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ORIGINAL ARTICLE

Identification and transcriptional analysis of *trans*-DCE-producing reductive dehalogenases in *Dehalococcoides* species

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During microbial reductive dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE), *trans*-1, 2-dichloroethene (*trans*-DCE) has been observed to be produced predominantly by certain mixed and pure cultures. However, the reductive dehalogenase (RDase) genes involved in *trans*-DCE generation remain elusive. In this study, identification and transcriptional analysis of RDases were conducted on *trans*-DCE-producing *Dehalococcoides* sp. strain MB. Two pairs of degenerate primers targeting the conserved regions of RDases in known *Dehalococcoides* species were applied to amplify the putative RDase genes of strain MB. Cloning and restriction analysis revealed the presence of seven unique RDase gene fragments (*dceA1 to dceA7*) that possess sequence identity to known RDase genes. Gene expression analysis of the PCE-grown culture MB exhibited 10-fold regulation of the RDase gene *dceA6* (designated *mbrA* gene), suggesting that it is involved in the production of *trans*-DCE. This is in agreement with the molecular size of the most abundant protein that is resolved on the denaturing protein gel. Complete sequence of the *mbrA* gene was obtained by chromosome walking, and the upstream of it is a regulator of transcription, indicating that the expression of this functional gene is tightly controlled in the microbe. The *mbrA* gene was subsequently found to be present in other *trans*-DCE-producing cultures containing *Dehalococcoides* sp. The new *mbrA* gene identified in this study may serve as an important biomarker for evaluating, predicting and elucidating the biological production of *trans*-DCE in the chloroethene-contaminated sites.
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Introduction

Tetrachloroethene (PCE) and trichloroethene (TCE) remain a critical environmental concern because of their poor disposal practices and accidental releases in various dry-cleaning and degreasing industries (Abelson, 1990; ATSDR, 1997a, 1997b). The released chloroethenes often contaminate groundwater, which in turn threatens drinking water supplies (Westrick *et al.*, 1984). Under anaerobic conditions, PCE and TCE can be reductively dechlorinated to the less chlorinated ethenes *cis*-1, 2-dichloroethene (*cis*-DCE) and vinyl chloride (VC) by a variety of dechlorinators or to the harmless ethene by *Dehalococcoides* species (Holliger *et al.*, 1998; Löffler *et al.*, 2000; He *et al.*, 2003; Luijten *et al.*, 2003; Müller *et al.*, 2004; Smidt and de Vos, 2004; He *et al.*, 2005; Hiraishi, 2008). However, in PCE-/TCE-contaminated sites, dechlorination often terminates at DCEs and VC. Although *cis*-DCE is the major DCE isomer produced by the currently characterized dechlorinators, reductive dechlorination of PCE or TCE to *trans*-1, 2-dichloroethene (*trans*-DCE) has also been observed in enrichment cultures and the pure culture of *Dehalococcoides* sp. strain MB (Löffler *et al.*, 1997; Griffin *et al.*, 2004; Miller *et al.*, 2005; Kittelmann and Friedrich, 2008; Cheng *et al.*, 2010; Cheng and He, 2009). Although reports on the generation of *trans*-DCE by microbial dechlorination are relatively fewer than studies on *cis*-DCE production, the microbial generation of *trans*-DCE is equally important because of its recalcitrant nature. The accumulation of *trans*-DCE at contaminated sites poses a serious problem, as it is known to be persistent and to disperse at the subsurface. Therefore, the remediation of chloroethenes remains a challenging task.

A number of PCE-to-*cis*-DCE reductive dehalogenases (RDases) and their encoding gene sequences have been characterized to understand the generation of *cis*-DCE, such as RDases in *Dehalospirullum multivorans* (Neumann *et al.*, 1996, 1998),

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Dehalobacter restrictus (Schumacher *et al.*, 1997; Maillard *et al.*, 2003), *Desulfitobacterium* sp. strain PCE-S (Miller *et al.*, 1998), *Clostridium bifermentans* (Okeke *et al.*, 2001) and *Dehalococcoides ethenogenes* strain 195 (Magnuson *et al.*, 1998; Magnuson *et al.*, 2000). Besides these PCE-RDases (PceA), *Dehalococcoides* species possess additional RDases that catalyze the dechlorination of DCEs to VC and ethene. For example, *D. ethenogenes* 195 contains an enzyme TceA, which is able to catalyze dechlorination of TCE and DCEs to VC and ethene (Magnuson *et al.*, 2000). Two other VC dechlorinating RDases, VcrA and BvcA, distinguish *Dehalococcoides* sp. strains VS and BAV1 from other strains of the same genus (Krajmalnik-Brown *et al.*, 2004; Müller *et al.*, 2004). The uniqueness of dehalorespiring bacteria is that all the dehalogenation reactions are catalyzed by RDases. RDases are characterized to have a molecular weight between 45–65 kDa when subjected to SDS-polyacrylamide gel electrophoresis, and to contain a corrinoid factor and iron sulfur (Fe_4S_4) clusters (Maillard *et al.*, 2003). Sequence similarity to other proteins is limited to the C-terminal part of the enzyme, which consists of the twin arginine translocation signal sequence and two Fe_4S_4 binding motifs with four conserved cysteines in each of the Fe_4S_4 clusters (Berks, 1996; Sticht and Rösch, 1998). As this class of RDases shares common features both at the biochemical and genetic levels, the sequence information of the known RDases is used for the design of degenerate primers to identify new RDase genes in this study.

To date, *Dehalococcoides* sp. strain MB is reported to be capable of reducing PCE/TCE to *trans*-DCE predominantly (Cheng *et al.*, 2010; Cheng and He, 2009). Microarray analysis reveals that the genes of strains MB and 195 are highly conserved, reflected by the fact that the genomic DNA of MB hybridizes with 1389 out of the 1579 (88%) probe sets on the microarray targeting genes of strain 195 and 10 of them are RDase genes (Cheng and He, 2009). Little is known about the novel RDases and their transcription in the *trans*-DCE-producing culture MB. Therefore, the aim of this study is to investigate the genetic make-up of RDase genes and to provide insights on their protein subunits in the *trans*-DCE-producing culture MB. Expression analysis of multiple RDase genes of strain MB will help to elucidate how the microbes catalyze dechlorination of PCE/TCE to the predominant *trans*-DCE and will provide an enzymatic basis of *trans*-DCE production in contaminated sites.

Materials and methods

Chemicals
The chlorinated ethenes, universal vectorette system and other chemicals were purchased from Sigma-Aldrich (Saint Louis, MO, USA). H_2 was obtained from a hydrogen generator (Schmidlin-DBS, AG, Neuheim, Switzerland). Lysozyme was purchased from Invitrogen (Carlsbad, CA, USA), and luciferase control RNA was obtained from Promega (Madison, WI, USA).

Culture and growth conditions
The *trans*-DCE-producing culture MB was grown in acetate (5 mM)-amended mineral salts medium spiked with 0.5 mM of PCE and a vitamin solution (Wolin *et al.*, 1963) comprising 0.05 mg l^{-1} of vitamin B_{12} (He *et al.*, 2007). H_2 was added as the electron donor (5 00 000 p.p.m.v by volume). All bottles were inverted and incubated in the dark at 30 °C without shaking. Dechlorination activity was monitored by a gas chromatograph (GC-6890, Agilent, Wilmington, DE, USA) equipped with a flame ionizing detector as previously described (Cheng and He, 2009).

Genomic DNA extraction and construction of DNA clone library
Genomic DNA was extracted from the cell pellet of 1.0 ml *trans*-DCE-producing culture MB with Qiagen DNeasy Blood and Tissue Kit (QIAGEN GmbH, Hilden, Germany) as described in the manufacturer's instructions, except that 45 µl of proteinase K (25 mg ml^{-1}), 20 µl of lysozyme (100 mg ml^{-1}) and 10 µl of achromopeptidase (25 mg ml^{-1}) were added to improve cell lysis. The extracted genomic DNA was then used as a template for the amplification of putative RDase genes with two pairs of degenerate primers (Table 1). In this study, primer pair 2 (RDH F1C and RDH R1C) was designed to cover ~90% of the known RDases detected in *Dehalococcoides* spp. The subsequent construction of clone libraries and screening of major clone types were conducted as previously described (Hölscher *et al.*, 2004).

RNA extraction and construction of cDNA clone library
Total RNA was extracted from the cell pellet of 1.5 ml pure culture MB by using the RNeasy extraction kit (QIAGEN GmbH) according to the manufacturer's instructions, except that the tubes holding the cells received 100 µl of 0.1 mm zirconia-silica beads (Biospec Products, Bartlesville, OK, USA) and were bead-beaten for 2 min to improve cell lysis.

Reverse transcription was carried out by using the two-step reverse transcription-PCR Sensiscript kit (QIAGEN GmbH). First, the corresponding cDNAs were synthesized by incubating 20–50 ng of extracted RNA in a 20 µl reaction mixture at 37 °C for 3 h. Then, the generated cDNAs were used as template for the amplification of RDase genes with degenerate primer pairs 1 and 2, respectively, as shown in Table 1. Two cDNA clone libraries were then established with the resulting amplicons using

Table 1 Specific primer sequences designed for culture MB reductive dehalogenase genes

Primer pair	*Primers*	*5′-3′ Sequence*	*Gene targeted*	*Temperature (°C)*	*Size (bp)*
1	RR2F[a]	SHMGBMGWGATTTYATGAARR	RDase genes	48	1500
	B1R[a]	CHADHAGCCAYTCRTACCA			
2	RDH F1C	TTYMVIGAYITIGAYGA	RDase genes	47	1200
	RDH R1C	CCIRMRTYIRYIGG			
3	*dceA1*F	CCGTACTGCCATCAGGTTTT	*dceA1*	48	556
	*dceA1*R	AAGCCCAAAGGGACAAGAAT			
4	*dceA2*F	AAGAACCCGTGACAACCAAG	*dceA2*	60	520
	dceA2R	GCAGTTTCCACCCACAAGTT			
5	*dceA3F*	CTGGTCATCCCCAATGTACC	*dceA3*	60	528
	dceA3R	GCACAGGGGCAGATTGTTAT			
6	*dceA4F*	TCTCTCCGGTTTTCCATGAC	*dceA4*	60	545
	dceA4R	ATCCTGGGGGTAGAGCATTT			
7	*dceA5F*	TCCGGACAGCTAATGAATCC	*dceA5*	60	590
	dceA5R	TGAATTAAGTGCGGGGGTAG			
8	*dceA6F (mbrAF)*	CCTGTAAACGACTCCCCAGA	*dceA6 (mbrA)*	60	427
	dceA6R (mbrAR)	GGATTGGATTAGCCAGCGTA			
9	*dceA7F*	GGATATCATGGTCCCACCAG	*dceA7*	60	148
	dceA7R	TTCAGCACAACCAGAGATGC			
	dceA1S	AAGCCCAAAGGGACAAGAAT	*dceA1*	60	
	dceA2S	GCAGTTTCCACCCACAAGTT	*dceA2*	60	
	dceA3S	GCACAGGGGCAGATTGTTAT	*dceA3*	60	
	dceA4S	TGGGCTCTTCTTCAAAGGAT	*dceA4*	58	
	dceA5S	CAGGCATCCGCACATTTAGT	*dceA5*	60	
10	*rdhA6VF*	GATGCCTGTCCCGGTAACGCGC	*dceA6*	72	
	rdhA6VR	TCCCACCGAGGGACACCAATGC			
11	*rdhA7VF*	GGCAGCTTTCCGCGCATATTTTGTA	*dceA7*	73	
	rdhA7VR	CTCAGTGCCATGCTGTTTGTCCTTT			
12	*rdhA1VF*	CTCCGGGGTTTCAAGGTCTGTTGAA	*dceA1*	72	
	rdhA1VR	GGAGGTAGGAAATATGTTTTTCGC			

[a]Primers from Krajmalnik-Brown *et al.*, 2004.

the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA). Screening and determination of the most abundant RDase genes were conducted by enzyme digestion using the *Hha*I and *Msp*I enzymes (New England Biolabs, Ipswich, MA, USA), followed by visualization of digested products on 2.5% agarose gel (Metaphor agarose, FMC BioProducts, Rockland, ME, USA) after being stained with ethidium bromide.

Sequence analysis of RDase genes

The seven dominant RDase gene inserts in the cDNA clone library were sequenced with an ABI 3100 Sequencer (Applied Biosystems, Foster City, CA, USA) using primers M13F-20 (5′-GTAAAACGAC GGCCAGT-3′) and M13R-24 (5′-GGAAACAGCTATG ACCATG-3′) (Invitrogen). Five of the RDase genes (*dceA1* to *dceA5*) required an additional internal primer to be designed (*dceA1S* to *dceA5S*) to obtain a complete sequence for each of the gene inserts (Table 1). Sequences were assembled and compared by using the BioEdit assembly software (http://www.mbio.ncsu. edu/BioEdit/ bioedit.html) (Hall, 1999) and BLASTX search tool, respectively. The deduced amino-acid sequences of the identified putative RDase genes were obtained with the TRANSLATE program (http://us.expasy.org/tools/dna.html) (Geneva, Switzerland). Phylogenetic trees were constructed with the online MAFFT program (http://align.genome.jp/mafft/) (Katoh *et al.*, 2002). FASTA program was used to compare the identity among the predicted amino-acid sequences of the identified putative RDases (http://fasta.bioch.virginia.edu/fasta_www2/fasta_www.cgi) (Pearson and Lipman, 1988).

Transcriptional analysis by quantitative real-time PCR

Gene expression analysis of the seven identified RDases in strain MB was conducted on an ABI 7500 fast quantitative real-time PCR (qPCR) (Applied Biosystems) by using SYBR green assays and their respective primers (primer pairs 3–9) (Table 1). Luciferase control RNA (Promega, Madison, WI, USA) was added as an internal reference transcript for mRNA losses during RNA isolation, reverse transcription and quantification (Johnson *et al.*, 2005), and *rpoB* was included in this study as a housekeeping gene (a positive control) for all qPCR assays. The housekeeping gene, *rpoB*, was chosen to serve as an indicator of the basal metabolic activity of the bacterial cells because it is highly conserved among *Dehalococcoides* spp. The transcriptional levels of RDase genes were normalized against the housekeeping gene, *rpoB*, before comparing with their respective *Dehalococcoides* 16S rRNA gene copies. SYBR green dye bound to all amplified double-stranded DNA during qPCR reactions and the fluorescently tagged DNA in turn would be detected by the qPCR system. The specificity of such

an assay was ensured by the use of the stringent HotStar Taq DNA polymerase (QIAGEN GmbH) as well as the inclusion of a melt curve analysis at the end of the entire amplification process. Reactions were performed in 20-μl volumes containing 10 μl of QuantiTect SYBR green PCR master mix (QIAGEN GmbH), 1.2 μl of each primer (5 pmol μl^{-1}), 6.6 μl of distilled water, and 1 μl of template DNA or cDNA. The thermocycling program was as follows: an initial step of 15 min at 95 °C, followed by 40 cycles of 15 s at 94 °C and 30 s at 55–59 °C depending on the primer annealing temperature (Table 1), and then 30 s at 72 °C. Fluorescence data were collected after each elongation step. To create qPCR standard curves, PCR products of individual genes amplified with the primers specified in Table 1 were ligated into the pCR2.1 vector (Invitrogen) and transformed into *Escherichia coli* TOP10 chemically competent cells (Invitrogen). The plasmids were obtained by using the QIAprep Spin Miniprep kit (QIAGEN GmbH). A calibration curve was obtained by using serial dilutions of known plasmid DNA concentrations. The qPCR experiments were carried out in triplicate along with appropriate controls (reference luciferase mRNA and housekeeping *rpoB* controls).

Genome walking of the RDase genes

The upstream DNA sequences of the three most abundant genes (*dceA1*, *dceA6* and *dceA7*) were mapped using the universal vectorette system (Sigma-Genosys, Saint Louis, MO, USA). A *Hin*dIII vectorette library was constructed using genomic DNA of strain MB according to the manufacturer's protocol. Gene-specific primers (VFs or VRs in Table 1) were designed to be used with the universal vectorette primers for the primary and secondary PCR. In addition to the above PCR reactions, three other reaction mixtures were set up as controls, which contained (1) the respective VF primer only, (2) the respective VR primer only and (3) the vectorette primer only. All amplified PCR products were then resolved on a 1% agarose gel (Invitrogen). Bands unique to the reaction that contained both vectorette primer and either VF or VR primer as compared with the above three controls were deemed as positive bands as either primer alone may prime/misprime at two sites generating a product. These positive bands were then gel purified with Nucleospin extract II kit (Macherey-Nagel, Düren, Germany) in accordance with the manufacturer's instructions. The gel-purified PCR products were then sequenced using the respective *dceA* gene-specific VF or VR primers and universal sequencing primers provided by the kit.

Gel electrophoresis, in-gel activity test and in-gel digestion for MALDI-TOF MS analysis

Cells from 300 ml of active culture MB were harvested by centrifugation at 23 000 × *g* for 20 min at 4 °C. The resulting cell pellet was resuspended in 3 ml of buffer 1 (Adrian *et al.*, 2007) and sonicated for 5 s with a 15 s cooling interval between each pulse on a Vibra Cell Sonicator (Sonics and Materials, Danbury, CT, USA). Cell disruption by sonication was carried out for a total of 10 min and kept in ice-cold slurry. The cell lysate was centrifuged at 10 000 × *g* for 30 min at 4 °C. The supernatant-containing cell extract was then collected and resolved on a native polyacrylamide gel. The positions of the respective excised protein bands were determined by the method as described by Adrian *et al.* (2007). Gel fragments were used for in-gel activity assays and the dechlorination activity of PCE was conducted as previously described (Neumann *et al.*, 2007). The protein band identified to show dechlorination activity was eluted and concentrated for a second gel electrophoresis step (Adrian *et al.*, 2007).

Denaturing SDS-polyacrylamide gel with a 10% resolving and 4% stacking gel were prepared as described by Läemmli (1970). The denaturing protein gels were run at 200 V for 2 h under aerobic conditions. After electrophoresis, the gel was stained with BioSafe Coomassie Stain (Bio-Rad Laboratories, Hercules, CA, USA).

The desired gel band was then excised using a scalpel and ground using a clean spatula. Tryptic gel digestion and the subsequent purification of digested protein fragments were performed as previously described (http://www.dbs.nus.edu.sg/research/facilities/ppc/index.htm). The digested peptides were analyzed with a Micromass Q-TOF Tandem mass spectrometer (Micromass, Manchester, UK). The map of peptide mass fingerprint generated was compared with the primary sequence database, MASCOT (Matrix Science, Boston, MA, USA).

Nucleotide accession number

The coding sequences of the putative RDase genes and putative B gene fragments were deposited in GenBank under accession numbers EU625398-625402 (*dceA1-5*) and GU120392 (*dceA7*). GenBank accession number GU120391 was assigned to the complete sequence of the identified *trans*-DCE-producing RDase gene, *dceA6* (designated the *mbrA* gene).

Results

Identification of RDase genes in culture MB

Two pairs of degenerate primers (RRF2–B1R, and RDH F1C–RDH R1C) were used to target the genomic DNA of strain MB. The primer pair 1 was designed on the basis of 17 RDase genes in the genome of strain 195 to target the conserved twin arginine motif (for RRF2) and WYEY motif (for B1R), which was used to identify the *bvcA* gene in strain BAV1 (Krajmalnik-Brown *et al.*, 2004). However, the

reverse primer B1R does not amplify *tceA*-like genes or other similar RDase genes, as it targets the conserved WYEY motif of the associated anchoring protein of nine RDase genes in strain 195 (Krajmalnik-Brown *et al*., 2004). Hence, to complement the RRF2-B1R primer pair, a second set of primers was designed according to the conserved twin arginine motif (for forward primer RDH F1C) and the conserved PIDD motif (for the reverse primer RDH R1C) in the RDase domain of *Dehalococcoides* species. When targeting the genomic DNA of strain MB, amplicons of expected size of 1.5–1.7 kb were obtained with primer pair 1 (RRF2 and B1R), whereas ~1.2 kb gene fragments were amplified by primer pair 2 (RDH F1C–RDH R1C). The amplicons were used to establish two clone libraries. Restriction fragment length polymorphism analysis of 120 clones from each clone library revealed seven distinct clone types, designated *dceA1* to *dceA7*, of which *dceA1, dceA6* and *dceA7* are the most abundant ones. The same RDase gene profiles were observed in all the DNA clone libraries established with DNA of enriched MB cultures and DNA of pure strain MB, indicating the high fidelity of the presence of RDases in these cultures. After sequencing the seven clone inserts, BLASTX analysis shows that strain MB shares three highly similar RDase genes (*dceA3*, *4* and *7*) with those of strain 195 (95–100% identity), three (*dceA1, 2* and *5*) with those of strain VS (54–97% identity), and one (*dceA6*) with that of strain CBDB1 (98% identity). None of the seven identified RDase genes of MB are similar to the TCE RDase genes in *Dehalococcoides* (for example, strains 195 and FL2). Phylogenetic analysis of the RDase genes in strain MB and other *Dehalococcoides* using MAFFT software generated phylogenetic trees that are similar in topology to previous studies (Hölscher *et al*., 2004; Waller *et al*., 2005) (Figure 1).

A similarity analysis was carried out with the translated amino-acid sequences of the RDase genes in strain MB by using protein software (TRANSLATE). Alignment of the seven amino-acid sequences (DceA1 to 7) by ClustalW shows that they all share the twin arginine translocation signal peptide, RRXFXK. The seven RDases share an amino-acid identity ranging from 28.8% to 68.8% when compared with each other (Table 2). The two most similar proteins are DceA6 (from primer pair 1 clone library) and DceA7 (from primer pair 2 clone library), sharing 72.1% amino-acid similarity. However, the presence of an early termination codon in the RDase domain of DceA7 distinguishes it from the other six putative RDase genes, though it shares 100% homology with the RDase gene DET 0088 in strain 195.

According to the seven RDase gene sequences of strain MB (Table 1), primer pairs 3–9 were designed

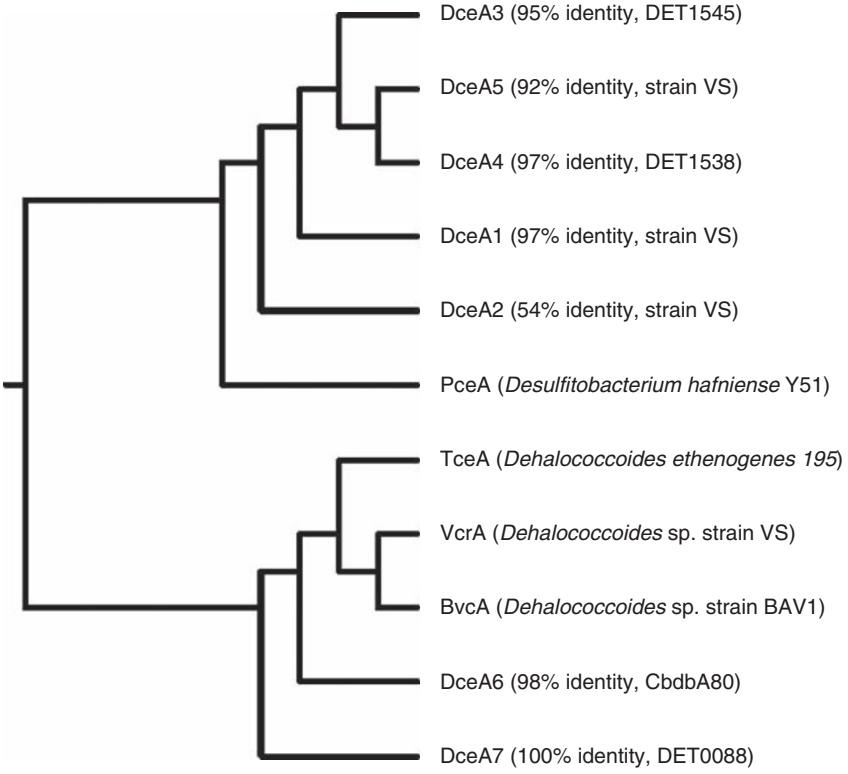

Figure 1 Comparison of amino-acid sequences of the reductive dehalogenase genes identified in *Dehalococcoides* sp. strains MB, VS, BAV1, 195 and *Desulfitobacterium hafniense* Y51. The percent identity compared with the sequences of the RDase genes from the NCBI database is shown in parentheses.

and specific bands were observed in the gel picture when targeted on the genomic DNA of culture MB (data not shown).

Transcriptional analysis of RDase genes in strain MB

The seven identified RDase genes were detected at DNA level in strain MB; however, the transcription of DNA to copies of mRNA might not occur for all seven genes. Therefore, transcriptional analysis was conducted to find out the functional RDase gene(s) among the seven identified ones in strain MB on exposure to PCE. After starving the MB cells for 72 h, three parallel cultures were spiked with PCE (0.22 mM of each). Total RNA was extracted from cultures at different time points, followed by reverse transcription and subsequent qPCR with the primer pairs designed specifically for the RDase genes (primers 3–9 in Table 1). At time zero, negligible amounts of cDNAs were detected by qPCR. After 72 h, *dceA6* and *dceA7* genes expressed eight- and fivefold, whereas *dceA6* gene reached its highest expression of 10-fold when compared with the 16S rRNA gene copy numbers at 120 h (Figure 2). The expressions of the other five identified RDase genes remained relatively low (less than twofold augmentation of gene copies) when compared with the 16S rRNA gene copies, suggesting little or no upregulation of the respective RDase genes. However, the gel picture of PCR showed positive bands when the genomic DNA of culture MB was targeted with the gene-specific primers, suggesting that these five genes were present in the genome of MB but were not expressing themselves (data not shown). Furthermore, the extracted RNA samples were not contaminated with genomic DNA, as confirmed by no visible amplicons from the amplification of RNA samples with the seven RDase gene-specific primers. Therefore, two (*dceA6* and *dceA7*) out

Table 2 Identity/similarity matrix of reductive dehalogenase gene translations in MB

Protein	*% Similarity/identity within MB proteins*						
	DceA1	*DceA2*	*DceA3*	*DceA4*	*DceA5*	*DceA6*	*DceA7*
DceA1		31.1	64.8	57.1	58.4	34.1	66.7
DceA2	54.1		55.8	68.8	59.4	35.7	—
DceA3	36.6	31.3		40.1	36.2	35.7	31.4
DceA4	30.4	42.4	62.5		63	28.8	—
DceA5	34.3	34.8	67.4	37.7		36.2	37.8
DceA6	61.4	65.1	62.9	55.6	60.5		36.5
DceA7	42.2	—	64.7	—	67.3	72.1	

The upper triangle (highlighted in lighter shade of grey) of the data represents the percent identity between two amino-acid sequences in a pairwise alignment, and the lower triangle (highlighted in darker shade of grey) of the data represents percent similarity. Similarity was calculated using BLOSUM matrix and a modified Smith–Waterman algorithm (Campanella *et al.*, 2003). Nearly complete translations of the RDase genes starting after the RRF2 and RDH F1C primers were used to generate the identity and similarity matrices.

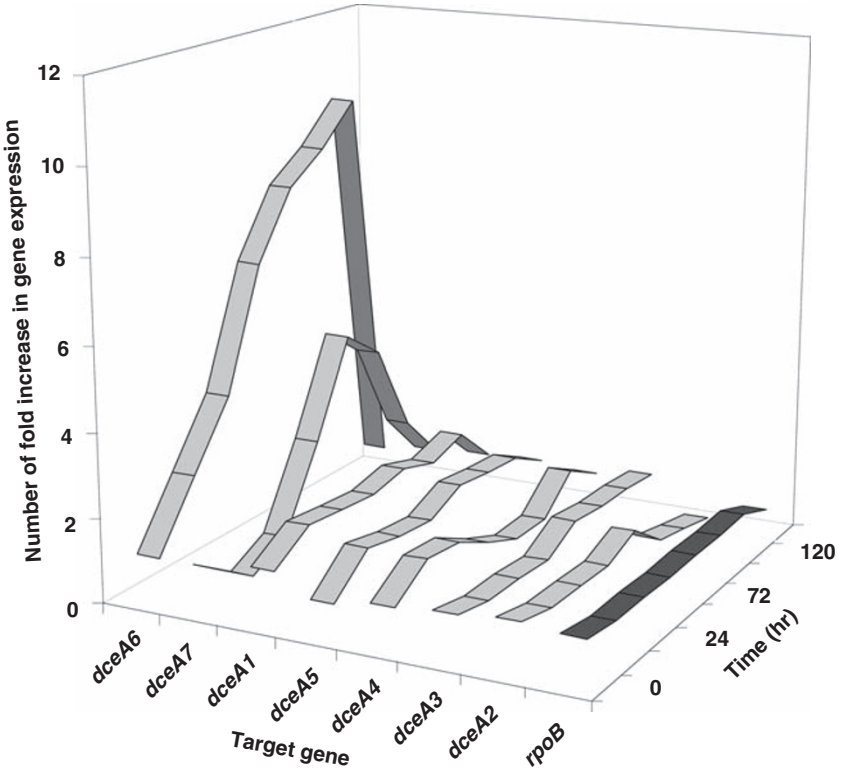

Figure 2 Expression profiles of the potential *trans*-1, 2-dichloroethene-producing reductive dehalogenase genes in *Dehalococcoides* sp. MB when fed with PCE. Transcripts corresponding to *dceA6* and *dceA7* are preferentially expressed.

of the seven identified RDase genes are actively expressed in strain MB when fed with PCE.

Genome walking of dceA6 and dceA7 genes

Two new pairs of sequencing primers (Table 1) were designed for genome walking to obtain sequence information on the 5′ and 3′ flanking regions of the expressed RDase genes *dceA6* and *dceA7*. Restriction enzymes, *Hin*dIII and *Rsa*I, were chosen as unique base cutters for the extension of *dceA6* and *dceA7* genes, respectively. Amplification of the ligated DNA with the designed primer and the universal vectorette primer resulted in amplicon sizes of approximately 0.6 and 1.2 kb for the *dceA6* gene and 0.8 and 2 kb for the *dceA7* gene. Finally, complete sequences (2.4 and 1.9 kb) were obtained for the RDase genes, *dceA6* and *dceA7* (Figure 3), and deposited in GenBank with accession numbers GU120391and GU120392, respectively. The upstream region of *dceA6* (~300 bp in length) was revealed to share 100% identity with the DNA-binding response regulator protein of CBDB1, and the *dceA6* gene is 98% similar to the putative RDase gene (*cbdbA80*) of *Dehalococcoides* sp. strain CBDB1. For the *dceA7* gene, its upstream region shares 91% identity with the radical *S*-adenosylmethionine (SAM) domain-containing protein of strain 195, whereas the *dceA7* RDase domain shares 100% identity with DET0088 of strain 195. Alignment of RDase gene sequences reveals that different groups of regulatory systems associate with the detected RDases. In addition to *dceA6* and *dceA7*, the full-length sequence of the *dceA1* (Cheng *et al*., 2010) gene was also obtained in this study. Comparison of the *dceA1* and *dceA6* genes shows that both RDases have an associated anchoring protein at the 3′ end (Figure 3).

Protein banding and in-gel dechlorination assay

From the Swiss-Prot (Geneva, Switzerland) software prediction, the size of the DceA6 RDase protein subunit is ~50 kDa. Protein extracts of culture MB fed with PCE were analyzed by native gel electrophoresis. A clear and reproducible gel pattern with one dominant band was observed on the Coomassie blue-stained native gel (Figure 4). Dechlorination activity of PCE to *trans*-DCE was detected after 24 h of incubating the corresponding unstained gel fragment. This protein band was subsequently eluted and concentrated for a second denaturing gel electrophoresis to verify the protein size. SDS-polyacrylamide gel electrophoresis analysis revealed that the size of the distinct band is ~50 kDa, which is located in the 36–55 kDa range of the pre-stained protein marker. MALDI-TOF MS analysis of the predominant protein mass of this band was found to be most similar to *cbdbA80*, a putative RDase of 592 amino-acids (equivalent to 1778 bp of nucleic acids) in strain CBDB1. RDase gene *cbdbA80* shows highest identity (98%) to the *dceA6* gene of strain MB. Besides being the most abundant protein in strain MB, the *dceA6* gene expressed 10-fold after exposure to PCE, suggesting

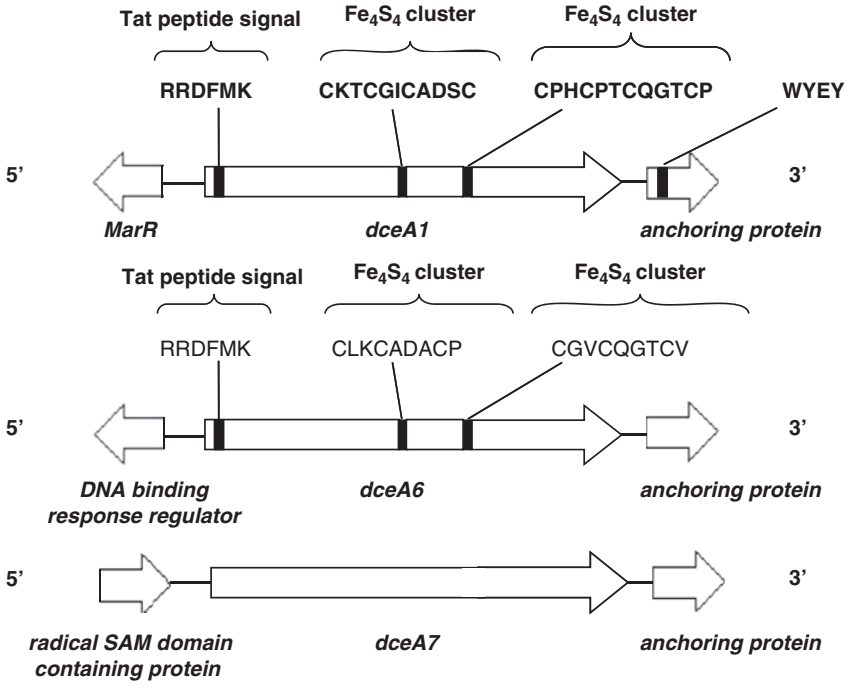

Figure 3 Arrangements of the potential *trans*-1, 2-dichloroethene-producing reductive dehalogenase genes and their corresponding anchoring protein. Also shown are the conserved dehalogenase features including the signal peptide RRDFMK, and two iron-sulfur (Fe_4S_4) clusters near the C-terminal end for *mbrA* (*dceA6*).

that *dceA6* is the RDase gene involved in *trans*-DCE production. The *dceA6* gene is designated the *mbrA* gene based on the name of strain MB.

Detection of the mbrA (dceA6) gene in various trans*-DCE-producing cultures*

Gene-specific primers, *mbrAF* (*dce6AF*) and *mbrAR* (*dce6AR*), were designed to determine whether the highly expressed *mbrA* (*dceA6*) gene could be an appropriate biomarker for distinguishing *trans*-DCE-producing cultures from other dechlorinating cultures generating a negligible amount of *trans*-DCE. The presence of the *mbrA* gene was tested on genomic DNA extracted from non-*trans*-DCE-producing *Dehalococcoides* isolate ANAS1 (Cheng and He, 2009) (negative control) as well as various *trans*-DCE-producing mixed cultures. The presence of DNA was first verified by detecting 16S rRNA genes in all the cultures. Results show the presence of the *mbrA* gene in 5 out of 10 *Dehalococcoides*-containing cultures that generated *trans*-DCE from reductive dechlorination of PCE/TCE (Figure 5). However, the other five *Dehalococcoides trans*-DCE-generating cultures do not contain the *mbrA* gene, suggesting the presence of the other *trans*-DCE-producing RDase genes in those cultures. The absence of the *mbrA* gene in the non-*Dehalococcoides trans*-DCE-producing cultures suggests the correlation of this newly identified RDase gene with the presence of the *Dehalococcoides* species. Specificity of the primer pairs, *mbrAF* and *mbrAR*, was further confirmed by a negative signal when targeting the genomic DNA of non-*trans*-DCE-producing cultures (for example, ANAS1) (Figure 5).

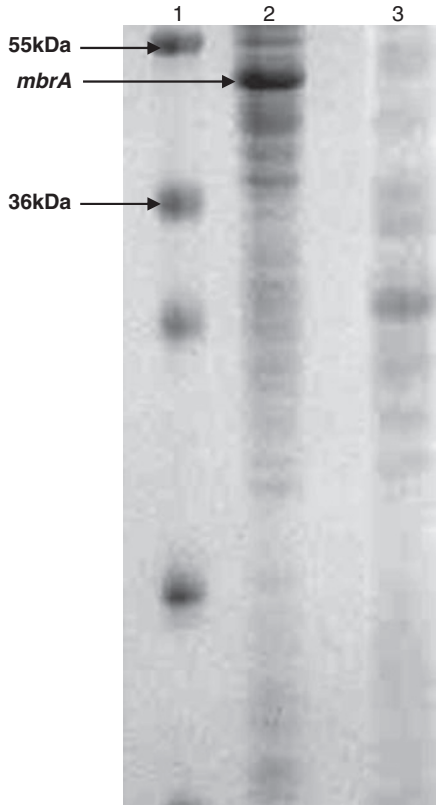

Figure 4 Whole cell proteins from the *Dehalococcoides* sp. strain MB separated by SDS-PAGE. Lane 1, PageRuler Plus Prestained Protein Ladder (Fermentas Canada Inc., Burlington, ON, Canada); lane 2, cell extract from strain MB fed with PCE; lane 3, cell extract from starved strain MB.

Discussion

Trans-DCE accumulation at chloroethene-contaminated sites has been an environmental concern because of their potential toxicity to humans (Mochida *et al*., 1995). However, molecular features of *trans*-DCE generation had been largely unknown. The *mbrA* RDase gene identified in this study is suggested to be involved in the reductive dechlorination of PCE/TCE to the major end product *trans*-DCE, corroborated by: (1) its elevated gene transcription level measured by qPCR; (2) protein expression observed on protein gels; and (3) in-gel PCE-to-*trans*-DCE dechlorination activity of the

Figure 5 (**a**) Genus-specific primers targeting the 16S rRNA genes of *Dehalococcoides* sp. (620 bp). (**b**) Detection of the *trans*-1, 2-dichloroethene (*trans*-DCE)-producing *mbrA* gene (427 bp) in various cultures. Lane 1: 100 bp DNA ladder (Promega); lanes 2–11: sediment-free *trans*-DCE-producing cultures established from soil sediments collected from various electrical waste landfill sites; lane 12: *trans*-DCE-producing enrichment culture MB; lane13: *trans*-DCE-producing culture from Sungei Buloh; lane14: pure culture MB; lane 15: non *trans*-DCE-producing culture *Dehalococcoides* sp. ANAS1; lane 16: water.

above most expressed protein. The increase in the *mbrA* gene expression leads to an augmented production of the functional protein, as the size of the mature MbrA (DceA6) RDase is estimated to be ~50 kDa, corresponding to the calculated molecular mass of 55 kDa from the length of the *mbrA* RDase gene. The identified RDase gene, *mbrA*, is detected in 5 out of the 10 *trans*-DCE-producing cultures, which were derived from microcosms established with environmental soil or sediments. All these observations indicate that environmental microbes are capable of generating *trans*-DCE as the end products. Hence, biotic degradation of PCE/TCE may explain the presence of high levels of *trans*-DCE at contaminated sites (ATSDR, 2007; Nijenhuis *et al.*, 2007). As for the *dceA7* gene, its fold of expression level is lower than that of the *mbrA* gene and its predicted molecular mass is ~17 kDa. Therefore, it is likely that the mRNA of *dceA7* is either not translated into the functional RDase enzyme or the translated protein might not be directly involved in the generation of *trans*-DCE.

Strain MB contains RDase genes that share high identity to genes in strains 195, VS and CBDB1, suggesting that these RDases might be acquired through the process of horizontal gene transfer (Regeard *et al.*, 2005). Although the identified *mbrA* gene consists of the same characteristics of typical *cis*-DCE-producing RDases (twin arginine translocation peptide signal and the Fe_4S_4 clusters), they differ functionally. Comparison of the amino-acid sequences of the newly identified *mbrA* gene and the known *tceA* gene reveals an identity of only 29%, thus indicating the uniqueness of the *trans*-DCE-producing *mbrA* gene. A previous microarray study also shows that strain MB shares high identity to 10 RDase genes of strain 195, among which three (*dceA3*, *dceA4* and *dceA7*) were also found in this study, indicating the reproducibility of this and previous findings (Cheng and He, 2009). Therefore, 14 putative RDase genes (7 from this study and 10 from microarray analysis, whereas 3 are overlapping ones) could be present in the *trans*-DCE-producing culture MB. Observations in this study are consistent with the hypothesis of the existence of a pool of RDases in each different strain with different combinations. The amplicons generated for primer pair 2 are much smaller in size (~1.2 kb) than those for primer pair 1, as the former targets the PIDD region of RDase genes. In this study, six intact coding sequences encode putative RDases (DceA1 to DceA6) with adjacent genes encoding for potential membrane-anchoring proteins. The *dceA7* gene (100% identity to DET0088) is truncated, and the 3′ anchoring protein is replaced by a hypothetical protein. Nevertheless, recent studies have reported that the expression of DET0088 was upregulated during PCE dechlorination in Alameda Naval Air Station enrichments and mixed cultures containing strain 195 (Rahm *et al.*, 2006; West *et al.*, 2008). Also, the *dceA7* gene was identified using the newly designed primer pairs, RDH F1C and RDH R1C, revealing inadequacies of the previous degenerate primer pair. In all, the presence of seven RDase genes provides an insight into the genetic makeup of strain MB for the first time, though the functions of the other non-expressing RDase genes identified in culture MB still remain to be elucidated.

The sequences of the two expressing RDase genes in strain MB possess two open reading frames, consisting of a dehalogenase-containing domain and an associated protein at the 3′ end (an anchoring protein and a hypothetical protein for *dceA6* and *dceA7*, respectively). The upstream regions of the two expressing RDase genes differ from each other. The upstream region of the RDase gene *mbrA* (*dceA6*) is a DNA-binding response regulator, whereas the 5′ region of the *dceA7* gene shares high identity with the SAM domain-containing protein as shown in strain 195. The transcription level of the *mbrA* gene is lower compared with some other known RDase genes such as the *tceA* gene, which is possibly due to the tight regulation of transcription by the upstream DNA-binding response regulator protein (Wagner *et al.*, 2009; Kube *et al.*, 2005). The upstream regions of the RDase genes have attracted much interest recently with current genome sequencing projects (Kube *et al.*, 2005; Seshadri *et al.*, 2005). Therefore, the DNA-binding response regulator upstream of the *mbrA* (*dceA6*) gene might be a part of the two-component regulatory system, whose function is to trigger downstream pathway cascades once it is activated by an extracellular signal (Fabret *et al.*, 1999). This signal will trigger the phosphorylated DNA-binding response regulator to bind to a transcriptional factor that will affect the dehalogenation processes by altering the level of transcription RDase genes (Fabret *et al.*, 1999). On the other hand, radical SAM proteins, such as the one detected in *dceA7*, are reported to catalyze unusual methylations, isomerization, sulfur insertion, ring formation, anaerobic oxidation and protein radical formation (Sofia *et al.*, 2001). They also function in the biosynthesis of DNA precursors, vitamins, cofactors, antibiotics and herbicides, and in the biodegradation pathways (Sofia *et al.*, 2001). Nonetheless, the function that the SAM radical protein has on its associated RDase gene *dceA7* remains to be determined.

In our previous study, one of the abundant putative RDase genes detected in strain MB is the *dceA1* gene (Cheng and He, 2009). Genome walking of the *dceA1* gene reveals that upstream of this RDase gene shares high identity with the transcriptional regulator belonging to the MarR (multiple antibiotic resistance) superfamily (Figure 3). It is oriented in the direction opposite to that of the associated RDase gene and its associated anchoring protein—a typical RDase locus associated with the MarR regulator (Kube *et al.*, 2005). MarR transcriptional regulators found in prokaryotes are often

involved in the catabolism of aromatic compounds as well as the modulation of multiple responses to other toxic molecules such as antibiotics (Alekshun *et al*., 2001). The presence of the MarR transcriptional regulator and the DNA-binding response regulator nearby suggests that the expressions of the RDase genes are tightly regulated. Despite the different origins (for example, strains 195 and CBDB1) from which MB might have inherited the upstream regions (of *dceA1*, *dceA6* and *dceA7*), these regions of the identified RDases should mainly function on the regulation of the downstream RDases.

In all, understanding the nature of the transcriptional regulators associated with the RDase genes is crucial to bioremediation applications as these regulatory elements often act as 'switches' for the microbial dechlorination activity. This study sheds light on the genetic pool of RDase genes present in the *trans*-DCE-producing cultures. The presence of the *mbrA* gene in other *trans*-DCE-producing cultures implicates the potential use of this gene as a functional biomarker, complementing the current inadequacies of the 16S rRNA gene approaches in assessing and monitoring *in situ* PCE/TCE dechlorination.

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