previous studies and our observations, anaerobic or semi-anaerobic environments polluted with organohalides may share a number of common phylogenetic and physiologic groups of bacteria irrespective of the kinds of chlorinated pollutants. Elucidation of the functions and ecological significance of these common groups of bacteria in organochlorine-dechlorinating communities awaits further study.

Total cell counting and real-time PCR experiments with different microcosms have shown that, while the total population of bacteria remained relatively unchanged among the microcosms at different levels of contamination, the population density of the "Dehalococcoides" group (\(P_{\text{adc}}\)) was di-
Fig. 9. Phylogenetic trees for the uncultured clones amplified by real-time PCR and their relatives. *Aquifex pyrophilus* is used as the outgroup to root the tree. Nodes showing a bootstrap percentage of more than 85% and of 50-84% (1,000 resamplings) are indicated by open circles and solid circles, respectively. Scale bar=2% substitution rate ($K_{sub}$). Clones with LS1-DHC and LS4-DHC (in bold faces) are those derived from microcosms LS1 and LS4, respectively.

Directly proportional to the concentration of PCDD/Fs ($C_{bas}$) present. This observation provides circumstantial evidence that members of the "*Dehalococcoides*" group were potent mediators for the dechlorination of PCDD/Fs in the microcosms. In fact, our attempt to isolate "*Dehalococcoides*" by anaerobic enrichment cultivation of the samples of sediment with thalalide and TCB as the terminal electron acceptor was successful. The ability of these new strains to reductively dechlorinate PCDD/Fs and other chlorinated compounds is now under investigation. The genome analysis of "*Dehalococcoides ethenogenes*" strain 195 has demonstrated the presence of 18 copies of putative reductive dehalogenase
genes, including the well-characterized tceA gene\(^{[6]}\). It has also been shown that the genome of “Dehalococcoides” sp. strain CBDB1 has 32 homologues of reductive dehalogenase genes but not tceA\(^{[5]}\). Further study of the distribution and expression of these genes in our isolates as well as in the microcosms should provide more definitive information on the involvement of the “Dehalococcoides” group in the transformation of PCDD/Fs.

An interesting finding of this study coupled with our previous observations\(^{[26,60]}\) is that most of the uncultured clones detected in the microcosms by real-time PCR formed different phylogenetic clusters from the typical “Dehalococcoides” assembly consisting of culturable strains such as “D. ethenogenes” strain 195, “Dehalococcoides” sp. strain CBDB1 and our new isolates. This finding suggests that these “Dehalococcoides”-related bacteria predominated as the dechlorinators and played more important roles in the dechlorination of PCDD/Fs in the microcosms. Similar assumptions have been made by Watts et al.\(^{[54]}\) and Yoshida et al.\(^{[60]}\), who detected diverse “Dehalococcoides”-related clones in PCB- and PCDD/F-dechlorinating microbial communities, respectively. At present, why we could isolate typical “Dehalococcoides” strains rather than their relatives possibly predominating in the microcosms is not known with certainty. A possible reason for this is a culture bias in that the concentration of chloroaromatics used for the enrichment of “Dehalococcoides” was on a molar basis, 50-fold higher than that of PCDD/Fs in the microcosms.

In conclusion, a complex microbial community including both PCDD/F-dechlorinating anaerobic bacteria and aerobic bacteria capable of oxidizing the dechlorinated products can develop under semi-anerobic polluted conditions and achieve apparently complete dechlorination of PCDD/Fs. In such environments, the transformation of PCDD/Fs may follow first-order kinetics with similar half-life coefficients, and a wide variety of “Dehalococcoides”-related bacteria that have not been obtained as culturable strains as yet may play the primary role in this process. These findings not only provide important insight into our understanding of the ecology of dioxin-transforming bacteria but also have important implications in the engineered bioremediation of organochlorine-polluted environments.

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