Bacteria of an anaerobic 1,2-dichloropropane-dechlorinating mixed culture are phylogenetically related to those of other anaerobic dechlorinating consortia

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A 16S-rDNA-based molecular study was performed to determine the bacterial diversity of an anaerobic, 1,2-dichloropropane-dechlorinating bioreactor consortium derived from sediment of the River Saale, Germany. Total community DNA was extracted and bacterial 16S rRNA genes were subsequently amplified using conserved primers. A clone library was constructed and analysed by sequencing the 16S rDNA inserts of randomly chosen clones followed by dot blot hybridization with labelled polynucleotide probes. The phylogenetic analysis revealed significant sequence similarities of several as yet uncultured bacterial species in the bioreactor to those found in other reductively dechlorinating freshwater consortia. In contrast, no close relationship was obtained with as yet uncultured bacteria found in reductively dechlorinating consortia derived from marine habitats. One rDNA clone showed >97% sequence similarity to *Dehalobacter* species, known for reductive dechlorination of tri- and tetrachloroethene. These results suggest that reductive dechlorination in microbial freshwater habitats depends upon a specific bacterial community structure.

Keywords: 16S rDNA clone library, bacterial diversity, 1,2-dichloropropane, reductive dechlorination, *Dehalobacter*

INTRODUCTION

Halogenated propanes, including 1,2-dichloropropane (DCP), are toxic and carcinogenic compounds which in the past have been used extensively as fumigants for a variety of crops (Löffler *et al.*, 1997). DCP is still used as a solvent, oil and paraffin extractant, metal-degreasing agent, paint and furniture finish remover, lead scavenger in antiknock fluids and textile stain remover. During the production of chlorinated compounds, such as carbon tetrachloride and tetrachloroethene, DCP is used as a chemical intermediate. Additionally, it is formed as a by-product during the synthesis of propyleneoxide and epichlorohydrine

(Hegemann *et al.*, 1998). In Germany, 78 000 tons of DCP per year are produced. DCP is only moderately soluble in aqueous systems and is slowly degraded by micro-organisms in natural habitats.

Under aerobic conditions partial microbial conversion of DCP by methanotrophic and nitrifying bacteria utilizing DCP co-metabolically has been reported by Oldenhuis *et al.* (1989) and Rasche *et al.* (1990). As shown by Vandenberg & Kunka (1988), *Pseudomonas fluorescens* PFL12 is able to grow aerobically in the presence of DCP as the only available carbon and energy source. However, DCP degradation products have not been detected. So far, complete dehalogenation of DCP to propene has only been reported for anaerobic microbial consortia enriched from freshwater sediments, which degrade DCP by reductive dechlorination (Löffler *et al.*, 1997). In our laboratory we have investigated the continuous anaerobic dechlorination of DCP in a fluidized bed bioreactor

Abbreviations: DCP, 1,2-dichloropropane; DIG, digoxigenin; SHA, Saale, Halle, library A.

The GenBank accession numbers for the 16S rDNA SHA sequences are AJ249094–AJ249114.

using a mixed microbial enrichment culture derived from sediment of the River Saale, Germany, as a first step towards a technical application of this process (Hauck *et al.*, 1999).

The efficient technical use of complex microbial communities is often hampered by their unknown population structure. The determination of microbial diversity is hence an important prerequisite for the bioremediation of DCP-polluted sites and wastewater streams. Conventional culture-based analyses of complex microbiota are strongly biased (Head *et al.*, 1998). Therefore, a direct culture-independent approach based on *in vitro* amplification of 16S rRNA genes has been chosen. The occurrence of 16S rDNA sequences closely related to cultivated dechlorinating bacteria and to as yet uncultured bacteria of other anaerobic, dechlorinating microbial communities provides strong evidence for a specific community structure. Such bacteria could be used as potential marker organisms, allowing an optimization of the transformation process by monitoring the community structure by rRNA-targeted fluorescence *in situ* hybridization or quantitative PCR analysis.

METHODS

Sampling and cultivation. In August 1997 samples were taken from sediment of the River Saale. The sampling site was located in front of a sewer of a propyleneoxide-producing plant (Buna-Werke, Schkopau) near Halle, Germany. Microbial consortia were cultivated anaerobically as sediment batch cultures at 30 °C in the dark in mineral salt medium (RAMM medium; Shelton & Tiedje, 1984) containing DCP (3 mg l⁻¹) and methanol (50 mg l⁻¹) until a stable dechlorination rate was reached. One of the dechlorinating batch cultures was immobilized subsequently on polyurethane foam cubes (78 d incubation; 100 cubes, each 1 cm³) and transferred into a 5 l fluidized bed bioreactor made of stainless steel. The reactor was supplied continuously with RAMM medium containing DCP (7 mg l⁻¹) and methanol (50 mg l⁻¹). Transformation of DCP to propene was analysed by head space GC once per day. Until the first sample was taken, the reactor was operated for 3 months under stable conditions (pH 6.2; redox potential -378 mV), transforming about 98.5% of supplied DCP (retention time, 24 h).

DNA extraction and purification. Community DNA was extracted from a single foam cube removed from the bioreactor 3 months after start-up. Cell lysis was achieved by a combined treatment using lysozyme, proteinase K, SDS and several freeze-thaw cycles, according to von Wintzingerode *et al.* (1999). After phenol/chloroform extraction, nucleic acids were ethanol-precipitated and dissolved in Tris/EDTA buffer. To pre-purify DNA, one gel filtration step with Microspin S-300 HR columns (Pharmacia Biotech) was performed. PCR inhibitors and fragmented DNA were removed from high-molecular-mass DNA by preparative agarose gel electrophoresis (Liesack & Stackebrandt, 1992) using the QIAquick gel extraction kit (Qiagen).

Amplification, cloning and sequence determination of 16S rDNA. Purified DNA (2 µl) was used for PCR amplification

in a total volume of 50 µl (2 mM MgCl₂). DNA was heated initially to 98 °C for 3 min, then cooled to 93 °C and 2.5 U AmpliTaq polymerase (Perkin-Elmer) was added. Each of the 28 amplification cycles included denaturation at 93 °C for 1 min, primer annealing at 53 °C for 1 min and extension at 72 °C for 5 min. Cycling was followed by a final extension step at 72 °C for 7 min. Amplification with *Bacteria*-specific forward primer TPU1 (5'-AGAGTTTGATCMTGGCTC-AG-3'; *Escherichia coli* positions 8-27) and reverse primer RTU8 (5'-AAGGAGGTGATCCANCCRCA-3'; *E. coli* positions 1522-1541) resulted in fragments of about 1500 bp. The amplicons were purified using the QIAquick gel extraction kit and ligated subsequently into vector pCR2.1 of the TA cloning kit (Invitrogen). *E. coli* TOP10F cells were transformed according to the manufacturer's instructions. Recombinant plasmids containing 16S rDNA inserts were extracted by an alkaline lysis procedure using the Amersham Pharmacia GFX Plasmid Preparation Kit. Inserts were PCR-amplified from plasmid DNA using insert-flanking M13 primers. PCR products were purified on a silica matrix (Boyle & Lew, 1995) and used as templates for direct cycle sequencing with the Thermo Sequenase Fluorescent Labelled Primer Cycle Sequencing Kit (Amersham Pharmacia) using fluorescent-dye-labelled universal M13 primers and different internal primers for sequencing of 16S rDNA inserts. Sequencing products were separated and detected on an automated LI-COR DNA 4000L sequencer (MWG).

Phylogenetic analysis. Initial sequence comparison with current 16S rDNA databases was done with the FASTA and gapped BLASTN search tool of the HUSAR software package (DKFZ, Heidelberg, Germany). Alignment and phylogenetic analysis of sequence data were performed by using the corresponding tools of the ARB software package (<http://www.mikro.biologie.tu-muenchen.de/pub/ARB/documentation/ARB.ps>). The phylogenetic tree shown in Fig. 1 was constructed from 822 positions by applying the neighbour-joining method (Saitou & Nei, 1987) with 1000 bootstrap resamplings and the Jukes & Cantor (1969) correction. A consensus mask omitting non-overlapping sequence parts was generated with the ARB program prior to phylogenetic analysis. Additionally, a phylogenetic tree was generated by applying the PHYLIP-parsimony method to verify branching points supported by low bootstrap values. The CHECK-CHIMERA tool (Larsen *et al.*, 1993) was used to search for chimaeric sequences.

Dot blot hybridization of recombinant clones. The approach described by von Wintzingerode *et al.* (1999), based on hybridization with digoxigenin (DIG)-labelled 16S rDNA fragments, allowed rapid screening of the gene library. Briefly, DIG-labelled rDNA fragments of analysed single clones were generated by nested PCR with primers TPU1 and RTU2 (5'-TGCCTCCCGTAGGAGTYTGG-3'; *E. coli* positions 334-353) from full-length 16S rDNA clone inserts, previously PCR-amplified from plasmid DNA using primers M13(-40)F and M13(-24)R. For hybridization, plasmid DNA was heat-denatured, spotted onto a positively charged nylon membrane (Roche Diagnostics) and fixed by UV cross-linking (3 min each side, 10 J cm⁻²). All hybridization experiments were done with the DIG hybridization system (Boehringer Mannheim), according to the manufacturer's instructions. Hybridization was done in standard hybridization buffer with 50% formamide at 62 °C for 4 h, followed by two washing steps with 0.1 × SSC containing 0.1% SDS at 68 °C for 25 min each.

RESULTS

The 16S rDNA clone library was designated SHA (Saale, Halle, library A). First, the library was screened with four DIG-labelled polynucleotide probes derived from frequently appearing rDNA clone sequences described previously for another reductively dechlorinating freshwater consortium (von Wintzingerode *et al.*, 1999). Hybridization with 16S rDNA fragments of clone SJA-19 led to positive results. For further analysis, randomly chosen SHA clones were sequenced. Eight of these SHA clones revealed high levels of similarity ($\geq 93.2\%$) to either WCHA, WCHB, WsCH and WFeA clones (Dojka *et al.*, 1998) or SJA clones (von Wintzingerode *et al.*, 1999). These sequence types have been found previously in habitats contaminated with different chlorinated compounds. Therefore, DIG-labelled polynucleotide probes were generated from these eight clones (marked by an asterisk in Table 1) to determine

Table 1. Sequence type distribution found within the 16S rRNA gene library obtained from the DCP-transforming bioreactor

Clones SJA have been reported by von Wintzingerode *et al.* (1999); clones WCHA, WCHB, WsCH, WFeA have been reported by Dojka *et al.* (1998). Inserts of clones marked by an asterisk (*) were used as templates to generate DIG-labelled polynucleotide probes for dot blot hybridization.

Clone family	No. of clones	Database match ($\geq 92\%$ identity)	Similarity (%)	Cluster
<i>Proteobacteria</i> (δ)				
SHA-207	1	SJA-162	97.9	SHA-I
SHA-22*	3	WsCH54	96.3	SHA-I
<i>Holophaga/Acidobacterium</i>				
SHA-18*	1	SJA-149	93.2	
Low G + C Gram-positives				
SHA-67*	1	SJA-19	99.2	SHA-IV
SHA-61*	4	WsCH5	96.1	SHA-V
SHA-219	1	WsCH5	97.2	SHA-V
SHA-33	4	WsCH5	95.1	SHA-V
Green non-sulfur related cluster				
SHA-56	4	WCHB1-43	97.9	SHA-II
SHA-24*	1	WCHB1-43	97.9	SHA-II
SHA-300	2	SJA-108	93.6	SHA-II
SHA-28*	1	SJA-58	94.5	
SHA-105	3	SJA-108	92.2	
SHA-53	1	SJA-61	96.5	
SHA-3	1	SJA-15	97.2	SHA-III
SHA-21*	9	SJA-51	93.6	SHA-III
Division level cluster OP8				
SHA-89	3	WFeA1-59	94.2	
<i>Cytophagales</i>				
SHA-54	1	WCHA1-01	96.7	SHA-VI
SHA-38	2	WCHA1-01	96.1	SHA-VI
SHA-25*	1	WCHA1-01	96.3	SHA-VI
SHA-7	1	WCHB1-29	93.4	

their distribution within a total of 300 clones of the SHA clone library by further dot blot hybridization. All SHA clones giving positive hybridization results were sequenced (550–1500 bp). The analysis revealed that a subset of 45 clones showed the highest levels of similarity ($>92\%$) to the rDNA sequences retrieved from the dechlorinating consortia mentioned above (Dojka *et al.*, 1998; von Wintzingerode *et al.*, 1999). Based on a sequence similarity of $\geq 97\%$ these 45 clones could be grouped into 20 SHA clone families, which were named after the representative full-length rDNA sequence. No sequence was found to be chimaeric.

Fig. 1 shows the phylogenetic relationships of 20 representative full-length SHA sequences to their most closely related 16S rDNA sequences within the domain *Bacteria*. Based on a sequence similarity $\geq 92\%$, suggesting a close taxonomic relationship, 14 clone families were grouped into six distinct monophyletic bacterial clusters termed SHA-I to -VI (Fig. 1). Each cluster contained at least three sequence families. Four of these clusters comprised exclusively 16S rDNA sequences obtained from reductively dechlorinating freshwater consortia (Dojka *et al.*, 1998; von Wintzingerode *et al.*, 1999; this study), whereas two clusters (SHA-IV and -VI) contained an additional three sequences found also in non-dechlorinating biotopes (SHA-IV, *Syntrophobotulus glycolicus*; SHA-VI, clone LD21, *Cytophaga* sp. strain BD1-16). Clusters SHA-I to -VI were clearly separated from phylogenetically related 16S rDNA sequences derived from both non-reductively dechlorinating freshwater habitats and reductively dechlorinating marine habitats as indicated by sequence divergence of up to 12% to the phylogenetically most closely related neighbours. The six SHA clone families which did not fall into clusters SHA-I to -VI (SHA-18, -89, -28, -105, -53 and -7) exhibited at least 9% variation to the next most closely related sequences found in other habitats. Within cluster SHA-IV, clone SHA-67 exhibited 97.9% similarity to *Dehalobacter restrictus* (Holliger *et al.*, 1998). *Dehalobacter restrictus* and *Dehalobacter* sp. strain TEA (Wild *et al.*, 1996) couple anaerobic growth to reductive dechlorination of tetrachloroethene and trichloroethene. Furthermore, SHA-67 was closely related to rDNA sequence SJA-19, which was derived from an anaerobic trichlorobenzene-dechlorinating consortium (von Wintzingerode *et al.*, 1999). Both rDNA sequences shared a nearly identical insertion between positions 75 and 84 (*E. coli* 16S rRNA numbering).

DISCUSSION

A large variety of different chlorinated compounds, used mostly as intermediates for chemical synthesis, have been developed by the chemical industry. In general, halogenated compounds are considered to be toxic molecules exhibiting long half-lives (Vogel *et al.*, 1987; Hileman, 1993). In anaerobic environments,

Fig. 1. Phylogenetic relationships of 20 representative full-length SHA sequences obtained from the DCP-dechlorinating bioreactor consortium to 16S rDNA sequences obtained from anaerobic, dechlorinating freshwater habitats [sequence prefixes WsCH, WCHA, WFeA and WCHB (Dojka *et al.*, 1998), and SJA (von Wintzingerode *et al.*, 1999)] and anaerobic,

microbial dehalogenation of halogenated compounds is generally initiated via the reductive route (Suflita *et al.*, 1982), defined by removal of a halogen substituent from a given molecule with concurrent addition of electrons (Mohn & Tiedje, 1992). The mixed anaerobic bioreactor culture analysed in this study is capable of dechlorinating DCP to propene by reductive dechlorination. The micro-organisms responsible for reductive dechlorination of DCP have not yet been obtained in pure culture, as is the case for many other chlorinated compounds. This is in contrast to the great number of complex environmental microbiota capable of reductive dechlorination. For technical purposes, i.e. large-scale bioremediation of contaminated aquifers and waste water streams, the application of these complex consortia may represent a promising technology as shown by several experiments using laboratory-scale bioreactors (Hauck *et al.*, 1999; Zou *et al.*, 1999). However, due to the unknown community structure these consortia have been treated as 'black boxes' in the past and a direct optimization of microbial biodegradation has been hampered. One strategy which allows a more optimized control of bioremediation processes is the continuous monitoring of indicator organisms (Power *et al.*, 1998; Stapleton *et al.*, 1998; Brockman, 1995). The aim of our study was to define groups of organisms which exclusively occur in reductively dechlorinating consortia by comparing recently published 16S rDNA sequences retrieved from dechlorinating marine and freshwater consortia. These organisms might serve as indicator organisms for the anaerobic, reductive dechlorination of chlorinated compounds.

The first 16S rDNA-based determination of microbial diversity of a reductively dechlorinating consortium was published by Dojka *et al.* (1998). The authors investigated the microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. They analysed rDNA clones obtained from samples of the methanogenic, methanogenic-sulfate-reducing and iron- or sulfate-reducing zones (WCHA, WCHB, WsCH, WFeA clones). The majority of their bacterial 16S rDNA sequences (70%) were phylogenetically associated with recognized bacterial divisions, mainly the green non-sulfur-related cluster, the low G+C Gram-positives and the *Proteobacteria*. Approximately 20% of the sequence types were affiliated with four division level clusters from a Yellowstone hot spring (OP5, OP8, OP10 and OP11) (Hugenholtz *et al.*, 1998). The remaining 16S rDNA sequences (10%) suggested the existence of six novel bacterial divisions (WS1–6) (Dojka *et al.*, 1998). In a second study, von

Wintzingerode *et al.* (1999) investigated the microbial diversity of an anaerobic, trichlorobenzene-dechlorinating microbial consortium derived from river sediment. Again, samples were taken from the River Saale, but the sampling site was located near Jena, approximately 200 km upstream from Halle. Several of the SJA sequences obtained were affiliated with the green non-sulfur related cluster, the *Proteobacteria*, the division level cluster OP10 (Hugenholtz *et al.*, 1998) and the spirochaetes and were closely related to rDNA sequences from the contaminated aquifer (Dojka *et al.*, 1998). In both studies, archaeal rDNA clone libraries were dominated by clone sequences nearly identical to *Methanosaeta concilii*.

In our study, analysing an anaerobic DCP-dechlorinating microbial consortium, at least 20 clone families within the SHA clone library were found to be phylogenetically most closely related to 16S rDNA sequences retrieved from the above-mentioned dechlorinating freshwater habitats. Six well separated clusters of closely related sequences were defined, which nearly exclusively comprised rDNA sequences from dechlorinating freshwater habitats. Within cluster SHA-IV, clone families SHA-67 and SJA-19 shared sequence similarities >97% to *Dehalobacter restrictus* (Holliger *et al.*, 1998) or *Dehalobacter* sp. strain TEA (Wild *et al.*, 1996), respectively. Both isolates are capable of reductive dechlorination of tri- and tetrachloroethene. Our findings indicate the presence of novel members of the genus *Dehalobacter*. Since known members of that genus share the same dechlorinating phenotype, it is reasonable to assume that as yet uncultured *Dehalobacter* spp. may contribute to the reductive dechlorination of DCP in the bioreactor described here.

In contrast to reductively dechlorinating consortia derived from freshwater habitats, the composition of corresponding consortia derived from marine habitats is different. A comparative 16S rDNA sequence analysis of a marine anaerobic microbial community degrading PCB by reductive PCB *ortho*-dechlorination (Pulliam Holoman *et al.*, 1998) revealed 16 predominant clone sequences (RFLP clones; Fig. 1), which were phylogenetically distant from clone sequences found in studies on reductively dechlorinating freshwater microbiota (Fig. 1). In a further study, Kengen *et al.* (1999) examined anaerobic enrichment cultures derived from polluted harbour sediments that reductively dechlorinate tetrachloroethene. Molecular analysis of these cultures indicated the presence of two dominant sequence types, both belonging to the low G+C Gram-positives (ST clones; Fig. 1). Highest similarities were found to the halo-respiring bacterium

dechlorinating marine habitats [sequence prefixes ST (Kengen *et al.*, 1999) and RFLP (Pulliam Holoman *et al.*, 1998)]. Clusters SHA-I to -VI comprise nearly exclusively rDNA sequences obtained from anaerobic, dechlorinating freshwater habitats. *Methanosaeta concilii* (X16932) and *Thermoplasma acidophilum* (M20822) were used as an outgroup. Branching points were supported by bootstrap values (●, 75%; ○, 50–74%). Branching points without circles were not resolved (bootstrap values <50%). The scale bar represents 10% sequence divergence.

Table 2. Comparison of 16S rDNA clone libraries obtained from habitats undergoing reductive dechlorination

Habitat	Dehalogenation compounds	Cell lysis procedure	Cloning system	Eubacterial PCR primers	Reference
River sediment, bioreactor	1,2-DCP	Enzymic, freeze-thaw, detergent	TA cloning	27f–1522r	This study
River sediment, bioreactor	Trichlorobenzene	Enzymic, freeze-thaw, detergent	LIC or TA cloning	27f–1522r	von Wintzingerode <i>et al.</i> (1999)
Aquifer, ground	Different hydrocarbons and chlorinated solvents	Bead beater, enzymic, detergent	TA cloning	27f–1492r 533f–1492r	Dojka <i>et al.</i> (1998)
Marine sediment	2,3,5,6-Tetrachlorobiphenyl	Bead beater, detergent	TA cloning	519f–1406r	Pulliam Holoman <i>et al.</i> (1998)
Marine sediment	Tetrachloroethene	Guanidinium thiocyanate, bead beater	TA cloning	8f–1512r	Kengen <i>et al.</i> (1999)

Dehalobacter restrictus (90%) and to members of the genus *Desulfotomaculum* (86%). Compared to 97.7% similarity found for clone SHA-67, these low sequence similarities indicate again that marine dechlorinating consortia reveal a different species composition to dechlorinating freshwater consortia.

However, it should be taken into account that comparison of rDNA clone libraries obtained from different habitats under different experimental conditions is limited (Head *et al.*, 1998). Bias can be introduced at various stages in the protocol, particularly during cell lysis, PCR amplification and cloning as reviewed by von Wintzingerode *et al.* (1997). Therefore, differences in experimental conditions regarding the habitat, the location of sample collection, cell lysis, cloning procedures and PCR primers used have been compiled (Table 2). Different cell lysis procedures and PCR conditions applied by von Wintzingerode *et al.* (1999) and in this study, but not in the work of Dojka *et al.* (1998), did not blur our main conclusion that a significant number of rDNA sequences of dechlorinating freshwater habitats were phylogenetically closely related, suggesting a similar population under comparable physiological conditions. In contrast, similar cell lysis and PCR protocols used by Pulliam Holoman *et al.* (1998) for marine habitats or by Dojka *et al.* (1998) for freshwater consortia did not reveal rDNA clone libraries of similar composition, indicating an overall low impact of the experimental conditions.

In conclusion the retrieval of 16S rDNA sequences closely related to those of cultivated and as yet uncultured bacteria within different anaerobic dechlorinating microbial communities strongly suggests a specific community structure for reductively dechlorinating freshwater habitats. Quantitative studies using primers or probes specific for the SHA clusters and clone families described in this study are currently under way to test our hypothesis on the usefulness of indicator organisms for reductive dechlorination to monitor the efficiency of bioremediation processes.

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