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Characterization of a Highly Enriched Microbial Consortium Reductively Dechlorinating 2,3-Dichlorophenol and 2,4,6-Trichlorophenol and the Corresponding *cpr*A Genes from River Sediment

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Abstract

Anaerobic reductive dechlorination of 2,3-dichlorophenol (2,3DCP) and 2,4,6-trichlorophenol (2,4,6TCP) was investigated in microcosms from River Nile sediment. A stable sediment-free anaerobic microbial consortium reductively dechlorinating 2,3DCP and 2,4,6TCP was established. Defined sediment-free cultures showing stable dechlorination were restricted to *ortho* chlorine when enriched with hydrogen as the electron donor, acetate as the carbon source, and either 2,3-DCP or 2,4,6-TCP as electron acceptors. When acetate, formate, or pyruvate were used as electron donors, dechlorination activity was lost. Only lactate can replace dihydrogen as an electron donor. However, the dechlorination potential was decreased after successive transfers. To reveal chlororespiring species, the microbial community structure of chlorophenol-reductive dechlorinating enrichment cultures was analyzed by PCR-denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments. Eight dominant bacteria were detected in the dechlorinating microcosms including members of the genera *Citrobacter*, *Geobacter*, *Pseudomonas*, *Desulfitobacterium*, *Desulfovibrio* and *Clostridium.* Highly enriched dechlorinating cultures were dominated by four bacterial species belonging to the genera *Pseudomonas*, *Desulfitobacterium*, and *Clostridium*. *Desulfitobacterium* represented the major fraction in DGGE profiles indicating its importance in dechlorination activity, which was further confirmed by its absence resulting in complete loss of dechlorination. Reductive dechlorination was confirmed by the stoichiometric dechlorination of 2,3DCP and 2,4,6TCP to metabolites with less chloride groups and by the detection of chlorophenol RD *cpr*A gene fragments in dechlorinating cultures. PCR amplified *cpr*A gene fragments were cloned and sequenced and found to cluster with the *cpr*A/*pce*A type genes of *Dehalobacter restrictus*.

Key words: 16S rDNA, chlorophenol, *cpr*A gene, DGGE, reductive dechlorination

Introduction

Chlorinated phenols (CPs) are common chemical pollutants harmful to human health that are found in ground water due to unmanaged industrial discharges (WHO, 1998). Chlorinated phenols have been extensively used in the manufacture of pesticides, herbicides and dyes and are therefore commonly found in sediments, soil and water resources near industrial wastewater effluents (WHO, 1989; Takeuchi *et al*., 2000). Concern about these compounds relates directly to their high toxicity and persistence in a variety of habitats to levels exceeding natural limits. Chlorinated phenols tend to accumulate in anaerobic habitats, evading aerobic microorganisms that are able to degrade them (Villemur, 2013). The biodegradation of CPs in anaerobic environments has been extensively studied (Häggblom, 1992; McAllister *et al*., 1996; Masunaga *et al*., 1996). Recently, much attention has been focused on anaerobic bacteria that can dechlorinate chlorinated hydrocarbons in a unique process called reductive dechlorination (El Fantroussi *et al*., 1998; Holliger *et al*., 2003). Reductive dechlorination is driven by the activity of a class of enzymes called reductive dehalogenases (RDs). In this process, organohalide-respiring bacteria couple dechlorination to energy generation and growth using chlorinated hydrocarbons as electron acceptors (Holliger, *et al*., 1998; Smidt *et al*., 2000; Löffler *et al*., 2003; Smidt and de Vos, 2004). Dehalorespiring bacteria are found in soil, sediments, and wastewater sludge, and are able to dechlorinate different types of chloroorganics *via* a reductive dechlorination process (Häggblom and Bossert, 2003). However, many of these bacteria remain uncultured. Dehalorespiration was first reported by Deweed *et al*. (1990) in *Desulfomonile tiedjei*, an organism that couples reductive dechlorination

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of 3-chlorobenzoate to formate oxidation. A number of dehalorespiring bacteria have since been described, however, those reducing chlorinated phenols are restricted to a few strains belonging to a few genera. A facultative anaerobic *Myxobacterium*, *Anaeromyxobacter dehalogenans* 2CP-1, has been described as a halorespiring bacteria reducing 2CP and 2,6-dichlorophenol (2,6DCP) (Cole *et al*., 1994; Sanford *et al*., 2002). In this case, dechlorination was found mostly to be site specific. *Desulfovibrio dechloracetivorans* SF3 showed *ortho* dechlorination of 2CP and 2,6DCP (Sun *et al*., 2000). The genus *Desulfitobacteria* was found to contain a relatively large number of strains capable of dechlorination of both aliphatic and aromatic hydrocarbons (Villemur *et al*., 2006). The first *Desulfitobacterium* strain, DCB-2, was isolated in 1992 (Madsen and Licht, 1992). *Desulfitobacterium dehalogenans* strain JW/IU-DC1, first documented by Utkin *et al.* (1994), was reported for its dechlorination of 2,3DCP, 2,6DCP, and 2,4,6TCP (Wiegel *et al*., 1999). Subsequently, other strains belonging to the genus *Desulfitobacterium* with dechlorination activity toward CPs were isolated and characterized, these included *Desulfitobacterium chlororespirans* strain Co23 (Sanford *et al*., 1996; Cupples *et al*., 2005), *Desulfitobacterium hafniense* strain DCB-2 (Christiansen and Ahring, 1996), strain TCP-A (Breitenstein *et al*., 2001), and strain PCP-1 (Bouchard *et al*., 1996; Villemur, 2013). The obligate anaerobic bacterium *Dehalococcoides ethenogenes* strain 195 known for its ability to dechlorinate tetrachloroethene (Maymo-Gatell *et al*., 1997) was recently reported to possess diverse dehalogenation ability. Genomic studies revealed the presence of 17 putative dehalogenase gene homologues in strain 195 that reflect its ability to dechlorinate different chlorinated aromatic hydrocarbons including different chlorophenol congeners (Fennell *et al*., 2004). Dehalogenation in the environment, particularly dechlorination activities in sedimentary environments, is an area of research interest due to its important ecological prospective (Häggblom and Bossert, 2003; Leys *et al*., 2013). Dechlorination of mono- and di-chlorophenol isomers in estuarine anaerobic sedimentary environments has been studied and chlorophenol-dechlorinating microbial communities in such environments have been characterized (Itoh *et al*., 2010). The sludge of related wastewater industrial effluents is a good candidate for studying reductive dechlorination of aryl halides. The dechlorination of pentachlorophenols was detected in the sludge from a pulp and paper mill (Karn *et al*., 2011). Reductive dechlorination of CPs, biphenyls, and benzenes has been reported in a number of sedimentary environments (Wu *et al*., 1998; Fagervold *et al*., 2005; Zanaroli *et al*., 2010; Kjellerup *et al*., 2014; Kuokkaa *et al*., 2014; Vandermeeren *et al*., 2014).

This study aims to assess whether the potential for microbial reductive dechlorination of aryl halides, such as CPs, exists in River Nile sediments and to characterize the bacterial community structure in reductive dechlorinating enrichment cultures developed for the establishment of a highly enriched bacterial consortium for reductive dechlorination of CPs.

Experimental

Materials and Methods

Sampling and anaerobic microcosms. Sediment samples were collected in 100-ml leak-proof stopper-supported screw-capped Duran bottles from the River Nile at active industrialized areas located in Helwan, Egypt (29°51′N, 31°20′E). Sediment samples were spiked with 2,3DCP and 2,4,6-trichlorophenol (2,4,6TCP), flushed with hydrogen, and stored anaerobically in the dark for almost one year prior to use. Screening for dechlorinating bacteria was performed after the establishment of active microcosms and enrichment cultures. Microcosms were initiated by the addition of 10 g of sediment slurry into 90 ml of basal mineral medium in 160 ml serum bottles capped with Teflon-coated butyl rubber stoppers and sealed with aluminum caps. The head space was flushed with hydrogen gas. 2,3DCP and 2,4,6TCP were separately added to triplicate microcosms to a final concentration of 100 μM. Original unamended sediment slurry was used as a control. Microcosms were kept at room temperature for monitoring purposes at different time intervals.

Anaerobic enrichment cultures. Microcosms and anaerobic enrichment cultures were established using defined mineral salts medium containing (per liter): NH_4Cl, 0.5 g; K_2HPO_4, 0.4 g; $MgCl_2 \cdot 6H_2O$, 0.49 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; KCl, 0.052 g; metal solution, 1 ml; vitamin solution, 20 ml. The metal solution had the following composition (per liter): $ZnSO_4 \cdot 7H_2O$, 0.001 g; $CuSO_4 \cdot 5H_2O$, 0.002 g; $MnSO_4 \cdot 5H_2O$, 0.001 g; $NaMoO_4.2H_2O$, 0.0005 g; $CoCl_2 \cdot 6H_2O$, 0.0005 g; $NiCl_2 \cdot \cdot 6H_2O$, 0.001 g; $Na_2SeO_4 \cdot 10H_2O$, 0.001 g; $FeSO_4 \cdot 7H_2O$, 0.005 g. The vitamin solution had the following composition (per liter): biotin, 0.002 g; folic acid, 0.002 g; pyridoxine-HCl, 0.01 g; thiamine-HCl, 0.005 g; riboflavin, 0.005 g; nicotinic acid, 0.005 g; p-aminobenzoic acid, 0.005 g; vitamin B12, 0.001 g. Acetate (5 mM) was used as the carbon source. The pH of the medium was adjusted to 7.0 using HCl. From the stock medium solution, 9 ml were dispensed into 22 ml anaerobic vials and 2,3DCP or 2,4,6TCP were added as the main terminal electron acceptor after autoclaving to a final concentration of 50 μM. Vials were capped with Teflon-coated butyl rubber stoppers and sealed with aluminum

caps. The head space was flushed with hydrogen gas. To remove dissolved oxygen and ensure anaerobic conditions, the medium was reduced with Ti(III)-citrate, with resazurin added as an indicator. Active cultures were maintained by transferring 1 ml into 9 ml of freshly prepared medium. Enrichment sediment-free cultures were obtained after at least five successive transfers of active cultures (10%) into new medium containing CPs as electron acceptors. All cultures were incubated inverted at 30°C in the dark without shaking and were monitored for dechlorination activity by gas chromatographic analysis. Cultures showing dechlorination were diluted in the same medium and reincubated under the same conditions until sediment-free cultures were obtained.

Tested electron donors and acceptors. Different electron donors including, hydrogen, formate, lactate, pyruvate, and acetate at 5-mM concentrations were tested. Chlorinated phenols, formate, lactate, pyruvate, acetate, and thiosulfate were tested as electron acceptors. When being used as a main electron acceptor, CPs were added to cultures at a final concentration of 50 μM.

Chemicals. Chlorinated phenol standards were obtained from Sigma-Aldrich (Deisenhofen, Germany) and were used as authentic samples to characterize unknown compounds.

Chemical analyses. Chlorinated phenols were analyzed after extraction from culture medium with methylene chloride using a gas chromatograph (GC-396B, GL Science, Japan) equipped with an Aquatic column (60 m × 0.32 mm I.D., 1.0 μm df; GL Science) with an online Tekmar Dohrman headspace autosampler. A GC-4000 gas chromatograph (GL Science) equipped with a J&W Scientific column (30 m × 0.53 mm I.D., 1.0 μm df) was also used for direct sample injection and analysis. The following program was used to achieve maximum separation between peaks: column oven temperature, 40°C for 3 min, 40°C to 80°C at a rate of 8°C/min, 80°C to 190°C at 44°C/min, 190°C for 5 min. Helium was used as the carrier gas. Chlorophenols concentrations were represented as means of three measurements from three independent experiments (±SD).

DNA extraction and PCR amplification. Total community DNA was extracted using an Ultra Clean Soil DNA purification kit (Mo Bio Laboratories, Solana Beach, CA, USA). About 0.5 g from established microcosms or harvested cells from enrichment cultures were transferred to bead beating tubes and vortexed horizontally for 2 min at room temperature. DNA was precipitated and purified according to the instruction manual. Amplification of the 16S rRNA genes for DGGE analysis was performed using GC-clamp primers (EUB341F-GC: 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAGCAG-3′ and EUB517R: 5′-ATTACCGCGGCTGCTGG-3′) (Muyzer *et al*., 1993). Amplification was performed in a 25 μl reaction containing: 2.5 μl of 10 × *Taq* buffer (100 mM Tris-HCl, pH 8), 1.25 mM $MgCl_2$, 100 μM dNTPs (Invitrogen, Carlsbad, CA, USA), 1.2 μM forward primer and reverse primer (Invitrogen), 0.5 U *Taq* DNA polymerase (Invitrogen), and about 5 ng of template DNA. PCR was performed in a Thermal Cycler (2720; Applied Biosystems, Foster City, CA, USA). A touchdown PCR program was implemented as follows: initial denaturation step at 95°C for 5 min; 5 cycles of 94°C for 40 s, annealing at 65°C for 40 s, and extension at 72°C for 40 s; 5 cycles of 94°C for 40 s, annealing at 60°C for 40 s, and extension at 72°C for 40 s; 10 cycles of 94°C for 40 s, annealing at 55°C for 40 s, and extension at 72°C for 40 s; 10 cycles of 94°C for 40 s, annealing at 50°C for 40 s, and extension at 72°C for 40 s were performed, followed by a final hold at 72°C for 7 min. Amplicons were analyzed by electrophoresis on 1% agarose gels with size markers (1 kb DNA ladder, Invitrogen) and visualized using ethidium bromide. Amplification of the *cpr*A genes was performed with a combination of forward (dehaloF4: 5′-(C/T)(C/A)T(G/T)GGTTA(C/T)(A/T)ATGC-3′) and reverse (dehaloR2: 5′-T(C/T)TGTACCATAGCC-3′) primers, which were designed from the conserved region in the *cpr*A gene of *D. dehalogenans* (von Wintzingerode *et al*., 2001). The amplification protocol included an initial denaturation step at 95°C for 5 min, 30 cycles of 94°C for 1 min, annealing at 50°C for 2 min, and extension at 72°C for 2 min, followed by a final elongation step at 72°C for 7 min. The amplified fragment was cloned into the pGEM-T Easy vector for sequencing.

DGGE. DGGE was performed using the Dcode Mutation Detection System (Bio-Rad Laboratories Ltd., Hertfordshire, UK). PCR products were electrophoresed on 8% polyacrylamide gels containing a 25% to 50% denaturing gradient of formamide and urea. Electrophoresis was performed in 0.5 × TAE buffer (1 × TAE buffer contains 0.04 M Tris base, 0.02 M sodium acetate, and 10 mM EDTA, pH 7.4) at 60°C for 5 h at 200 V. Gels were stained with SYBR Green I nucleic acid gel stain (Cambrex Bio Science, Rockland, MA, USA) and analyzed for DGGE band profiles with a UV Gel Doc XR documentation system (Bio-Rad Laboratories Inc., Hercules, CA, USA). Selected DGGE bands were excised and eluted by incubation in 100 μl of TE buffer at 100°C for 5 min. The supernatant was used as a template for reamplification of the 16S rRNA genes using bacterial primers EUB314F, without the GC clamp, and EUB517R, prior to sequencing.

Cloning and sequencing. PCR products were ligated to the pGEM-T Easy vector (Promega, Madison, WI, USA) and used to transform *Escherichia coli* JM109 (Takara, Tokyo, Japan) according to the manufacturer's recommendations. Recombinant *E. coli* clones were

screened for correct inserts by colony PCR. Recombinant plasmid DNA of positive clones was extracted using the Wizard Plus SV Miniprep DNA purification system (Promega) and used for sequencing. BigDye terminator cycle sequencing (Sanger *et al*., 1977) was performed with an ABI 310 genetic analyzer (Applied Biosystems) using dye terminator-based PCR products amplified with the vector-specific forward primer M13F according to the instruction manual.

Sequence and phylogenetic analysis. Obtained sequences were processed for analysis using the *Genetyx-Win* MFC application software version 4.0. Sequences were identified and compared with their nearest phylogenetic neighbors using the BLAST search program, National Center for Biotechnology Information (NCBI), National Library of Medicine, USA (www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*., 1997). Sequence alignments were performed using *Clustal W*1.83 XP software (Thompson *et al*., 1997) and phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei, 1987) and *MEGA6* software (Kumar *et al*., 2004).

Nucleotide sequence accession numbers. Sequences of the 16S rDNA gene identified in this study have been deposited in the GenBank database under the accession numbers (LC010626-LC010637). The *cpr*A gene sequences have been deposited under the accession numbers (LC010638-LC010645).

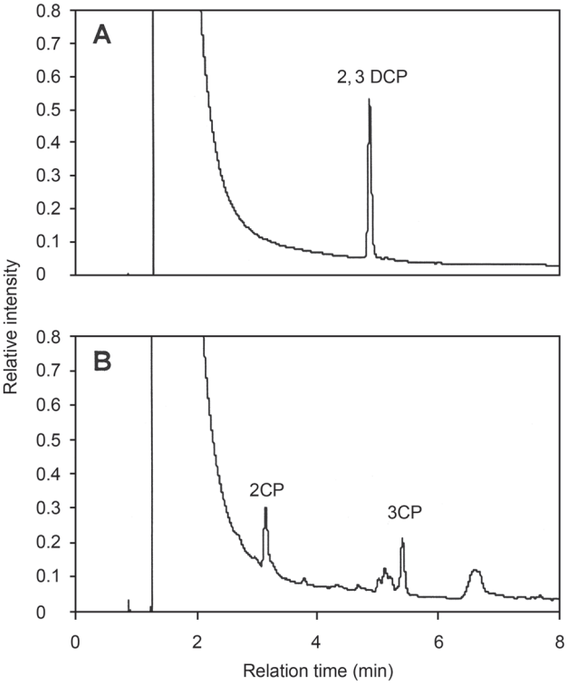

Fig. 1. Detection of dechlorination activity in established microcosms.

(A) GC chromatogram showing 2,3DCP as a starting compound spiked to microcosms. (B) GC chromatogram showing transformation of 2,3DCP to 2CP and 3CP after prolonged time of incubation as a result of dechlorination activity.

Results

Dechlorination in established anaerobic cultures. Microcosms and enrichment cultures maintained under optimum conditions of temperature and pH showed a dechlorination activity against CPs. Figure 1 shows the detection of 2CP and 3CP as dechlorination products from microcosms amended with 2,3DCP. In enrichment cultures, complete dechlorination of 50 μM 2,3DCP to 3CP and 2,4,6TCP to 2,4DCP, 4CP within 3 weeks of incubation was observed (Fig. 2). Dechlorination activity was favored with *ortho* chlorine, however, detection of trace amounts of 2CP in 2,3DCP-dechlorinating cultures revealed the weak dechlorination activity of *meta* chlorine. Dechlorination was found to be mainly site specific. 2,4,6TCP exhibited dechlorination of *ortho*-chlorine producing mainly 2,4DCP. Dechlorination beyond 2,4DCP yield 4CP mainly with trace amounts of 2CP. Figure 3 shows the proposed reductive dechlorination routes for both 2,3DCP and 2,4,6TCP in established cultures. Dechlorination and growth were found to be dependent on the presence of acetate as a carbon source in the culture medium. Hydrogen was found to be necessary as an electron donor for efficient dechlorination of 2,3DCP and 2,4,6TCP. Among different organic acids tested, only lactate can replace hydrogen as an electron donor. However, the rate of dechlorination was decreased and cultures lost dechlorination activity after successive transfers.

Bacterial community structure in dechlorinating cultures. The DGGE fingerprints obtained in this study with metagenomic DNA from microcosms amended with 2,3DCP and 2,4,6TCP showed changes in the bacterial community structure from the original sediment. Community fingerprints of microcosms supplemented with either 2,3DCP or 2,4,6TCP were stable and showed no significant changes in the DGGE profiles even after a prolonged incubation time. The DGGE profiles for 2,3DCP or 2,4,6TCP dechlorinating microcosms showed the predominance of eight major Operational Taxonomic Units (OTUs) (Fig. 4A). Sequencing of the dominant DGGE bands of 16S rRNA gene assigned these OTUs to members of the genera *Citrobacter*, *Geobacter*, *Pseudomonas*, *Desulfitobacterium*, *Desulfovibrio*, and *Clostridium* (Table I). Different DGGE profiles were observed when enrichment cultures were compared with dechlorinating microcosms. Enrichment resulted in loss of at least four of the microcosm bacterial members, represented by bands A1 (*Citrobacter* sp.), A2 (*Geobacter* sp.), A6 (*Desulfovibrio* sp.), and A7 (*Clostridium* sp.). Highly enriched dechlorinating

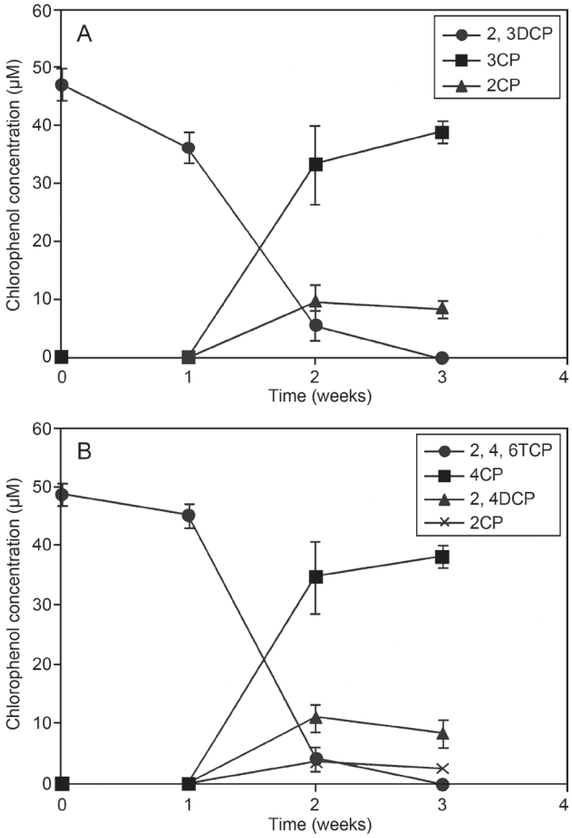

Fig. 2. Changes in chlorophenol concentration in enrichment cultures over time.

(A) Dechlorination of 2,3DCP to 3CP and 2CP. (B) Dechlorination of 2,4,6TCP to 2,4DCP with further dechlorination to 4CP and 2CP. Each bar represents the mean and standard deviation of three replicates samples.

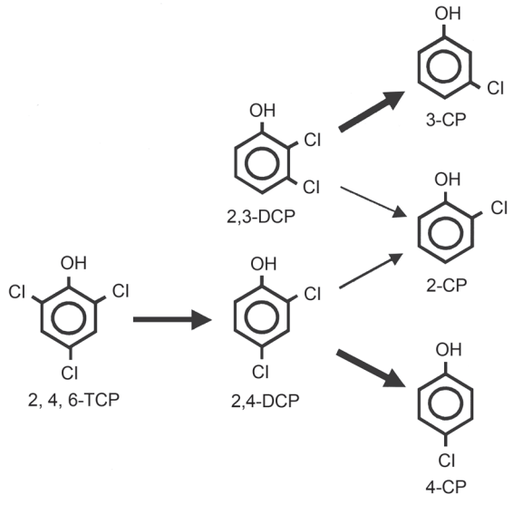

Fig. 3. Proposed pathway for 2,3DCP and 2,4,6TCP dechlorination in highly enriched cultures.

Dechlorination was favored at *ortho* position. Low dechlorination activity was observed at *meta* position of 2,3DCP, or *para* position of 2,4,6TCP. TCP was dechlorinated at *ortho* position successively giving 2,4-DCP and finally 4-CP. Small amount of 2-CP was detected.

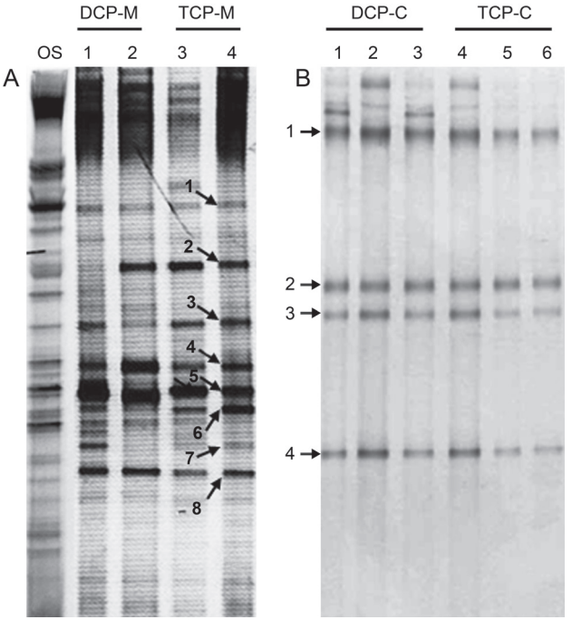

Fig. 4. DGGE fingerprints of 16S rRNA gene fragments from established dechlorinating microcosms (A) and enrichment cultures (B).

Lane OS represents bacterial fingerprint of original sediment. Lane 1,2 represent fingerprints obtained from genomic DNA of DCP dechlorinating microcosms while lane 3,4 represent those from TCP dechlorinating microcosms. Lane 1,2,3 corresponds to bacterial populations of highly enriched DCP dechlorination cultures, while lane 4,5,6 correspond to those of TCP dechlorination cultures.

cultures comprised four major OTUs (Fig. 4B), which were identified as members of the genera *Pseudomonas*, *Desulfitobacterium* and uncultured *Clostridium*.

Identification of dechlorinating bacteria. This study also investigated the microorganisms responsible for dechlorination in sediment microcosms or enrichment cultures. The DGGE microcosm OTU A4 is similar to OTU B2, which was detected in enriched dechlorinating cultures. Both OTUs have been assigned to the genus *Desulfitobacterium*, which is known for its ability to couple dechlorination to growth *via* halorespiration. The DGGE pattern of cultures lacking dechlorination activity revealed the absence of OTU B2, indicating its direct involvement in dechlorination. Direct microscopic examination of highly enriched dechlorinating cultures showed the predominance of curved rod-shaped bacteria, a characteristic morphology of *Desulfitobacterium*. Taken together, these results provide compelling evidence that both OTUs A4 and B2 identified as *Desulfitobacterium* sp. were responsible for dechlorination of 2,3DCP and 2,4,6TCP, lowing the concentration of CPs.

Detection of the chlorophenol RD gene (*cprA*). To detect the chlorophenol RD encoding gene (*cprA* gene) from microcosms and dechlorinating cultures, PCR amplification using the primer set dehaloF4/

Table I
OTUs identified in established microcosms and enrichment cultures with their phylogenetic affiliations.

DGGE bands	Accession No.	Closest match			Phylogenetic affiliation
		Identity	Accession No.	Similarity (%)	
A1	LC010626	*Citrobacter freundii* MW-D 2220	KC835086	98	Gammaproteobacteria
		Citrobacter sp. F3-7	EF491835	98	Gammaproteobacteria
		Enterobacter sp. 18A	HQ289882	98	Gammaproteobacteria
		Klebsiella sp. SUS9K	KF991505	98	Gammaproteobacteria
		Uncultured Enterobacteriaceae bacterium C146500177	JX531197	98	Gammaproteobacteria/Environmental sample
A2	LC010627	*Geobacter lovleyi* Geo7.1A	JN982204	99	Deltaproteobacteria
		Geobacter thiogenes K1	NR028775	99	Deltaproteobacteria
		Uncultured *Geobacter* sp. clone MFC-1-L16	JX944529	99	Deltaproteobacteria/Environmental sample
		Uncultured delta proteobacterium clone CF_05	EF562566	99	Deltaproteobacteria/Environmental sample
		Geobacter sp. enrichment culture clone MC18B6-5	JQ256498	99	Deltaproteobacteria/Environmental sample
A3	LC010628	*Pseudomonas fulva* Z47	KF835747	100	Gammaproteobacteria
		Pseudomonas putida P1	KJ960183	99	Gammaproteobacteria
		Pseudomonas aeruginosa P17	KJ960182	99	Gammaproteobacteria
		Pseudomonas stutzeri 54DMH1	KM025368	99	Gammaproteobacteria
		Pseudomonas sp. IMARCUG-3	KJ918745	99	Gammaproteobacteria
A4	LC010629	*Desulfitobacterium* sp. CR1	AB299028	100	Clostridia
		Desulfitobacterium sp. RPf35Ei	AY548779	97	Clostridia
		Desulfitobacterium dichloroeliminans LMG P-21439	NR102483	96	Clostridia
		Desulfitobacterium hafniense DCB-2	NR121797	95	Clostridia
		Desulfitobacterium dehalogenans JW/IU-DC1	NR044641	95	Clostridia
A5	LC010630	*Pseudomonas putida* strain P1	KJ960183	99	Gammaproteobacteria
		Pseudomonas aeruginosa P17	KJ960182	99	Gammaproteobacteria
		Pseudomonas stutzeri 54DMH1	KM025368	99	Gammaproteobacteria
		Pseudomonas sp. IMARCUG-3	KJ918745	99	Gammaproteobacteria
		Pseudomonas sp. JS-C55	KJ921736	99	Gammaproteobacteria
A6	LC010631	*Desulfovibrio vulgaris* Miyazaki F	CP001197	100	Deltaproteobacteria
		Desulfovibrio sp. wpp1	KF601938	100	Deltaproteobacteria
		Uncultured *Desulfovibrio* sp. clone immo-6	AB936633	100	Deltaproteobacteria/ Environmental sample
		Desulfovibrio sp. enrichment culture clone 2	JF714419	100	Deltaproteobacteria/ Environmental sample
		Uncultured delta proteobacterium clone SRB4	DQ069228	100	Deltaproteobacteria/ Environmental sample
A7	LC010632	Uncultured *Clostridium* sp. clone BacBotHigh001	JF731763	100	Clostridia/Environmental sample
		Clostridium sp. M-43	AB504378	99	Clostridia
		Uncultured bacterium clone PSM25	AB639316	99	Environmental sample
		Clostridium sp. S11-3-10	AB838978	98	Clostridia
		Uncultured *Clostridium* sp. clone XT41	KF511913	97	Clostridia/Environmental sample
A8	LC010633	Uncultured *Clostridium* sp. DGGE-GZ-C7	KC529382	100	Clostridia/Environmental sample
		Uncultured *Clostridium* sp. clone A05	JX545163	100	Clostridia/Environmental sample
		Clostridium sp. enrichment culture DGGE-gz-5d-15	JX470312	100	Clostridia/Environmental sample

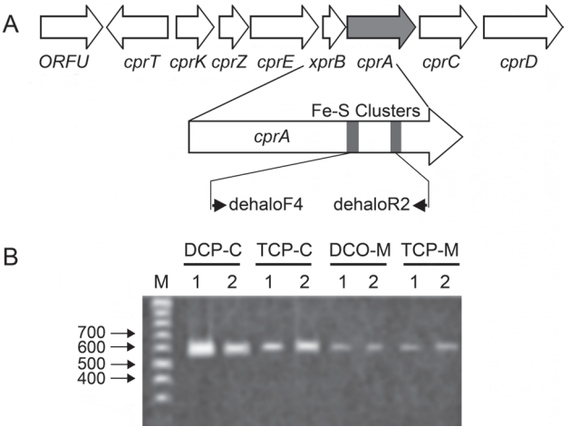

Fig. 5. Detection of chlorophenol reductive dehalogenase encoding gene, *cpr*A in Nile river sediment microcosms and established enrichment cultures.

(A) Schematic representation of the *cpr* gene locus in *D. dehalogenans* (Smidt *et al.*, 2000) showing target *cpr*A gene and the sequenced region in this study covering up the Fe-S cluster. (B) PCR amplification of Fe-S cluster region of *cpr*A clones from DCP and TCP cultures as well as dechlorinating microcosms. Lane M represents the size marker (1 Kb DNA ladder, Invitrogen, USA).

dehaloR2 was performed with an expected product size of approximately 600 bp (Fig. 5B). Eight clones were recovered from DCP and TCP dechlorinating cultures. Sequence analysis of the obtained clones from DCP and TCP dechlorinating cultures showed sequence similarity of 99% to the *cpr*A gene from uncultured bacterium DCB and 96–98% to the *cpr*A gene from *Dehalobacter restrictus*.

Phylogenetic analysis of the *cpr*A genes. When aligned to available sequences in the public databases,

Table I continued

DGGE bands	Accession No.	Closest match			Phylogenetic affiliation
		Identity	Accession No.	Similarity (%)	
A8	LC010633	*Clostridium* sp. enrichment culture clone N6	JQ420069	100	Clostridia/Environmental sample
		Uncultured bacterium clone LBAC143	KJ601180	100	Environmental sample
B1	LC010634	*Pseudomonas stutzeri* YC-YH1	KJ786450	99	Gammaproteobacteria
		Pseudomonas pseudoalcaligenes	LK391695	99	Gammaproteobacteria
		Pseudomonas sp. NEAU-ST5-5	JQ762269	99	Gammaproteobacteria
		Uncultured *Pseudomonas* sp. clone 1-22-20	KJ650703	99	Gammaproteobacteria/Environmental sample
		Uncultured Pseudomonadaceae bacterium c:S2-8094	KF786991	99	Gammaproteobacteria/Environmental sample
B2	LC010635	*Desulfitobacterium* sp. CR1	AB299028	100	Clostridia
		Desulfitobacterium sp. RPf35Ei	AY548779	97	Clostridia
		Desulfitobacterium dichloroeliminans LMG P-21439	NR102483	96	Clostridia
		Desulfitobacterium hafniense DCB-2	NR121797	95	Clostridia
		Desulfitobacterium dehalogenans JW/IU-DC1	NR044641	95	Clostridia
B3	LC010636	*Pseudomonas putida* strain P1	KJ960183	99	Gammaproteobacteria
		Pseudomonas aeruginosa P17	KJ960182	99	Gammaproteobacteria
		Pseudomonas stutzeri 54DMH1	KM025368	99	Gammaproteobacteria
		Pseudomonas sp. IMARCUG-3	KJ918745	99	Gammaproteobacteria
		Pseudomonas sp. JS-C55	KJ921736	99	Gammaproteobacteria
B4	LC010637	Uncultured *Clostridium* sp. DGGE-GZ-C7	KC529382	100	Clostridia/Environmental sample
		Uncultured *Clostridium* sp. clone A05	JX545163	100	Clostridia/Environmental sample
		Clostridium sp. enrichment culture DGGE-gz-5d-15	JX470312	100	Clostridia/Environmental sample
		Clostridium sp. enrichment culture clone N6	JQ420069	100	Clostridia/Environmental sample
		Uncultured bacterium clone LBAC143	KJ601180	100	Environmental sample

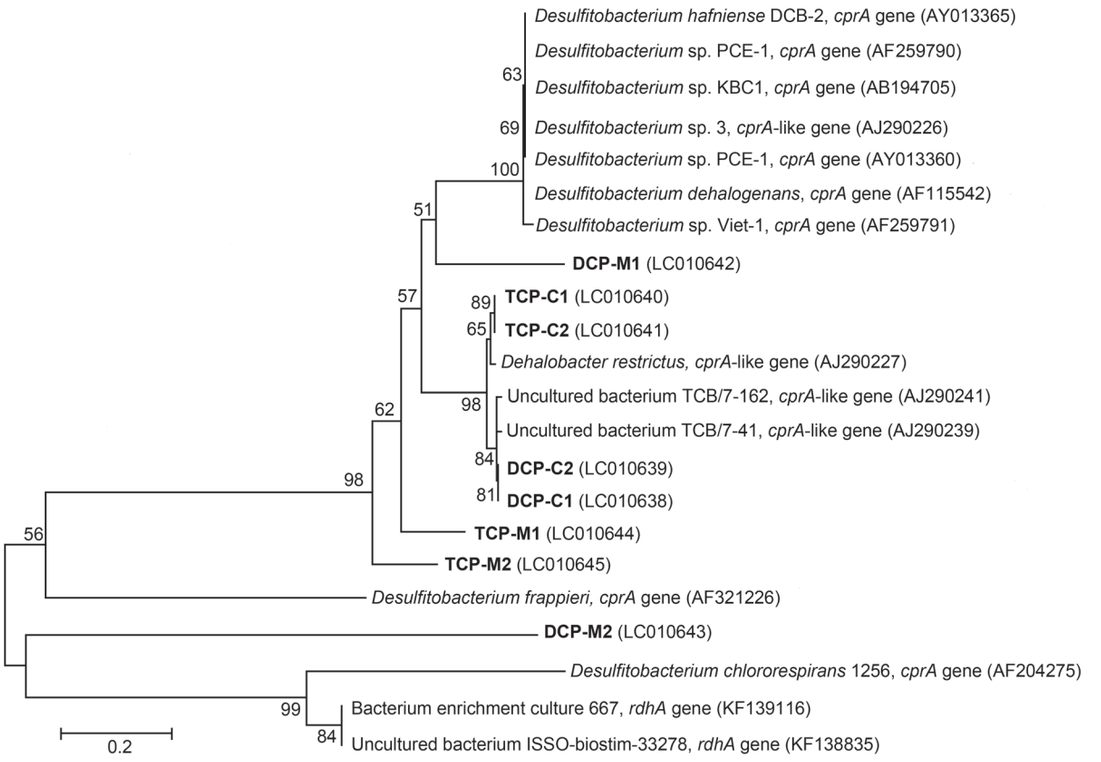

Fig. 6. Neighbour-joining tree showing the phylogenetic distribution of *cpr*A gene fragments from microcosms and enrichment cultures. The dendrogram was constructed from a *ClustalW* alignment and *Mega 3* analysis. Nodes supported by bootstrap values greater than 50 are indicated. Accession numbers of clones (boldface) and reference sequences from GenBank are indicated in parentheses. The scale bar represents substitutions per nucleotide.

the *cpr*A genes cloned from microcosms was closely related to the *cpr*A gene from uncultured bacterium TCB (97%), *D. restrictus* (96%), *Desulfitobacterium frappieri* (82%), *D. hafniense* (82%), and *D. chlororespirans* (84%). The *cpr*A genes cloned from enriched cultures were closely related to those from uncultured bacterium DCP (99%), *D. restrictus* (98%), *D. chlororespirans* (85%), *D. hafniense* (82%), and *D. frappieri* (86%). Figure 6 shows the phylogenetic relationships between the detected *cpr*A clones and those from reported dechlorinating bacteria.

Discussion

Organohalide respiration is a unique metabolic process that is implicated in the global halogen cycle and is of environmental significance for remediation purposes. As part of anaerobic dehalogenation processes, reductive dechlorination has triggered considerable interest for the potential detoxification of anthropogenic groundwater contaminants (Hug and Edwards, 2013). Anaerobic reductive dechlorination has been studied in a number of sedimentary environments (Lake *et al.*, 1992; Alder *et al.*, 1993; Wu *et al.*, 1998; Fagervold *et al.*, 2005; Fung *et al.*, 2009; Itoh *et al.*, 2010). These investigations were made possible using laboratory microcosms. Anaerobic reductive dechlorination of chlorinated benzenes for example was investigated in microcosms containing chloroaromatic-contaminated river sediment (Vandermeeren *et al.*, 2014). Dechlorination activity in enrichment cultures showed at least three different dechlorination reactions. A distinct *ortho*-dechlorinating path for 2,3DCP, 2,4DCP, and 2,4,6TCP was predominant. However, *meta*- and *para*-dechlorination paths also existed with slow rates and restriction to 2,3DCP and 2,4DCP, respectively. Although some strains were reported to exhibit chlorophenols dechlorination of all three substituent (*ortho*, *meta*, and *para*) positions (Thibodeau *et al.*, 2004; Gauthier *et al.*, 2006), this pattern of dechlorination was restricted to the *ortho* position, consistent with dechlorination reported for the anaerobic bacterium, strain PCE1 (Gerritse *et al.*, 1996). In enrichment cultures, dechlorination beyond monochlorophenols was not detected. This pattern of dechlorination is most similar to that reported for *D. chlororespirans*, which was able to dechlorinate *ortho* chlorines of polysubstituted phenols to monohalophenol end products (Sanford *et al.*, 1996). However, the chlorophenol concentration may

represent a limiting factor for dechlorination activity as a higher concentration of CPs may result in reduction of dechlorination activity due to the cytotoxicity of these compounds. It has been reported that bacteria may develop some traits to overcome the cytotoxicity of higher CP concentrations. Surfactant production was found to have a role in overcoming the toxicity of chlorophenols (Chrzanowski *et al*., 2009). Rhamnolipids, in particular, were found to reduce the toxicity of CPs due to their accumulation in biosurfactant micelles and hydrophobic interactions with rhamnolipid-based dissolved organic carbon (Chrzanowski *et al*., 2011).

Dechlorination activity could only be serially transferred in enrichments containing hydrogen as an added electron donor. Dechlorinating cultures have a strict requirement for hydrogen. A variety of alternative electron donors have been reported to support reductive dechlorination (Gerritse *et al*., 1999), however, in this study, electron donors other than hydrogen failed to support dechlorination. Lactate has been found to be used by a number of dehalorespiring bacteria as an electron donor (Li *et al*., 2013). *D. chlororespirans* strain Co23 was able to grow by coupling the oxidation of lactate to the reductive dechlorination of 3-chloro-4-hydroxybenzoate and 2,3-DCP (Sanford *et al*., 1996). However, it was found in this study that cultures growing with lactate were not stable and lost dechlorination activity after successive transfers. Different organic acids are used as the electron donors by some bacterial species; however, hydrogen is thought to be the most important electron donor for dehalorespiration, because of the high affinity of dehalorespiring bacteria for hydrogen (Luijten *et al*., 2004). Competition for hydrogen is known to exist among hydrogen-utilizing microorganisms in anoxic environments. In such habitats, where the hydrogen concentration is limited, the threshold ecosystem hydrogen concentration is controlled by microbial populations that couple hydrogen oxidation to thermodynamically favorable electron accepting reactions, including reductive dehalogenation (Mazur and Jones, 2001; Mazur *et al*., 2003). The establishment of sediment-free highly enriched reductive dechlorinating cultures would require hydrogen amendment to replace that provided by co-cultured bacteria. The presence of acetate was found to be important for sustaining stable dechlorinating cultures. It is suggested that acetate may play an important role as a carbon source to support the growth of dechlorinating bacteria and other associated bacteria. This differs from the reported role of acetate as an electron donor for growth coupled to reductive dechlorination (Sun *et al*., 2000).

The diversity of organohalide-respiring bacteria has not yet been fully described. Ongoing research in this field may lead to the discovery of novel strains, species and even new genera that could be implemented in bioremediation processes (Hug *et al*., 2013). The metagenomes of microbial communities are currently under investigation to reveal the metabolic interactions within organohalide-respiring consortia. Some consortia have been developed and characterized for organohalide-respiring populations and associated bacteria (Duhamel and Edwards, 2007; Rowe *et al*., 2008; Brisson *et al*., 2012; Hug, 2012; Maphosa *et al*., 2012). PCR-DGGE analysis using 16S rRNA genes usually yields patterns that reflect the composition of dominant microorganisms, including the nonculturable members (Gelsomino *et al*., 1999). The DGGE patterns obtained in this study with total community DNA showed changes in the dominant bacterial populations of native river sediment compared with CP amended microcosms. However, the bacterial community structure of 2,3DCP and 2,4,6TCP dechlorinating microcosms were similar and have remained largely intact over time. The DGGE profile for 2,3DCP or 2,4,6TCP dechlorinating microcosms revealed the predominance of eight major OTUs identified as members of the genera *Citrobacter*, *Geobacter*, *Pseudomonas*, *Desulfitobacterium*, *Desulfovibrio*, and *Clostridium*. Based on these detected genera, dechlorination has been assigned to *Desulfitobacteria* due to its reported ability to dechlorinate a variety of aryl halides and couple dechlorination to growth for its respiration (Smidt *et al*., 2000; Löffler *et al*., 2003; Holliger *et al*., 1998). The presence of *Desulfovibrio* in dechlorinating microcosms suggests its possible indirect role in dechlorination *via* syntrophic association with existing dehalorespiring bacteria (Drzyzga *et al*., 2001). *Geobacter*, as well as being detected in established dechlorinating microcosms, has also been suggested to play a role in organohalide respiration (Wagner *et al*., 2012). Cultures devoid of the distinct DGGE band assigned to Desulfitobacterium, lack any dechlorination activity, providing clear evidence that *Desulfitobacterium* sp. play a key role in the dechlorination of 2,3DCP and 2,4,6TCP. *Desulfitobacterium* spp. have been cited in many studies as dechlorinating bacteria (Bouchard *et al*., 1996; Breitenstein *et al*., 2001; Cupples *et al*., 2005; Villemur *et al*., 2006). Highly enriched cultures showed the prevalence of curved rods, a characteristic feature of *Desulfitobacterium* spp. (Villemur *et al*., 2006). Although some physiological characteristics of *Desulfitobacterium* spp. were reported, they did not match the dechlorination requirements in established dechlorinating cultures, suggesting a novel type of *Desulfitobacterium* with alternative physiological requirements. The requirements for dechlorination in this study were found to be similar to those reported for Dehalobacter sp. strain TCP1 when dechlorinating 2,4,6-TCP to 4-CP with hydrogen as the sole electron donor and acetate as the carbon source (Wang *et al*., 2014). Further attempts to isolate dechlorinating

bacteria were unsuccessful indicating the possible existence of syntrophic interactions, as co-culture consortia, within dechlorinating communities.

Reductive dehalogenase (RD) encoding genes (*rdh*A) have been identified in a wide variety of strictly anaerobic aryl halide respiring bacteria (Löffler *et al*., 1996; Christiansen *et al*., 1998; van de Pas *et al*., 1999; Thomas *et al*., 2008). Hug and Edwards (2013) reported that the RD complement within an organism, enrichment culture, or contaminated site, is of critical importance for determining the potential dechlorination activity. In this study, *cpr*A RD genes were detected in dechlorinating microcosms and enrichment cultures (Fig. 5A, B), showing similarity to the *cpr*TKZEBACDA operon of *D. dehalogenans* (Smidt *et al*., 2000). The *cpr*A gene was not detected in cultures lacking dechlorination activities suggesting its key role in catalyzing orthochlorine removal from either 2,3 DCP or 2,4,6-TCP. Analysis of *cpr*A gene clones retrieved from both DCP and TCP dechlorinating cultures revealed eight distinct sequences, some with high sequence similarity to *D. restrictus* and some with relatively low sequence similarly to *Desulfitobacterium* spp. DGGE profiling revealed only one OTU affiliated to *Desulfitobacterium* sp., suggesting the presence of different copies of the *cpr*A gene. *D. hafniense* strain PCP-1 was reported to possess two RDs encoded by *crd*A and *cpr*A5 (Villemur *et al*., 2002). These two RD genes have also been found in several other *Desulfitobacterium* strains (Gauthier *et al*., 2006). The fact that several RD encoding genes exist in *Desulfitobacterium* spp. suggests the presence of distinct but related enzymes to achieve the dehalogenation of several chlorinated compounds.

Conclusion

The current study demonstrated the potential for reductive dechlorination of CPs in the sediment from a contaminated site of the River Nile by naturally occurring bacteria. Highly enriched, stable dechlorinating cultures were established using acetate as the carbon source, hydrogen as the electron donor, and CPs as electron acceptors. Metagenomic analysis of a highly enriched chlorophenol-reductive dechlorinating consortium revealed the predominance of *Pseudomonas*, *Desulfitobacterium*, and two uncultured *Clostridia*. Dechlorination was mainly assigned to *Desulfitobacterium*, as its absence resulted in complete loss of dechlorination. Chlorophenol RD *cpr*A gene fragments were retrieved from dechlorinating cultures. The *cpr*A gene was only detected by amplification in active dechlorinating cultures confirming dehalorespiration of chlorophenols. Several copies of the *cpr*A genes were detected with high similarity to the RD of *D. restrictus*. Phylogenetic analysis based on *cpr*A sequences indicated that dechlorinating cultures had a wide distribution of *cpr*A types. Collectively, these results provide valuable insights into potential bioremediation applications especially in anaerobic environments.

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