Enrichment of Dioxin-Dehalogenating Bacteria by a Two-Liquid-Phase System with 1,2,3-Trichlorobenzene

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Abstract

Enrichment cultures capable of 1,2,4-trichlorodibenzo-p-dioxin (1,2,4-TrCDD) dechlorination were derived from dioxin-contaminated Spittelwasser sediments by successive transfers to fresh defined mineral salts medium. The number of dioxin-dehalogenating bacteria was low and represented about 0.007 % of the total cell number. Addition of 1,2,3-trichlorobenzene (1,2,3-TrCB) dissolved in hexadecane to cultures supplied with a mixture of organic acids as electron donors resulted in a dechlorinating culture that transformed 1,2,3-TrCB to 1,3-dichlorobenzene (1,3-DiCB). Amendments with hydrogen instead of organic acids resulted in significantly lower dechlorination activity. The most-probable-number technique demonstrated that during 1,2,3-trichlorobenzene dechlorination, the number of 1,2,4-trichlorodibenzo-p-dioxin dechlorinating bacteria increased tremendously from 2.5 x 10^3 cells ml^-1 to 1.1 x 10^7 cells ml^-1. The latter number accounted for 11 % of the total cell number indicating that 1,2,3-TrCB can serve as an alternative chlorinated electron acceptor for dioxin dechlorinators. Restriction fragment length polymorphism (RFLP) analysis of 16S rDNA clone libraries from the initial dioxin-dechlorinating culture and from the two enrichments with trichlorobenzene revealed changes in community composition. Compared to the initial culture, the community structure of the culture containing TrCB plus "organic acids" showed a proportional increase of ten different restriction patterns that might reflect the enrichment of microorganisms with dioxin-dechlorinating capabilities. Two of these patterns (5 and 6) increased remarkably - each accounting for 6 % of the investigated clones - and represented a Dehalococcoides strain
and an organism of the Bacteroidetes phylum, respectively. Inhibition of methanogens enhanced the rate of chlorobenzene dehalogenation. 1,2,3,4-Tetrachlorodibenzo-p-dioxin (1,2,3,4-TeCDD) applied in hexadecane was also rapidly dechlorinated in the absence of methanogens.

INTRODUCTION

Environmental contamination with polychlorinated dibenzo-p-dioxins and -furans (PCDDs and PCDFs) has raised significant concern due to their toxicity and persistence in the environment. PCDD/Fs are generally considered as environmental contaminants of anthropogenic origin. Although this is true for many large scale industrial sites all over the world (see http://www.epa.gov/superfund/sites/npl/npl.htm for the U.S. National Priorities List [NPL]), it became evident that pre-industrial (35) and unidentified natural sources exist, e.g. in lake sediments (39), in clay from the U.S. and Germany (38), and in coastal sediments of Queensland, Australia (21). The formation of dioxins and other haloorganic compounds occurs in nature through biogenic (22, 45) or geogenic processes (22) and bacteria may have evolved mechanisms to cope with these substances or even gain energy by metabolizing halogenated compounds (26).

Microbial growth linked to the mineralization of chlorinated dioxins and furans has been demonstrated under aerobic conditions (53). However, polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/F) commonly accumulate under anaerobic conditions; thus, anaerobic subsurface environments constitute the ultimate sinks for PCDD/Fs released into the environment. Within the last decade, research has been conducted to study the ability of anaerobic microorganisms to dehalogenate chlorinated dioxins. Microbially mediated anaerobic biotransformation of dioxins can undergo sequential reductive dechlorination, in
which chlorine removal and substitution with hydrogen produces a reduced organic compound with fewer chlorines. The reductive dechlorination of spiked and historically present dioxins has been documented in a variety of microcosms and cultures from anaerobic freshwater and estuarine sediments (1, 3, 7, 8, 10, 13, 47).

To derive energy from reductive dechlorination during anaerobic respiration, dechlorinating microorganisms have been proposed to use PCDD/Fs as terminal electron acceptors by coupling the reaction to the oxidation of organic compounds or molecular hydrogen. Thermodynamic calculations have shown that energy available from reductive dechlorination of PCDD/Fs is sufficient to support the growth of dechlorinating bacteria (28).

A recent study identified several members of a dioxin-dechlorinating community from river Saale sediment and detected different bacteria known for dehalogenation activity (6). Nevertheless, little is known about the overall composition of PCDD-dechlorinating communities and their population dynamics during the transformation of dioxins and other chlorinated compounds. Despite efforts to isolate the responsible organisms, up to now only one pure culture, which was originally isolated as a chlorobenzene dehalorespiring bacterium (2), has been described that grows by the reductive dechlorination of dioxins (12). Such isolation failures might be explained by difficulties in culturing the respective bacteria. Many of the dehalogenating organisms tend to grow slowly or seem to require additional growth factors, such as amino acids, vitamins, or filtered supernatant of preceding cultures (25, 33). However, the main problem in cultivating and isolating dioxin-dechlorinating bacteria are low cell numbers that are primarily due to limitations in the amount of available dioxin in these cultures (43). For example, Ballerstedt obtained a cell number of just $2.5 \times 10^3$ ml$^{-1}$ for 1,2,4-TrCDD dechlorinating bacteria within a community containing a total cell number of $>10^8$ ml$^{-1}$ (5).
16S rRNA-based molecular approaches are often considered the cultivation-independent method of choice to describe bacterial communities. However, since dechlorinating bacteria are not predominant in most dioxin-dechlorinating communities, often representing less than 1 of 10,000 cells, they may not be easily detected by molecular approaches that are based on conserved primers for initial PCR amplification (e.g., DGGE/TGGE, RFLP/T-RFLP).

One way to overcome these limitations is to increase the cell numbers of dechlorinators by adding alternative halogenated compounds. "Priming" has been used to stimulate PCB dechlorination in general, as well as to selectively enrich distinct PCB dechlorinating populations. Addition of specific PCB congeners such as brominated biphenyls or halogenated benzoates has been shown to stimulate certain types of PCB dechlorination (17, 46, 54). It is presumed that the priming effect can be attributed to enrichment of dechlorinating populations, which metabolize these alternative halogenated electron acceptors. The ability of organohalogens to enrich PCB dechlorinators has been clearly shown by Cho et al. (14) and Wu et al. (54). Such enrichment is probably due to the fact that many dehalogenating organisms and enzymes are not specific for a single compound and that the added substrates might be more bioavailable due to more favorable physical-chemical properties. In addition, these effects might be caused by the increased bioavailability of freshly added compounds rather than "aged" contaminants from the site.

Reductive dechlorination of highly hydrophobic polychlorinated dioxins is probably a slow process, especially under field conditions, and appears to occur only to a limited extent (9). Therefore, stimulating bacterial dioxin dechlorination is desirable, but an effect of priming on the biotransformation rates of dioxins has only been indicated by a few authors. The ability to reduce chlorinated dioxins can be stimulated by the addition of 2-monobromo-
dibenzo-\textit{p}-dioxin (3) and brominated phenols (47). However, potential effects on the dehalogenating microbial community have not been studied in detail.

Here we show reductive dechlorination of 1,2,3-trichlorobenzene by a dioxin dechlorinating mixed culture. Additionally, we demonstrate that the number of dioxin-dechlorinating bacteria can be increased during cultivation with the more water-soluble substrate 1,2,3-trichlorobenzene, supplied via a hexadecane phase. Using molecular approaches that target the 16S rRNA genes, we report the community structure of the initial 1,2,4-trichlorodibenzo-\textit{p}-dioxin dechlorinating culture prior to exposure to 1,2,3-trichlorobenzene. Furthermore, comparing two derived 1,2,3-trichlorobenzene enrichments with the initial culture, we describe changes in the composition of the dechlorinating communities according to the enrichment conditions. We also study the influence of specific inhibitors of methanogens and Gram-positive bacteria on the dechlorination of 1,2,3-trichlorobenzene, and demonstrate enhanced dechlorination of 1,2,3,4-TeCDD in the two-liquid-phase system.

**MATERIALS AND METHODS**

**Origin of the enrichment cultures.** Anoxic PCDD/F-contaminated sediment (0 cm to 10 cm core) was obtained from the creek Spittelwasser site, Germany. The 1,2,4-trichlorodibenzo-\textit{p}-dioxin (1,2,4-TrCDD)-dechlorinating enrichment culture was derived from primary slurry microcosms spiked with 50 \(\mu\text{M}\) 1,2,3,4-tetrachlorodibenzo-\textit{p}-dioxin (1,2,3,4-TeCDD) (13). The following subcultures were enriched for the ability to dechlorinate 1,2,4-TrCDD and transformed 1,2,4-TrCDD to 1,3-dichlorodibenzo-\textit{p}-dioxin (1,3-DiCDD) and 2-monochlorodibenzo-\textit{p}-dioxin (2-MCDD). Consecutive transfers (10% [vol/vol]) in bicarbonate-buffered defined mineral salts medium amended with acetate, a mixture of organic acids (formate, benzoate, pyruvate, fumarate) as electron donors, and 1,2,4-TrCDD as the
putative electron acceptor yielded stable dioxin-dechlorinating cultures (12). The
dehalogenating activity was maintained without the addition of sterilized sediment.

**Culture media and growth conditions.** Defined, reduced anaerobic mineral salts "medium
204" was prepared as follows: mineral salts were added under a continuous stream of N₂/CO₂
(80%/20%) to deionized water after it had been boiled and cooled to room temperature under
N₂/CO₂ (80%/20%) (in grams per liter, unless indicated otherwise): MgCl₂ x 6H₂O, 0.054;
MgSO₄ x 7H₂O, 0.068; CaCl₂ x 2H₂O, 0.12; NH₄HCO₃, 0.41; yeast extract (Difco), 0.05;
resazurin, 0.001; 1 ml of selenite- and tungstate solution (0.5 g NaOH, 3 mg Na₂SeO₃ x 5
H₂O, 4 mg Na₂WO₄ x 2 H₂O per liter of deionized water); 1 ml of trace element solution SL10
(51). The pH was adjusted to 7.2 using NaHCO₃. After autoclaving, 1 ml of filter-sterilized
vitamin solution described by Holliger *et al.* (25), modified by the addition of 63 µM of
1,4-naphthoquinone, sodium/potassium phosphate buffer (final concentration 3.33 mM, pH
7.2) and titanium(III)-nitrilotriacetic acid (final concentration, 0.1 mM) was added by syringe.
Amorphous ferrous sulfide (11) (0.15 mM, according to McCue *et al.* (34)) together with
sodium sulfide (1 mM) promoted dechlorination and was used as a reducing agent for
maintaining highly reduced conditions over long time periods. Transformation of
trichlorobenzenes (TrCB) was tested using 60 µM of 1,2,3-TrCB and 1,2,4-TrCB, respectively,
added directly to the medium from 1 M stock solutions in acetone. These cultures received a
10 % (vol/vol) inoculum from the fourth transfer of the Spittelwasser culture "B (0-10cm)"
(13) which had transformed 1,2,4-TrCDD to 2-MCDD. Inoculum autoclaved on three
consecutive days was used for preparation of sterile controls, and incubations without the
addition of culture material served as chemical controls.
Two-liquid phase cultures were prepared and incubated in autoclaved 60-ml serum bottles containing 30 ml of sterile culture volume and sealed with Viton stoppers and aluminium crimps. The bottles were purged with N₂/CO₂ (80 %/20 %) to remove any residual air prior to the addition of anaerobic medium. Material from the fifth transfer from the Spittelwasser culture described above served as the inoculum (10% (vol/vol)). Finally, 1.5 ml of 200 mM 1,2,3-TrCB dissolved in hexadecane was added, resulting in a nominal concentration of 10 mM 1,2,3-TrCB. Cultures were grown with 5 mM acetate as the carbon source. Electron donors (formate, benzoate, fumarate, and pyruvate) were added from sterile anoxic stock solutions to give initial concentrations of 1.25 mM each in "organic acid enrichment cultures". After 68 days of incubation, another 1.25 mM was added for each of these compounds. For "hydrogen enrichment cultures", 2.5 ml (dissolved hydrogen concentration: 65 µM) of H₂ was added as the electron donor at the beginning and after 68 days. Dissolved hydrogen concentrations were calculated as previously described by Löffler et al. (32). All serum bottles were incubated stationary at room temperature in the dark for 35 weeks, during which they were periodically sampled in duplicates (500 µl) and analyzed to determine the extent of dechlorination. For the inhibitor studies, bromoethanesulfonic acid (BES) and vancomycin were added from filter sterilized, anoxic stock solutions in deionized water to final concentrations of 5 mM and 5 mg l⁻¹, respectively. The two-phase cultures were amended with BES, BES plus vancomycin, or no inhibitors and were grown with medium 204, the mixture of organic acids (1.25 mM each), and 5 mM acetate. Preparation of the cultures followed procedures described above; the only exceptions were (i) the two-phase enrichment culture with 1,2,3,4-TeCDD in which a nominal concentration of 3.3 mM of dioxin was supplied, (ii) a control with hexadecane but without chlorinated substrates and (iii) the inoculum (5 % v/v)
which was obtained from the preceding trichlorobenzene plus organic acids enrichment culture.

**MPN determination.** MPN tubes were spiked with 1,2,4-TrCDD as an electron acceptor with 50-µl Hamilton syringes from stock solutions in acetone to final concentrations of 25 µM (7). The test tubes were supplemented with medium 204 containing 2.5 mM each of formate, benzoate, fumarate, and pyruvate. All MPN tubes contained 5 mM acetate as carbon source. The cultures were incubated in 15-ml Hungate tubes with a total volume of 3 ml for 2 months in the dark at 20°C with shaking (130 rpm), and the headspace was analyzed for dechlorination products of 1,2,4-TrCDD by solid-phase microextraction and gas chromatography as described previously (12). A three-tube MPN procedure was used to estimate the number of dioxin dechlorinators. A dilution series (10⁻¹ to 10⁻⁹ in reduced mineral medium) of each sample was prepared by serially transferring 0.5 ml-portions of the cultures at time point zero and at the end of the experiment (244 days). The MPN test vials were inoculated with 300 µl of each dilution in the 10⁰ to 10⁻⁹ range. Total cell numbers were determined using fluorescence microscopy (Axioplan, Carl Zeiss, Germany) by counting 4’,6-diamidino-2-phenylindole (DAPI)-stained cells. The number of dioxin dechlorinators was estimated by assaying the dechlorination of 1,2,4-TrCDD in the MPN test vials. MPN vials were counted as positive when dechlorination occurred within 8 weeks of incubation regardless of the concentrations of 1,3-DiCDD and 2-MCDD. The instrument detection limit for 1,3-DiCDD and 2-MCDD analysis was below 0.78 µM.

1,2,4-TrCDD, 2,3-DiCDD, 2-MCDD and 1-MCDD were obtained from Amchro (Hattersheim, Germany) at the highest available purities. 1,3-DiCDD was provided by John R. Parsons, Amsterdam, The Netherlands; and authentic standards for 1,2-DiCDD, 1,3-DiCDD and
1,4-DiCDD were provided by Roland Weber, Tübingen, Germany. Chlorobenzenes (99 % purity) were purchased from Sigma-Aldrich (Steinheim, Germany).

**Isolation.** For isolation of colonies from the trichlorobenzene enrichment cultures, mineral medium 204 was completed by 0.88 % (wt/vol) high-purity Noble agar (Difco, Detroit, MI). The medium contained 5 mM acetate and 5 mM formate. After autoclaving, the temperature of the medium was held at 55°C. Hungate tubes used for the agar shakes were spiked with 7.5 µM 1,2,4-TrCDD (final concentration) and acetone was evaporated under a sterile stream of N₂/CO₂ (80%/20%). Dilution series of the enrichment cultures were prepared after 244 days (see above) and used to inoculate the agar shakes (10 ml total volume). After solidification, 3 ml of hydrogen was added to the headspace of the vials. Single colonies from 10⁻⁵ to 10⁻⁸ dilutions were picked after 21 weeks and transferred into 3-ml liquid cultures supplemented with 5 mM acetate, 2.5 ml H₂ and 25 µM 1,2,4-TrCDD. The colonies were picked under a gentle stream of sterile N₂/CO₂ using 1-ml single-use syringes and needles (0.6 x 80 mm, Braun, Melsungen, Germany) that had been flushed with N₂/CO₂. The cultures were analyzed for dechlorination products (12) after 150 days and positive cultures were subcultured with 5 mM acetate, a mixture of 1,2,3-TrCDD and 1,2,4-TrCDD (25 µM each), and hydrogen (2.5 ml) or organic acids (2.5 mM each for formate, benzoate, fumarate and pyruvate) as electron donors.

**Chlorobenzene analysis.** Hexane extracts were analyzed for 1,2,3-TrCB and its dechlorination products using a Shimadzu GC 14A equipped with a flame ionization detector (FID) connected to a DB608 column (30 m length, 0.331 mm i.d., 0.5 µm film thickness; J&W Scientific; Folsom, CA, USA). The make-up gas was nitrogen, and helium was used as the carrier gas at a flow of 2.2 ml min⁻¹. 2,4-Dichlorotoluene was the recovery standard and
1,3,5-tribromobenzene was used as the internal standard. Chlorinated benzenes were separated using the following temperature program which allowed baseline separation of all dichlorinated benzenes: initial hold at 50°C for 1.1 min, 40°C per min to 70°C (1.9 min), increase to 140°C at a rate of 20°C per min (1.2 min), 40°C per min to 160°C (1.2 min), increase to 220°C at a rate of 25°C per min (0.5 min), 40°C per min to 280°C, final hold at 280°C for 5 min. Injector and detector temperatures were 250 and 300°C, respectively. The chlorobenzenes were identified by matching the retention times with those of authentic standards. Identification of chlorobenzenes was confirmed with a Hewlett-Packard model 6890 gas chromatograph equipped with a HP5973 mass selective detector and a HP-5ms capillary column (30 m length, 0.25 mm i.d., 0.25 µm film thickness; Agilent Technologies).

**Chloride release measurement.** Chloride release was measured to monitor dechlorination in the two-liquid phase cultures. Samples (500 µl in duplicates) were periodically withdrawn from the aqueous phase of the cultures by syringe and centrifuged at 9,500 x g for 5 min to remove particles. The supernatants were analyzed for chloride concentration using a Chlor-o-counter MKII (Flohr Instrumenten, Nieuwegein, The Netherlands). The variability among duplicate samples was generally less than 8 %.

**Extraction of DNA.** Cells were harvested from 1 ml of culture by centrifugation at 9,500 x g for 20 min. The procedure for bead-based cell disruption was essentially performed as described by Kuske et al. (31). The resulting community DNA was precipitated with ethanol and sodium acetate (40), purified according to standard procedures (40), and dissolved in sterile deionized water.

**Restriction Fragment Length Polymorphism (RFLP).** PCR was performed with bacterial domain specific 16S rDNA primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') (50). The 16S rRNA genes were amplified using
approximately 50 ng of genomic DNA in reaction mixtures containing 1 x Taq PCR buffer (Promega), 1.8 mM MgCl₂, 250 µM of each deoxynucleoside triphosphate, 210 nM of each forward and reverse primer, and 0.025 U of Taq DNA polymerase (Promega) per µl reaction volume. Reaction mixtures were incubated in a "PCR Sprint" thermocycler (Thermo Hybaid, Germany). Conditions for PCR were as follows: an initial denaturation step at 94°C for 2 min was followed by 30 cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min. The last 10 elongation steps were performed with a 20 s time increment per cycle. The resulting PCR products were purified using the QIAquick PCR purification Kit (Qiagen, Hilden, Germany), then ligated into the pGEM T-Easy-vector (Promega, Madison, WI, USA), and transformed into E. coli XL1-Blue (Stratagene, La Jolla, CA). The cloned 16S rDNA fragments were reamplified from recombinant clones with reaction mixtures similar to those described above, but a primer set specific to the polylinker region of the pGEM T-Easy-vector (PG1f: 5'-TGGCGGCCGCGGGAATTC-3', PG2r: 5'-GGCCGCGAATTCACTAGTG-3'). The PCR reaction was performed using an initial denaturation step at 94°C for 3 min, 30 cycles consisting of 94°C for 45 s, 57°C for 45 s, and 72°C for 1 min, and a final extension at 72°C for 5 min. Fifteen µl of reamplified 16S rDNA (DNA concentration approximately 200 ng/µl) were digested overnight at 37°C with 1.7 units per µl of Msp I and Rsa I (MBI Fermentas, St. Leon-Rot, Germany). Digested fragments were separated on a 4 % agarose gel (Agarose NEEO, Carl Roth, Karlsruhe, Germany) in 1 x TBE (40).

**Sequence analysis of 16S rDNA clone libraries.** Cycle sequencing was performed using the automated laser fluorescence DNA sequencer ABI Prism 377, version 4.0 (PE Applied Biosystems, Langen, Germany), universal bacterial primers that recognized conserved regions of the 16S rDNA (41, 50), and the vector-specific primers PG1f and PG2r. Closest relatives were identified using the Fasta33 program at EMBL-EBI and RDP's Sequence Aligner.
Obtained sequences were checked for chimeras using RDP's Chimera Check and the Bellerophon server (http://foo.maths.uq.edu.au/~huber/bellerophon.pl). Sequences were imported and aligned in the ARB software package (http://www.arb-home.de/) and added to an alignment of about 8600 bacterial sequences (ARB alignment by Philip Hugenholtz, January 2002, http://rdp.cme.msu.edu/html/alignments.html) for phylogenetic tree reconstruction. For addition to the maximum parsimony tree, filters excluding the most variable positions and terminal sequences were used. Phylogenetic analyses were performed using maximum parsimony and maximum-likelihood methods.

**Nucleotide sequence accession numbers.** GenBank accession numbers for the sequences used to construct phylogenetic trees are as follows: *Clostridium lituseburense* ATCC 25759^T^, M59107; *Clostridium putrefaciens* DSM 1291^T^, Y18177; *Cytophaga fermentans* ATCC 19072^T^, M58766; *Dehalococcoides ethenogenes* 195, AF004928.2; *Dehalococcoides* sp. CBDB1, AF230641; *Desulfonema limicola* DSM 2076^T^, U45990; *Sedimentibacter saalensis* ZF2^T^, AJ404680; *Synergistes jonesii* ATCC 49833^T^, L08066; *Syntrophus gentianae* HQgö1^T^, X85132; *Trichococcus pasteurii* KoTa2^T^, X87150; strain S2551, AF177428; clone IIIB-28, AJ488099; clone BA053, AF323776; clone DCE29, AJ249260; clone DCEH2, AJ249262; clone SHA-300, AJ249112; clone SJA-58, AJ009468; and clone WCHB1-69, AF050545.

The determined 16S rRNA gene sequences representing RFLP types 1, 2, 3, 4, 5, 6, 7, 10, 14, and 26 were submitted to GenBank under the accession numbers ... .

**RESULTS**

**Chlorobenzene dechlorination.** Enrichment cultures established with material from the fourth serial transfer of an actively 1,2,4-TrCDD-transforming culture from Spittelwasser sediment showed complete reductive dechlorination of 60 µM 1,2,3-trichlorobenzene...
Enrichment of Dioxin-Dehalogenating Bacteria

(1,2,3-TrCB) to 1,3-dichlorobenzene (1,3-DiCB) within 16 weeks; however, dechlorination of 1,2,4-trichlorobenzene did not occur over this same time period. The dechlorination pathways for 1,2,4-TrCDD and for 1,2,3-TrCB are shown in Fig. 1. Controls without inoculum or with autoclaved inoculum did not show any significant dechlorination of trichlorobenzenes.

![Figure 1 Pathways for reductive dechlorination of 1,2,4-trichlorodibenzo-p-dioxin (1,2,4-TrCDD) in cultures from Spittelwasser sediment and of 1,2,3-trichlorobenzene (1,2,3-TrCB) observed in the following enrichment cultures. DiCDD, dichlorodibenzo-p-dioxin; MCDD, monochlorodibenzo-p-dioxin; DiCB, dichlorobenzene.](image)

A two-phase-system originally described by Holliger et al. (25) was used to study the effect of 1,2,3-TrCB dechlorination on dioxin dechlorinating bacteria from the fifth transfer of a 1,2,4-TrCDD-dechlorinating Spittelwasser culture. 1,2,3-TrCB (200 mM) was supplied in hexadecane, providing a constant flow of chlorinated substrate to the dechlorinating bacteria and avoiding inhibitory effects caused by high concentrations of chlorinated benzenes. The nominal 1,2,3-TrCB concentration corresponded to 10 mM.

Two enrichment cultures were established. One culture received organic acids, while the other received hydrogen as electron donor. Dechlorination was qualitatively checked for the presence of dechlorination products in the water phase and was quantitatively monitored by
measuring the chloride concentration. After 168 days, 4.2 mM chloride was released into the aqueous phase of the culture containing organic acids corresponding to the conversion of 42 mol % of 1,2,3-TrCB to 1,3-DiCB (Fig. 2). 1,3-DiCB was also found as the dechlorination end product in cultures that received H₂ as the electron donor. The lag time before dechlorination started was equal to that of the organic acids-amended enrichment culture (Fig. 2), but 1,2,3-TrCB dehalogenation was slower and only approximately half the amount of chloride was released after 168 days. At the final sampling point after 244 days, 5.5 and 2.6 mM of chloride were released into the aqueous phase of the organic acids culture and hydrogen culture, respectively.

![Figure 2](image-url)  
*Figure 2* Time course of reductive dechlorination of 1,2,3-trichlorobenzene to 1,3-dichlorobenzene by the two-phase enrichment cultures. Each data point represents the mean of duplicate samples. Samples for MPN analysis and RFLP analyses were taken at day 0 and after 244 days.

**MPN estimation.** The number of 1,2,4-TrCDD dechlorinating bacteria was estimated in the chlorobenzene-amended two-phase culture containing organic acids by the MPN technique in the beginning and after 244 days. Dechlorination of the spiked compound 1,2,4-TrCDD
followed the previously observed sequential removal of chlorine, and 1,3-DiCDD and 2-MCDD were the dechlorination end products after 2 months of incubation. The initial level of 1,2,4-TrCDD dechlorinators increased by 3 orders of magnitude, from $2.5 \times 10^3$ to $1.1 \times 10^7$ cells ml$^{-1}$. The total number of cells increased from initial $3.7 \times 10^7$ to $1.0 \times 10^8$ cells ml$^{-1}$ indicating that the abundance of dioxin dechlorinators in the community had increased from 0.007 % to 11 %.

**Community structure.** Restriction fragment length polymorphism (RFLP) analysis (Fig. 3) and sequencing of amplified 16S rDNA was used to study the community composition of the two trichlorobenzene enrichment cultures and of the 1,2,4-TrCDD-spiked culture which served as the inoculum. A total of 295 clones containing 16S rDNA inserts were analyzed and 39 different patterns were identified. Selected cloned fragments were sequenced. Seventeen of these patterns appeared only once in one of the libraries and suggested that the respective organisms may not be essential for the dechlorination process.

**Figure 3** RFLP analysis of 16S rDNA inserts of 18 clones selected from the clone library obtained from the trichlorobenzene-dechlorinating culture grown with organic acids. The predominant restriction patterns 1, 2 and 3 as well as pattern 5 and 6 are highlighted (for phylogenetic assignment see Fig. 5). The fragments ($\leq 676$ base pairs) of the size standard pGEM-DNA marker/Hinf I, Rsa I, Sin I (Promega) are shown in lanes "M".
The clone libraries were dominated by three sequence types, represented by the restriction patterns 1, 2 and 3 (Fig. 4). The numerically dominant restriction pattern was pattern 2 (up to 49 % of the clone library from the organic acids enrichment). The 16S rDNA sequence of the pattern 2 representative shared 99.9 % identity with *Trichococcus collinsii* strain 37AN3 (GenBank accession no. AJ306612) and 99.8 % with *Trichococcus pasteurii* strain KoTa2^T^, formerly described as *Lactosphaera pasteurii* (30). A similar *Trichococcus* strain, designated Coc4, was isolated previously from organic acids amended dioxin-dechlorinating subcultures from the Saale River but showed no dioxin-transforming activity (6). However, these bacteria are known to ferment pyruvate to formate and acetate (30).

RFLP type 1, the second most predominant sequence type, accounted for about 16 % in the clone library of the initial culture and 15 % in the library of the trichlorobenzene plus organic acids enrichment. Interestingly, this sequence type was not observed in the clone library from the hydrogen enrichment. The 16S rDNA sequence was 98 % identical to the sequence of *Syntrophus gentianae* strain HQgö1 (49). Physiologically, organisms of the genus *Syntrophus* are known to obtain energy from the anaerobic oxidation of benzoate (e.g. (49)) and other organic acids (29) to acetate and hydrogen in syntrophic association with hydrogen-consuming partners. Pattern 3, the last of the most abundant RFLP types, represented a bacterium most closely related to *Clostridium lituseburense* ATCC 25759 (M59107). The 16S rRNA genes shared 97 % identity.

RFLP and sequence analysis, performed using double-stranded 16S rRNA gene sequencing, revealed shifts in the community composition of the trichlorobenzene-spiked culture as compared to the initial culture grown with 1,2,4-trichlorodibenzo-*p*-dioxin. Despite the dominance of patterns 2 and 3, the three libraries were markedly different from each other (Fig. 4). Enriched sequence types might represent organisms potentially involved in
chlorobenzene dechlorination, which could also explain the increased cell number of 1,2,4-TrCDD dechlorinators during chlorobenzene cultivation found by MPN analysis. Comparing the clone libraries of the initial dioxin-dechlorinating culture and the organic acids plus 1,2,3-TrCB enrichment, the relative abundance of ten patterns (2, 4, 5, 6, 7, 10, 11, 14, 16, and 26) increased during cultivation with trichlorobenzene.

Figure 4 Community structure of the trichlorobenzene-dehalogenating two-phase enrichment cultures and of the 1,2,4-TrCDD-dechlorinating initial culture. Each column represents the relative abundance of each RFLP type in the respective clone library. A total of 295 randomly chosen clones (organic acids enrichment: 138 clones, hydrogen enrichment: 73 clones, initial culture: 84 clones) was analyzed by restriction fragment length polymorphism (RFLP). *The respective 16S rDNA inserts were entirely sequenced. For phylogenetic affiliation see text and Fig. 5.

The phylogenetic affiliation based on full length sequences of the three predominant and seven enriched 16S rDNA sequence types is shown in Fig. 5. The enriched patterns 11 and 16 were not included in Fig. 5 because only partial 16S rDNA sequences were available. Both 16S rDNA sequence types were similar to members of the genus Clostridium; the sequence of pattern 11 was related to Clostridium putrefaciens DSM 1291 (92 %, AF127024) while pattern
16 showed similarity to *Clostridium hastiforme* DSM 5675 (95.5 %, X80841). Sequences representing patterns 4 and 26 were also found to be most closely related to bacteria of the *Clostridiaceae*. The 16S rDNA sequence from pattern 14 organisms aligned closely with clone sequences of uncultured bacteria of the *Deferribacteres* phylum including bacterium DCE 29 (98 %) obtained from a chloroethene-dehalogenating mixed culture. *Synergistes jonesii* was the closest cultivated relative (86 % identity, L08066). Sequences of clones representing pattern 7 grouped with members of the δ-Proteobacteria, whereas the 16S rDNA from pattern 10 showed the highest sequence similarity to unidentified green non-sulfur bacteria found in cultures capable of dehalogenating chlorinated compounds such as 1,2-dichloropropane (42) and trichlorobenzene (48).

Four of the ten restriction patterns that were enriched in the organic acids culture increased also in the hydrogen-fed culture (Pattern 2, 6, 14, and 16; Fig. 4). One pattern, designated pattern 35, was exclusively enriched in the hydrogen culture.

The abundance of pattern 5 increased remarkably accounting for nearly 6 % of all sequence types found in the organic acid culture. The insert of one clone was completely sequenced on both strands. It showed a 16S rRNA gene sequence identical to that of *Dehalococcoides* sp. strain CBDB1 and strain FL2, except for one mismatched base. Sequences of eight additional clones could not confirm the sequence difference at this position suggesting that the mismatch was most likely caused by PCR artifacts. Therefore, we conclude that the 16S rRNA genes represent a single *Dehalococcoides* population. The novel *Dehalococcoides* strain, designated DCMB5, belongs to the Pinellas group, a phylogenetic branch within the *Dehalococcoides* group (green non-sulfur bacteria) (23).

Similarly to pattern 5, pattern 6 became one of the most numerous sequence types in the trichlorobenzene plus organic acids culture. Its abundance increased to 6 % of all clones.
Enrichment of Dioxin-Dehalogenating Bacteria

examined in this culture, whereas the RFLP type was not encountered in the library from the initial culture. The 16S rDNA showed highest similarity (99 %) to an uncultured bacterium of the *Bacteroidetes* phylum. Clone IIIB-28 (AJ488099) was derived from a bacterial consortium removing predominantly singly flanked chlorine substituents from chlorobenzenes. The 16S rDNA sequence grouped also with other sequences of uncultured "Flexibacteraceae" obtained from contaminated sites including clone WCHB1-69 (97.0 %) (18) and from dehalogenating mixed cultures including clone SHA-5 from a 1,2-dichloropropane-dechlorinating culture (91 % identity, AJ306736). The next cultivated relatives were members of the *Sphingobacteriaceae* (*Pedobacter saltans*, 85 %, AJ438173.2) and "Flexibacteraceae" (*Cytophaga fermentans*, 84.5 %, M58766).

**Figure 5** 16S rRNA phylogenetic tree of predominant sequence types (pattern 1, 2, and 3) and enriched RFLP types in the clone library from the 1,2,3-trichlorobenzene plus organic acids enrichment, expanded with reference sequences of representative bacteria. The tree is based on the results of a maximum-parsimony analysis, as implemented in the ARB software package. A similar tree topology was generated for a phylogenetic tree constructed using maximum-likelihood methods. Bar = 0.1 base changes per position.
Isolation Efforts. Attempts to isolate the dioxin-dechlorinating population(s) by cultivating in solid medium containing 0.88 % (w/v) high-purity agar were performed with inoculum from the two 1,2,3-trichlorobenzene enrichments after 244 days of cultivation. One hundred twenty-five single colonies were picked from $10^{-5}$ to $10^{-8}$ dilutions of the original enrichment cultures and were transferred back into liquid medium spiked with 25 $\mu$M 1,2,4-TrCDD. Dechlorination activity of 1,2,4-TrCDD in defined basal salts medium amended with hydrogen and 5 mM acetate was recovered from 4 colonies derived from the organic acids culture at dilutions of $10^{-5}$, $10^{-6}$ and $10^{-7}$. Only one of them could be successfully transferred into a second liquid subculture with hydrogen and acetate. It transformed a mixture of 1,2,3- and 1,2,4-TrCDD exclusively to 1,3-DiCDD. 2-Monochlorodibenzo-$p$-dioxin was not detected as a dechlorination product. To describe the microbial constituents of the actively dechlorinating culture, we conducted an RFLP approach. The community consisted of at least six different populations. The previously detected RFLP pattern 6 was represented by 11 % of 37 clones examined. Patterns 2, 3, 16, 26 and 32 were also identified. Attempts to detect *Dehalococcoides*-like microorganisms by RFLP in the dechlorinating cultures from agar shakes have failed so far, probably due to the fact that this bacterium cannot grow in semisolid medium with agar. In this regard, it should be noted that Adrian *et al.* (2) reported dichlorobenzene formation for strain CBDB1 only in soft media containing 0.3 % of a low melting agarose, but not in media with standard agarose or with agar.

**Contribution of methanogens and Gram-positive bacteria to the dechlorination process.**

Strong $F_{420}$ fluorescence was observed (data not shown) in the two-liquid phase cultures indicating the presence of methanogens. The lower dechlorination rate in the hydrogen-amended enrichment culture (Fig. 2) suggested competition for hydrogen between dechlorinating bacteria and methanogens or other hydrogen-consuming bacteria.
To specifically inhibit hydrogenotrophic populations, bromoethanesulfonic acid (BES) was added either separately or in combination with vancomycin to a set of two-liquid phase cultures grown with the organic acid mixture and 1,2,3-trichlorobenzene. The preceding organic acids enrichment was used as inoculum. Suppression of methanogenesis by BES resulted in an enhanced dechlorination rate as compared with that of the control (Fig. 6). However, the addition of vancomycin partly reversed the stimulating effect. Another two-liquid phase culture was amended with BES and received a nominal concentration of 3.3 mM 1,2,3,4-tetrachlorodibenzo-p-dioxin instead of 10 mM trichlorobenzene (Fig. 6). The release of chloride at a rate of about 16 µmol chloride l\(^{-1}\) d\(^{-1}\) between day 10 and 66 from the dioxin molecule was apparent and demonstrated that the ability to dechlorinate dioxins was not lost during the two-liquid-phase cultivation with trichlorobenzene.

**Figure 6** Reductive dechlorination of 1,2,3-TrCB (10 mM) and 1,2,3,4-TeCDD (3.3 mM) in two-liquid phase cultures amended with BES and vancomycin as indicated, and organic acids as electron donors. The inoculum (10 % vol/vol) originated from the "organic acids" plus 1,2,3-TrCB enrichment culture.
DISCUSSION

Reactive dechlorination of different chlorinated substrates including tetrachloroethene, chlorinated benzenes (6) and chlorinated biphenyls (data not shown) by dioxin-dechlorinating mixed cultures has been tested in our laboratory. 1,2,3-Trichlorobenzene was found to be rapidly transformed by dioxin-dechlorinating mixed cultures from Spittelwasser sediment. 1,2,3-TrCB is by three orders of magnitude more soluble in water than the trichlorinated dioxin model compound 1,2,4-TrCDD (25, 43). Due to its partition coefficient between hexadecane and water (logK_{HW} 4.04; (25)), about 20 \( \mu \)M of 1,2,3-TrCB were expected to have partitioned into the water phase. The application of dioxins via a decane phase was described by Barkovskii and Adriaens (8). The authors demonstrated that dioxins - despite their higher logs of partition coefficients (e.g., logK_{Octanol/Water} of 1,2,4-TrCDD is 6.35; (43)) are available for reductively dechlorinating bacteria in a biphasic system. However, high costs and the toxicity risk of dioxins might have so far precluded application of dioxins in the millimolar range via a solvent phase to enrich for dioxin-dehalogenating bacteria. Here, we exemplarily demonstrated that 1,2,3,4-TeCDD dissolved in hexadecane served as a substrate for dechlorination with a very high rate, which exceeded 300fold the rates reported for dechlorination of 1,2,3,4-TeCDD supplied as undissolved crystals (7, 10). Nevertheless, dechlorination of 1,2,3-TrCB was four times faster, accounting for about 50 \( \mu \)mol chloride \( \text{l}^{-1} \text{d}^{-1} \).

Our results show that the number of dioxin-dechlorinating microorganisms increased significantly during 1,2,3-TrCB cultivation. Thus, it appears that at least some populations utilizing chlorobenzenes are also involved in dioxin dechlorination. This observation indicates that trichlorobenzene dechlorination is directly coupled to the growth of dechlorinating microorganisms and is consistent with the hypothesis that these dehalogenating organisms also consume dioxins as part of their energy metabolism by using dioxins as terminal electron
acceptors. Comparable results were obtained for PCB dechlorination where the addition of structurally similar (54) as well as structurally non-related halogenated compounds (14) promoted growth of PCB dechlorinators.

The increased number of dioxin dechlorinators should be represented by changes in the community composition. The parent dioxin-dechlorinating and the derived chlorobenzene-dechlorinating mixed cultures were compared by RFLP of 16S rDNA clone libraries. The organic acids enrichment had received the same organic acids, but at a somewhat reduced total amount (15 vs. 25 mM) than the parent culture. Despite comparable growth conditions employed in both cultures, a striking difference existed between the concentration of the halogenated electron acceptors (nominal concentration 10 mM of 1,2,3-TrCB vs. 25 µM of 1,2,4-TrCDD). Since anaerobic oxidation of hexadecane is only known under sulfate-reducing or denitrifying conditions (15, 19, 44), its contribution as an electron donor might be negligible in our cultures. Therefore, we conclude that the enriched sequences represent organisms that might be involved in chlorobenzene transformation and might be associated with dioxin-dechlorinating activity as well.

Two populations were enriched remarkably in the presence of 1,2,3-TrCB in the more active organic acids culture. Based on the 16S rDNA, the new *Dehalococcoides* strain is closely related to the strains FL2 and CBDB1. *Dehalococcoides* sp. strain CBDB1 is the first described bacterium capable of dehalogenating dioxins and chlorobenzenes in pure culture, but preliminary results obtained with *Dehalococcoides ethenogenes* strain 195 indicate that this capability is probably more distributed throughout the *Dehalococcoides* cluster (20). Whereas strains DCMB5 and CBDB1 belong to the Pinellas group, strain 195 is a member of the Cornell group of *Dehalococcoides* (23), indicating that specific dehalogenation capabilities cannot be used for classification of *Dehalococcoides* species. The second bacterium belongs to
a group poorly represented by cultivated organisms within the *Sphingobacteria* lineage of the *Bacteroidetes* phylum. It shares 16S rDNA similarity with an unidentified bacterium (AJ488099) detected in another chlorobenzene dechlorinating culture and with other unidentified bacteria including sequences that have been found in several dechlorinating communities. Physiological predictions on the basis of phylogenetic affiliation alone are highly speculative; thus, attempts to infer physiological properties from rDNA sequences might fail, even when comparing species that are very closely related by 16S rRNA genes. However, studying relative changes in the abundance of 16S rDNA sequences in clone libraries, we demonstrated that distinct bacteria were enriched within the communities. The observed changes in community structure suggest a link between the 16S rDNA data and the physiological properties of the cultures. It is very likely that strain DCMB5 is involved in reductive dechlorination of chlorobenzenes and dioxins. It remains to be determined whether the pattern 6 bacterium participates directly in the dechlorination process or rather plays a role in cosubstrate utilization.

1,2,3-Trichlorobenzene was reductively dechlorinated to 1,3-dichlorobenzene in both enrichment cultures, although the addition of H₂ resulted in lower transformation rates compared to the culture amended with a mixture of organic acids. In principle, hydrogen is thought to be the prevailing electron donor for the reductive dechlorination process. Thus, it was surprising that substitution of organic acids with hydrogen in the two-phase cultures resulted in a decreased dechlorinating activity (Fig. 2). Similar inhibiting effects of higher hydrogen concentrations on the reductive dechlorination of dioxins and PCBs were also observed by Ballerstedt *et al.* (6) and Wiegel and Wu (52), respectively. The PCB-dechlorinating bacterium *o*-17 was even lost from the culture under a high H₂ partial pressure (16). The direct application of hydrogen at high initial levels might favor the growth
of acetogenic bacteria and methanogens, which are efficient competitors for hydrogen, especially at higher concentrations (32). This may limit access to hydrogen for the dehalogenating bacteria, but probably also interferes with the availability of other essential constituents of the medium such as vitamins and trace elements. Circumstantial evidence for an involvement of methanogens in cosubstrate or electron donor utilization (i.e. hydrogen and acetate) came from the observation that addition of BES as an inhibitor of methyl-coenzyme M reductase strongly enhanced the dechlorination activity. In our mixed cultures, the fermentation of organic acids might have supplied a suitably slow and continuous release of hydrogen. Clostridia and related bacteria, which are known to ferment pyruvate or fumarate were identified by 16S rDNA sequences (patterns 14, 16, 26) in one or both trichlorobenzene-enriched cultures. The continuous formation of hydrogen is beneficial for dehalogenating bacteria, which possess a high affinity for hydrogen at nanomolar concentrations (32) and can compete for it immediately after its release.

Since H₂ production from organic acids such as benzoate and acetate is thermodynamically favourable only at a low hydrogen partial pressure, this reaction must be coupled to the consumption of hydrogen by hydrogen-scavenging bacteria. Due to the low hydrogen threshold, dechlorinators constitute ideal hydrogenotrophic bacteria in such syntrophic relationships by increasing the energy yield for both partners. *Syntrophus*-like organisms were detected in the more active organic acids enrichment using our 16S rDNA approach. Strains of *Syntrophus* are capable of syntrophic benzoate-oxidation and have been isolated from a number of stable consortia (4, 27, 37, 49). In addition, *Syntrophus* sp. have been found in several organochlorine-contaminated aquifers (18) and dehalogenating consortia (36) and it was argued that they played an important role as syntrophic partners for reductive dechlorination. In our study, the *Syntrophus* species was completely lost in the H₂-fed culture.
Furthermore, in contrast to the more active organic acids culture, restriction pattern 5 could not be found in the clone library of the hydrogen-amended culture. Therefore, it can be speculated that with organic acids as electron donors, a well-balanced syntrophic association between hydrogen-utilizing bacteria (possibly dechlorinators) and organic acids-oxidizing, hydrogen-producing organisms existed, which resulted in a productive 1,2,3-trichlorobenzene dechlorination. These results may emphasize the involvement of a syntrophic network for such transformation reactions and might further underline the difficulties in isolating dioxin-dechlorinating bacteria from dechlorinating communities. Our results indicate that growth stimulation of PCDD dechlorinating bacteria is possible using trichlorobenzene. This might aid future attempts to enrich and isolate dioxin-dechlorinating bacteria from mixed cultures. It also highlights the potential of priming reductive dechlorination, although alternative less toxic compounds that are acceptable for application to dioxin contaminated sites remain to be discovered.

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