

Reductive dechlorination of β -hexachlorocyclohexane (β -HCH) by a *Dehalobacter* species in coculture with a *Sedimentibacter* sp.

Wim van Doesburg*, Miriam H.A. van Eekert, Peter J.M. Middeldorp,
Melike Balk, Gosse Schraa, Alfons J.M. Stams

Laboratory of Microbiology, Wageningen University and Research Centre, Hesselink van Suchtelenweg 4, 6703 CT Wageningen, The Netherlands

Received 27 October 2004; received in revised form 24 February 2005; accepted 5 March 2005

First published online 31 March 2005

Abstract

An anaerobic coculture was enriched from a hexachlorocyclohexane (HCH) polluted soil. The coculture reductively dechlorinates the β -HCH isomer to benzene and chlorobenzene in a ratio of 0.5–2 depending on the amount of β -HCH degraded. The culture grows with H_2 as electron donor and β -HCH as electron acceptor, indicating that dechlorination is a respiratory process. Phylogenetic analysis indicated that the coculture consists of two bacteria that are both related to gram-positive bacteria with a low G + C content of the DNA. One bacterium was identified as a *Dehalobacter* sp. This bacterium is responsible for the dechlorination. The other bacterium was isolated and characterized as being a *Sedimentibacter* sp. This strain is not able to dechlorinate β -HCH. The *Dehalobacter* sp. requires the presence of *Sedimentibacter* for growth and dechlorination, but the function of the latter bacterium is not clear. This is the first report on the metabolic dechlorination of β -HCH by a defined anaerobic bacterial culture. © 2005 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Hexachlorocyclohexane biotransformation; Reductive dechlorination; Halorespiration; Coculture; *Dehalobacter*; *Sedimentibacter*

1. Introduction

Approximately 10 million tons of technical HCH have been applied worldwide as an insecticide since the 1940s [1]. The production procedure renders a mixture of usually five (α -, β -, γ -, δ -, and ϵ -) stable hexachlorocyclohexane (HCH) isomers of which the γ -isomer (lindane, usually 8–15% of mixture) is known for its insecticidal activity [2]. At first the mixture as a whole was applied, but since the late 1970s the active γ -isomer was separated from the other inactive isomers and the latter were discarded at waste sites. HCH production and use has been banned or restricted in many countries during the past two decades [3] but at many different locations worldwide, soil, ground water and the atmos-

phere (through volatilization) have been contaminated with HCH [1]. Although all the HCH isomers are acutely and chronically toxic, β -HCH (5–14% in technical HCH mixture) is considered to be the most dangerous isomer due to its resistance to biodegradation and its estrogenic effects in mammals [1,4].

Microbial degradation of HCH in soils and sediments has been studied both under aerobic and anaerobic conditions (e.g. [5–8]) and β -HCH has been found to be the most persistent isomer presumably due to the equatorial positioning of all the chlorine atoms [9]. Limited transformation of β -HCH was observed under aerobic conditions with *Bacillus circulans*, *Bacillus brevis* and *Pseudomonas* sp. [10,11]. Transformation of β -HCH under anaerobic conditions has been observed to occur via two subsequent dichloroelimination reactions (removal of two chlorine atoms and formation of a double bond) yielding dichlorocyclohexadiene, followed by either a

* Corresponding author. Tel.: +31 317 484099; fax: +31 317 483829.
E-mail address: wim.vandoesburg@wur.nl (W. van Doesburg).

(presumably abiotic) dehydrochlorination to chlorobenzene or a third (biotic) dichloroelimination to benzene (e.g. [8]). So far, only cometabolic dechlorination of β -HCH has been observed by anaerobic or facultative anaerobic pure cultures like, e.g., *Clostridium* spp. and *Citrobacter freundii* [12] and mixed cultures originating from contaminated soils (e.g. [8]) or anaerobic (sewage) sludges (e.g. [6,13]).

It was suggested that the ability to dechlorinate γ -HCH is strongly correlated to the ability of some fermentative microorganisms to generate H_2 [12]. It has also been postulated that HCH and related compounds act as alternative electron acceptors for the Stickland reaction in *Clostridium rectum*, thus coupling the dechlorination of HCH to the generation of ATP [14].

Microorganisms that are able to conserve energy via the dechlorination of chlorinated compounds were first observed with *Desulfomonile tiedje* [15]. This process is since then referred to as (de)halorespiration. In the past two decades many other halorespiring microorganisms have been identified of which *Dehalobacter restrictus* was the first bacterium known to transform tetrachloroethene [16]. Bacteria in other genera, like *Dehalococcoides ethenogenes* and *Desulfitobacterium*, have also been found to be capable of halorespiration. This process seemed to be confined to the dechlorination of chlorinated ethenes and chloroaromatic compounds since, e.g., chlorinated alkanes were found to be converted solely via cometabolic processes [17]. Recently several bacteria have been found that are able to use the energy generated from the dechlorination of chlorinated alkanes for growth. *Dehalococcoides ethenogenes* strain 195 is able to reductively dechlorinate 1,2-dichloroethane partially to ethane [18]. Wildeman et al. isolated *Desulfitobacterium dichloroeliminans*, which is also able to halorespire 1,2-dichloroethane to ethene exclusively via the process of dichloroelimination [19]. Furthermore, a strain closely related to *D. restrictus* was found to grow via the reductive hydrogenolysis (reductive elimination of one chlorine atom at the time) of 1,1,1-trichloroethane to stoichiometric amounts of chloroethane [20]. To our knowledge, a microorganism able to sustain its growth via the dechlorination of β -HCH has not been isolated yet.

Here, we describe the transformation of β -HCH by a bacterial coculture of a *Dehalobacter* species and a *Sedimentibacter* species enriched from contaminated soil.

2. Materials and methods

2.1. Chemicals

The HCH isomers (>99% purity) were obtained from C.N. Schmidt B.V. (Amsterdam, The Netherlands). Chloroform (99% pure) was from Fluka (Zwijndrecht,

The Netherlands). Wilkins–Chalgren anaerobe broth (WCB) and peptone were purchased at Oxoid (Basingstoke, UK). 2-Bromoethane sulfonic acid (BESA) was acquired at Fisher Scientific Nederland. Gas mixtures (N_2/CO_2 , H_2/CO_2) were obtained at Hoekloos (Schiedam, The Netherlands). All other chemicals used were of the highest available purity and purchased from Sigma (Zwijndrecht, The Netherlands) and Merck (Amsterdam, The Netherlands). Primers were synthesized by MWG Biotech AG (Ebersberg, Germany).

2.2. Enrichment procedure

Sandy soil samples were taken from an HCH-polluted location in Hengelo, The Netherlands [13], and stored under nitrogen at 4 °C until use. About 4 g (wet weight) of soil were added in a anaerobic glove box to 120-ml serum bottles containing 20 ml of methanogenic mineral medium as described by Holliger et al. [21], but without the addition of fermented yeast extract (FYE). The bottles were closed with viton stoppers (Maag Technik, Dübendorf, Switzerland) and aluminium crimp caps and the headspace was replaced with N_2/CO_2 (80:20 (v/v); 140 kPa). Approximately 0.1 ml of β -HCH solution (1 g l⁻¹, dissolved in acetone) was added to the bottles resulting in a final concentration of 17 μ M. Sodium lactate (50 mM, final concentration), sodium acetate (75 mM), sodium propionate (25 mM) or methanol (100 mM) were added in separate bottle sets from sterilized stock solutions. The bottles were incubated statically at 30 °C in the dark. β -HCH degradation in time was monitored by measurement of the benzene and chlorobenzene concentration in the headspace. After complete removal of β -HCH, the culture was transferred (5% inoculum size) into fresh medium. Unless mentioned otherwise the β -HCH degradation was also checked in blanks that were not inoculated or blanks that were autoclaved for 20 min at 120 °C.

The cultures incubated with lactate as the electron donor were used for further enrichment of the β -HCH degrading culture. FYE [21] (0.2 g l⁻¹) was added as a nutrient supplement to the enrichment to sustain dechlorination. BESA (10 mM) was added in initial incubations to inhibit methanogenesis, but was omitted after methanogenic activity had ceased. Subsequently, the β -HCH concentration was increased (0.3 ml of 35 g l⁻¹ β -HCH solution, final concentration 1.8 mM), and lactate was replaced by H_2 and acetate (2 mM). It was confirmed that the dechlorinating activity was not inhibited by the acetone present in the incubations by comparing the lag phases before dechlorination occurred in the presence of increasing amounts of acetone. FYE was replaced by peptone (0.05 g l⁻¹) and the medium was slightly adjusted (see Section 2.3). Ultimately, the different bacterial strains in the enrichment were separated via Percoll gradient centrifugation developed by Beaty et al.

[22]. This method is based on the separation of the microorganisms caused by differences in their relative density. Here, a slightly modified method was used [23]. The mixed culture was centrifuged and resuspended in 50 mM sodium phosphate pH 7.5 containing 50% Percoll (v/v). The cells were separated by generating a Percoll gradient in airtight centrifuge tubes (8 ml) at 21,000g and 10 °C for 30 min in a Centrikon T-1065 centrifuge (Kontron Instruments Inc., Everett, MA). Four distinct, separated bands were observed after centrifugation. The four bands of cells were separated and transferred in fresh anaerobic mineral medium with H₂/CO₂ and β-HCH or WCB in a dilution series to isolate pure cultures. WCB is a rich medium for the general growth of anaerobes and was used with a headspace of N₂/CO₂ (80:20 (v/v); 140 kPa).

The microbial composition of the enrichment cultures was regularly checked with phase-contrast light microscopy and with molecular ecological methods as described in Section 2.8 (denaturing gradient gel electrophoresis (DGGE)).

2.3. Cultivation of the coculture

Applying the above-mentioned techniques, we obtained a coculture that consists of two bacteria. This culture was grown in 120-ml serum bottles containing 20 ml of methanogenic mineral medium under H₂/CO₂ (80:20 (v/v); 140 kPa) headspace. The medium composition was slightly modified to create optimal growing conditions for the dechlorinating bacteria [16]. The amount of the vitamin cyanocobalamin was increased 10-fold (final concentration in the medium: 50 µg l⁻¹) and the vitamins biotin, folic acid, pyridoxine, riboflavin, nicotinamide, *p*-aminobenzoic acid, lipoic acid, and pantothenic acid were omitted. Peptone and acetate were added to a final concentration of 0.05 g l⁻¹ and 2 mM, respectively. Approximately 0.2 ml of β-HCH stock solution (15 g l⁻¹, dissolved in acetone) was added to the bottles resulting in an average final concentration of 510 µM. The bottles were inoculated with 5% (v/v) of the coculture and incubated statically in the dark at 30 °C. Quadruple tests were performed for the HCH degradation experiment and autoclaved bottles (20 min at 120 °C) were prepared as negative controls. Benzene, chlorobenzene, β-HCH, and chloride were measured on a regular basis. For equilibration, benzene and chlorobenzene were not measured before day one.

2.4. Dechlorination of chlorinated compounds

To investigate the dechlorination of other HCH isomers (α-, γ- and δ-HCH) by the coculture, 0.1 ml of acetone stock solutions (10 g l⁻¹ α-, β-, γ-, δ-HCH) was added to duplicate 120-ml serum bottles (final concentration of HCH was approximately 170 µM). The bot-

tles were inoculated with 5% (v/v) of culture and subsequently incubated statically at 30 °C in the dark. Benzene and chlorobenzene in the headspace were followed in time.

The degradation of other chlorinated compounds like tetrachloroethene (PCE) and 1,2-dichloroethane (12DCA) was investigated in the medium described in Section 2.3. The compounds were added dissolved in anaerobic water (PCE and 12DCA) or dissolved in hexadecane (PCE) as described by Holliger et al. [21].

2.5. Analytical methods

Benzene, chlorobenzene, acetone, 12DCA and PCE were analyzed by injecting 0.4 ml of headspace gas into a Chrompack 436 gas chromatograph (Chrompack, Bergen op Zoom, The Netherlands) equipped with a flame ionization detector (FID) connected to a Sil 5CB capillary column (25 m × 0.32 mm, 1.2 µM film) and a split injection (ratio 1:50). The operation temperature of the oven was 60 °C for 4 min, and then increased by 15 °C min⁻¹ to 105 °C, and held at 105 °C for 1 min. The temperature of the injector and detector were 250 and 300 °C, respectively. Calibration standards were prepared by adding a known amount of the compound to 120-ml serum bottles containing 20 ml of medium to give the same liquid-to-headspace ratio as that for the cultures.

The HCH concentration was determined in the liquid phase after extraction of 1 ml of sample with 1 ml of chloroform. The extraction mixture was then sonified for 15 min followed by a 24-h extraction in an end-over-end shaker. The HCH concentration was determined in the chloroform extracts with a gas chromatography–mass spectrometry method using external HCH standards [8]. Chloride concentration was determined using HPLC as described previously [24] with fluoride as internal standard. Volatile fatty acids concentrations were determined by HPLC as described by Middeldorp et al. [8].

2.6. DNA isolation

A bead–bead and phenol–chloroform based DNA extraction method was used to extract DNA from the dechlorinating enrichment cultures and the coculture. Cells of the liquid culture (4 ml) were collected by centrifugation (13,000 rpm, 10 min) and resuspended in 1 ml Tris–EDTA buffer in a 2-ml screw-cap tube containing 150 µl of TE-buffered phenol (Invitrogen, Breda, The Netherlands) and 0.3 g of 0.1 mm zirconium beads (Bio-Spec Product, Bartlesville, OK). After two cycles of 30 s bead beating and cooling on ice the lysate was extracted with phenol–chloroform–isoamyl alcohol two to three times until the top layer was clear. Nucleic acids were overnight precipitated in static conditions at –20 °C

by adding 0.1 volume of 3 M sodium acetate and 0.6 volume of isopropanol. After centrifugation (13,000 rpm, 20 min), the DNA pellet was washed with 70% ethanol, air dried, and resuspended in 100 µl of Tris–EDTA buffer.

2.7. Amplification of the 16S rRNA gene, cloning and sequencing

The total 16S rRNA gene was amplified with a GeneAmp PCR System 2400 thermocycler (Perkin–Elmer Cetus, Norwalk, CT). After preheating to 94 °C for 5 min, 35 amplification cycles of denaturation at 94 °C for 30 s, primer annealing at 48 °C for 20 s and elongation at 68 °C for 40 s were performed. A final extension of 7 min at 68 °C was performed. The PCR mixtures (50 µl) contained 20 mM Tris–HCl (pH 8.4); 50 mM KCl; 1.5 mM MgCl₂; 200 µM of each deoxynucleoside triphosphate; 10 µM of primers 7f and 1492r [25]; 2.5 U of *Taq* DNA polymerase (Invitrogen, Breda, The Netherlands); and 1 µl of template DNA. Size and yield of PCR products were estimated by 1.2% agarose gel (w/v) electrophoresis and ethidium bromide staining.

PCR products were purified by the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and cloned into *Escherichia coli* JM109 (Invitrogen, Breda, The Netherlands) by using the pGEM-T Easy vector system (Promega, Madison, WI) with ampicillin selection and blue/white screening according to the manufacturer's manual. Positive clones (white colonies) were taken up with a sterile toothpick and transferred into 0.2-ml PCR tubes containing 50 µl of Tris–EDTA buffer, which was heated for 15 min at 95 °C to release the DNA. The 16S rRNA gene inserts from recombinant clones were reamplified by PCR with the vector specific primers T7 and Sp6 (Promega, Madison, WI). PCR products were screened by restriction fragment length polymorphism (RFLP), using the restriction endonucleases *AluI*, *CfoI* and *MspI* (Promega, Madison, WI). Aliquots (5 µl) of crude reamplified 16S rRNA gene PCR products were digested with 2.5 U of each of the enzymes in 1× buffer B (Promega, Madison, WI) in a final volume of 10 µl, for 1.5 h at 37 °C. Digested products were separated by 4% agarose gel electrophoresis and visualized by ethidium bromide staining. PCR products with different RFLP patterns were purified by the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and used as a template in sequencing reactions.

For sequence analysis, the Sequenase sequencing kit (Amersham, Slough, UK) was used according to the manufacturer's instructions with IRD800 (MWG-Biotech, Ebersberg, Germany) labeled sequencing primers Sp6, T7 (Promega, Madison, WI), 533f and, 1100r [25]. The sequences were automatically analyzed on a LI-COR (Lincoln, NE) DNA sequencer 4000L and cor-

rected manually. Total 16S rRNA gene sequences were compared to sequences deposited in publicly accessible databases using the NCBI Blast search tool at <http://www.ncbi.nlm.nih.gov/blast/>.

2.8. DGGE analysis

Purified DNA from the dechlorinating enrichment cultures, coculture or cultured transformants was used as PCR template. A DGGE-suitable 16S rRNA gene amplicon was generated with 35 cycles of 94 °C for 30 s, 56 °C for 20 s, and 68 °C for 40 s. The PCR reaction mixtures were the same as for the total 16S rRNA gene PCR, except for the two primers; F-968-GC and R-1401 [26]. The PCR products were separated by DGGE according to the specifications of Muyzer et al. [27] by using the Dcode system (Bio-Rad Laboratories, Hercules, CA). Linear gradients of 30–60% of denaturant (100% denaturant consisted of 7 M urea and 40% formamide) were used in 8% (v/v) polyacrylamide (37.5:1 acrylamide–bisacrylamide) gels according to the modifications of Heilig et al. [28]. Electrophoresis was performed at a constant voltage of 85 V and a temperature of 60 °C for 16 h. After electrophoresis, the gels were stained with AgNO₃ according to the method of Sanguinetti et al. [29].

2.9. GenBank accession numbers

The 16S rRNA gene nucleotide sequences determined in this study have been deposited into the GenBank database under Accession Nos. AY673991, AY673992 and AY766465 for the *Dehalobacter* species and AY673993, AY766466 and AY766467 for the *Sedimentibacter* species.

3. Results and discussion

3.1. Enrichment, isolation and identification of the coculture

The enrichment of β-HCH-degrading microorganisms was started in methanogenic medium with a soil sample from a polluted site in The Netherlands. β-HCH was completely transformed within two weeks with lactate or acetate as the electron donor. In the presence of propionate and methanol, the complete removal of β-HCH took three and four weeks, respectively (Table 1). There was a stoichiometric increase of the benzene and monochlorobenzene concentration in all the incubations, irrespective of the electron donor used. There was no dechlorination observed in uninoculated or autoclaved controls. The acetone that was present in the batches as the initial solvent of β-HCH was not metabolized. The dechlorinating activity could not be

Table 1

Average benzene and chlorobenzene concentration (\pm SD) (μ M) in the incubations after complete transformation of β -HCH (initial concentration $\pm 17 \mu$ M as calculated from stock solution addition) in combination with different electron donors

Electron donor	Days after start of incubation	Benzene	Chlorobenzene
Acetate	11	5.4 \pm 0.28	15.0 \pm 1.06
Lactate	11	11.0 \pm 1.91	13.4 \pm 0.64
Propionate	19	3.4 \pm 0.21	14.8 \pm 0.07
Methanol	28	12.3 \pm 2.26	9.9 \pm 0.71

Values are means ($n = 2$).

transferred into fresh medium with acetate, propionate or methanol as the electron donor. The incubations with lactate could be repeatedly transferred, but the lag phase increased to a maximum of three weeks. After the addition of FYE the lag phase decreased again to less than one week. No dechlorination occurred when the addition of lactate was omitted indicating that FYE alone could not support the dechlorination. Propionate and methane could be detected as products of lactate fermentation. The repeated addition of the methanogenic inhibitor 2-bromoethane sulfonic acid (10 mM) in subsequent transfers led to the loss of the methanogenic activity in the enrichment cultures but the dechlorinating activity was not affected. After seven transfers with the addition of BESA the methanogenic activity could no longer be measured and the routine addition was stopped.

Several morphologically different types of microorganisms could be distinguished, even after increasing the β -HCH concentration to 1.8 mM and replacing the lactate by H_2 as the electron donor and FYE (at a concentration of 0.6 g l^{-1}) as the carbon source. Further adjustments of the medium like replacing the FYE with 0.05 g l^{-1} peptone led to an increase of the lag phase to 1–2 weeks. Finally, after the addition of acetate (2 mM) as a carbon source the lag phase decreased again to several days. Attempts to grow the β -HCH dechlorinating bacterium on agar containing medium failed. An enrichment culture consisting of four morphologically different microorganisms was ultimately obtained. Separation in a 50% Percoll gradient followed by a dilution series resulted in four different microbial bands. One of the bands consisted of a bacterium that was identified as a *Bacillus* sp., which was not involved in the dechlorination. Two Percoll gradient bands were identified, after subculturing in WCB medium, DGGE analysis and sequencing, as containing the same bacterium. This bacterium is closely (97% homology) related to *Sedimentibacter saalensis* and belongs to a genus that also contains *Sedimentibacter hydroxybenzoicus*. This bacterium was previously classified as *Clostridium hydroxybenzoicum*, but has recently been reclassified [30]. *S. saalensis* cells can occur in long filaments or in single cells and

in our isolate each form apparently has a different density, leading to the appearance of two separate bands in the Percoll gradient. The *Sedimentibacter* strain grew only in dilution series made in WCB medium or in methanogenic mineral medium supplemented with 5 g l^{-1} yeast extract, and was considered a pure culture after three transfers in dilution series and upon microscopy analysis. The DGGE analysis revealed that the pure culture of *Sedimentibacter* gave a distinct DGGE profile consisting of several amplicons with different intensity (Fig. 1). Three different RFLP patterns (data not shown) of the clones containing the total 16S rRNA gene of this culture were sequenced. In a DGGE analysis of the cloned inserts, the amplicons appeared as single bands (Fig. 1), and corresponded with the three most intensive bands of the pure culture. Twenty positions of variable nucleotides were detected by comparing the three sequences of the cloned inserts, indicating heterogeneities in the 16S rRNA genes in *Sedimentibacter* as reported before within single microorganisms [26,31]. *Sedimentibacter* alone was not able to dehalogenate β -HCH in mineral methanogenic medium with or without extra supplementation of all vitamins, yeast extract or filter sterilized coculture (data not shown). This is in contrast with earlier observations for *Clostridium* sp. and *C. freundii* that cometabolically dechlorinate β -HCH [12]. The use of γ -HCH as a terminal electron acceptor by *C. rectum* has also been reported [14].

One microorganism in the Percoll gradient could not be obtained in pure culture. When the Percoll band, containing a short rod, was diluted in mineral medium with

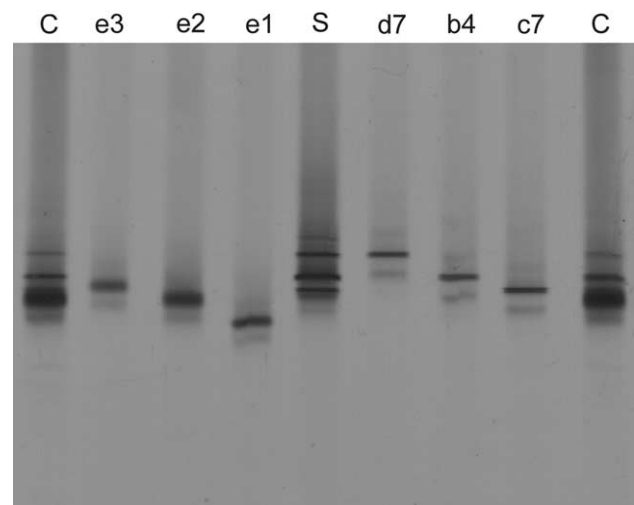


Fig. 1. DGGE analysis of amplified 16S rRNA genes (V6–V8 region) derived from the coculture (C) and the isolated *Sedimentibacter* species (S). The amplicons of the sequenced clones (e3, e1, e2) are derived from the coculture, which correspond to the *Dehalobacter* species. Lanes d7, b4 and c7 contain the amplicons of clones derived from the isolated *Sedimentibacter* species, respectively. The GenBank access numbers for clone e3 is AY673992, e1 is AY766465, e2 is AY673991, d7 is AY766467, b4 is AY673993 and for c7 is AY766466.

H₂, and β -HCH, dechlorination was observed until the 10⁵ dilution. Analysis under the microscope revealed two morphologically different types of organisms, and one of them resembled the *Sedimentibacter* species (Fig. 2). However, when this Percoll band was diluted in the same medium to which the *Sedimentibacter* had been added, dechlorination to the 10⁷ dilution was observed. Total 16S rRNA genes of this 10⁵ dilution culture were amplified, cloned and analyzed by RFLP. Inserts of clones with different RFLP patterns (data not shown) were sequenced. They consisted of the *Sedimentibacter* sp. and a strain that was 99% homologous with *D. restrictus* [16]. Twenty clones with the same RFLP pattern belonging to the *Dehalobacter* species were checked on DGGE. The results revealed that there were three distinct fragments for the *Dehalobacter* species (Fig. 1, lanes C–e2). Seventeen of the clones did have an amplicon (Fig. 1, lane e1), which corresponded with the thick band in the DGGE pattern of the coculture. Sequences derived of the two other plasmid inserts belonging to the amplicons e2 and e3 (Fig. 1), had three positions of variable nucleotides in comparison with the sequence derived of clone e1. This could be due to heterogeneity in 16S rRNA genes of *Dehalobacter*, but errors introduced by PCR cannot be excluded. Further dilution series in the mineral methanogenic media supplemented with all vitamins and FYE did not result in a pure culture that could sustain dechlorination (data not shown). The obtained β -HCH dechlorinating culture was considered to be a coculture after the lack of growth in our medium without β -HCH supplemented with lactate, glucose or pyruvate (data not shown), DGGE analysis (Fig. 1) and phase-contrast light microscopy (Fig. 2) examination.

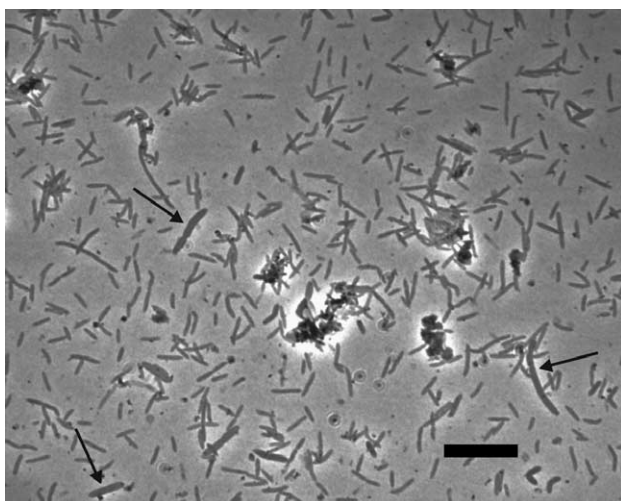


Fig. 2. Phase-contrast micrograph of the coculture grown on H₂/CO₂, peptone and β -HCH. Arrows indicate some of the *Sedimentibacter* bacteria present in the coculture. Bright areas presumably represent HCH crystals. Bar = 10 μ M.

3.2. Dechlorination of β -HCH by the coculture

The dechlorination of β -HCH by the coculture occurred after a lag phase of up to one day. Acetate could be omitted from the medium, but in that case the lag phase increased up to nine days (data not shown). Stoichiometric amounts of benzene, chlorobenzene and chloride were formed as the end products (Fig. 3). Usually the benzene:chlorobenzene ratio varied between 0.5 and 2.5 and it increased with the amount of β -HCH degraded (data not shown). This dechlorination pattern is similar to that found before in a degradation experiment with a contaminated soil [8]. β -HCH was dechlorinated via two subsequent dichloroelimination reactions to dichlorocyclohexadiene followed by a third dichloroelimination leading to the formation of benzene or a dehydrochlorination through which chlorobenzene is formed (Fig. 4). Benzene and chlorobenzene are end products of the β -HCH dechlorination and our coculture was not able to transform or mineralize these compounds. Although anaerobic degradation of benzene has been observed under different redox conditions [32], so far only two bacteria have been isolated that are capable of benzene degradation (under denitrifying conditions

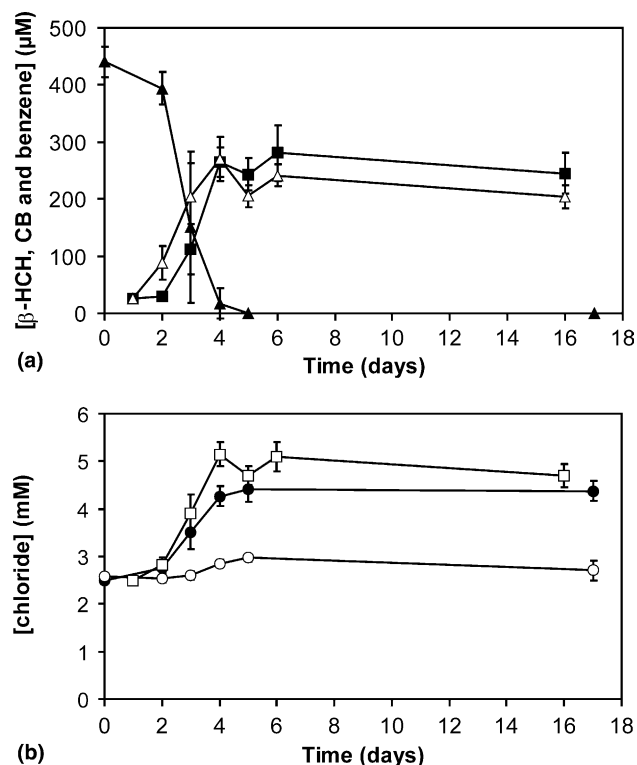


Fig. 3. Dechlorination of β -HCH by the coculture at 30 °C with the formation of benzene and chlorobenzene (a) and chloride (b). \blacktriangle , β -HCH; \blacksquare , benzene; \triangle , chlorobenzene (CB); \bullet , chloride; \square , theoretically calculated chloride; and \circ , chloride in sterile control. Bars indicate SDs ($n = 4$).

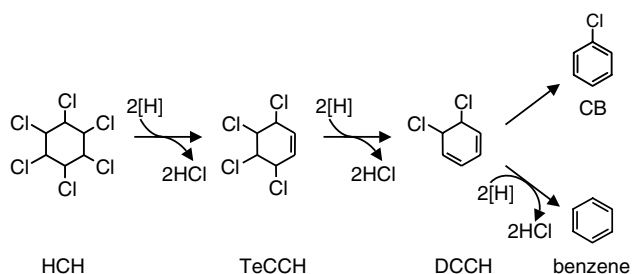


Fig. 4. Transformation pathway of β -HCH redrawn from Middeldorp et al. [8]. Transformation takes place via tetrachlorocyclohexene (TeCCH) and dichlorocyclohexadiene (DCCH) to benzene and chlorobenzene (CB).

[33]). Anaerobic degradation of chlorobenzene has not yet been documented.

The dechlorination of some of the other isomers, with a calculated initial concentration of 170 μM , was investigated (Fig. 5). α -HCH was transformed at rates that were equal to those found for β -HCH while the complete transformation of γ -HCH to benzene and chlorobenzene took roughly 130 days. δ -HCH dechlorination was not observed within 220 days after start of the experiment. Results were the same irrespective of the manner of incubation, i.e., precultivation on β -HCH followed by addition of the other isomers in the same batch or direct transfer of 5% (v/v) β -HCH grown coculture into fresh medium containing the respective HCH isomers. The results as a whole seem to suggest that specific enzymes are involved in the dechlorination and that there is a preference for the α - and β -HCH dechlorination. Optical densities of the cocultures during the dechlorination of β -HCH and the other isomers could not be measured, because the addition of HCH in acetone led to a turbid medium. Only after complete transformation of HCH did the medium become clear again.

The fact that the dechlorinating activity of β -HCH could be successfully transferred for more than 3 years

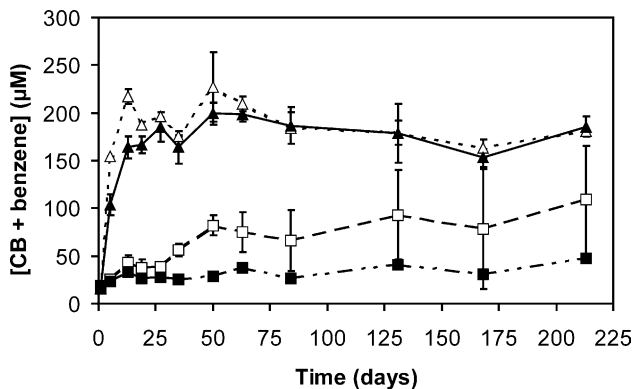


Fig. 5. Sum of benzene and chlorobenzene (CB) formation through reductive dechlorination of different HCH isomers by the coculture. Δ , α -HCH; \blacktriangle , β -HCH; \square , γ -HCH; and \blacksquare , δ -HCH. Bars indicate SDs ($n = 2$).

with only 5% (v/v) transfer of old cultures to fresh medium confirms our finding that the *Dehalobacter* species is able to grow via the process of halo-respiration. The benzene:chlorobenzene ratio after transformation was 0.3, 0.7, and 0.2 for α -HCH, β -HCH, and γ -HCH, respectively (data not shown). An explanation for these different benzene:chlorobenzene ratios may be that the conformation of the chlorine atoms in the HCH isomers is not the same. β -HCH does not contain axial chlorine atoms, but δ -, α - and γ -HCH have one, two, and three axial chlorine atoms, respectively. Our experiments show that the benzene:chlorobenzene ratio decreases with increasing number of axial chlorine atoms in the HCH molecule.

The dechlorination of other chlorinated compounds like 1,2-dichloroethane and tetrachloroethene was also investigated with the coculture. 12DCA (50 and 250 μM) was not dechlorinated by the coculture during an incubation of 120 days. The coculture was also not able to dechlorinate PCE (90 μM and 10 mM) within five months. Apparently, the *Dehalobacter* in the coculture differs from *D. restrictus* [16]. This *Dehalobacter* was initially enriched in our laboratory [21] and it is able to reductively dechlorinate tetrachloroethene and trichloroethene with H_2 as electron donor. Recently, a bacterium related to *Dehalobacter* was found that is capable to reductively dechlorinate trichloroethane to chloroethane [20]. The identification of our strain as a *Dehalobacter* species indicates that the dechlorination capacity of members of this genus is not restricted to chlorinated ethenes and ethanes.

3.3. Role of *Sedimentibacter*

The role of the *Sedimentibacter* in the coculture has not yet been clarified. *Sedimentibacter* is not able to dechlorinate. Moreover, growth of *Sedimentibacter* alone was not observed in the mineral medium as used in the experiments here (data not shown). *S. hydroxybenzoicus* and *S. saalensis* require the presence of yeast extract for growth [30], thus the absence of this compound may explain its low growth yield in our medium and the lack of growth in the presence of lactate, glucose or pyruvate (data not shown). When grown in coculture with *Dehalobacter* ultimately about 10% of the bacteria present are *Sedimentibacter* (Fig. 2). On the other hand, *Dehalobacter* is not capable of β -HCH dechlorination in the absence of *Sedimentibacter* indicating that the presence of the latter is essential for the growth of *Dehalobacter*. We suggest that *Sedimentibacter* stimulates the transformation of β -HCH via the excretion of growth factors like vitamins, amino acids or other compounds. The nature of the stimulating compound(s) is not known. It is remarkable that *Sedimentibacter* related species have more often been found in dechlorinating cultures [34,35].

Acknowledgements

We thank Hans Heilig for help with the molecular analyses. Akzo Nobel Chemicals BV is acknowledged for kindly providing the soil sample. Funding was provided by the Dutch Technology Foundation NWO-STW (Project No. WBI.4483).

References

- [1] Li, Y.F. (1999) Global technical hexachlorocyclohexane usage and its contamination consequences in the environment: from 1948 to 1997. *Sci. Total Environ.* 232, 121–158.
- [2] The Pesticide Manual (incorporating the Agrochemicals Handbook 1994) Crop Protection Publications (Tomlin, C., Ed.) Bath Press, Bath.
- [3] Breivik, K., Pacyna, J.M. and Münch, J. (1999) Use of α -, β - and γ -hexachlorocyclohexane in Europe, 1970–1996. *Sci. Total Environ.* 239, 151–163.
- [4] Ulrich, E.M., Caperell-Grant, A., Jung, S.-H., Hites, R.A. and Bigsby, R.M. (2000) Environmentally relevant xenoestrogen tissue concentrations correlated to biological response in mice. *Environ. Health Persp.* 108, 973–977.
- [5] Bachmann, A., Walet, P., Wijnen, P., de Bruin, W., Huntjens, J.L.M., Roelofsen, W. and Zehnder, A.J.B. (1988) Biodegradation of alpha- and beta-hexachlorocyclohexane in a soil slurry under different redox conditions. *Appl. Environ. Microbiol.* 54, 143–149.
- [6] Buser, H. and Muller, M.D. (1995) Isomer and enantioselective degradation of hexachlorocyclohexane isomers in sewage sludge under anaerobic conditions. *Environ. Sci. Technol.* 29, 664–672.
- [7] Haider, K. and Jagnow, G. (1975) Abbau von ^{14}C -, ^3H - und ^{36}Cl -markierten γ -hexachlorocyclohexan durch anaerobe bodenmikroorganismen. *Arch. Microbiol.* 104, 113–121.
- [8] Middeldorp, P.J.M., Jaspers, M., Zehnder, A.J.B. and Schraa, G. (1996) Biotransformation of α -, β -, γ -, and δ -hexachlorocyclohexane under methanogenic conditions. *Environ. Sci. Technol.* 30, 2345–2349.
- [9] Beurskens, J.E.M., Stams, A.J.M., Zehnder, A.J.B. and Bachmann, A. (1991) Relative biochemical reactivity of three hexachlorocyclohexane isomers. *Ecotox. Environ. Safe.* 21, 128–136.
- [10] Gupta, A., Kaushik, C.P. and Kaushik, A. (2000) Degradation of hexachlorocyclohexane (HCH; α , β , γ and δ) by *Bacillus circulans* and *Bacillus brevis* isolated from soil contaminated with HCH. *Soil Biol. Biochem.* 32, 1803–1805.
- [11] Sahu, S.K., Patnaik, K.K., Bhuyan, S., Sreedharan, B., Kurihara, N., Adhya, T.K. and Sethunathan, N. (1995) Mineralization of α -, γ -, and β -isomers of hexachlorocyclohexane by a soil bacterium under aerobic conditions. *J. Agric. Food Chem.* 43, 833–837.
- [12] Jagnow, G., Haider, K. and Ellwardt, P. (1977) Anaerobic dechlorination and degradation of hexachlorocyclohexane isomers by anaerobic and facultative anaerobic bacteria. *Arch. Microbiol.* 115, 285–292.
- [13] van Eekert, M.H.A., van Ras, N.J.P., Mentink, G.H., Rijnaarts, H.H.M., Stams, A.J.M., Field, J.A. and Schraa, G. (1998) Anaerobic transformation of β -hexachlorocyclohexane by methanogenic granular sludge and soil microflora. *Environ. Sci. Technol.* 32, 3299–3304.
- [14] Ohisa, N., Kurihara, N. and Nakajima, M. (1982) ATP synthesis associated with the conversion of hexachlorocyclohexane related compounds. *Arch. Microbiol.* 131, 330–333.
- [15] DeWeerd, K.A., Mandelco, L., Tanner, S., Woese, C.R. and Sulflita, J.M. (1990) *Desulfomonile tiedje* gen. nov. and sp. nov., a novel anaerobic, dehalogenating, sulfate-reducing bacterium. *Arch. Microbiol.* 154, 23–30.
- [16] Holliger, C., Hahn, D., Harmsen, H., Ludwig, W., Schumacher, W., Tindall, B., Vazquez, F., Weiss, N. and Zehnder, A.J.B. (1998) *Dehalobacter restrictus* gen. nov. and sp. nov., a strictly anaerobic bacterium that reductively dechlorinates tetra- and trichloroethene in an anaerobic respiration. *Arch. Microbiol.* 169, 313–321.
- [17] El Fantroussi, S., Naveau, H. and Agathos, S.N. (1998) Anaerobic dechlorinating bacteria. *Biotechnol. Progr.* 14, 167–188.
- [18] Maymó-Gatell, X., Chien, Y.T., Gossett, J.M. and Zinder, S.H. (1997) Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. *Science* 276, 1568–1571.
- [19] de Wildeman, S., Diekert, G., van Langenhove, H. and Verstraete, W. (2003) Stereoselective microbial dehalorespiration with vicinal dichlorinated alkanes. *Appl. Environ. Microbiol.* 69, 5643–5647.
- [20] Sun, B., Griffin, B.M., Ayala-del-Rio, H.L., Hashsham, S.A. and Tiedje, J.M. (2002) Microbial dehalorespiration with 1,1,1-trichloroethane. *Science* 298, 1023–1025.
- [21] Holliger, C., Schraa, G., Stams, A.J.M. and Zehnder, A.J.B. (1993) A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. *Appl. Environ. Microbiol.* 59, 2991–2997.
- [22] Beaty, P.S., Wofford, N.Q. and McInerney, M.J. (1987) Separation of *Syntrophomonas wolfei* from *Methanospirillum hungatii* in syntrophic cocultures by using Percoll gradients. *Appl. Environ. Microbiol.* 53, 1183–1185.
- [23] de Bok, F.A.M., Luijten, M.L.G.C. and Stams, A.J.M. (2002) Biochemical evidence for formate transfer in syntrophic propionate-oxidizing cocultures of *Syntrophobacter fumaroxidans* and *Methanospirillum hungatei*. *Appl. Environ. Microbiol.* 68, 4247–4252.
- [24] Scholten, J.C.M. and Stams, A.J.M. (1995) The effect of sulfate and nitrate on methane formation in a freshwater sediment. *Antonie Leeuwenhoek* 68, 309–315.
- [25] Lane, D.J. (1991) 16S/23S rRNA sequencing In: *Nucleic Acid Techniques in Bacterial Systematics* (Stackebrandt, E. and Goodfellow, M., Eds.), pp. 115–175. Wiley, Chichester.
- [26] Nübel, U., Engelen, B., Felske, A., Snaird, J., Wieshuber, A., Amann, R.I., Ludwig, W. and Backhaus, H. (1996) Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *J. Bacteriol.* 178, 5636–5643.
- [27] Muyzer, G., de Waal, E.C. and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59, 695–700.
- [28] Heilig, H.G.H.J., Zoetendal, E.G., Vaughan, E.E., Marteau, P., Akkermans, A.D.L. and de Vos, W.M. (2002) Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. *Appl. Environ. Microbiol.* 68, 114–123.
- [29] Sanguinetti, C.J., Dias Neto, E. and Simpson, A.J.G. (1994) Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. *Biotechniques* 17, 915–919.
- [30] Breitenstein, A., Wiegel, J., Haertig, C., Weiss, N., Andreesen, J.R. and Lechner, U. (2002) Reclassification of *Clostridium hydroxybenzoicum* as *Sedimentibacter hydroxybenzoicus* gen. nov., comb. nov., and description of *Sedimentibacter saalensis* sp. nov. *Int. J. Sys. Evol. Microbiol.* 52, 801–807.
- [31] Ueda, K., Seki, T., Kudo, T., Yoshida, T. and Kataoka, M. (1999) Two distinct mechanisms cause heterogeneity of 16S rRNA. *J. Bacteriol.* 181, 78–82.
- [32] Lovley, D.R. (2000) Anaerobic benzene degradation. *Biodegradation* 11, 107–116.

- [33] Coates, J.D., Chakraborty, R., Lack, J.G., O'Connor, S.M., Cole, K.A., Bender, K.S. and Achenbach, L.A. (2001) Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of *Dechloromonas*. *Nature* 411, 1039–1043.
- [34] Breitenstein, A., Saano, A., Salkinoja-Salonen, M., Andreesen, J.R. and Lechner, U. (2001) Analysis of a 2,4,6-trichlorophenol-dehalogenating enrichment culture and isolation of the dehalogenating member *Desulfitobacterium frappieri* strain TCP-A. *Arch. Microbiol.* 175, 133–142.
- [35] Gu, A.Z., Hedlund, B.P., Staley, J.T., Strand, S.E. and Stensel, H.D. (2004) Analysis and comparison of the microbial community structures of two enrichment cultures capable of reductively dechlorinating TCE and *cis*-DCE. *Environ. Microbiol.* 6, 45–54.