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ORIGINAL PAPER

Reductive dechlorination of tetrachloroethene by a stepwise catalysis of different organohalide respiring bacteria and reductive dehalogenases

Julien Maillard · Marie-Paule Charnay · Christophe Regeard · Emmanuelle Rohrbach-Brandt · Katia Rouzeau-Szynalski · Pierre Rossi · Christof Holliger

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Abstract The enrichment culture SL2 dechlorinating tetrachloroethene (PCE) to ethene with strong trichloroethene (TCE) accumulation prior to *cis*-1,2-dichloroethene (*cis*-DCE) formation was analyzed for the presence of organohalide respiring bacteria and reductive dehalogenase genes (*rdhA*). *Sulfurospirillum*-affiliated bacteria were identified to be involved in PCE dechlorination to *cis*-DCE whereas "*Dehalococcoides*"-affiliated bacteria mainly dechlorinated *cis*-DCE to ethene. Two *rdhA* genes highly similar to tetrachloroethene reductive dehalogenase genes (*pceA*) of *S. multivorans* and *S. halorespirans* were present as well as an *rdhA* gene very similar to the trichloroethene reductive dehalogenase gene (*tceA*) of "*Dehalococcoides ethenogenes*" strain 195. A single strand conformation polymorphism (SSCP) method was developed allowing the simultaneous detection of the three *rdhA* genes and the estimation of their abundance. SSCP analysis of different SL2 cultures showed that one *pceA* gene was expressed during PCE dechlorination whereas the second was expressed during TCE dechlorination. The *tceA* gene was involved in *cis*-DCE dechlorination to ethene. Analysis of the internal transcribed spacer region between the 16S and 23S rRNA genes revealed two distinct sequences originating from *Sulfurospirillum* suggesting that two *Sulfurospirillum* populations were present in SL2. Whether each *Sulfurospirillum* population was catalyzing a different dechlorination step could however not be elucidated.

Keywords Reductive dechlorination · Organohalide respiration · Anaerobic degradation · Chlorinated solvents

J. Maillard · M.-P. Charnay · C. Regeard · E. Rohrbach-Brandt · K. Rouzeau-Szynalski · P. Rossi · C. Holliger (✉)
Ecole Polytechnique Fédérale de Lausanne (EPFL), ENAC IIE—Laboratory for Environmental Biotechnology, Station 6, 1015 Lausanne, Switzerland
e-mail: christof.holliger@epfl.ch

Present Address:
M.-P. Charnay
Université de Lausanne, Ecole de Biologie, Quartier UNIL-Sorge, Bâtiment Biophore, 1015 Lausanne, Switzerland

Present Address:
C. Regeard
CNRS UMR 8621, Institut de Génétique et Microbiologie, Bat. 409, Université Paris Sud, 91405 Orsay, France

Present Address:
K. Rouzeau-Szynalski
Product Technology Center-Nestlé, Konolfingen, Switzerland

Present Address:
P. Rossi
EPFL ENAC IIE CEL, Station 2, 1015 Lausanne, Switzerland

Introduction

Reductive dechlorination of tetrachloroethene (PCE), one of the most often encountered groundwater pollutants, is an interesting microbial process from a fundamental as well as application point of view. This naturally occurring process can be used to bioremediate chloroethene-contaminated aquifers either by natural attenuation, by biostimulation or also called enhanced natural attenuation, and, if necessary, by bioaugmentation (Christ et al. 2005; Da Silva et al. 2006; Hood et al. 2008). Different bacteria have been identified that use chloroethenes as terminal electron acceptor in anaerobic respiration (Holliger et al. 2003; Smidt and de Vos 2004). Besides the identification of yet unknown bacterial isolates of new bacterial genera and species, a novel biochemistry of corrinoid-containing enzymes has been discovered involving electron instead of alkyl transfer (Neumann et al. 1996; Kräutler et al. 2003; Maillard et al. 2003; Costentin et al. 2006).

Some isolates of the genera *Desulfitobacterium*, *Dehalobacter*, *Sulfurospirillum*, *Geobacter*, and *Desulfuromonas* can dechlorinate PCE to *cis*-DCE whereas only isolates of "*Dehalococcoides*" spp. have the capability to dechlorinate chloroethenes to ethene in a co-metabolic (Maymo-Gatell et al. 2001; He et al. 2005) or metabolic, respiratory process (Cupples et al. 2003; He et al. 2003; Duhamel et al. 2004; Sung et al. 2006). *Dehalococcoides* spp. have frequently been detected in aquifer samples, especially at sites where dechlorination has gone beyond DCE (Hendrickson et al. 2002; Rahm et al. 2006; Imfeld et al. 2008; Dowideit et al. 2010), as well as in different enrichment cultures (Duhamel et al. 2004; Freeborn et al. 2005; Yang et al. 2005; Grostern and Edwards 2006; Daprato et al. 2007; Rouzeau-Szynalski et al. 2011). A detailed analysis of the enrichment culture KB1 indicated that several *Dehalococcoides* spp. populations have been present probably catalyzing the complete PCE dechlorination with several reductive dehalogenases (Duhamel et al. 2002; Waller et al. 2005). In order to enumerate the number of genes, to follow reductive dehalogenase (*rdhA*) gene expression, and to differentiate between *Dehalococcoides* spp. populations, a quantitative real-time PCR technique has been developed (Johnson et al. 2005) and applied to enrichment cultures (Holmes et al. 2006; Lee et al. 2006) and aquifer samples (Lee et al. 2008). Also these studies have indicated that more than one *Dehalococcoides* population have been involved in complete chloroethene dechlorination.

Besides this co-existence of different *Dehalococcoides* populations probably responsible for the catalysis of different dechlorination steps, the co-existence of organohalide respiring bacteria belonging to different bacterial genera has also been reported. A PCE-dechlorinating culture enriched on different electron donors contained members of the bacterial genera *Desulfitobacterium* and *Dehalococcoides* (Yang et al. 2005). The members of the former genus have dechlorinated PCE to *cis*-DCE whereas the ones of the latter could be associated with the formation of ethene (Bunge et al. 2007). In enrichment cultures from aquifer samples, *Dehalobacter* spp. have been shown to dechlorinate 1,1,2-trichloroethane to vinyl chloride (VC) via a dehalo-elimination reaction whereas *Dehalococcoides* spp. have dechlorinated VC to ethene (Grostern and Edwards 2006). Co-existence of up to four organohalide respiring bacteria has been found in different enrichments dechlorinating PCE with methanol as electron donor with a predominance of *Dehalobacter* spp. and *Dehalococcoides* spp. (Daprato et al. 2007). In a chloroethene and chloroethane-dechlorinating culture forming ethene, *Geobacter* spp. and *Dehalococcoides* spp. were responsible for TCE dechlorination whereas only *Dehalococcoides* dechlorinated beyond DCE and formed ethene (Duhamel and Edwards 2007). Similar results have been found in a bioaugmented laboratory sand column where *Geobacter lovleyi* has been identified as the major PCE-dechlorinating population and *Dehalococcoides* spp. as the ethene-forming one (Amos et al. 2009).

Hence, although single strains of bacteria having the capability to completely dechlorinate PCE exist, such as *Dehalococcoides ethenogenes* strain 195, ethene formation in PCE-contaminated aquifers has probably been the result of multiple organohalide respiring populations catalyzing the different dechlorination steps. In order to better understand the involvement of multiple organohalide respiring bacteria in stepwise catalysis of PCE dechlorination, a detailed analysis of an enrichment culture designated SL2 and containing *Sulfurospirillum* spp. and *Dehalococcoides* spp. has been carried out. For this purpose, a molecular fingerprinting technique based on single strand conformation polymorphism (SSCP) using capillary

electrophoresis for the analysis of reductive dehalogenase genes was developed.

Materials and methods

Chemicals

All chemicals were analytical grade and used without purification. Tetrachloroethene (PCE) (99%) and *n*-hexadecane (99%) were purchased from Acros Organics, Geel, Belgium. Due to the possible presence of inhibiting compounds in synthetic *cis*-DCE, biogenic *cis*-DCE prepared as described by Maymo-Gatell et al. (2001) was used, however *Desulfitobacterium hafniense* strain TCE1 was used as PCE-dechlorinating bacterium. All gases (N_2, CO_2, H_2) were purchased from SLGas, Sauerstoffwerk Lenzburg, Switzerland.

Cultivation technique and source of inoculum

The inoculum biomass of the consortium described in this study originated from a fixed-bed bioreactor treating groundwater that was pumped to surface at a PCE-contaminated site. PCE was completely dechlorinated to ethene in this reactor and the sample was kindly provided by Bioclear B.V., Groningen, The Netherlands, in the framework of the EU FP5 project Maroc. The cultivation of the enrichment cultures was carried out in 100 or 500 ml serum bottles (VWR international AG, Merck, Dietikon, Switzerland) containing 50 or 250 ml of anaerobic medium, and was performed with a two-liquid-phase system that has been described previously (Holliger et al. 1998). The bottles were sealed with Viton® or butyl rubber stoppers and the gas phase of the bottles was replaced with a H_2/CO_2 mixture (4:1, v:v, 1.0 bar overpressure) using a gas exchange system (Druva Sonderventie GmbH, Eppelheim, Germany). PCE was dissolved in hexadecane at a concentration of 100 mM. An aliquot of 5 ml per 50 ml medium was added to inoculated culture bottles by syringe. Acetate (5 mM) was added as carbon source and the cultures were incubated at 30°C in the dark on a rotary shaker at 100 rpm. When mixtures of organic compounds were used as electron donors, H_2 was replaced by N_2 and no acetate was added as carbon source.

Analytical methods

The dechlorination activity of the cultures was followed by measuring the concentration of chloride in the aqueous phase by silver ion titration with a Chloro-counter (Flohr Instrument, Nieuwegein, Netherlands). Chloroethenes and methane were analyzed by gas chromatography as previously described (Maillard et al. 2003). The volatile fatty acids were analyzed using a HPLC (Varian Star 9100, Varian AG, Zug, Switzerland) with a refractive index (RI) detector (ERC-7415 A, Varian). Samples of 1.2 ml were deproteinated by adding 150 µl $Ba(OH)_2$ 0.15 M and 150 µl $ZnSO_4$ 0.15 M, centrifuging 10 min at 11,000×*g*, and filtering with a 0.2 µm filter (Sarsted, Sevelen, Switzerland). The HPLC was equipped with a guard column connected to an organic acid column maintained at 60°C (ORH-801, InterAction INC, Varian). The solvent was 50 mM H_2SO_4 with a flow rate of 0.61 ml/min.

DNA extraction

The kit UltraClean™ Soil DNA Isolation from MoBio Laboratories, Inc. (BIOzym, Landgraaf, Netherlands) was used for the DNA extraction. Aliquots of 15 ml of the enrichment cultures were centrifuged at 29,000×*g* for 5 min at 4°C, the pellet resuspended in 300 µl 0.1 M sodium phosphate buffer (pH 8.0), and the suspension transferred in the extraction tubes provided by MoBio. Some modifications were introduced in the protocol of the Kit MoBio as described previously (Rouzeau-Szynalski et al. 2011). The extracted DNA was quantified with a fluorometer TD-700 (Turner Designs) using the PicoGreen dsDNA quantitation reagent Kit P-7581 from Molecular Probes.

Total RNA extraction and reverse transcription

The SV Total RNA Isolation System Z3101 from Promega was used for total RNA extraction. Cells were harvested from aliquots of 15 ml of the enrichment cultures by centrifugation at 29,000×*g* for 5 min at 4°C. Reverse transcription was performed as follows: 10.5 µl of the RNA extract (70–100 µg/ml) and 0.5 µl of 10 µM reverse primer on the target gene were mixed, incubated for 5 min at 70°C, and cooled on ice. Then 5 µl of AMV RT 5× reaction buffer (Promega), 2.5 µl of 10 mM dNTPs, 11 µl of RNase-free water and 1 µl of

AMV Reverse Transcriptase (30 units) were added. The reaction mixture was incubated for 90 min at 42°C, followed by enzyme denaturation for 15 min at 70°C. Standard PCR conditions were used to amplify cDNA as described below.

PCR amplification of *rdhA* genes and cloning and sequencing of PCR products

All primers used were synthesized by Microsynth GmbH (Balgach, Switzerland). For cloning of the 16S rRNA gene the eubacterial primers Eub-8F (5′-AGAG TTTGATCCTGGCTCAG-3′; position 8–27) and Eub-534R (5′-ATTACCGCGGCTGCTGGC-3′; positions 534–516) were used. Degenerate and specific PCR were performed for the isolation of *rdhA* genes with the methods and primers described previously (Regeard et al. 2004), except for the specific detection of *vcrA* and *bvcA* where the methods and primers vcrA880f/vcrA1018r and bvcA277f/bvcA523r described by Behrens et al. (2008) were used. For cloning, the PCR products obtained with degenerate primers were purified using the PCR Purification Minelute Kit (Qiagen GmbH, Hilden, Germany). PCR products were directly cloned into pGEM-T easy (Promega), and selected using colony PCR with T7 and SP6 standard primers. The detailed cloning and sequencing procedure has been described previously (Regeard et al. 2004).

Terminal restriction fragment length polymorphism (T-RFLP) analysis

The 16S rRNA genes of the microbial communities present in the DNA samples were amplified with eubacterial primers Eub-8F and Eub-534R, the former being fluorescently labeled with HEX (hexachloro-6-carboxy-fluorescine) at the 5′ position. Amplification reactions were performed with a thermocycler T3 (Biometra, Biolabo, Châtel-St-Denis, Switzerland). The details of the whole procedure have been previously described (Rouzeau-Szynalski et al. 2011).

Single strand conformation polymorphism (SSCP) analysis of *rdhA* genes

The two primer sets SpSm1f/SpSm1r and SpDe1f/SpDe1r (Regeard et al. 2004) were used to amplify the approximate 200 bp fragments. The reverse primer SpSm1r was labeled with HEX, the reverse primer SpDe1r with FAM (6-carboxy-fluorescine). The PCR was carried out under the following conditions: 25 µl PCR mixture contained 2.5 µl of Taq DNA Polymerase 10× buffer, 1.0 µl of 2.5 mM dNTPs, 1 µl of 10 µM each primer, 0.2 µl of Taq DNA Polymerase, 100 ng of DNA. The DNA was amplified with the following program: 3 min initial denaturation at 94°C, 26 cycles of 1 min denaturation at 94°C, 30 s of primers annealing at the 52°C, and 1 min of elongation at 72°C. A final extension step of 10 min at 72°C was included. PCR products were purified before further use.

The PCR or RT-PCR products diluted in sterile water at ratios of 1:10 to 1:100 (1 µl) were added to 10.5 µl of HiDi formamide and 0.5 µl of internal size standard GeneScan-400 Rox (ABI Applied Biosystems, Rotkreuz, Switzerland). The samples were denatured for 2 min at 90°C and cooled at 4°C before placing them on ice. Electrophoresis was performed using a ABI Prism® 3100 CE system equipped with capillary tubes (36 cm × 50 µm) filled with a polymer composed of 5.5% GeneScan polymer (ABI Applied Biosystems), 10% glycerol and 1× TBE. After preparation of running buffer, polymer and instrument, electrophoresis was carried out at 15 kV and 25°C for 25 min per 16 samples.

Analysis of the internal transcribed spacer (ITS) region

For the analysis of ITS region between the 16S and 23S rRNA genes of *Sulfurospirillum* spp. the primers ITS-f (5′-GTCGTAACAAGGTAGCCGTA-3′) and ITS-r (5′-GCCAAGGCATCCACC-3′) were used (Cardinale et al. 2004). After a PCR with a temperature gradient, 54°C was chosen as optimal annealing temperature. The reaction mixture, amplification conditions and cloning/sequencing procedures were the same as the ones used for the amplification of the *rdhA* genes. For accurate separation and semi-quantitative analysis of ITS PCR products, the Bioanalyzer (Agilent Technologies) was used with the DNA 1000 kit.

Nucleotide sequence accession number

All sequences obtained in this study were deposited in Genbank under the following accession numbers: 16S rRNA genes (HM745914–HM745916), *rdhA*

genes (HM745917–HM745923), as wells as ITS sequences (HM745924–HM745926).

Results

Dechlorination of PCE by culture SL2

The enrichment SL2 dechlorinating PCE to ethene and obtained from a biomass sample of a fixed-bed bioreactor treating PCE-contaminated groundwater was initially fed with a mixture of seven electron donors containing acetate, formate, propionate, butyrate, ethanol, lactate, and pyruvate. When testing individual electron donors, hydrogen was the electron donor resulting in the most rapid dechlorination rates. Therefore hydrogen was used in the following for routine transfer of SL2 but acetate had to be provided as carbon source. The dechlorination pattern of SL2 was rather peculiar due to the strong initial accumulation of TCE upon PCE dechlorination without formation of *cis*-DCE (Fig. 1). Only when the PCE concentration was low, TCE dechlorination and formation of *cis*-DCE and VC started. PCE dechlorination to VC was completed after 20 days and VC was subsequently dechlorinated only very slowly to ethene.

In order to get indications about the bacteria involved in the different dechlorination steps, SL2 was transferred on PCE as well as *cis*-DCE. The latter transfer line was designated SL2-DCE. On PCE, two transfer lines were created. In one transfer line, designated SL2-PCEa, the culture was only transferred on fresh medium when a large percentage of PCE was dechlorinated to ethene. In the other transfer line, designated SL2-PCEb, transfers were done when TCE dechlorination was almost completed. In addition, a serial dilution was done and only the highest dilution that showed dechlorination activity was used for further transfers on fresh medium. This procedure allowed loss of *cis*-DCE dechlorination activity and thus having only the bacteria present catalyzing the first two dechlorination steps.

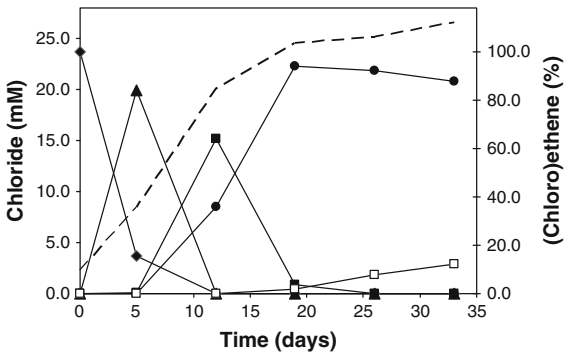

Fig. 1 Stepwise reductive dechlorination of PCE via TCE, *cis*-DCE, and VC to ethene by the enrichment culture SL2. (*Filled diamonds*) PCE, (*filled triangles*) TCE, (*filled squares*) *cis*-DCE, (*filled circles*) VC, (*open squares*) ethene, (*dashed lines*) chloride

Identification of organohalide respiring bacteria and reductive dehalogenases present

T-RFLP analysis of the three culture lines showed the predominant presence of two terminal restriction fragments (T-RFs) depending on the dechlorination steps occurring (Table 1). In SL2-PCEb, T-RF 254 was predominant. Cloning and sequencing of the 16S rRNA genes of this transfer line identified this population as *Sulfurospirillum* spp. with 97% sequence identity with *S. deleyianum* (DSM 6946; Genbank CP001816). The second predominant population showed a T-RF length of 168 bp and was identified as *Dehalococcoides* spp. The clones shared the highest sequence identity with strain BAV1 (99%; Genbank CP000688). There was one abundant additional T-RF with a length of 270 bp but it only appeared when dechlorination was completed in SL2-PCEb cultures (data not shown). *Wolinella* spp.-affiliated populations (sequence identity of 99% with *W. succinogenes* strain ATCC 29543; Genbank BX571656) were identified as representatives of this operational taxonomic unit.

PCR detection of specific reductive dehalogenases showed the presence of genes similar to *pceA* of *Sulfurospirillum multivorans* and *tceA* of *D. ethenogenes*, and thus confirmed the presence of these two genera in SL2. PCR detection of *pceA* of *Dehalobacter-Desulfitobacterium* did not yield a positive signal indicating the absence of this gene in SL2, and also no signal was obtained for the known VC reductive dehalogenases *vcrA* and *bvcA* (data not shown).

Cloning and sequencing of PCR products obtained from the three transfer lines of SL2 with the *rdhA* degenerate primer set (ceRD2L-f and RD7-r) showed that two clades of *pceA* genes were present, one showing 98% identity to *pceA* of *S. multivorans* (in the following referred to as *Smul-pceA* homolog), the

Table 1 T-RFLP and reductive dehalogenase clone library analysis of different SL2 cultures

Terminal restriction fragment[a]/ reductive dehalogenase gene[b]	Relative peak area of T-RFs 168 and 254 and number of clones containing either *pceA* or *tceA* in different SL2 cultures[c]		
	SL2-PCEa (PCE to ethene)	SL2-PCEb (PCE to *cis*-DCE)	SL2-DCE (*cis*-DCE to ethene)
T-RF 168	42%	0	64%
T-RF 254	24%	93%	21%
Deth-tceA	6	0	10
Sul-pceA	23	11	6

A specific SL2 culture dechlorinating PCE to *cis*-DCE was obtained by serial dilution and regular transfer when *cis*-DCE was dechlorinated and no vinyl chloride was yet formed

[a] The terminal restriction fragment T-RF 168 corresponds to a *Dehalococcoides* spp. population present in the consortium, T-RF 254 to a *Sulfurospirillum* spp. population

[b] The reductive dehalogenase genes were cloned from DNA isolated from culture SL2. *Sul-pceA* cloned *rdhA* gene with high similarity to *pceA* of *S. multivorans* and *S. halorespirans*, *Deth-tceA* cloned *rdhA* gene with high similarity to *tceA* of *D. ethenogenes*

[c] Metagenomic DNA was isolated of the different cultures at the end of the incubation period

other with 96% identity to *pceA* of *Sulfurospirillum halorespirans* (*Shal-pceA* homolog) (Fig. 2). In the gene fragment amplified with the degenerate primers the *Smul-pceA* homolog presented a twelve nucleotide insertion which is absent in the *Shal-pceA* homolog allowing a clear distinction between the two *pceA* clades (data not shown). Some clones contained a PCR product with high similarities (98% identity) to a putative reductive dehalogenase gene previously found in *S. multivorans* (*Smul-rdhA*) and *S. halorespirans* (*Shal-rdpA*). In addition, *rdhA* sequences similar to *tceA* of *D. ethenogenes* strain 195 (94–95% sequence identity) were also identified (*Deth-tceA* homolog) (Fig. 2).

Clones for both *pceA* clades and for the *Deth-tceA* homolog were isolated from SL2-PCEa and SL2-DCE, while only clones belonging to the *Smul-pceA* homolog clade were identified in SL2-PCEb. This suggested that SL2-PCEb did not contain any *Dehalococcoides* spp. anymore whereas SL2-DCE

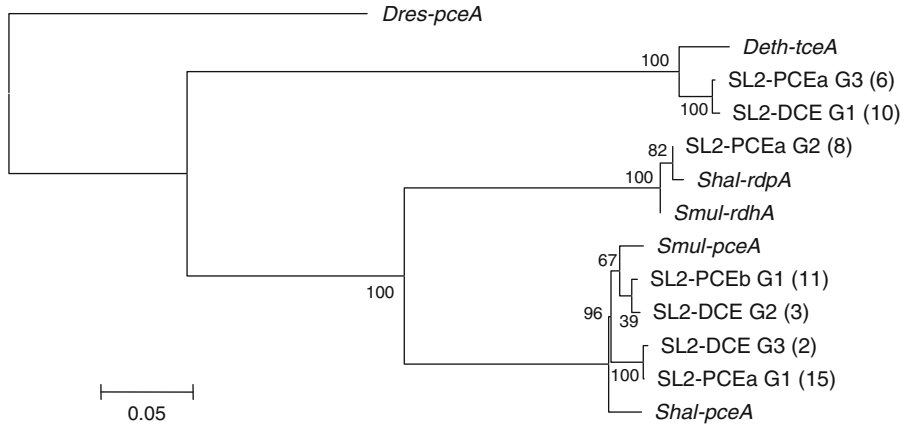

Fig. 2 Phylogenetic analysis of clones obtained by a PCR approach designed for detection of reductive dehalogenase genes. *SL2-DCE GX* (*Y*) group X of clones from culture SL2-DCE with (Y) number of clones, *SL2-PCEa GX* (*Y*) group X of clones from culture SL2-PCEa with (Y) number of clones, *SL2-PCEb GX* (*Y*) group X of clones from culture SL2-PCEb with (Y) number of clones, *pceA* tetrachloroethene reductive dehalogenase, *Dres Dehalobacter restrictus* (Genbank AJ439607), *tceA* trichloroethene reductive dehalogenase, *Deth D. ethenogenes* strain 195 (CP000027); *rdhA*, *rdpA* putative reductive dehalogenase, *Smul S. multivorans* (*pceA*: AF022812; *rdhA*: AJ539530), *Shal S. halorespirans* (*pceA*: AY013367; *rdpA*: AY013368)

still contained *Sulfurospirillum* spp. (Table 1). This was not surprising since the hexadecane containing biogenic *cis*-DCE obtained as described in Materials and Methods often contained some PCE.

Development of a SSCP approach for reductive dehalogenase analysis

The short PCR products of approximately 200 bp obtained with the specific primers for *rdhA* gene detection (Regeard et al. 2004) made them very suitable for a SSCP analysis approach. Initially developed for *rdhA* gene diversity assessment in environmental samples (unpublished results), it was adapted in this study for the analysis of the dynamics of specific genes in enrichment cultures, in particular SL2 that contained two almost identical *pceA* genes. After optimizing different parameters such as run temperature, non-denaturing polymer and glycerol concentrations, a reproducible analysis of standard samples was obtained with very precise mobility values for the three different clones identified earlier (Table 2). Mixing the three samples and performing a multiplex SSCP showed the same mobility values for the three genes. Performing analyses of DNA samples isolated from different SL2 cultures showed peaks with mobility values of 558–559, 579–580, and 585–586, precisely the ones obtained with the three standards. Performing this analysis with dilution series of the standard DNA mixture showed linearity of the observed peak height and area depending on the concentration of the sample DNA indicating that the SSCP approach not only allowed the detection of the different genes but also an estimation of their abundance.

Table 2 Mobility values of cloned reductive dehalogenase genes in SSCP analysis

Reductive dehalogenase gene[a]	Individual mobility value[b, c]	Mobility value in mixture[d]
Smul-pceA homolog	584.9 ± 0.5	585.1 ± 0.1
	587.4 ± 0.5	587.2 ± 0.2
Shal-pceA homolog	580.0 ± 0.1	580.1 ± 0.1
	582.4 ± 0.1	582.6 ± 0.2
Deth-tceA homolog	558.5 ± 0.1	558.5 ± 0.2
	560.0 ± 0.5	560.0 ± 0.3

[a] The reductive dehalogenase genes were cloned from culture SL2 DNA. *Smul-pceA homolog* cloned *rdhA* gene with high similarity to *pceA* of *S. multivorans*, *Shal-pceA homolog* cloned *rdhA* gene with high similarity to *pceA* of *S. halorespirans*, *Deth-tceA homolog* cloned *rdhA* gene with high similarity to *tceA* of *D. ethenogenes*

[b] The mobility value was determined in relation to the size standard used. The value does not represent the actual fragment size, but 1/10 of the data point value obtained for each peak

[c] Each gene appeared as a double peak on the electropherograms, a large peak first followed by a small peak. For semi-quantitative data the peak height and area of the large peak was taken into account

[d] Plasmid DNA of the three clones was mixed and a multiplex SSCP analysis was performed on this mixture including the initial PCR with the mixture of the two primer pairs SpSm1f–SpSm1r and SpDe1f–SpDe1r

Analysis of SL2-PCEa by SSCP

Samples of a culture of transfer line SL2-PCEa were taken after different periods of incubation to investigate on DNA level the dynamics of the three reductive dehalogenase genes during PCE dechlorination (Fig. 3). After 1 week of incubation, mainly the *Smul-pceA* homolog was detected. Eight days later, also the *Deth-tceA* homolog started to be detected and increased in abundance during the following week of incubation when dechlorination to VC was occurring. Hence, the dechlorination to VC seemed to be catalyzed by a *Dehalococcoides*-affiliated population containing the *Deth-tceA* homolog. The *Shal-pceA* homolog was not detected in this culture which might have been the consequence of the different electron donor used. For this culture it was not the routinely used hydrogen but a mixture of propionate, butyrate and ethanol. Analysis of SL2-PCEa cultures with hydrogen clearly showed that both *pceA* genes were present also in this transfer line (data not shown).

Analysis of SL2-PCEb by SSCP

The presence of two possibly different *pceA* genes and the unusual accumulation of TCE prior to *cis*-DCE formation that has not been observed so pronounced in *Sulfurospirillum* spp. cultures previously were quite intriguing. A time-course analysis of a SL2-PCEb culture where PCE was only dechlorinated to *cis*-DCE showed that first the *Shal-pceA* homolog was transcribed during PCE dechlorination to TCE followed by the *Smul-pceA* homolog upon TCE dechlorination to *cis*-DCE (Fig. 4). Data on

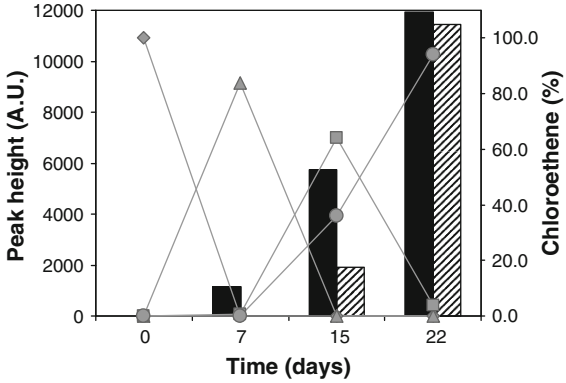

Fig. 3 Dynamics of the *Smul-pceA* and *Deth-tceA* homologues during PCE dechlorination by SL2-PCEa with a mixture of propionate, butyrate and ethanol as electron donors. (*Filled diamonds*) PCE dechlorinated, (*filled triangles*) TCE formed, (*filled squares*) *cis*-DCE formed, (*filled circles*) VC formed, *black bars Smul-pceA* homolog, *hatched bars Deth-tceA* homolog, *AU* arbitrary units

DNA extracted of the same samples indicated that the copy number of the *Shal-pceA* homolog increased during PCE dechlorination but continued to increase during TCE dechlorination. The mRNA of this gene was however less abundant during TCE to *cis*-DCE dechlorination than during the first dechlorination step. The transcripts of the *Smul-pceA* homolog were not very abundant during the first 4 days of incubation but started to increase from day 5 on and also the gene copy number increased strongly in abundance between days 5 and 8.

Analysis of the ITS regions of bacteria in SL2-PCEb

The patterns of abundance and transcription of the two *pceA* genes suggested that there might be two *Sulfurospirillum* populations present in SL2. The internal transcribed spacer (ITS) region of the 16S–23S rRNA genes was therefore analyzed in order to get indications for the presence of multiple strains of *Sulfurospirillum* spp. Three bands with different length were obtained after PCR with ITS-specific primers and subsequent analysis with the Bioanalyzer (Fig. 5a). Cloning and sequencing of ITS regions present in the DNA of SL2-PCEb at day 2 revealed two *Sulfurospirillum*-affiliated ITS (*Sul*-ITS1 and -ITS2), and one *Wolinella*-affiliated ITS (*Wol*-ITS) (Fig. 5b). The analysis of the isolated ITS clones with

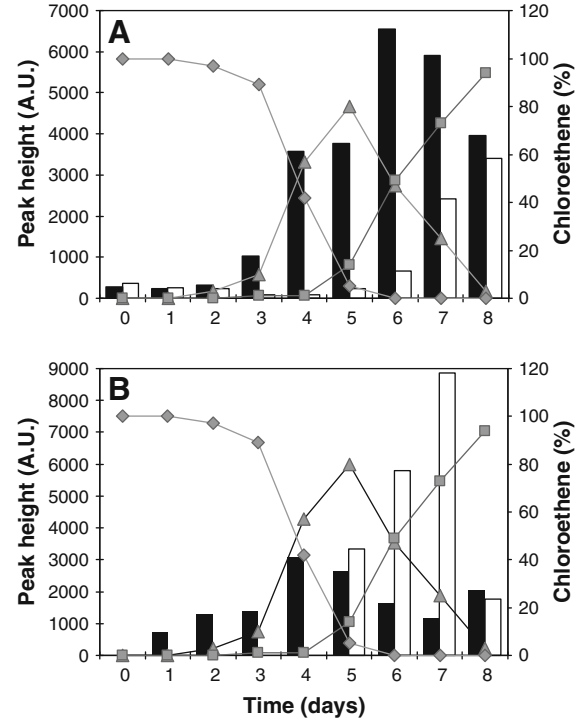

Fig. 4 Dynamics of **a** the *Shal-pceA* and *Smul-pceA* homologues and **b** their transcripts during PCE dechlorination to *cis*-DCE by SL2-PCEb. The relative peak heights obtained in SSCP analysis were multiplied by the relative DNA or RNA concentration with the highest concentration extracted at day 8 being 100%. (*Filled diamonds*) PCE dechlorinated, (*filled triangles*) TCE formed, (*filled squares*) *cis*-DCE formed, *black bars Shal-pceA* homolog, *white bars Smul-pceA* homolog, *AU* arbitrary units

the Bioanalyzer was in complete agreement with the ITS population in SL2-PCEb (Fig. 5a). Both *Sulfurospirillum* ITS were clearly different as *Sul*-ITS1 (646 nt) was very similar to the ITS region of *S. multivorans* (*Smul*-ITS) in length and sequence, while *Sul*-ITS2 (552 nt) was characterized by a 94 nucleotide long deletion. The genome of *Sulfurospirillum deleyianum* (Genbank CP001816) contains three copies of the 16S–23S rRNA genes, however all with an identical ITS region (*Sdel*-ITS, 630 nt). It was therefore concluded that the ITS analysis of SL2-PCEb suggested the presence of two *Sulfurospirillum* populations.

The dynamics of ITS sequences during stepwise dechlorination of PCE to *cis*-DCE was then investigated (Fig. 6). The *Wolinella*-affiliated ITS was detected in the inoculum and during the first 2 days of cultivation, but disappeared in the following,

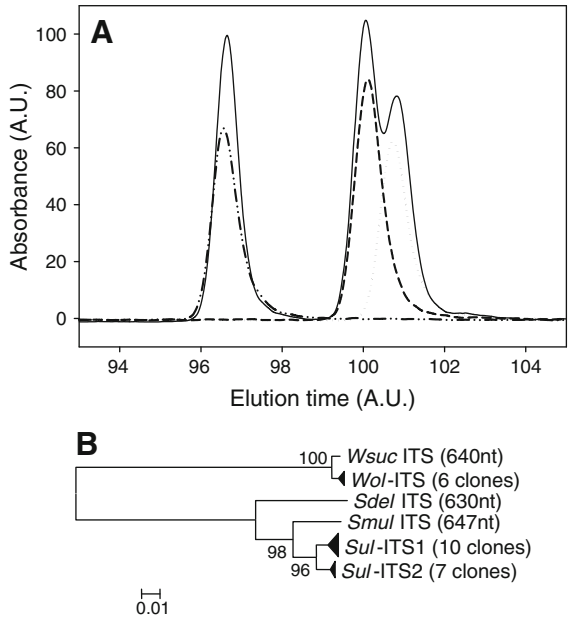

Fig. 5 Identification of sequences of the intergenic transcribed spacer region between the 16S rRNA and 23S rRNA genes (ITS) present in the DNA sample coming from SL2-PCEb culture at day 2. **a** Analysis with the Bioanalyzer of the three distinct ITS sequences in SL2-PCEb (*solid line*) and from selected clones after sequencing: *dotted line*, *Sul*-ITS1; *dash-dot-dot line*, *Sul*-ITS2; *dashed line*, *Wol*-ITS. **b** Phylogenetic relationship of ITS sequences identified in this study, compared to reference ITS: *Wsuc*-ITS, ITS from *Wolinella succinogenes* genome (Genbank BX571656); *Smul*-ITS, ITS from *S. multivorans* (DQ834670); *Sdel*-ITS, ITS from *Sulfurospirillum deleyianum* genome (CP001816)

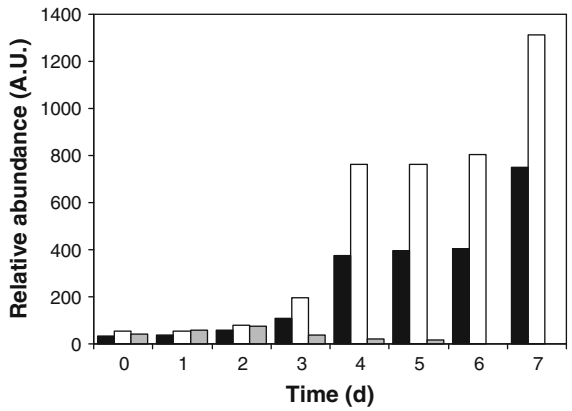

Fig. 6 Dynamics of *Sulfurospirillum* and *Wolinella* ITS sequences during PCE dechlorination by SL2-PCEb. The relative peak area obtained in the analysis with the Bioanalyzer were multiplied by the relative DNA concentration with the highest concentration extracted at day 7 being 100%. *Black bars*, *Sulfurospirillum*-affiliated ITS1 (*Sul*-ITS1); *white bars*, *Sul*-ITS2, *grey bars*, *Wolinella*-type ITS (*Wol*-ITS); *AU* arbitrary units

suggesting that *Wolinella* was largely present in the inoculum but not involved in dechlorination. Both *Sulfurospirillum*-affiliated ITS sequences seemed to evolve similarly with a clear increase in relative abundance starting from day 3. Hence, these results did unfortunately not allow elucidating whether the first dechlorination step from PCE to TCE was catalyzed by a *Sulfurospirillum* population expressing the *Shal-pceA* homolog and the second by another *Sulfurospirillum* spp. population expressing the *Smul-pceA* homolog.

Discussion

The enrichment culture SL2 obtained from a bioreactor treating PCE-contaminated groundwater contained mainly *Sulfurospirillum*- and *Dehalococcoides*-affiliated bacteria. The creation of different transfer lines similar to the subcultures described for culture KB1 (Waller et al. 2005) and another PCE-dechlorinating consortium (Yang et al. 2005) allowed to obtain first indications about the roles of the two populations in the dechlorination of PCE to ethene. *Sulfurospirillum*-affiliated bacteria were mainly involved in PCE dechlorination to *cis*-DCE whereas *Dehalococcoides* spp. was mainly but probably not exclusively dechlorinating *cis*-DCE to VC and ethene. T-RFLP analysis of the transfer line SL2-PCEb dechlorinating PCE to *cis*-DCE only contained the T-RF corresponding to *Sulfurospirillum* spp. and no clones containing *Deth-tceA* homologues were obtained from this transfer line. The transfer procedure applied for SL2-PCEb was apparently very selective and allowed only *Sulfurospirillum*-affiliated bacteria to remain in these cultures. That does however not exclude that *Dehalococcoides*-affiliated bacteria were not also dechlorinating PCE and certainly TCE when both populations were present like in transfer line SL2-PCEa. A similar phenomenon has been described for a consortium containing *Desulfitobacterium* spp. and *Dehalococcoides* spp. (Yang et al. 2005).

Whereas other studies described PCE-dechlorinating consortia composed of either different strains of *Dehalococcoides* spp. (Duhamel et al. 2002; Holmes et al. 2006), *Desulfitobacterium* spp. and *Dehalococcoides* spp. (Szynalski 2003; Yang et al. 2005; Bunge et al. 2007), and *Dehalobacter* spp. and

Dehalococcoides spp. (Daprato et al. 2007), this seems to be the first consortium to be described where *Sulfurospirillum* spp. and *Dehalococcoides* spp. dechlorinate PCE to ethene in a concerted action.

The need of acetate as carbon source if molecular hydrogen was provided as electron donor corresponds with the physiological properties of organohalide respiring members of the two bacterial genera detected in SL2. Both *S. multivorans* and also *S. halorespirans* need an organic carbon source upon growth with hydrogen (Scholz-Muramatsu et al. 1995; Luijten et al. 2003). All members of *Dehalococcoides* spp. are so far described as organohalide respiring bacteria restricted to hydrogen as electron donor and needing acetate as carbon source (Tas et al. 2009). In the inoculum originating from a bioreactor treating PCE-contaminated groundwater and in the first enrichment cultures thereof, *Dehalobacter* spp. that has the same physiological characteristics as *Dehalococcoides* spp. has been detected as well (Rouzeau-Szynalski et al. 2011). However, this organohalide respiring population was apparently eliminated during the regular transfers and could not maintain itself in the consortium.

The presence of *rdhA* genes which were very similar to *pceA* of *S. multivorans* and *S. halorespirans* and to *tceA* of *D. ethenogenes* strain 195, corroborated the analysis of 16S rRNA genes revealing that *Sulfurospirillum* spp. and *Dehalococcoides* spp. were present in SL2. The presence of these three genes also suggested that at least two genes were present in SL2 possibly involved in dechlorination of PCE and three in dechlorination of TCE. The time series experiment on DNA level with SL2-PCEa indicated that the *pceA* genes were responsible for PCE dechlorination since they increased in abundance during this dechlorination step. The abundance of the *tceA* gene that started to increase during TCE and certainly *cis*-DCE dechlorination indicated that this gene was involved in TCE dechlorination and responsible for the *cis*-DCE dechlorination observed. The dechlorination pattern observed in SL2 with a very slow dechlorination of VC to ethene suggested that specific genes of VC reductive dehalogenases were not present in SL2 as indicated by PCR detection with specific primers for the known VC reductive dehalogenase genes *vcrA* and *bvcA* that did not result in a positive response in DNA samples of SL2. Hence, *tceA* was probably the *rdhA* gene responsible for dechlorination beyond *cis*-DCE in SL2. In a TCE enrichment designated ANAS where two *Dehalococcoides* populations have been identified, evidence has been presented that a *tceA*-containing population was responsible for TCE and *cis*-DCE dechlorination whereas a *vcrA*-containing population was dechlorinating VC to ethene (Holmes et al. 2006).

In other studies where dechlorinating consortia have been characterized for the presence of organohalide respiring bacteria and reductive dehalogenase genes, multiple strains and multiple *rdhA* genes have been detected and different approaches have been chosen to assign a specific role to the individual strains and *rdhA* genes. In the ANAS enrichment mentioned above two *Dehalococcoides* populations, one containing *tceA* and the other *vcrA*, have been identified by the use of quantitative PCR for 16S rRNA and reductive dehalogenase genes and by creating subcultures on individual chloroethenes similar to what was done in this study (Holmes et al. 2006). Transcription analysis applied to *rdhA* genes of subcultures of ANAS on individual chloroethenes has shown that both *tceA* and *vcrA* have been expressed simultaneously, even during dechlorination of TCE to *cis*-DCE (Lee et al. 2006). The mixed culture KB1 has been described to contain multiple *Dehalococcoides* populations too based on subcultures on different chloroethenes and the characterization of their substrate utilization patterns and bacterial community composition determined by DGGE (Duhamel et al. 2002). Transcription analysis of the different *rdhA* genes present in KB1 did however not allow elucidating which *rdhA* gene was involved in which dechlorination step (Waller et al. 2005). In the present study, the time series experiments together with the SSCP approach on DNA and RNA level allowed obtaining indications about the involvement of the three major *rdhA* genes present in SL2. The rather atypical dechlorination pattern with strong TCE accumulation prior to *cis*-DCE formation could thus be described as a process involving the subsequent transcription of two *pceA* genes, one responsible for PCE dechlorination to TCE, the other, possibly together with the first one, for the dechlorination of TCE to *cis*-DCE. The accumulation of TCE has been reported during growth of *S. multivorans* (Scholz-Muramatsu et al. 1995) and also in cell suspensions of the same bacterium (Neumann et al.

1995), but never to the extent observed in SL2 cultures. Although the ITS analysis provided indications for the presence of two *Sulfurospirillum* populations in SL2, it was unfortunately not possible to assign the first dechlorination step to one population and the second to the other.

The SSCP approach developed and presented here is a useful tool to rapidly analyze environmental and culture samples for individual *rdhA* genes and to obtain qualitative and semi-quantitative information on the presence and transcription of these genes. Although qPCR is a more quantitative mean to study the transcription of individual genes, it would probably have been impossible to distinguish the two *pceA* genes in SL2 by qPCR because of their very similar gene sequences. The SSCP approach developed here for the analysis of three specific *rdhA* genes can certainly be adapted to the analysis of other *rdhA* genes and thus be used as rapid analytical tool for large scale screening of sites undergoing natural attenuation as well as biostimulated or bioaugmented in situ bioremediation.

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