NEW *DEHALOCOCCOIDES* SPECIES DECHLORINATE CHLOROETHENES WITH UNUSUAL METABOLIC PATHWAYS

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Summary

Chlorinated organic solvents are pervasive groundwater and soil contaminants due to their extensive usage (as solvents, detergents or degreasers), improper disposal and accidental spills. Under anaerobic conditions, chloroethenes such as tetrachloroethene (PCE) and trichloroethene (TCE) can be reductively dechlorinated to the less chlorinated ethenes, *cis*-1,2-dichloroethene (*cis*-DCE) by a variety of dechlorinators, and to vinyl chloride (VC) or ethene only by *Dehalococcoides* species. Although the generation of *cis*-DCE are much more commonly observed than its isomer, *trans*-1,2-dichloroethene (*trans*-DCE), the accumulation of *trans*-DCE at contaminated sites poses a serious problem due to its recalcitrant nature. Currently, there is no information available on the *Dehalococcoides* isolates that generate *trans*-DCE as the main end product. Furthermore, the available isolates *Dehalococcoides* sp. strains BAV1 and FL2 that are able to dechlorinate *trans*-DCE to ethene cannot metabolically detoxify TCE or PCE to ethene. Therefore isolates that could detoxify TCE and *trans*-DCE completely to ethene still remain elusive and complete detoxification of PCE remains a challenging task at chloroethene-contaminated sites.

The main purpose of this study is to elucidate mechanisms involved in the generation and detoxification of *trans*-DCE in PCE/TCE-contaminated sites. Another objective is to achieve complete detoxification of PCE to ethene for efficient bioremediation. The enrichment process of several microcosm studies demonstrated that microorganisms within Cornell subgroup of *Dehalococcoides* could generate more *trans*-DCE than *cis*-DCE and terminate the reductive dechlorination of PCE or TCE at DCEs for the first time. Pure culture *Dehalococcoides* sp. strain MB was isolated from environmental sediments. It reductively dechlorinates PCE to *trans*-DCE and *cis*-DCE at a ratio of 7.3 (± 0.4) : 1. Although strain MB shares 100% 16S

rRNA gene sequence identity with the first isolate of the same genus, *Dehalococcoides ethenogenes* strain 195, these two strains possess different dechlorinating pathways. Microarray analysis revealed that 10 out of 19 putative reductive dehalogenase (RDase) genes present in strain 195 were also detected in strain MB. Transcriptional analysis of RDase genes in strain MB grown with PCE shows that one RDase gene, designated *mbrA*, exhibited 10-fold up-regulation, higher than the rest of RDase genes. The highly expressed RDase gene, *mbrA* gene may serve as an important biomarker for evaluating, predicting, and elucidating the biological production of *trans*-DCE in the chloroethene-contaminated sites.

Subsequently, another strictly anaerobic bacterium, designated as *Dehalococcoides* sp. strain 11a, was isolated in defined medium. Strain 11a rapidly and consistently dechlorinated TCE, 1,1-DCE, *trans*-DCE, *cis*-DCE, VC, and 1,2-dichloroethane metabolically to ethene with an average dechlorination rate of 53.1, 22.5, 21.6, 24.8, 86.5, and 16.7 μmol L^{-1} day^{-1} respectively. The complete detoxification of PCE to ethene for the contaminated groundwater could be achieved with a co-culture of strain 11a and a PCE-dechlorinating isolate *Sulfurospirillum multivorans*. Although strain 11a shares 100% 16S rRNA gene sequence identity with the first VC-dechlorinating isolate *Dehalococcoides* sp. strain BAV1, strain 11a showed broader substrate range than strain BAV1.

To summarize, the successful cultivation of strain MB indicates that biotic processes could contribute significantly to the generation of *trans*-DCE in chloroethene-contaminated sites, while the isolation of strain 11a enhances our understanding of the evolution of this unusual microbial group - genus of *Dehalococcoides*. This study also provides a promising cost-effective bioremediation solution to the chloroethene-contaminated sites.

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List of Abbreviations

PCE	tetrachloroethylene or percholroethylene
TCE	Trichloroethylene
VC	vinyl chloride
DCE	Dichloroethene
cis-DCE	*cis*-dichloroethene
1,1-DCE	1,1-dichloroethene
trans-DCE	*trans*-dichloroethene
ETH	ethylene, ethene
PCB	polychlorinated biphenyls
PBDE	polybrominated diphenyl ethers
PCDD/Fs	polychlorinated-dibenzo-*p*-dioxins (DD)/dibenzofurans
PCP	Pentachlorophenol
TCP	Trichlorophenol
RDase	Reductive dehalogenase
NAPL	Non aqueous phase liquid
DNAPL	dense non aqueous phase liquid
pceA	PCE reductive dehalogenase encoding gene
tceA	TCE reductive dehalogenase encoding gene
vcrA	VC reductive dehalogenase encoding gene
bvcA	BAV1-VC reductive dehalogenase encoding gene
tceA	TCE reductive dehalogenase encoding gene
cbrA	Chlorobenzene reductive dehalogenase encoding gene
tceA	TCE reductive dehalogenase encoding gene

GC-FID	Gas chromatography-flame ionization detector
16S rRNA	16S Ribosomal RNA
TAE buffer	Tris-acetate-EDTA buffer
dNTP	Deoxyribonucleotide triphosphate
BSA	Bovine serum albumin
bp	Nucleotide base pairs
U	Enzyme units of activity
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
PCR	Polymerase chain reaction
DGGE	Denaturing gradient gel electrophoresis
T-RFLP	Terminal restriction fragment length polymorphism
SNuP	Single-nucleotide primer extension assay
Confocal LSM	Confocal laser scanning microscopy
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
FISH	Fluorescence in situ hybridization
SEM	Scanning electron microscope
AFM	Atomic force microscope

Chapter I Introduction

Clean groundwater is important to meet public (e.g. drinking water), agricultural (e.g. irrigation), and industrial demands. Groundwater is increasingly threatened by organic, inorganic, and radioactive contaminants that have been improperly (e.g. agricultural applications, improper disposal) or accidentally released into the environment. Among the contaminants, halogenated organic compounds are the most widely distributed, which include chloroethenes, chloroethanes, chlorophenols, chlorobenzenes, polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs), and polychlorinated-dibenzo-*p*-dioxins/dibenzofurans (PCDD/Fs) (Holliger et al., 2003; Bayen et al., 2005) (Fig 1.1). Out of these halogenated compounds, halogenated organic solvents, e.g. tetrachloroethene (PCE), trichloroethene (TCE), 1,2-dichloroethane (1,2-DCA), and 1,1,1-trichloroethane (TCA) have been widely used in various industries, e.g. metal processing, degreasing, electronics, dry cleaning and paint, paper and textile manufacturing (Abelson, 1990). They can be found not only in water, but also in air, soil, and sediment.

Most of chloroethenes and chloroethanes are synthetic compounds, having a structure different from naturally occurring compounds, which makes them recalcitrant to be transformed in the natural environments. Since the beginning of the 20^{th} century, large quantities of halogenated organic solvents have been released into the environment and contaminated groundwater, marine, and soil. They are among the most commonly found groundwater pollutants and are detected at approximately 80% of all superfund sites in the USA.

Chlorinated ethenes vary in the number of chlorine atoms on each molecule; from polychlorinated, PCE, TCE and dichlorothenes (DCEs), to the monochlorinated

vinyl chloride (VC). PCE and TCE are found in groundwater most frequently and in highest concentration, due to extensive usage, illegal and improper disposal and accidental spills (Doherty, 2000a, b; Bradley, 2003). DCEs (mainly *cis*-DCE) occur in groundwater primarily as the result of in situ microbial transformation (Bradley, 2003). VC contamination of groundwater results primarily from microbial reduction of DCE and TCA under anaerobic conditions or as the result of releases from PVC manufacturing operations (Hartmans, 1995).

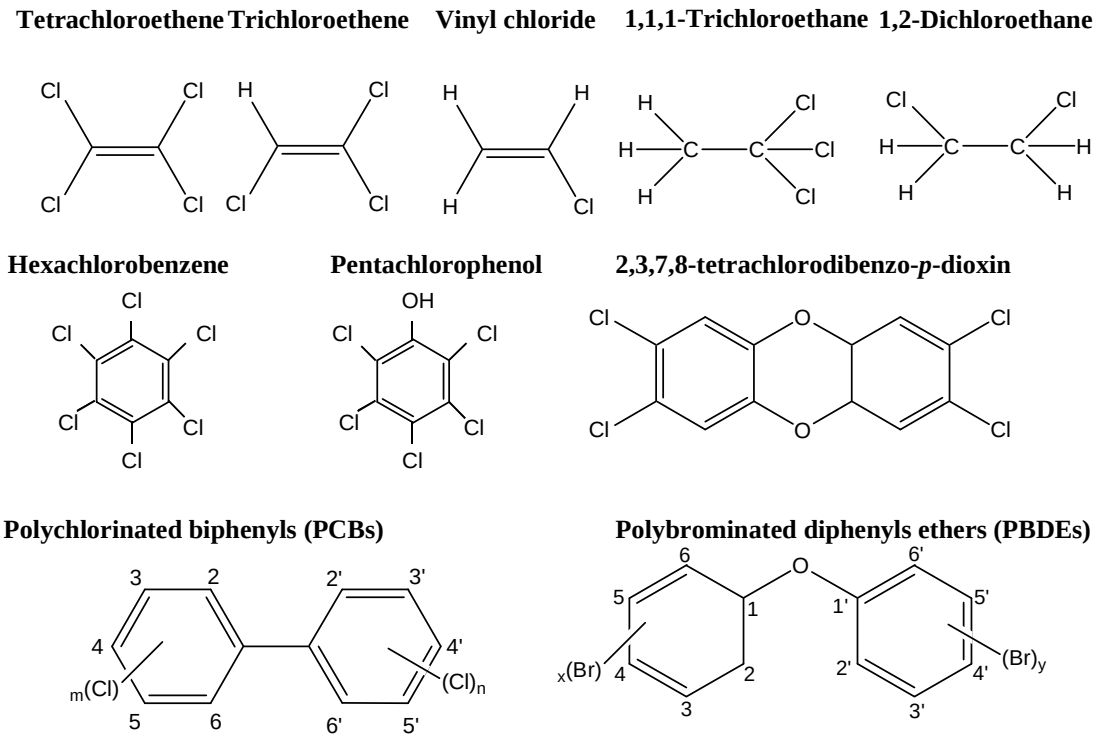


Fig 1.1 Structures of typical halogenated compounds in subsurface.

1.1 Chlorinated solvent contamination in the environment

The two-carbon chlorinated solvents (C_2) that are of major commercial importance are PCE, TCE, and 1,1,1-trichloroethane (TCA), and 1,2-DCA. They are widely used in industry due to their rapid evaporation rates, low flammability and reactivity, and excellent ability to quickly and efficiently dissolve a wide range of organic substances (Doherty, 2000a, b). PCE is most commonly known for its use in

the dry cleaning industry and peak usage in the U. S. occurred in 1980, whereas TCE is used as degreasing solvent (an extraction agent for fats). TCE was first prepared in 1864 by E. Fischer but received little attention until TCE was commercialized in Germany in 1910 (Ullmann, 2005). In 1912, TCE was applied in large scale for laundries, textiles and varnishes and as an extraction agent for fats. However, the toxicity of TCE was shown when used as a defatting agent for soybean to feed animal. It was recognized as the source of cattle poisoning, which resulted in extensive losses of cattle in Europe between 1923 and 1925 (Doherty, 2000a). Production of TCE in the United States started as a replacement for petroleum distillates in the dry-cleaning industry in the early 1920s and TCE became a good alternative of solvent for vapor degreasing in the 1930s. TCE has been used primarily as a solvent in industrial degreasing operations. Other uses have been as a solvent in dry cleaning and food processing, as an ingredient in printing inks, paints, etc. However, the use of TCE as a degreaser decreased in the 1960s as a result of more stringent environmental regulations and increasing popularity of trichloroethane (TCA) (Doherty, 2000a) although the production rate of TCE increased steadily with a peak in the 1970s (Ullmann, 2005).

Due to improper handling, storage, or disposal, large quantities of PCE/TCE have extensively contaminated soils and groundwater supplies in the various industrial, residential, and military sites. In military sites, PCE/TCE contamination can be due to toxic solvents, oils, greases, corrosives, fuels, and unexploded ordnance, emitted or discharged directly into soil, air, or water by the military yearly. Currently, there are over 22,000 contaminated sites in 3,300 active and former military installations in the United States. Many of these are included in the so-called "Superfund" list of the most contaminated and dangerous sites. This problem extends

to overseas US bases including bases in Asia and the Pacific (Bayona and Albaigés, 2006; Kurisu, 2008). A recent report shows that about 25,500 tonnes per year of TCE are sold in Europe for metal cleaning in 2007 although TCE is classified as a category 2 carcinogen (http://www.manufacturingtalk.com/news/ssg/ ssg101.html).

1.2 Toxicity of chlorinated solvents

Chlorinated solvents, like PCE, TCE and TCA have become common environmental contaminants, resulting from discharge to surface waters and groundwater by industrial or individual consumers, evaporative losses during use, incidental addition of TCE during food production, leaching from hazardous waste landfills into groundwater. On the other hand, more and more studies show that excess incidences of liver cancer, kidney cancer, or non-Hodgkin's lymphoma are found to be associated with occupational exposure to the chlorinated solvents (Wartenberg et al., 2000; Sullivan and Krieger, 2003). These health issues have helped the public dramatically raise their awareness of the man-made solvents' side effects. A number of studies on the commonly used solvents have been carried out in terms of their possible carcinogenic effects, safe-handling procedures, clean-up technologies, global effects, environmental regulations, etc (Apfeldorf and Infante, 1981; Abelson, 1990; ATSDR., 1993, 1997; Kielhorn et al., 2000; United Nations Environment Programme, 2000; Davis et al., 2002; National Environment Agency, 2002; Holliger et al., 2003; Sullivan and Krieger, 2003; Garcia, 2005; Ritalahti et al., 2005; ATSDR., 2006; Löffler and Edwards, 2006; ATSDR., 2007; Bhatt et al., 2007; EPA, 2007).

The U. S. National Toxicology 11th report on Carcinogens (revised Feb 2009) lists the following six halogenated solvents as suspected carcinogens: carbon

tetrachloride (CCl_4), chloroform ($CHCl_3$), 1,2-DCA, dichloromethane (DCM), and PCE, and TCE as "reasonably anticipated to be human carcinogens", whereas VC is a proven carcinogen (http://ntp.niehs.nih.gov/). As compared with other chloroethenes, VC has the lowest median lethal concentration (LC50) as shown in Table 1.1, which agrees well with its proven carcinogenic effect to human beings.

As for TCE, it was reclassified as a category 2 carcinogen by the European Union (EU) in January 2001 for the convincing evidence that it may cause cancer to human beings (http://www.hse.gov.uk/foi/internalops/fod/oc/200-299/294_49.pdf). Additionally, EPA also notes that TCE has the potential to induce neurotoxicity, immunotoxicity, developmental toxicity, liver toxicity, kidney toxicity, endocrine effects. Therefore, it is highly likely to produce cancer in humans.

Besides chlorinated ethenes, chlorinated ethanes are also prevailing groundwater and soil contaminants due to extensive usage, illegal and improper disposal and accidental spills. 1,2-DCA is commonly used as an intermediate of industrial polyvinyl chloride (PVC) production. Over 209 tons of 1,2-DCA were discharged to groundwater during 1987-1993 (www.epa.gov/enviro/html/tris/ez.html). Due to leakage and improper disposal, 1,2-DCA represents one of the world's most important toxic C2 chlorinated aquifer pollutants.

Table 1.2 shows that PCE, TCE, VC, 1,2-DCA, and chloroethane (CA) have been found to be the top five chlorinated C_1-C_2 solvents in 2008 based on an survey on the average annual underground releases for "1988 Core Chemicals" in the United States (see "toxics release inventory" in 2008 at U.S. Environmental Protection Agency website, http://www.epa.gov/triexplorer/chemical.htm). As one of the co-contaminant of the above listed solvents, TCA is also listed as an ozone-depleting chemical worldwide. These pollutants pose dispersing and long-lasting hazards

Table 1.1 Toxicoogical review of chloroethenes

Chemical	Condition- 4 hrs	Concentration (mg L^{-1})	Concentration (μM)	Molecular Weight (g mol^{-1})	Source
PCE	LC50 (mice)	36.0	217.1	165.83	Friberg et al, 1953
TCE	LC50 (rats)	67.2	511.2	131.4	http://www.epa.gov/ttnatw01/hlthef/tri-ethy.html
	LC50 (mice)	45.4	345.6		
1,1-DCE	LC50 (rats)	25.2	259.7	96.95	http://www.epa.gov/ttnatw01/hlthef/di-ethyl.html
trans-DCE	LC50 (rats)	95.4	984.0		Toxicological review of *cis*- and *trans*-1,2-DCE (2010) (www.epa.gov.iris)
cis-DCE	LC50 (mice)	54.2	559.1		
trans- & *cis*-DCE	LC50 (cats)	50-72	515.7-742.7		Lehmann (1911)
VC	LC50 (rabbits)	0.588	9.4	62.5	http://www.epa.gov/ttnatw01/hlthef/vinylchl.html
	LC50 (mice)	0.299	4.8		

Table 1.2 Chlorinated ethenes and ethanes in 2008 based on average annual underground releases for "1988 Core Chemicals" in the United States

Commonly used chlorinated solvents		Total on-site disposal or other releases (pounds)	Main application
C_1	Carbon tetrachloride	165,683	Chemical intermediate (Freon 11, Freon 12), fire extinguishing agent, grain fumigant, suppressant, paint manufacturing, gasoline additive, semiconductor production, refrigerant
	Chloroform	539,938	Chemical intermediate (Freon 22), cleaning agent, insecticidal fumigant, solvent
	Chloromethane	1,723,065	Chemical intermediate, methylating and chlorinating agent, greases, oils and resins, herbicide
	Dichloromethane	4,423,504	Degreasing agent, paint/varnish remover, blowing agent for urethane foams, solvent extractant (drugs, coffee), fumigant (grain, fruits)
C_2-chlorinated ethanes	1,1,2,2-Tetrachloroethane	3,644	Solvent, mothproofing textiles, paint/varnish/rust remover, immersion liquid (crystallography)
	1,1,1-Trichloroethane	87,514	Metal degreasing, Cleaning solvent, electrical machinery, plastics, textile spotting fluid, chemical intermediate, coolant, lubricant, inks, drain cleaners
	1,1,2-Trichloroethane	19,100	Intermediate
	1,2-Dichloroethane	454,675	Vinyl chloride (VC) production, chemical intermediate, lead scavenger, solvent
	Chloroethane	691,601	Chemical reagents, binder in paints commestic and similar products
C_2-chlorinated ethenes	Tetrachloroethene	1,592,814	Metal degreasing, Grain fumigation, chemical intermediate (chlorofluorocarbons), veterinary anthelmintic, heat exchange fluid
	Trichloroethene	3,596,806	Solvent, Vapor degreasing, typewriter correction fluid, low temperature heat transfer fluid, fire retardant, chemical intermediate, polyvinyl chloride manufacturing, extractant, lacquer/adhesive
	1,2-Dichloroethenes	4,282	Chemical intermediate, low temperature extractant (cafferine, perfumes), solvent (printed circuits, fats, phenol, camphor, natural rubber, labquer, thermoplastics)
	Vinyl chloride	385,429	PVC production

(suspected carcinogens) for humans and environments and tend to accumulate in reduced aquifers (De Wildeman and Verstraete, 2003). Pump-and-treat technologies are too expensive and time-intensive for the expanded pollution plumes, while oxidative degradation is not suitable for the prevailing (De Wildeman et al., 2003) conditions on site and the presence of multiple chlorinated compounds may inhibit the dechlorination process. PCE and TCE could be easily dechlorinated by cheap Fe(0) or bimetallic particles, but 1,2-DCA is totally resistant to metals (Gillham and O'Hannesin, 1994; Zhang et al., 1998). Therefore, much attention has been given to the cost-effective, controlled, fast and complete in situ bioremediation technologies simutaneously for these co-existing solvents (De Wildeman and Verstraete, 2003; GeoSyntec Consultants., 2005).

1.3 Extended toxicity of chlorinated solvents

The extensive usage of these chlorinated solvents consequently possesses a high potential to contaminate the environment, particularly water and soil, not only because of these solvents' toxicity and widespread distribution in aquifers but also the toxicity caused by their degradation products, e.g. DCEs and VC (Vogel and McCarty, 1985; Vogel et al., 1987; Vogel and McCarty, 1987; Abelson, 1990). They tend to sink and accumulate in groundwater sources (known as dense nonaqueous phase liquids, DNAPLs) because these halogenated solvents are generally denser than water. These toxic wastes can adversely disrupt the ecosystem by reducing bio-diversity, even extinguishing some sensitive species, or devastating natural restorative processes. The long-term, low-dose exposure to different kinds of toxic solvents may bring the potential hazards to the human health, ranging from disorders of the lungs, liver, kidneys, and other organs, or adverse effects on the immune, reproductive, or central

nervous systems, as well as mutations of genes and a variety of cancers (Wartenberg et al., 2000; Sullivan and Krieger, 2003). Acute overexposure to halogenated solvents can cause severe health effects to humans, including possible nervous system damage, heart failure, and increased rates of cancer (Sullivan and Krieger, 2003). Several halogenated solvents, e.g. PCE, TCE, 1,2-DCA, are considered as potential carcinogens for humans and have been shown to cause cancer in laboratory animals (Prince, 1998). 1,2-DCA is mainly used in the production of vinyl chloride (VC), which is the precursor to PVC but a proven carcinogen to humans (Kielhorn et al., 2000). Much of VC originates from higher chlorinated ethenes and ethanes in industry. Alternatively, as VC has higher solubility than the other chloroethanes, it also exists in groundwater systems, probably as the transformation product of PCE or TCE. In addition, these solvents including TCA, have been found to be particularly hazardous to the ozone layer (http://www.hse.gov.uk/foi/internalops/fod/oc/200-299/294_49.pdf). Therefore, complete and efficient removal of these solvents is of utmost importance to human beings and the environment.

1.4 Regulation of halogenated solvents

For many volatile organic compounds, different strategies have been attempted to prevent their misuse. For instance, the US EPA has also regulated maximal concentration level (MCL) for drinking water contaminants at 5 μg L^{-1}, 5 μg L^{-1}, 70 μg L^{-1}, 100 μg L^{-1}, 7 μg L^{-1}, 2 μg L^{-1}, 5 μg L^{-1} for PCE, TCE, *cis*-DCE, *trans*-DCE, 1,1-DCE, VC, and 1,2-DCA, respectively (http:// www.epa.gov/safewater/ contaminants/index.html). The usage of TCA was severely restricted and regulated by European Pollutant Emission Register (EPER) reporting requirements, United Nations Environment Programme (UNEP) Montreal Protocol on Substances that

Deplete the Ozone Layer in 1987 (which required the withdrawal of TCA by 2015 for developing countries) (United Nations Environment Programme, 2000). As a hazardous substance, importation and sale of TCA is strictly banned in UK and Singapore, since 2000 and 2002, respectively (National Environment Agency, 2002). In the United States, no TCA is supposed to be applied for domestic use after Jan 1, 2002 (ATSDR., 2006).

1.5 Problem statement

The solubilized chlorinated ethenes (e.g., PCE, TCE, *trans*-DCE and *cis*-DCE) and chlorinated ethanes (e.g., TCA and 1,2-DCA) were commonly detected in the contaminated sites. Much attention has been paid to the remediation of halohydrocarbons at contaminated sites, mainly chloroethenes and chloroethane (Zinder, 2010) through microbial reductive dehalogenation. Under anaerobic conditions, PCE can be reductively dechlorinated to TCE primarily via *cis*-DCE, VC to ethene (Vogel and McCarty, 1985; Zhang and Bennett, 2005) catalyzed by the specific reductive dehalogenases (RDases). One study conducted by U.S. Geological Survey (Garcia, 2005) during 2000-2005 identified chlorinated ethenes as one major source of volatile organic compounds through the subsurface occurrence characterization in the U.S. The highest concentration for PCE, TCE, *cis*-DCE and *trans*-DCE were 500-920 μg L^{-1}, 10,000-920,000 μg L^{-1}, 5,000-710,000 μg L^{-1}, 1,000-1,700 μg L^{-1} at one landfill site (Garcia, 2005). Generally, the most common degradation products of PCE and TCE are *cis*-DCE instead of *trans*-DCE; typically, *cis*-DCE is the predominant product by a 30-to-1 ratio compared with *trans*-DCE (Murphy and Morrison, 2002). Further reductive dechlorination of DCEs to VC and ethene is likely when the *cis*-DCE concentration is larger than 80% of the total

1,2-DCEs' concentration (total 1,2-DCEs are equivalent to the sum of *cis*-DCE and *trans*-DCE). However, when the *trans*-DCE concentration is 50% or more of the total DCE concentration, there is considerable likelihood that *trans*-DCE would be released directly into the environment because of the shortage of *trans*-DCE dechlorinating microbes (Byl and Williams, 2000). Recently, more and more reports have been published on the large accumulation of more *trans*-DCE than *cis*-DCE (Garcia, 2005; ITRC, 2007). During microbial reductive dechlorination of PCE/TCE, although reports on the generation of *trans*-DCE are relatively fewer than studies on *cis*-DCE generation, the accumulation of *trans*-DCE at contaminated sites poses a serious problem owing to its recalcitrant nature at subsurfaces. Studying the fate of *trans*-DCE is equally important as *cis*-DCE.

In addition, the incomplete sequential dechlorination of PCE under anaerobic conditions could generally result in accumulation of TCE, *cis*-DCE, and/or VC (Krumholz, 1997; Maymó-Gatell et al., 1997; Miller et al., 1997; Holliger et al., 1998; Luijten et al., 2003; Sung et al., 2003; He et al., 2005). However, as the isomers of *cis*-DCE, *trans*-DCE or 1,1-DCE have also been observed and accumulated as the predominant end product during microbial reductive reduction of PCE and TCE (Griffin et al., 2004; Miller et al., 2005; Shouakar-Stash et al., 2006; Zhang et al., 2006). Skeen et al. observed the formation of significant amount of *trans*-DCE, *cis*-DCE as well as 1,1-DCE in a methanogenic consortium in 1995 (Skeen et al., 1995). In 1997, Christiansen et al. demonstrated that *trans*-DCE was formed as the main DCE isomers over *cis*-DCE and 1,1-DCE in an upflow anaerobic sludge blanket reactor with the ratio of *trans*-DCE to *cis*-DCE at 1.2-2.2 with trace amount of 1,1-DCE in the transformation of PCE (Christiansen et al., 1997). Meanwhile, the formation of *trans*-DCE and *cis*-DCE (ratio-2.45) were also found from a sediment-

free, nonmethanogenic culture which was initially cultivated in 1,2-dichloropropane (Löffler et al., 1997b). In 2004, Griffin et al. reported the generation of *trans*-DCE to *cis*-DCE from PCE dechlorination at a ratio of 3.0(±0.5) :1 from six different microcosms which probably contained Pinellas groups of *Dehalococcoides* spp. (Griffin et al., 2004). Besides this unique *Dehalococcoides* group, a polychlorinated biphenyl-dechlorinating bacterium, *Dehalobium chlorocoercia* DF-1, was reported to be capable of transforming PCE to *trans*-DCE and *cis*-DCE at a ratio of 1.2-1.7 (Miller et al., 2005). It shared 89% similarity of 16S rRNA gene sequence with *D. ethenogenes* strain 195. However, culture DF-1 failed to grow in a fully defined medium as it required the presence of the cell extract from *Desulfovibrio* spp. Thus, besides *cis*-DCE and 1,1-DCE, *trans*- DCE has been shown to be the major intermediate and end product during the dechlorination of PCE/TCE and no dechlorination beyond *trans*-DCE through *Dehalococcoides* sp. was observed in some contaminated sites.

In summary, currently little is known on the microbes involved in the production of *trans*-DCE in the contaminated sites. The mechanism of *trans*-DCE generation in natural environment is also poorly understood. Due to its recalcitrant nature, the accumulation of *trans*-DCE in the environment hinders its further transformation to the harmless product, ethene, thus posing a potential threat to human health. This accumulation will subsequently result in an incomplete dechlorination of PCE or TCE, thus leading to inadequate bioremediation in the contaminated sites.

In addition, the functional genes responsible for production of significant amounts of *trans*-DCE remain unidentified. Several highly enriched cultures in our lab are showing sequential dechlorination of PCE via TCE to *trans*-DCE and *cis*-DCE

as the major and minor degradation products, respectively. Thus, characterization and isolation of such microbes followed by studying the reductive dehalogenase (RDase) genes specific for *trans*-DCE production are greatly required for understanding the mechanism of *trans*-DCE formation.

Complete dechlorination of PCE to ethene, the desired end point, often does not occur naturally because of insufficient electron donors, slow reaction kinetics, the absence of dehalogenating bacteria, or other environmental factors (Cupples et al., 2004). The transformations of PCE are frequently incomplete, often resulting in the accumulation of VC, a more hazardous and mobile compound (Haston and McCarty, 1999). Dechlorination of VC often occurs cometabolically, which will also increase the difficulty of bioremediation. The resulting slow transformation rate of VC would increase the treatment cost tremendously, or even result in unsuccessful bioremediation of chloroethene-comtaminated site.

1.6 Thesis hypothesis

Previous studies suggest the presence of novel microorganisms involved in *trans*-DCE production, mainly belonging to the genus of *Dehalococcoides*, which is capable of dechlorinating PCE to *trans*-DCE predominantly at many contaminated sites and in laboratory cultures. This novel strain should differ primarily in the range of dechlorinating substrates and functional RDase genes from previously discovered *Dehalococcoides* spp. Microcosms around the area with large accumulation of *trans*-DCE may have the potential to further remove the generated *trans*-DCE as there is a higher likelihood that these microorganisms can adapt to this contaminant. Therefore, it will be of great interest to explore the microorganisms that can produce *trans*-DCE as well as the microorganism that can eliminate *trans*-DCE in the contaminated

groundwater.

Given that high levels of *trans*-DCE may cause central nervous system depression in humans, the isolation of the *trans*-DCE producing culture may contribute to further investigations on the application of *Dehalococcoides* species. Therefore, exploring robust culture(s) that can detoxify PCE completely under anaerobic conditions is urgent in order to stimulate fast dechlorination in *situ*. The mechanisms underlying this special dechlorination process will also be studied for better understanding of the bioremediation process while providing right source of inocula for successful bioaugmentation. This would be the final objective of this project, complete detoxification of chloroethenes. Results from this study will significantly improve the treatment efficiency towards halogenated compound-contaminated sites.

1.7 Objectives

The main objective of this study is to achieve the complete detoxification of PCE and to elucidate the formation of *trans*-DCE by *Dehalococcoides* spp.

The specific objectives of the current study are to:

1) Explore the diversity of microorganisms in the *trans*-DCE producing microcosms collected from natural environments and characterize PCE-to-*trans*-DCE dechlorinating enrichment cultures.

2) Characterize and isolate novel *trans*-DCE producing microbes, and understand the functional genes specific for *trans*-DCE production.

3) Elucidate how the microbes catalyze dechlorination of PCE to the predominant *trans*-DCE through expression analysis of multiple RDase genes from the *trans*-DCE producing culture.

4) Cultivate robust dechlorinators capable of completely detoxifying PCE to ethene metabolically and understand the role of specific functional genes involved in individual dechlorinating isolate(s).

1.8 Thesis outline

To achieve these objectives, research work has been carried out mainly in six stages for this thesis as shown in Fig 1.2.

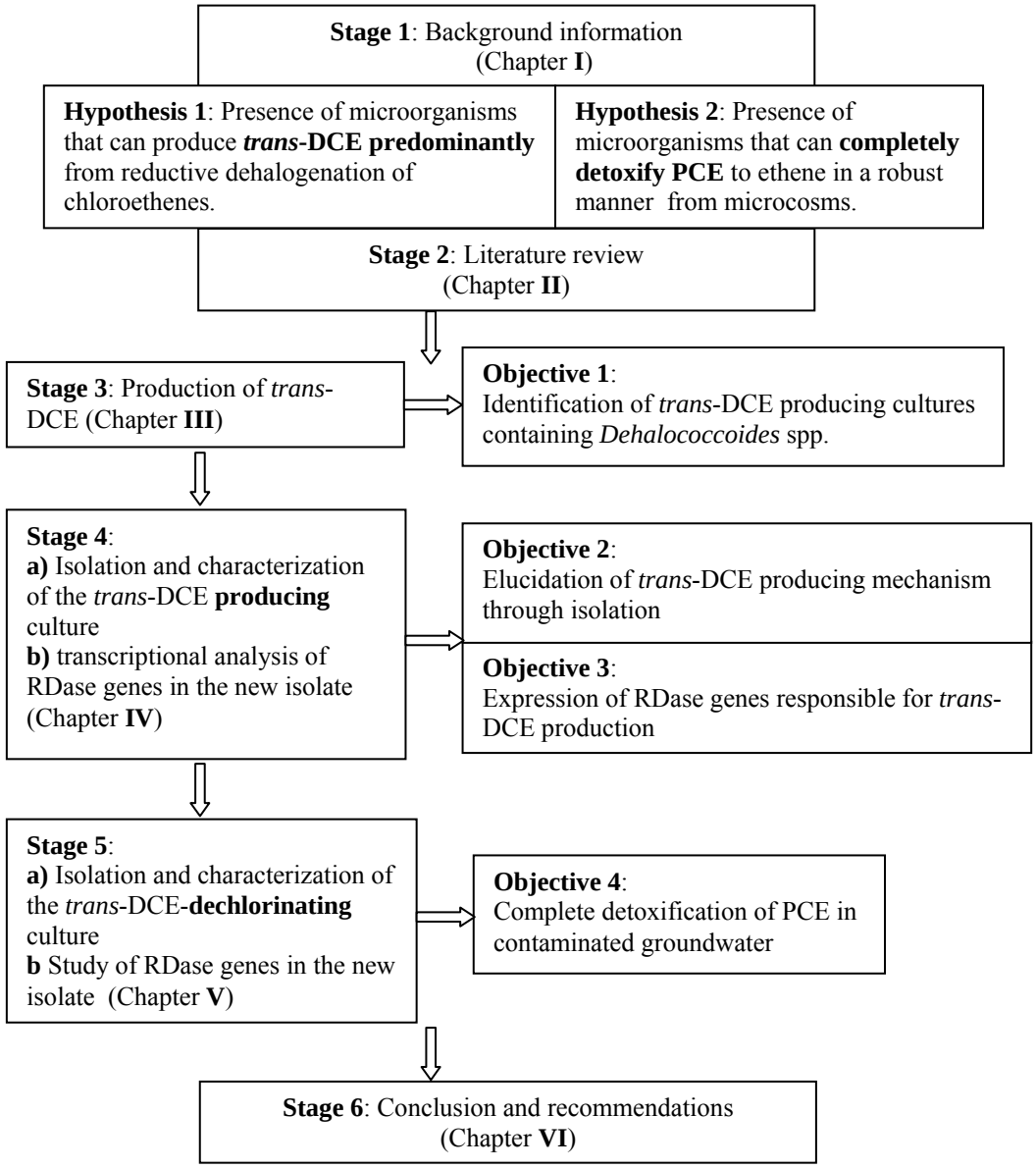


Fig 1.2 The stages and objectives of this study

Chapter II offers a comprehensive literature review on the microbial reductive dechlorination processes mainly for chlorinated ethenes, application of bioremediation studies, and the main dehalorespirators as characterized by the molecular tools. Chapter III describes study on the enrichment and identification of microorganisms involved with *trans*-DCE production from reductive dechlorination of PCE and TCE. Chapter IV is the detailed study on the isolation of this novel *trans*-DCE producing culture and the transcriptional analysis of RDase genes in this isolate. Chapter V focuses on the isolation of a novel microbe capable of completely dechlorinating all the chloroethenes to ethene including the recalcitrant *trans*-DCE. The application of this isolate is also investigated for the bioremediation of PCE-contaminated groundwater. Chapter VI presents the major conclusions of this study and states recommendation for future studies.

Chapter II Literature Review

Anthropogenic compounds can be transformed in the environment naturally through microbial mediated processes. During these processes, organic compounds are oxidized for energy and growth, with oxygen as the electron acceptor (Pavlostathis et al., 2003). However, man-made halogenated organic solvents present unique challenges to determining their fate in the environment because of their physiochemical properties (Wisconsin Department of Natural Resources, 2003). They are more oxidized due to the presence of electronegative halogen substituent, which makes them relatively stable for better usage but resistant to biodegradation under aerobic conditions. Therefore, in the subsurface environment, reduction of these solvents is more likely to occur than oxidation in particular for polyhalogenated compounds. These polyhalogenated compounds and their byproducts have a tendency to accumulate in the ecosystem including sediments, sludge, soils, and groundwater. Numerous studies have shown that halogenated compounds are recalcitrant molecules resistant to mineralization, but can be transformed by anaerobic mechanism into harmless products, or into relatively lightly halogenated compounds, like vinyl chloride (VC) or chloroethane (CA) that are further degradable by aerobic microorganism (Holliger et al., 2003). These reactions have triggered the request for microbial populations to play a role in reductive dehalogenation or detoxification in these environments. Various molecular tools have been developed to understand this dehalogenation process.

2.1 Transformation of halogenated compounds

Polyhalogenated compounds are relatively stable in the aerobic environments, probably due to the high reactivity of halogens and stable structure of carbon-halogen

bond (Reineke, 2001). When released into soil, these aerobically persistent polyhalogenated compounds can form dense nonaqueous phase liquids (DNAPLs) and often exist in deep subsurface environments, which are anaerobic. Depending on their ultimate fate, those chlorinated solvents may be degraded to harmless byproducts or they may exert harmful effects through toxicity, biomagnification and/or persistence in the environment. Their harmful impact on the biota may be direct (e.g. toxicity) or indirect, such as by destruction of the protective ozone layer in the stratosphere by atmospheric halocarbons.

Of great importance is the microbial reductive dechlorination of chlorinated solvents in the natural environments (Smidt and de Vos, 2004). With the removal of halogens during reductive dehalogenation, the less halogenated products tend to be less hydrophobic, more mobile, more volatile, and more soluble than the parent compounds by many orders of magnitude. However, as halogens are removed sequentially, dehalogenation reactions tend to occur extremely slowly in particular when reaching di- or mono-halogenated state (Pavlostathis et al., 2003). Generally, two basic mechanisms are involved with reductive dehalogenation, abiotic (or cometabolic), and biotic (metabolic) conversion. The former is proposed to be catalyzed mainly by metal ion-containing heat-stable tetrapyrroles or enzymes. During this process, these compounds are incorporated as cofactors and do not serve as a source of carbon or energy for microbial growth thus additional energy is required (Holliger et al., 2003; Smidt and de Vos, 2004). Cometabolism is particularly referred to the simultaneous degradation of two compounds, in which the degradation of the second one depends on the presence of the first substrate (Jitnuyanont et al., 2001). Examples of cometabolism include the reductive dechlorination of PCE by sulfate reducers (Cole et al., 1995), methanogens (Fathepure

and Boyd, 1988; Freedman and Gossett, 1989), or acetogens (Terzenbach and Blaut, 1994). Therefore, halogenated compounds might be degraded by fermentative, oxidative, or reductive pathways, depending on the prevailing environmental conditions. In general, most abiotic transformations are slow, but can still be significant within the time scales commonly associated with the movement of groundwater. In contrast, biotic transformations typically proceed much faster, provided that there are sufficient substrate and nutrients and a microbial population that can mediate such transformations (Vogel et al., 1987). Although the role of cometabolic conversion cannot be excluded for the destruction of halogenated compounds, the metabolic conversion is the primary mechanism for the transformation of chlorinated solvents in the contaminated sites (Zinder, 2010). Halogenated compounds can serve in three different metabolic functions in anaerobic bacteria: i) as carbon or energy source or both, ii) as substrate for cometabolic activity, and iii) as terminal electron acceptor in an anaerobic respiration process (Holliger et al., 2003). The last respiration process, also termed as microbial reductive dehalogenation which contributes to the primary metabolism (Zinder, 2010), can be further divided into two groups, hydrogenolysis, and dihaloelimination. Hydrogenolysis refers to the displacement of a halogen substituent with hydrogen, while dihaloelimination refers to replacement of two halogen-carbon bonds with a carbon-carbon bond. The transfer of electrons from an external electron donor is essential for both groups of reactions. In natural environment, hydrogenolysis occurs more frequently than dihaloelimination except 1,2-dichloroethane (1,2-DCA). Thus reductive dehalogenation has been predominantly referred to the term hydrogenolysis.

2.2 Dehalorespiration process

In the reductive dehalogenation process, halogenated compound serves as terminal electron acceptor resulting in energy production for microbial growth, which is known as (de)halorespiration. A number of studies have found some halogenated compounds are commonly used by bacterial species as growth substrates, e.g., chloroethenes, chloroethanes, chlorophenols, chlorobenzenes, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and polychlorinated-dibenzo-*p*-dioxins (DD)/dibenzofurans (PCDD/Fs) (Zhang and Bennett, 2005; Bayona and Albaigés, 2006; Häggblom et al., 2006; Bunge and Lechner, 2009; Lee and He, 2010).

Previous studies have shown that certain naturally occurring microorganisms have evolved to break down these contaminants. Originally, reductive dehalogenation was found to be a cometabolic side reaction in anaerobes, such as methanogens, sulfate-reducers, and acetogens (Bouwer and McCarty, 1983; Vogel and McCarty, 1985, 1987; Fathepure and Boyd, 1988; Freedman and Gossett, 1989; Terzenbach and Blaut, 1994; Cole et al., 1995). Most cometabolic transformations are slow but they can still be significant within the time scales associated with groundwater migration. Since early 1980s, considerable evidences have shown that metabolic reductive dechlorination of PCE, TCE, DCEs, VC, TCA, 1,2-DCA have arisen from anaerobic microcosms, enrichment cultures, and pure cultures and these metabolic processes proceeded much faster than the cometablic reactions (Vogel et al., 1987; DiStefano et al., 1991; Holliger et al., 2003). With recent development of rapid and inexpensive molecular techniques, bioremediation industry developed rapidly for the PCE, TCE or TCA-contaminated sites/soil/groundwater, including i) the identification and isolation

of the specific bacteria mediating the dehalorespiration process, ii) essential nutrient requirements, and the proper approaches for stimulating the desired reactions but minimizing undesirable microbial activities, iii) development of biomarkers to evaluate and predict the *in situ* activities, iv) exploration of potential application of current halorespirators for halogenated compounds other than chlorinated solvents.

Over the past two decades, numerous mixed and pure culture studies have revealed that predominantly reductive dehalogenation processes, in addition to oxidative and fermentative mechanisms, are responsible for the initial attack and degradation of a wide range of halogenated compounds in the absence of molecular oxygen (Häggblom and Bossert, 2003; Holliger et al., 2003; Janssen et al., 2005). Furthermore, significant attention has been given to characterization, isolation of the main dehalorespirators, and identification of the main enzyme(s)-reductive dehalogenase(s) responsible for the dehalorespiration process.

2.3 Specific bacteria mediating the dehalorespiration process

With the advent of molecular techniques, the dehalorespiration process for halogenated solvents has been understood and shown to be carried out mainly by three distinct groups of microorganisms, 1) genera *Dehalobacter* and *Desulfitobacterium* in the *Peptococcaceae* family in the Firmicutes, 2) members (*Anaeromyxobacter*, *Desulfuromonas*, *Geobacter*, *Desulfomonile*, *Geobacter*, *Desulfononile*, *Desulfovibrio*, and *Sulfurospirillum*) of the delta (δ) and epsilon (ε) subphyla of the *Proteobacteria*, and 3) the *Dehalococcoides*-predominant group in the Chloroflexi (Taş et al., 2009a; Zinder, 2010). Among these three groups, the majority of these bacteria transform PCE or TCE to *cis*-DCE, only *Dehalococcoides* spp. are a unique group that is capable of completely dechlorinating PCE to ethene (Bombach

et al., 2010) and only *Dehalobacter* spp. have been reported to dechlorinate TCA metabolically (Sun et al., 2002; Grostern and Edwards, 2009). Due to the extensive usage of chlorinated solvents and their related potential carcinogenicity, dechlorination of PCE, TCE, TCA and 1,2-DCA, carried out by *Dehalococcoides* and *Dehalobacter*, is of great oncern to be covered in this section.

2.3.1 Major dechlorinating microorganisms for chlorinated ethenes

Chloroethene dechlorination isolates that belong to the genera *Anaeromyxobacter*, *Desulfitobacterium*, *Sulfurospirillum*, *Desulfomonile*, *Desulfuromonas*, *Desulfovibrio*, and *Trichlorobacter* are metabolically versatile with respect to their spectrum of electron donors and acceptors; while limited isolates appeared as highly specialized bacteria that strictly depend on halorespiration for growth, in most cases coupled to hydrogen as the electron donor (Smidt and de Vos, 2004). These pure cultures capable of coupling growth to this process via halorespiration include *Desulfitobacterium* spp., *Desulfuromonas* spp., *Sulfurospirillum multivorans*, *Dehalobacter* spp., and *Dehalococcoides* spp. (Holliger et al., 2003) (see Fig 2.1).

The majority of them could dechlorinate PCE and TCE to *cis*-DCE. The first microorganism that coupled PCE dechlorination to growth was *Dehalobacter restrictus* PER-K23 (gram-negative bacteria), dominant in an enrichment culture, which dechlorinated PCE and TCE to *cis*-DCE found in 1993 (Holliger et al., 1993) and obtained in pure culture in 1998 (Holliger et al., 1998). *Dehalobacter restrictus* TEA, a gram-positive, rod-shaped, motile bacterium, was isolated from an anaerobic charcoal reactor originally inoculated with contaminated groundwater, capable of dechlorinating PCE and TCE to *cis*-DCE. It required H_2 as the electron donor, and

acetate or CO_2 as carbon source. The 16S rRNA gene sequence of strain TEA is 99.7% similar to the sequence of strain PER-K23 (Wild et al., 1996). Isolate belonging to genus of *Desulfitobacterium* could also dechlorinate PCE to *cis*-DCE, e.g., *Desulfitobacterium* sp. strain PCE-S (Miller et al., 1997), *Desulfitobacterium* sp. strain B3e31 (Yoshida et al., 2007). However, there are also bacteria capable of dechlorinating PCE predominantly to TCE rather than *cis*-DCE, eg. *Desulfitobacterium* sp. strain PCE1 (Gerritse et al., 1996).

Generally, the above mentioned isolates often result in the accumulation of *cis*-DCE during reductive dechlorination of PCE or TCE (Bradley, 2003). Although reductive dechlorination of PCE and TCE was first recognized as early as 1983 (Bouwer and McCarty, 1983), but the first isolate that dechlorinates PCE completely to ethene in a step-wise manner was *Dehalococcoides etheneogenes* strain 195, obtained in 1997 (Maymó-Gatell et al., 1997).

Since then, it is well established that PCE can be reductively dechlorinated by microorganisms to TCE, DCE isomers, and VC but complete dechlorination of PCE to ethene by pure culture was only observed for *Dehalococcodies ethenogenes* strain 195 (Maymó-Gatell et al., 1997). Considerable efforts have been expanded in understanding and improving the microbial dehalogenation process of PCE/TCE or chlorinated ethanes after the isolation of *D. ethenogenes* strain 195 (Maymó-Gatell et al., 1997; Magnuson et al., 1998; Magnuson et al., 2000). For instance, dechlorination of VC is found to be the rate-limiting and the last step for complete detoxification of PCE to ethene in anaerobic condition for this member of Cornell subgroup of *Dehalococcoides*. This VC-dechlorinating step follows first-order reaction and is also a rate-limiting step for other *Dehalococcoides* spp., such as strain FL2 within the Pinellas subgroup (He et al., 2005). In some cases, VC can also be

degraded in aerobic systems with VC-oxidizing microorganisms, e.g. methanotrophs, etheneotrophs, or VC-assimilating bacteria (Chuang et al., 2010).

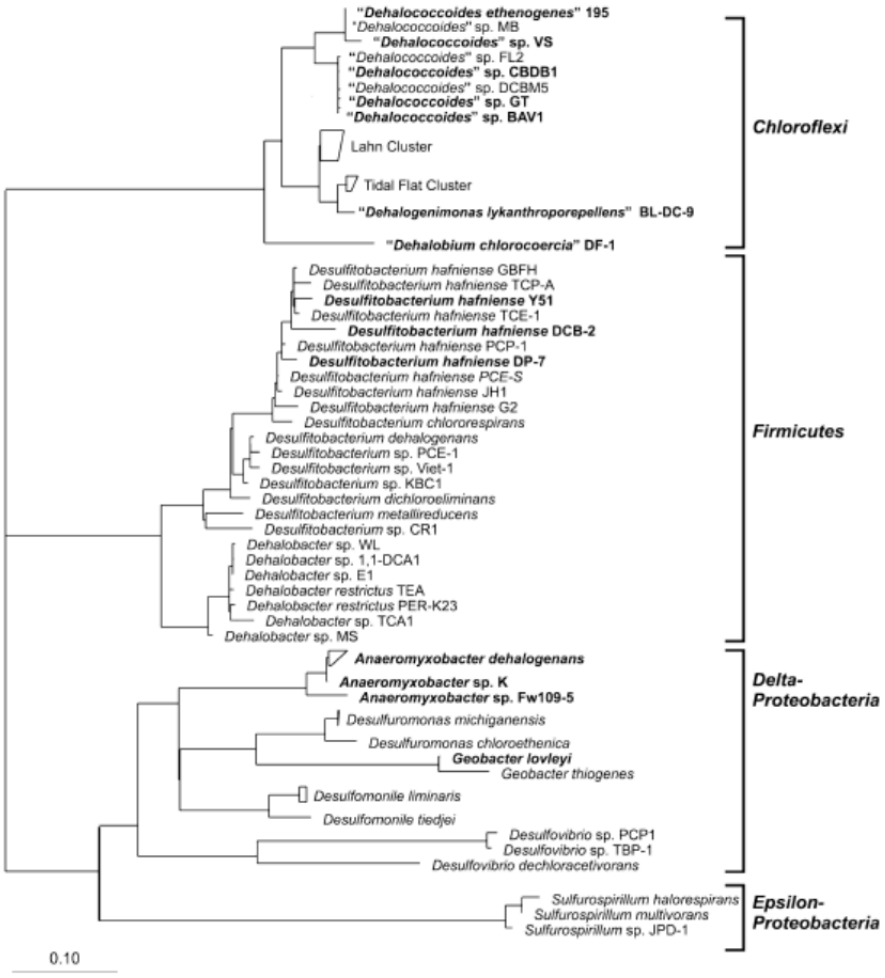


Fig 2.1 Phylogenetic tree of major dechlorinating bacteria constructed based on the 16S rRNA gene sequences. The reference bar indicates the branch length that represents 10% sequence divergence (Taş et al., 2009a).

To date microbial community analyses of dehalogenating bacteria largely focused on chlorinated ethene-contaminated groundwater or soils by *Dehalococcoides* spp. (Taş et al., 2009b). The presence of *Dehalococcoides* spp. in pristine and contaminated (with PCE, TCE, or VC) sites from North America, Europe, and Japan was reported elsewhere (Löffler et al., 2000; Hendrickson et al., 2002; Kittelmann and Friedrich, 2008a, b). To date, only members of the genus of *Dehalococcoides* have

been reported to be capable of dechlorination past DCEs to VC and ethene (Smidt and de Vos, 2004) (as shown in Fig 2.2).

Fig 2.2 Dechlorination process of PCE or TCE by the defined cultures.

Numerous studies have shown that *Dehalococcoides* genus is widely distributed in nature and is able to dehalogenate a wide variety of halogenated compounds, like chlorinated benzenes, biphenyls, naphthalenes, dioxins, and ethenes (see Table 2.1) (Adrian et al., 2000; Bradley, 2003; Zhang and Bennett, 2005; Häggblom et al., 2006; Adrian et al., 2007b; Bunge et al., 2007; Bunge et al., 2008; Adrian et al., 2009; Bunge and Lechner, 2009; Zinder, 2010). There are currently 5 members of known *Dehalococcoides* isolates, *Dehalococcoides ethenogenes* 195 (Maymó-Gatell et al., 1997; Maymó-Gatell et al., 1999; Seshadri et al., 2005), CBDB1 (Adrian et al., 2000), *Dehalococcoides* isolate BAV1 (He et al., 2003b), FL2 (He et al., 2005) and GT (Sung et al., 2006a). Among them, *D. ethenogenes* 195 is the first isolate to completely dechlorinate PCE to ethene, although the last step of dechlorination of VC was done co-metabolically (Maymó-Gatell et al., 1997) (Fig 2.2). Recently, further exploration of strain 195 suggests that its substrate range was not only limited to chloroethenes, but was also involved with other halogenated compounds, e.g., 2,3-dichlorophenol, 1,2,3,4,7,8-hexachlorodibenzofuran, and octa-brominated diphenyl

ether (Ahn et al, 2008; He et al., 2006; Fung et al 2007). Stain BAV1 could grow with all the DCE isomers and VC as substrate (He et al., 2003a; He et al., 2003b), but it used TCE and PCE co-metabolically. Strain FL2, on the other hand, was able to

Table 2.1 *Dehalococcoides* spp. and their metabolic substrates

Name of the culture	Halogenated compounds reduced	End-products	References
Dehalococcoides ethenogenes strain 195	PCE, TCE, *cis*-DCE, 1,1-DCE	VC (ethene)	Maymó-Gatell et al (1997)
	1,2-DCA,	Ethene	Maymó-Gatell et al (1999)
	1,2,3,4-tetrachlorodibenzo-*p*-dioxin	1,2,4-trichlorodibenzo-p-dioxin, 1,3-dichlorodibenzo-*p*-dioxin	Fennell et al. (2004)
	2,3,4,5,6-pentachlorophenyl	2,3,4,6-, or 2,3,5,6-tetra-chlorobiphenyl, 2,4,6-tri-chlorobiphenyl	
	Hexachlorobenzene (HCB)	1,2,3,5-tetrachlorobenzene, 1,3,5-trichlorobenzene	
	2,3-DCP, 2,3,4-TCP, 2,3,6-TCP	Lower chlorinated phenols (ortho chlorine removed)	Adrian et al. (2007)
Dehalococcoides sp. strain BAV1	*trans*-DCE, *cis*-DCE, 1,1-DCE, VC, 1,2-DCA	Ethene	He et al. (2003a)
Dehalococcoides sp. strain CBDB1	HCB	1,3-DCB, 1,4-DCB, and 1,3,5-TB	Adrian et al. (2000)
	2,3-DCP, all six TCPs, all three triCPs and penta-CP	Lower chlorinated phenols	Adrian et al. (2007)
	Polychlorinated dioxins	Dichloro-dioxins	Bunge et al (2003)
	Polychlorinated biphenyls (Aroclor 1260)	various	Adrian et al (2009)
Dehalococcoides sp. strain FL2	TCE, *trans*-DCE, *cis*-DCE, 1,1-DCE	VC (ethene)	He et al. (2005)
Dehalococcoides sp. strain GT	TCE, *cis*-DCE, 1,1-DCE, VC	Ethene	Sung et al. (2006)
Dehalococcoides sp. strain DCMB5	1,2,4-Trichlorodienzo-*p*-dioxin	2-Monochlorodizenbo-*p*-dioxin	Bunge et al. (2008)
	1,2,3-TCB	1,3-DCB	
Dehalococcoides spp. (VS, mixed culture)	TCE(slow), *cis*-DCE, 1,1-DCE, VC	Ethene	Cupples et al. (2003)
Dehalococcoides spp. (KB-1, mixed culture)	TCE, *cis*-DCE, VC	Ethene	Duhamel et al. (2004)
Dehalococcoides spp. (ANAS, mixed culture)	TCE, *cis*-DCE, 1,1-DCE, VC	Ethene	Holmes et al. (2006)

dechlorinate TCE, *trans*-DCE and *cis*-DCE to VC as the end product (He et al., 2005). Strain GT was identified to use TCE, *cis*-DCE, 1,1-dichloroethene (1,1-DCE) and VC as the growth supporting substrates (Sung et al., 2006). Strain CBDB1 metabolically dechlorinated chlorobenzenes (Adrian et al., 2000), chlorophenols (Adrian et al., 2007b), commercial PCB mixture Aroclor 1260 (Adrian et al., 2009) and some polychlorinated dibenzodioxin congeners (Bunge et al., 2003) but exhibited no TceA activity on chlorinated ethenes biotically (Hölscher et al., 2004).

2.3.2 Major dechlorinating microorganisms for chlorinated ethanes

Although a number of studies have been conducted on the removal of chlorinated ethenes from contaminated sites (Fig 2.3a), remediation of chlorinated ethanes, such as 1,1,1-TCA and 1,2-DCA, remains problematic and these chlorinated ethanes can even inhibit the restoration of chloroethenes-contaminated site.

1,1,1-TCA may undergo slow abiotic degradation to acetic acid and 1,1-DCE or co-metabolic biotransformation (Bradley, 2003). A growth-linked dehalorespiratory process of 1,1,1-TCA is only limited to strain TCA1, closely related to *Dehalobacter restrictus*, which could reductively dechlorinate 1,1,1-TCA to 1,1-DCA and CA (Sun et al., 2002). Similar to strain TCA1, a mixed anaerobic microbial culture, named MS/H_2, mainly consisting of *Dehalobacter*, enriched from 1,1,1-TCA contaminated sites also demonstrated its halorespiring capacity to dechlorinate 1,1,1-TCA to 1,1-DCA but not any further (Grostern and Edwards, 2006). These strains of *Dehalobacter* are unable to dechlorinate TCE. Great inhibition of TCE removal by *Dehalococcoides*-containing enrichment culture KB-1 was also noticed in the presence of chlorinated ethanes (Grostern and Edwards, 2006).

To date, reductive dechlorination of chloroethenes and chloroethanes mostly focused on *Dehalococcoides* and *Dehalobacter* species. Although strain 195 could dechlorinate 1,2-DCA to ethene (Maymó-Gatell et al., 1999), this strain requires the presence of unknown bacterial extracts. Additionally, strain BAV1 can dechlorinate 1,2-DCA, all the DCE isomers, and VC coupling for growth but not PCE or TCE (He et al., 2003b). Only a few field studies focused on chlorinated ethanes. A recent study on the dechlorinating potential of 1,2-DCA by sediments collected from three different European rivers shows that biodegradation of 1,2-DCA occurred only in the sediments instead of liquid phase under anaerobic conditions (van der Zaan et al., 2009). Generally, anaerobic removal of 1,2-DCA was observed under 1) methanogenic, 2) denitrifying, and 3) iron-reducing conditions. Reductive dechlorination of 1,2-DCA to ethene occurred under the first conditions, while oxidation of 1,2-DCA was slowly observed under the denitrifying or iron-reducing conditions (van der Zaan et al., 2009) to CO_2 (Fig 2.3b).

De Wildeman et al. (2003) successfully isolated *Desulfitobacterium dichloroeliminans* strain DCA1 which could dichloroeliminate 1,2-DCA and 1,2-dichloroprapane. The novel reductive dehalogenase (RDase) gene *dcaA*, isolated from this strain DCA1 shows that the open reading frame (ORF) encoding the catalytic subunit of *dcaA*, showed only 94% nucleotide and 90% amino acid identity with *pceA* of strain DSMZ 9455T.

A new *Dehalobacter*-containing coculture (WL DCA/H_2) was later identified to be able to dihaloeliminate 1,2-DCA only with the presence of a non-dechlorinating *Acetobacterium* sp. strain. A new putative RDase gene, WL *rdhA1* was also identified from this study and transcribed specifically upon exposure to 1,2-DCA and found to be different from the RDase genes identified from strain DCA1.

2.4 RDase genes in dehalorespiration process

RDase is the key enzyme for halorespiration, which catalyzes the substitution of a halogen substituent (e.g., chlorine, bromine or iodine atom) with a hydrogen atom. The halogen removal by RDases drives the halorespiration process by helping to form a proton gradient on either side of the bacterial inner membrane (Habash et al., 2004). Membrane-bound hydrogenases and/or formate hydrogenases produce H^+ on the periplasmic side of the inner membrane, whereas H^+ is utilized by a soluble or membrane-associated RDase on the cytoplasmic side. The presence of RDase at the end of the electron transport chain allows halogenated substrates to serve as a terminal electron acceptor. Therefore, halorespiration performs two important functions, which was first proposed by Mohn and Tiedje (Mohn and Tiedje, 1990) based on studies with *Desulfomonile tiedjei* strain DCB-1. The first function is to form a proton motive force that result in H^+ movement from the periplasmic to cytoplasmic sides of the membrane through an ATP-generating ATPase, while the other function is the dehalogenation of halogenated organic compounds.

Previous reports show that *Dehalococcoides* species have been involved in the reductive dechlorination of PCE and TCE past DCEs to VC and ethene (Maymó-Gatell et al., 1997; Smidt and de Vos, 2004). Most of these enzymes share a common feature of being membrane-bound, and containing a corrinoid cofactor and Fe_4S_4 clusters (Müller et al., 2004). Among these RDases, PceA (AAW40342) and TceA (AF228507), are the first two RDases that were partially purified from an anaerobic microbial enrichment culture containing *Dehalococcoides ethenogenes* strain 195 (Magnuson et al., 1998; Magnuson et al., 2000). The identified RDase genes, *pceA* and *tceA* genes are responsible for dechlorination of PCE to TCE and TCE to *cis*-DCE,

VC and ethene, respectively; whereas *bvcA* and *vcrA* genes play major roles for VC dechlorination to ethene (Magnuson et al., 1998; Krajmalnik-Brown et al., 2004; Müller et al., 2004) (Table 2.2). Currently, *bvcA* gene was only found in strain BAV1-like culture, encoding the reductive dechlorination of all DCEs isomers and VC (Krajmalnik-Brown et al., 2004).

D. ethenogenes strain 195 exploits a separate RDase gene (*pceA*) for the dechlorination of PCE to TCE (Hendrickson et al., 2002). The *tceA* gene, present in both strains 195 (Seshadri et al., 2005) and FL2 (He et al., 2005), has been shown to participate in the sequential transformation of TCE to *cis*-DCE and VC metabolically and in the reduction of VC to ethene co-metabolically (Magnuson et al., 2000; Magnuson et al., 1998). This RDase dechlorinates TCE, *cis*-DCE, and 1,1-DCE at rates ranging from 5 to 12 μmol min^{-1} (mg of protein) $^{-1}$, dechlorinates VC and *trans*-DCE at substantially lower rates of 0.04 to 0.45 μmol min^{-1} (mg of protein) $^{-1}$, but cannot dechlorinate PCE (Magnuson et al., 2000). The VC reduction rate of TceA was less than 1% of its activity of TCE and *cis*-DCE reduction.

The *vcrA* gene was found to be involved in both strain VS (Müller et al., 2004) and strain GT (Sung et al., 2006) in the metabolism of VC. Müller et al. found that the enzyme VcrA reduced VC and all DCE isomers at high rates, but not PCE or TCE (Müller et al., 2004). Similarly, the reduction of TCE to *cis*-DCE by VcrA was very slow and occurred at only 5% of the reduction rate for *cis*-DCE to VC, indicating the possibility of a cometabolic process for TCE reduction (Cupples et al., 2003). This VC RDase activity was sensitive to air exposure with an activity half-life of 5(±3) min.

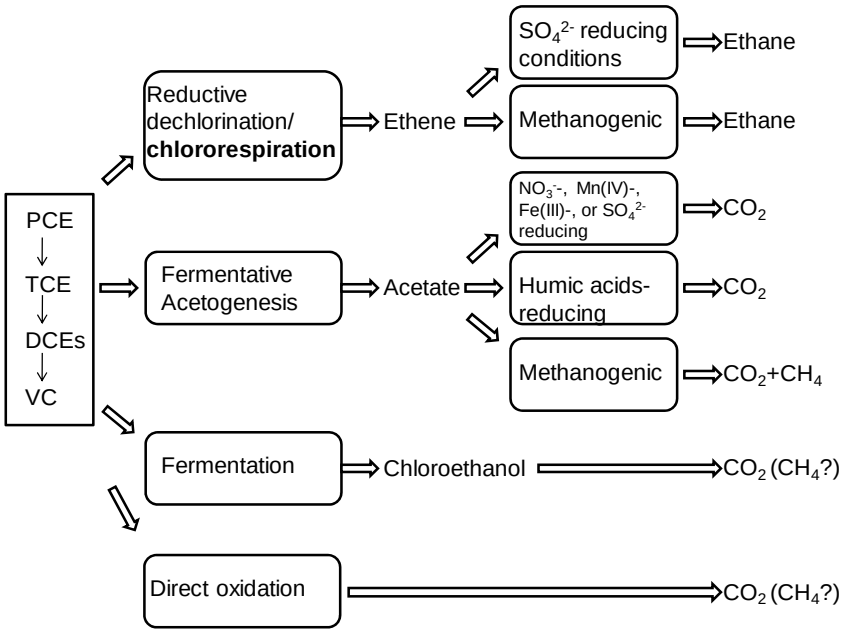
Table 2.2 Functionally identified reductive dehalogenase (RDase) genes from *Dehalococcoides*

Organism	Enzyme, gene (accession No.)	Molecular weight	Substrate based on in vitro assay	Reference
Dehalococcoides ethenogenes strain 195	PCE RDase, *pceA* (AAW40342)	51 kDa	PCE	Magnuson *et al*., 1998
Dehalococcoides ethenogenes strain 195	TCE RDase, *tceA* (AF228507)	61 kDa	TCE, *cis*-DCE, *trans*-DCE, 1,1-DCE, VC	Magnuson *et al*., 1998; Magnuson *et al*., 2000
Dehalococcoides sp. strain CBDB1	Chlorobenzene RDase, *cbrA* (CAI82345)	51.2 kDa	1,2,3,4-TeCB, 1,2,3-TCB, pentachlorobenzene	Adrian *et al*., 2007a; Jayachandran *et al*., 2004
Dehalococcoides sp. strain VS	VC RDase, *vcrA* (YP_003330719)	62 kDa	All DCE isomers, VC	Müller *et al*., 2004
Dehalococcoides sp strain BAV1	VC RDase, *bvcA* (AAT64888)	57.4 kDa	-	Krajmalnik-Brown *et al*., 2004

2.5 Remediation biotechnologies

There are many technological options to remove the toxic chlorinated ethenes and ethanes (Fig 2.3), such as physical, chemical, biological methods, or combined technologies (Kurisu, 2008). Physical methods include pump and treat, adsorption technology (by granular activated carbon (GAC) or resin adsorption), filtration technology, volatilization technology (e.g., air sparging, soil vapour extraction), washing/extraction technology, treatment walls (barriers) (Narayanan et al., 1993; ITRC 2008). Physical treatment technology generally does not destroy wastes but is a means of separating hazardous contaminants from soils, sludges, and sediments, thus reducing the volume of the hazardous wastes. An example of chemical treatment is chemical reduction of chlorinated compounds by zero-valent iron (ZVI) (Kurisu, 2008) (Gillham and O'Hannesin, 1994); biological treatments employ the biodegradation or bioaccumulation of the contaminants by microorganisms or plants (de Bruin et al., 1992; Tartakovsky et al., 2005). Whether abiotic or microbial dechlorination of chlorinated solvents will take place largely depends on field conditions, such as the abundance of dechlorinating bacteria, soil properties, and the mass loading of reactive

(a)



(b)

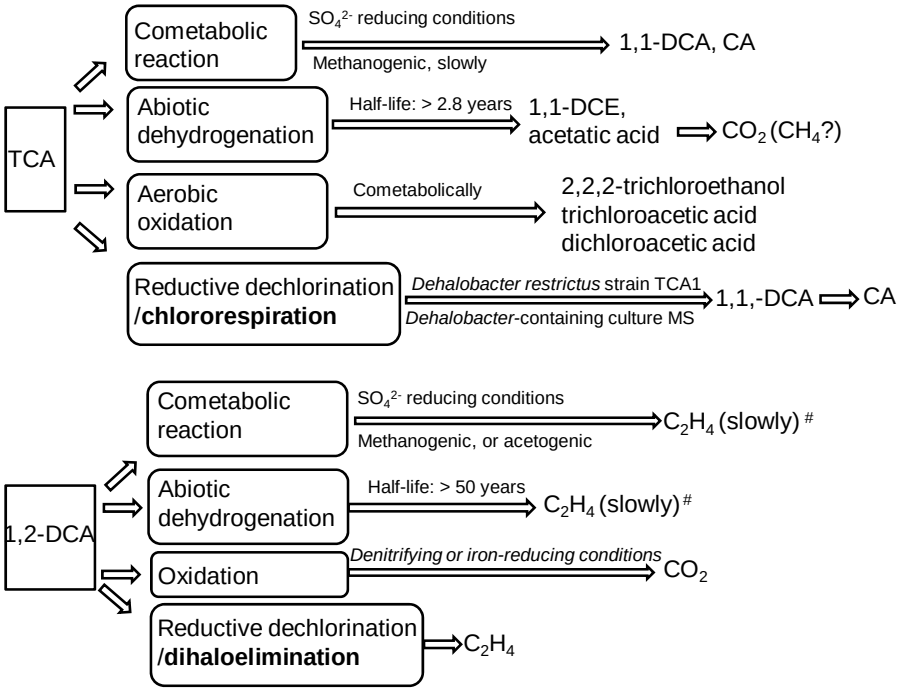


Fig 2.3 Pathway of anaerobic biodegradation of chloroethenes (a) and chlorinated ethanes (b). Note: *, Bradley et al., 2003. #, Slowly, dechlorinate rate up to 0.07 nmol Cl^- min^{-1} mg^{-1} $_{protein}$ (Reference: Holliger et al., 1990); normal dechlorinate rate, 25 times higher than cometabolic conversion (De Wildeman et al., 2003).

minerals, electron donors (Dong et al., 2009).

A combination of features of the site makes it an interesting case study for the exploration of remediation options. In a recent study conducted by Plagentz et al. (2006), both chemical and physical methods are applied to treat multiple contaminants simultaneously in two-sequential column using ZVI and granular activated carbon (GAC) (Plagentz et al., 2006). In addition, Lampron et al. (2001) have studied the impact of microorganisms on the performance of Fe(0) barriers treating TCE-contaminated groundwater. It is reported that abiotic and biological process in the Fe(0)-cell system compete for the TCE available. The maximal amounts of *cis*-DCE and VC generated were greater in reactors containing cells and H_2 (no iron) than in reactors containing cells and Fe(0) as shown in the Fig 2.4 (Lampron et al., 2000). With the presence of hydrogen-utilizing, dechlorinating population in an iron barrier, TCE reduction will result in accumulation of VC in large quantities, greater than what would be expected in an abiotic system. The mobile product (VC) may introduce a greater tendency to break through the iron barrier and alter the permeability, which will damage the iron barrier.

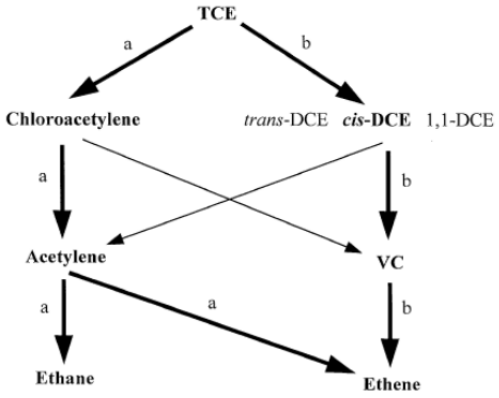


Fig 2.4 Reduction pathway for the abiotic (a) and biological (b) transformation of TCE in Fe(0)-cell system.

It is *Dehalococcoides ethenogenes* strain 195, the first pure culture that demonstrates growth and energy production coupled to complete reduction of PCE to ethene, termed as dehalorespiration (Maymó-Gatell et al., 1995; Maymó-Gatell et al., 1997). Since then, bioremediation technologies become more and more popular among many other approaches in that it has advantages over other physicochemical methods in terms of cost and environmental impact (Bradley, 2003). The major advantages of bioremediation have been attributed to its capability in completely destroying the contaminants, less expensive than other remediation options. Additionally it can treat both dissolved and sorbed contaminants and can move with the contaminant plume, which makes it not limited to a fixed area, typical of chemical flushing or physical technologies. Although bioremediation usually proceeds at modest rate and it may take relatively longer time to complete the cleanup than other methods, more and more practices have found that bioremediation is suitable for the treatment of a widely spread contamination of chlorinated solvents with low concentration, particularly those after the treatment of the core contamination by other methods (ITRC, 2005).

2.6 Bioremediation of chlorinated solvents

Microbial transformations of chlorinated solvents have been studied extensively, with specific attention for the microbial community analysis (Zinder, 2010), *in situ* bioremediation strategies (Ritalahti et al., 2005; ITRC, 2007), enzymatic systems through various eco-genomic toolboxes (Maphosa et al., 2010). Microorganisms belonging to genera of *Dehalococcoides* spp., *Dehalobacter* spp., *Desulfitobacterium* spp. and *Sulfurospirillum* spp. have been found as the main dechlorinators on site (Zinder, 2010).

"Bioremediation refers to a managed or spontaneous process in which biological, especially microbiological, catalysis acts on chemical compounds, thereby detoxifying or eliminating environmental contaminants" (Major and Cox, 1992). Bioremediation can be achieved through one of the following three different approaches, biostimulation, bioaugmentation, and monitored natural attenuation (MNA), largely depending on the existence and capacity of the dechlorinating microorganisms. Biostimulation is to activate the existing microorganisms by introducing some compounds to contaminated sites. The ultimate goal of biostimulation is to speed up and activate the dechlorination by providing chemicals, such as nutrients, electron donors, or reductants to maintain the desired redox conditions. It is mainly suitable when the dehalogenators exist in the site but dechlorination takes place slowly.

In the absence of essential dehalorespirators, bioaugmentation is needed through the addition of microorganisms with known dehalogenating capabilities. The microorganisms to be added can be pure cultures, or mixed consortia but should not be pathogenic. Post evaluation is greatly required to monitor the microbial ecology, concentration reduction. The efficacy will be largely dependent on the microorganisms provided. Bioaugmentation and biostimulation have been mainly chosen for the enhanced dechlorination of chloroethenes by targeting the *Dehalococcoides* 16S rRNA genes and RDase genes, e.g. *pceA*, *tceA*, *bvcA*, *vcrA* by qPCR (GeoSyntec Consultants., 2005; Ritalahti et al., 2005; Schaefer et al., 2009). A passive remediation, MNA is occurring in the contaminated sites where sufficient microorganisms to dechlorinate the pollutants are found and dechlorination process can be proceeded within the tolerance period as projected. However, remediation of groundwater is an expensive and time-consuming endeavour. When cost is an issue,

MNA might be eventually chosen by regulators. Frequent site monitoring and diagnostics are always required to warrant the success of bioremediation, or active remediation method (biostimulation or bioaugmentation) can be introduced afterwards.

2.6.1 Bioremediation technologies

Current bioremediation technology can be divided into three separate processes, designated as off-site, on site and *in situ* (Kurisu, 2008). Off-site biological treatment includes methods practiced at waste treatment facilities or sewage treatment plants. On-site biological treatment usually needs excavation of soils or pumping of groundwater to remove contaminants, followed by immediate treatment at contaminated sites. It may involve composting or bioreactors operated in engineered systems, such as bioventing, biosparging, direct injection method, groundwater circulation, permeable reactive barriers, photoremediation. *In situ* bioremediation (ISB) refers to the enhancement of biological activity in place. It may involve manipulation of eco-environmental conditions, such as introduction of selected inocula (bioaugmentation) or pumping of groundwater for better hydrogeological control or essential nutrients (biostimulation) but without engineered systems.
The cleanup technology for the remediation of DNAPLs includes a list of remedial approaches, e.g. *in situ* chemical oxidation/reduction, surfactants (solvent-enhanced flushing), thermal treatment, extraction (dual phase, water flood, or pump and treat), *in situ* air sparging, and *in situ* bioremediation (ISB). ISB is the newest application, including bioaugmentation, biostimulation, biopolishing, or enhanced reductive dechlorination (ERD).

Table 2.3 represents about one-third of ISB studies available in U.S. (ITRC, 2007). ISB is an attempt to work with nature through natural biological activity in the

subsurface and aims to degrade DNAPLs contaminants into less toxic or, ideally, harmless substances. Significant progress has been achieved in the deployment of ISB against DNAPLs, particularly for those chlorinated ethenes (ITRC, 2007). Nowadays, with the development of molecular tools and identification of more robust dehalorespirators, ISB shows great potential in achieving measurable results within required time frames in an economical manner.

Multiple molecular tools are usually combined to explore the microbial dechlorinating communities for the environmental samples (Maphosa et al., 2010). For instance, three molecular tools, terminal-restriction fragment length polymorphism (T-RFLP), RFLP with clone sequencing, quantitative PCR (qPCR), were compared to assess the effectiveness of bioremediation through surveying the microbial differences between two contaminated sites, INEEL, and Seal Beach (Rahm et al., 2006). The first two methods failed to differentiate the microbial communities for these two sites and *Dehalococcoides* was not targeted for these two sites by either T-RFLP or RFLP with sequencing methods. However, qPCR targeting the 16S rRNA gene of *Dehalococcoides* strains which are known for their unique DCE-dechlorinating capacity shows a significant proportion of *Dehalococcoides* in INEEL but no detectable *Dehalococcoides* at Seal Beach. This result accounted for the different TCE-dechlorinating activities at bioremediation sites (Rahm et al., 2006).

In a bench study for the bioremediation of PCE DNAPL source zone at Dover Air Force Base, DE. (Table 2.3), two bioremediation strategies were compared to evaluate the effect of PCE bioremediation. One was only biostimulation through addition of electron donor, e.g. methanol, sodium acetate, or ethanol (Sleep et al., 2006). The other was biostimulation first and then followed by bioaugmentation with dechlorinating culture KB-1. Growth of iron-reducing bacteria, *Geobacter* was found

for both systems through biostimulation strategy-addition of electron donor, probably due to the high iron content of the Dover soil. Methanogenesis became predominant activity with prolonged biostimulation after available iron was exhausted but not dechlorination activity despite of presence of native *Dehalococcoides* species. Dechlorination of PCE to ethene was only observed for the bioaugmented system with KB1. The dechlorination activity was also effectively monitored by PCR-DGGE and qPCR, particularly in soil samples. The results show that concentrations of both *cis*-DCE and *Dehalococcoides* reached the first peak after 48 days of bioaugmentation, indicating the remarkable growth of *Dehalococcoides* with PCE. The diverted distribution of electron donor around the PCE DNAPL zone was found to limit its dechlorination significantly. This study also suggests three methods to enhance electron donor efficiency, 1) to recirculate PCE-laden water to inhibit methanogenesis, 2) to inoculate cultures capable of transforming PCE efficiently, and 3) to choose electron donor with low hydrogen partial pressure limitations. The last one, effective electron donor delivery to the dechlorinating microorganisms was found to be the most challenging part of this pilot-scale study.

To date, limited information is available for bioremediation of chlorinated ethanes. One example is to use air stripper and soybean emulsion oils to generate hydrogen, which helps in stimulating specific bacteria capable of carrying out the transformation of chlorinated ethenes and ethanes (Solutions-IES, 2006). More than 75% reduction of 1,1,1-TCA was observed in this study and significant increases of 1,1-DCA and 1,1-DCE was found, possibly in a partial dechlorination manner. Eventually, complete detoxification of chlorinated contaminants (e.g. TCE, TCA,

Table 2.3 Summary of six case studies of in situ bioremediation of chlorinated ethenes DNAPL source zones

ID	Field study	Description
1	Location	Test Area North (TAN) site at Idaho National Engineering and Environmental Laboratory)
	Source	TCE-residual source area, 2-mile long dissolve-phase plume (total TCE ranging from 350-35,000 gallons)
	Problems	A superfund site. Three sections: the hot spot (residual TCE source zone); the medial zone; the distal zone
	Approaches	Pump-and-treat as default remedy. Development of 5 innovative technologies: 1) metal-enhanced reductive dechlorination, 2) monolithic confinement, 3) in situ chemical oxidation, 4) enhanced in situ bioremediation, 5) monitored natural attenuation (MNA)
	Observations and lessons learned	Organic content of the sludge injected to the site have high amount of TCE and PCE (3% by weight). Complete dechlorination of TCE to ethene was achieved as a result of electron donor injections in the residual source area. Innovative monitoring have been conducted periodically: 1) compound-specific stable carbon isotopes to differentiate between the effects of groundwater transport, dissolution of DNAPLs at the source, and enhanced bioremediation; 2) various molecular techniques, e.g., PLFA analysis, qPCR for *Dehalococcoides* species and T-RFLP (Rahm et al 2006, Macbeth et al., 2004 & 2006)
	Parameters monitored	Electron donor and nutrient distribution, redox-sensitive parameters (dissolved oxygen-DO), VOC contaminants and degradation products (chloroethenes, ethene and ethane), biological activity indicators (alkalinity-bicarbonate production), and water quality parameters (temperature, pH, specific conductance, and oxidation-reduction potential-ORP). Monthly monitoring of radiological parameters.
	Remarks	It is critical to apply high-concentration electron donor solutions for rapid reductive dechlorination, as referred to Bioavailability Enhancement Technology (B.E.T., U.S. Patent 6,783,678)
2	Location	Dover Air Force Base (AFB) National Test Site (DNTS), Dover Delaware
	Source	PCE DNAPLs source, plus JP-4 jet propellent (LNAPL), toluene, xylene, TCE, and chlorobenzene. 99g total hydrocarbon, 1.75 kg total BTEX, 40 g TCE, <115 g PCE, and 40 g of chlorobenzene.
	Problem	The groundwater there is slightly acidic with low dissolved-solid contents.

Table 2.3 Summary of six case studies of in situ bioremediation of chlorinated ethenes DNAPL source zones (continued)

ID	Field study	Description
2	Approaches	Bioaugmentation
	Observations and lessons learned	Three methods to enhance electron donor efficiency: 1) to recirculate PCE-laden water to inhibit methanogenesis, 2) to inoculate cultures capable of transforming PCE efficiently, and 3) to choose electron donor with low hydrogen partial
	Parameters monitored	VOC, dissolved hydrocarbon gases, VFAs, anions, stable carbon isotopic analysis (SCIAs), *Dehalococcoides*-PCR and qPCR
	Remark	Accumulation of *cis*-DCE and VC was observed. (Sleep et al 2006)
3	Location	Launch Complex 34 (LC34) at Cape Canaveral (the Kennedy space center)
	Source	Up to 40, 000 kg of TCE in the aquifer below LC34
	Problems	Centuries may be needed to restore groundwater using intrinsic remediation processes. Indigenous *Dehalococcoides* was detected but electron donor was limited. *Cis*-DCE was detected as 5% of the total chloroethenes (molar basis)
	Approaches	Enhanced in situ bioremediation with 3 phases of study, 1) recirculation of raw groundwater, 2) recirculation of electron donor-amended groundwater, 3) bioaugmentation with recirculation of electron donor- amended groundwater. Treatment system consisted of a network of injection, extraction, and monitoring wells.
	Observations and lessons learned	Complete 3 phases of study. Dechlorination of TCE (1,220 mg/L) by KB-1 was sustained in the absence of continuous electron donor addition.
	Parameters monitored	About 2,120 mg/L of total dissolved solids including about 285 mg/L of sulfate. Neutral pH with an alkalinity of about 400 mg/L. ORP in the surface aquifer (76-171 mV) above the range associated with reductive dechlorination. Several geochemical parameters are monitored as well.
	Remark	Rapid and complete dechlorination occurred for high concentration of TCE (1,220 mg/L). Dechlorination activity by KB-1 was sustained and established in the absence of electron donor addition.

Table 2.3 Summary of six case studies of in situ bioremediation of chlorinated ethenes DNAPL source zones (continued)

ID	Field study	Description
4	Location	ARCADIS (confidential)
	Source	PCE source area (more than 20 years) in a shallow, unconsolidated aquifer of low permeability and shallow hydraulic gradients
	Problems	Site geology is relatively unfavorable.
	Approaches	Enhanced bioremediation through carbohydrate-driven biostimulation. ERD system consisted of extraction wells, vacuum-enhanced recovery (VER), monitoring well network, injection well layout. Two electron donor injection approaches (target TOC source=45,000 mg/L): ①lines of donor injection applied for moderate/fast flowing groundwater system, and ② injection grid for slow-flowing systems
	Observations and lessons learned	The successful application relies on its capacity to draw NAPL mass into solution. High morality of *cis*-DCE and VC indicates that dechlorination process is not complete. Introduction of highly fermentable carbohydrates helped induce desorption and dissolution of nonaqueous mass and support dechlorination metabolism.
	Parameters monitored	TOC, chloroethenes and ethanes, filed parameters (pH, ORP)
	Remarks	The low aquifer permeability limited the movement of liquid contaminants.
5	Location	Portland, Oregon dry cleaner site located in a strip mall
	Source	Dry cleaning contact water saturated with PCE (150,000 μg/L), and pure phase PCE discharges to a utility trench. Leaks from them negatively impacted soils and groundwater.
	Problems	Pump-and-treat, in situ vacuum extraction (SVE), and air sparging (AS) effective in reducing the BTEX (benzene, toluene, ethylbenzene, and xylenes) but not TCE.
	Approaches	Source area remediation, accelerated bioremediation using hydrogen release compound (HRC) or HRC-X (extended-release, highly concentrated version of HRC) within the plume and source area as it needs modest site access and minimal operation activity
	Observations and lessons learned	HRC was considered as the most favorable technology for the site due to its documented success in stimulating complete detoxification of PCE to ethene and minimal operation and maintenance. Significant accumulation of *trans*-DCE (from 160 μg/L prebaseline to maximum of 543 μg/L) was detected in addition to *cis*-DCE and VC.

Table 2.3 Summary of six case studies of in situ bioremediation of chlorinated ethenes DNAPL source zones (continued)

ID	Field study	Description
5	Parameters monitored	VOCs, VFAs (metabolic acids), chloroethenes, geochemical parameters, e.g. dissolved iron and manganese, sulfate, sulfide.
	Remarks	HRC is an ester of glycerol (a three-carbon polyalcohol) and polylactate (a tetramer of lactic acid) which will slowly release lactic acid once injected into an aquifer.
6	Location	The Tarhell Army Missile Plant (TAMP) in Burlingtong, North Carolina
	Source	TCE source with concentration up to 2,600 μg/L, other chlorinated organic compounds (CVOCs), BTEX.
	Problems	Active remediation like pump-and-treat is effective in reducing BTEX but not dissolved-phase TCE.
	Approaches	Active remediation using emulsified oil substrate (EOS) to target CVOCs (including TCE), consisting of food-grade soybean oil, surfactants, macro-and micronutrients, and vitamins blended to form uniformly sized oil droplets.
	Observations and lessons learned	This approach provides good contact between the slowly biodegradable organic substrate and the contaminants. Accumulation of TCE, *cis*-DCE, and VC was observed. There a lack of information collected on molecular biology or stable isotope ratios.
	Parameters monitored	Monitoring program includes the depth to water, EOS accumulation, DO, ORP, pH, specific conductance, CVOCs light hydrocarbons, electron acceptors, TOC, VFAs, and inorganics (dissolved iron, manganese, and chloride), etc.
	Remarks	EOS has a negative surface charge.

DCA, and VC) to non-toxic end-products (ethene and ethane) has not been achieved, probably due to the lack of appropriate dechlorinating microorganism capable of complete reduction of 1,1,1-TCA to non-toxic end products.

2.6.2 Microorganisms involved with commercial applications

Since late 1980s, the growing acceptance of bioremediation has led to intense exploration of dehalorespirators for bioaugmentation. A number of observations suggest that reductive dechlorination of chloroethenes in groundwater systems is often attributable to the activities of cooperative consortia of microorganisms rather than to a single species (Bradley, 2003). These interesting enrichment cultures have been well characterized before available for commercial use and their value has been demonstrated under field conditions according to the white paper "Bioaugmentation for remediation of chlorinated solvents: Technology Development, Status, and Research Needs" published by Environmental Security Technology Certification Program (ESTCP, Department of Defense, U.S.A.) in 2005 (GeoSyntec Consultants., 2005). These cultures include KB-1^{TM} (developed at University of Toronto and commercialized by SiREM), the Bachman Road culture (the source for both Regenesis's BioDechlor INOCULUM^{TM} and the BC2 inoculum marked by BioAug LLC), the Pinellas culture (developed by GE and licensed to Terra Systems), SDC-9 (developed by Shaw Environmental, Inc.) and several other cultures marked by Bioremediation Consulting Inc (see Table 2.4) (Major and Cox, 1992; GeoSyntec Consultants., 2005).

Table 2.4 Characterization of commercial bioaugmentation inocula for chloroethene - contaminated sites

Companies	Specific dechlorinating microorganisms	Major contaminants to be treated	Source of inoculum
SBP Technologies, Inc., Technical Resources, Inc.,	*Pseudomonas cepacia*	TCE	TCE-contaminates sites
BioTrol, Inc.	*Methylosinus* trichosporum, *Pseudomonas cepacia*	TCE	In-house culture collections
SiREM	*Dehalococcoides* spp. (*vcrA*-containing culture and *bvcA*-containing culture), acetogens and methanogens	PCE, TCE	KB-1, developed at University of Toronto
Regenesis	*Dehalococcoides* spp., *Sulfurospirillum* multivorans comb. nov.	PCE, TCE	Bio-Dechlor INOCULUM Plus, identified in George Tech
BioAug LLC	*Dehalococcoides* spp.	Chloroethenes	BC2 inoculum, George Tech
Terra Systems	*Dehalococcoides* spp.	Chloroethenes	The Pinellas, developed by GE
Shaw Environmental Inc.	*Dehalococcoides* spp.	Chloroethenes	SDC-9, developed by Shaw Environmental Inc.

2.6.3 Problems/Challenges to be addressed in bioremediation

Firstly, remediation time frame is an area of active research and debate among the remediation community. Secondly, there is a lack of cost evaluation system, remediation goal (end point concentrations) within the duration timeline in the field of bioremediation applications. It is commonly suggested by various reviewers in the bioremediation community that both cost and time frame to complete the detoxification play a major role in resolving the utility of a technology (ITRC, 2007).

The expert panel from the Interstate Technology & Regulatory Council (ITRC) concluded that bioremediation of chlorinated ethene source zones is a viable remediation option. A more quantitative comparison is needed to justify the expenditure of the application of corresponding bioremediation technology. Therefore, the first challenge is to decide the best biological amendments (or to stimulate those already present) that will accelerate the site restoration and use it as an energy source.

1) Bioaugmentation

Bioaugmentation is often associated with issues, such as competing with indigenous microorganisms due to different ecological factors. It is difficult to assess the applicability and effectiveness of the inocula added. This is because the lab-scale studies can only be used as a reference and cannot guarantee their metabolic function by themselves.

2) Biostimulation

Bioremediation through injection of high concentration of electron donor solutions has significantly enhanced depletion of TCE in the residual source and accelerated biodegradation rate of TCE to ethene from the first example as shown in Table 2.3. The large volume of the contaminated area requires huge injection volumes of electron donors, which indicates the huge life-cycle costs of the project. In order to reduce the treatment cost, one effective way is to minimize the introduction of TCE from the source (sludge) or to treat the sludge sample before it is injected to the contaminated sites.

The project cost in a source area bioremediation study (located at a Portland, Oregon dry cleaner site) was estimated as the total of two parts, installation cost (installation labor, injection points, substrates [e.g., HRC, HRC-X, shipping], baseline

sampling, surveying, completion report) plus sum of annual operation costs within project time frame (mobilization, direct labor, sampling equipment and supplies, laboratory analysis, and project planning and reporting) (ITRC, 2007). This cost estimation method could certainly serve as an important case history to the bioremediation community but the cost per unit volume of the material should be provided.

In accordance to the first case study of the report published by the Bioremediation of DNAPLs Team, reducing all contaminants of concern to below maximul contaminant levels (MCLs) regulated by EPA for drinking water could be served as the ultimate target to restore the contaminated groundwater.

Additionally, accumulation of *trans*-DCE is one of the challenging chloroethenes for bioremediation community probably due to their recalcitrant properties. Site investigations plus a number of laboratory studies demonstrate microbial production of *trans*-DCE during reductive dechlorination of PCE/TCE (Griffin et al., 2004; Garcia, 2005; Miller et al., 2005; Kittelmann and Friedrich, 2007). For the bioremediation of chlorinated ethenes, particularly those sites with significant accumulation of *trans*-DCE, special care needs to be given on the choice of proper inoculum source as there is a lack of microorganisms capable of metabolically dechlorinating TCE and *trans*-DCE (Kittelmann and Friedrich, 2007; Cheng and He, 2009; Chow et al., 2010).

Finally, it will be of great interest to explore the enrichment cultures capable of complete detoxification of both chlorinated ethenes and ethanes in that they tend to co-exist in the contaminated sites, such as PCE, TCE, *trans*-DCE, TCA, 1,2-DCA. The isolation and characterization of these cultures would be beneficial to humans and earth essentially.

2.7 Molecular tools used in bioremediation community

Microbial reductive dehalogenation occurs in strictly anaerobic conditions, involving with diverse anaerobic microorganisms from different genera. Of note with respect to microbiological characterization is the use of newer monitoring techniques, such as nested PCR, T-RFLP, PCR-denaturing gradient gel electrophoresis (PCR-DGGE), single-nucleotide primer extension assay (SNuP), quantitative real-time PCR (qPCR), carbon isotope, comparative genomics through microarray analysis, sequencing of genome, transcriptional analysis, proteomics (Bombach et al., 2010; Zinder, 2010).

2.7.1 Characterization of microbial populations based on 16S rRNA genes

For bioaugmentation, PCR-based tools are important to monitor the fate of microorganisms added into the contaminated sites. Nested PCR targeted with genus-specific method has been one of the most commonly used diagnostic tools. Nested PCR has been firstly developed and applied in environmental biota, e.g. paddy soil, in fish, sediments, and water in 1995, using primers which are internal to the first-amplified DNA fragment (Arias et al., 1995; Tsushima et al., 1995). The rapid and sensitive detection method was immediately developed and applied in monitoring of anaerobic dechlorinating bacteria in 1997 for *Desulfomonile tiedjei*, *Desulfitobacterium dehalogenans* (el Fantroussi et al., 1997). On the basis of their 16S rRNA gene sequence differences, *Dehalococcoides* can be divided into three sub-groups, namely, the Cornell, Victoria, and Pinellas groups (Hendrickson et al., 2002). This method has been employed gradually among more and more genera, including *Dehalococcoides*, *Desulfuromonas*, *Desulfitobacterium*, and *Dehalobacter* spp.

(Löffler et al., 2000; Ritalahti and Löffler, 2004). This method is particularly sensitive and reproducible to the template with relative low concentration and it can also effectively prevent false negative signal. One limitation of this method is that the template concentration should be in an optimized range. Otherwise, it may result in underestimation of certain genus with low concentration by showing false negative result.

Other PCR-based tools, have also been commonly used as 16S rRNA gene based finger-printing methods in the identification of dechlorinating microorganisms, in particular for the phylogenetically closely-related *Dehalococcoides* spp. in the bioremediation field, such as PCR-DGGE, T-RFLP, clone library, SNuP (Duhamel et al., 2004; Gu et al., 2004; Sung et al., 2006a; Nikolausz et al., 2008; Wu et al., 2009).

Although the *Dehalococcoides* 16S rRNA gene is one obvious marker (McDonald et al., 2008), numerous studies have found that *Dehalococcoides* strains with different dechlorinating activities share similar or identical 16S rRNA gene sequences. Furthermore, the sole presence on the basis of the *Dehalococcoides* 16S rRNA gene in an environment may not guarantee the dechlorinating activity of a specific pollutant. For instance, strain BAV1 would not generate ethene without H_2 and strain CBDB1 cannot dechlorinate DCEs to ethene. Therefore, the high conservation of the 16S rRNA gene in *Dehalococcoides* made it insufficient to represent this unique group, not even to differentiate the dechlorination capacities among different strains of *Dehalococcoides* (Bhatt et al., 2007). Consequently, molecular tools that target metabolic activities of the entire communities in the environment are needed to have an accurate assessment for *in situ* bioremediation.

2.7.2 Identification of RDase genes

Microbial reductive dehalogenation is found to be catalyzed by an unique enzyme systems- RDases, which are linked to an anaerobic respiratory chain (Smidt and de Vos, 2004). Understanding the role of RDases has attracted many researchers' attention for the past two decades, and many molecular techniques have been developed and applied to study the microbial composition, ecology of the *Dehalococcoides*, comparison of their behaviours, which will be discussed here. Current chloroethene-dehalorespiration process has been found to be encoded mainly by four RDase genes, namely, *pceA*, *tceA*, *bvcA*, and *vcrA* for chloroethenes (Bhatt et al., 2007). Table 2.5 lists the primers which have been commonly used to target those chloroethene-RDase genes.

The corresponding RDase genes have been largely used as a good biomarker in bioremediation field as they narrow down the investigation to the studied functional group and enable a much higher sensitivity of detection in complex environmental samples, e.g. uncultivated members (Krajmalnik-Brown et al., 2004; Fields et al., 2006; Lee et al., 2006). Similar to the *Dehalococcoides* 16S rRNA gene, the *tceA* or *vcrA* gene copies also represented the cell number of common TCE-to-VC dechlorinators or VC-to-ethene cultures in that one single *Dehalococcoides* cell usually has one single copy of 16S rRNA gene and these functional RDase genes (Behrens et al., 2008).

The dechlorinating capabilities have been well addressed together with the identification, quantification, and transcriptional analysis of the known chloroethene RDase genes from *Dehalococcoides* spp. (Waller et al., 2005; Holmes et al., 2006; Sung et al., 2006a). Holmes et al. (2006) successfully applied qPCR to discriminate

the *Dehalococcoides* strains in the enrichment culture ANAS by quantifying *tceA* and *vcrA* gene copies, and *Dehalococcoides* 16S rRNA gene copies (Holmes et al., 2006). It is suggested that at least two different *Dehalococcoides* strains should exist in this culture (ANAS) during the dechlorination of TCE to ethene.

Although the presence of the functional RDase genes, such as *bvcA*, *tceA*, *vcrA*, *pceA*, may help identify the dechlorinating potential and quantify the exact amount of specific subgroup of *Dehalococcoides* in the contaminated sites (Cupples, 2008), the role of other cofactors cannot be ignored as well. A *pceA*-containing strain, *Sulfurospirillum* multivorans stain N failed to present its PCE-dechlorinating ability. However, as strain N's closest relative, *Sulfurospirillum* multivorans stain K which was originated from the same source as strain N, can dechlorinate PCE rapidly to TCE and *cis*-DCE. The identical *pceA* gene sequence was obtained for both strains N and K, but a novel corrinoid was missed in strain N as compared to strain K. The missing corrinoid cofactor (Neumann et al., 2002) was reported to be the major reason for the displayed dechlorinating activity by reverse transcription PCR (RT-PCR) and western blot analysis under the same experimental conditions (Siebert et al., 2002). The genes encoding for the PCE dehalogenase namely *pceA* and *pceB* were present in both strains by PCR, but the expression of *pceA* in strain N was much weaker than that of strain K by RT-PCR and the apoprotein of the dehalogenase was only missing in strain N revealed by western blot hybridization (Siebert et al., 2002). Therefore, the expression profiles of functional RDase genes or other unidentified RDase genes are also closely tracked for most dechlorinating studies.

Table 2.5 Specific primer sets for the detection of known chloroethene/chloroethane reductive dehalogenase genes

ID	Targeted RDase gene	Primer name	Sequences (5'-3')	Annealing temperature (ºC)	Amplicon (bp)	Targeted group	References
1	*pceA*-1	Forward P1	TTG GAT GAG GCC TTG AAC GC	55	618	*D. restrictus* and *Desulfitobacterium* spp. strains TCE1, PCE-S, and Y51	Regeard et al. 2004
		Reverse P1	GCG CTG CAT AAT AGC CAA GC				
2	*pceA*-2	Forward P2	TCG TTG CAG GTA TCG CTA TG	52	194	*Sulfurospirillum multivorans*	Regeard et al. 2004
		Reverse P2	TTC AAC AGC AAA GGC AAC TG				
3	*pceA*-3	Forward P3	ATG GTG GAT TTA GTA GCA GCG GTC	52	204	*Dehalococcoides ethenogenes* strain 195	Fung et al. 2007
		Reverse P3	ATC ATC AAG CTC AAG TGC TCC CAC				
4	*tceA*-1	Forward T1	GCT TTG GCG GTG ATG ATA AG	50	194	*Dehalococcoides ethenogenes* strain 195	Regeard et al. 2004
		Reverse T1	GTT ATA GCC AAG GCC TGC AA				
5	*tceA*-2	Forward T2	ACG CCA AAG TGC GAA AAG C	50	1693	*Dehalococcoides spp.*	Magnuson *et al.*, 2000
		Reverse T2	TAA TCT ATT CCA TCC TTT CTC				
6	*bvcA*	Forward B1	TGC CTC AAG TAC AGG TGG T	52	839	*Dehalococcoides spp.*	Krajmalnik-Brown *et al.*, 2004
		Reverse B1	ATT GTG GAG GAC CTA CCT				
7	*vcrA*-1	Forward V1	TGC TGG TGG CGT TGG TGC TCT	55	441	*Dehalococcoides spp.*	Müller *et al.*, 2004
		Reverse V1	TGC CCG TCA AAA GTG GTA AAG				
8	*vcrA*-2	Forward V2	CTA TGA AGG CCC TCC AGA TGC	60	1482	*Dehalococcoides spp.*	Müller *et al.*, 2004
		Reverse V2	GTA ACA GCC CCA ATA TGC CAA GTA				

1) Reverse transcriptional analyses of RDase genes

The detection of specific RDase genes has allowed for better assessment of the dechlorinating potential in contaminated sites, as compared to conventional 16S rRNA gene-based tools. Attempts to trace these RDase genes' transcriptional profile by reverse transcription quantitative PCR (RT-PCR) could further predict the dechlorinating activities thus preventing ambiguous results. RT-PCR has been largely applied to genus of *Dehalococcoides* by introducing luciferase control RNA as an internal reference mRNA during sample preparation (cell lysis, RNA isolation, and DNA removal) in 2005 (Johnson et al., 2005a). This application was first developed in Alvarez-Cohen's group and the accuracy for the absolute quantification of *tceA* transcripts was evaluated on a TCE-to-ethene dechlorinating microbial enrichment (Johnson et al., 2005b). Expression level was quantified by qPCR as the number of *tceA* transcripts per *tceA* gene. It is found that the expression of *tceA* gene increased about 40-fold when the chloroethenes-starved cells were exposed to its growth-supporting substrates, TCE, *cis*-DCE, 1,1-DCE but not observed for PCE or VC. It is also interesting to note that *tceA* expression level increased about 30-fold when exposed to the non-metabolic substrate, *trans*-DCE (Johnson et al., 2005b). The level of *tceA* expression was found to be independent of the concentration of chloroethanes or electron donor (H_2), which generally have much higher concentration than the half-velocity coefficients. Johnson et al. (2005) also found the strong correlation of *tceA* expression and incubation temperature (Johnson et al., 2005b).

With the identification of more and more RDase genes, the transcriptional profile of multiple RDase genes has been well characterized to better understand the dechlorinating potential (Waller et al., 2005). With the isolation of another VC-RDase gene *vcrA* in 2004 (Müller et al., 2004), significant expression of *tceA* and

vcrA during the first 24-hour contact with TCE for the starved cells was also found in a mixed enrichment culture that contains both of *tceA* and *vcrA* genes, suggesting that elevated RDase-encoding transcript numbers could serve as a biomarker to indicate the physiological ability of *Dehalococcoides* spp. (Lee et al., 2006).

Previous findings have also supported that RDase-encoding genes are expressed in the presence of a variety of chlorinated organics and under different environmental conditions (Johnson et al., 2005b; Lee et al., 2006; Fung et al., 2007; Behrens et al., 2008; Lee et al., 2008). But the correlation between activities and expression has not been studied in-depth in particular for pure culture(s).

2) Degenerate primers

The availability of sequences in a database is the key to identifying marker genes suitable for use in microbial ecology studies. The identification of multiple RDase genes could have facilitated the estimation of dechlorination potential on site. Regeard et al. (2004) designed the first set of degenerate primer to target the putative chloroethene RDase genes (Regeard et al., 2004). Krajmalnik-Brown et al. (2004) designed a pair of degenerate primer for targeting conserved regions of RDase genes in *Dehalococcoides*, RRF2 (5'-SHM GBM GWG ATT TYA TGA ARR-3') and B1R (5'-CHA DHA GCC AYT CRT ACC A-3') (Krajmalnik-Brown et al., 2004). Abbreviations for degenerate nucleotide positions are as follows: R = A or G; K = G or T; M = A or C; S = C or G; W = A or T; Y = C or T; B = C, G, or T; D = A, G, or T; V = A, C, or G; H = A, C, or T. Through this pair of degenerate primer, the VC RDase gene (*bvcA*) was identified in both the gDNA and the cDNA of *Dehalococcoides* sp. strain BAV1. Several non-identified putative RDase genes were also probed for other TCE-dechlorinating cultures (Hölscher et al., 2004; Krajmalnik-Brown et al., 2004; Waller et al., 2005).

The presence as well as expression of these RDase genes by qPCR provides clues to predicting the activity of the current microorganisms before reaction occurs regardless of their general specialization to reductive dechlorination. For instance, the presence of *tceA* gene suggests a metabolic dechlorinating capacity of TCE to DCEs and possible accumulation of VC. However, the abundance of *bvcA* and *vcrA* genes implicates the detoxification of VC, which is the essential step for complete detoxification of chloroethenes. The recent investigation of the RDase genes has offered a wealth of sequence data.

To date, the degenerate primer set followed by genome walking has been commonly used to probe the RDase genes from a number of mixed cultures containing *Dehalococcoides* (Hölscher et al., 2004; Krajmalnik-Brown et al., 2004; Waller et al., 2005). Clone library targeted with degenerate primer set has allowed for identification of multiple new RDase genes in both pure cultures and enrichment cultures, including strain BAV1 (7 homologues) (Krajmalnik-Brown et al., 2004), strains FL2 and CBDB1 (at least 14 homologues for each) (Hölscher et al., 2004), and mixed culture KB-1 (14 homologues) (Waller et al., 2005). For culture KB-1, multiple RDase genes including *vcrA* and *bvcA* gene, were simultaneously transcribed in the dechlorination of different substrates, such as TCE, *cis*-DCE, VC, and 1,2-DCA. However, only genes that are specific to functions of interest, such as *bvcA*, *tceA*, *vcrA* genes, can serve as useful biomarkers whose quantification can enable effective monitoring of different *Dehalococcodies* activities (Lee et al., 2008). However, one drawback of current degenerate primer sets is their limited covering range. On one hand, the current degenerate primer sets cannot target all functionally-important RDase genes, for example, primer pair RRF2 and B1R cannot target *tceA* gene. On the other hand, these primer sets mainly target RDase genes involved with

chloroethene dechlorination instead of other broad range of halogenated organics. Future study on the identification and functional investigation of other putative RDase genes may provide new insights into the dehalogenation process and thus design new degenerate primers to standardize current diagnosis methods for bioremediation.

3) Problems to be addressed

Although multiple RDase genes have been identified from cultured representatives of *Dehalococcoides*, very little information is available about the regulation and specific functions of RDase genes during the reductive dechlorination of *trans*-DCE. Given that *trans*-DCE tends to persist in anoxic environment, studying the genetic makeup of the *trans*-DCE dechlorinating cultures may provide an enzymatic basis of *trans*-DCE production in contaminated sites. The expression analysis of genes from those *trans*-DCE producing cultures will help to elucidate how the microorganisms catalyze dechlorination of PCE/TCE to the predominant *trans*-DCE.

2.7.3 Disclosures of *Dehalococcoides* spp. genomes

Despite previous identification of several RDase genes for *Dehalococcoides* activity, it remains unclear about the *Dehalococcoides* biology probably due to limited information about the role of various RDase genes. The use of shotgun metagenome microarrays has allowed further investigation of the PCE/TCE-dechlorinating communities, such as enrichment culture KB-1 (Waller, 2009). With the developments of high-throughput sequencing technologies, more and more full genomic sequences have been revealed for those functionally-important chloroethene-dechlorinating enrichment cultures, such as *Desulfitobacterium hafniense* Y51 (Nonaka et al., 2006), and several members of *Dehalococcoides* (e.g. strain 195,

BAV1, VS, and GT) (Seshadri et al., 2005; McMurdie et al., 2007; McMurdie et al., 2009; Taş et al., 2009a) (Table 2.6). The common features for those *Dehalococcoides* are that these genomes are among the smallest in all free-living bacteria and each genome includes only one copy of rRNA gene (Taş et al., 2009a).

The genome sequence provides not only the diversity of RDase genes, but also offers insight into the microorganism's unique nutritional requirements and its commitment to the dehalorespiratory process. The genome of strain 195 also suggests that an ancestor for *Dehalococcodies* was a nitrogen-fixing autotroph (Seshadri et al., 2005; Lee et al., 2009). The first reported complete genome sequence of dehalogenators is *Dehalococcoides ethenogenes* strain 195 (Seshadri R *et al*., 2005), which composes a 1,469,720 base pair (bp) circular chromosome that contains 1,591 predicted coding sequences (CDSs). The genome possesses large duplicated regions and several integrated elements (IEs). The sequence result also shows that pathways for the synthesis of some cofactors appear incomplete, which supports the observation that *Dehalococcoides ethenogenes* 195 requires corrinoid vitamin B_{12} in large amounts for growth (Maymó-Gatell et al., 1997; He et al., 2007a). *D. ethenogenes* strain 195 has been shown not only capable of reductively dechlorinating chloroethenes through dehalorespiration, but also a number of other halogenated compounds, e.g. 1,2-DCA, 1,2,3,4-tetrachlorodibenzo-*p*-dioxin, 2,3,4,5,6-pentachlorophenyl, hexachlorobenzene (HCB), and chlorophenols (Maphosa et al., 2010).

Table 2.6. Comparison of the genomes of five halorespiring strains

Parameter	*Desulfitobacterium hafniense* Y51	*Dehalococcoides* ethenogenes strain195	*Dehalococcoides* sp. strain CBDB1	*Dehalococcoides* sp. strain BAV1	*Dehalococcoides* sp. strain VS
Taxonomic	Clostridia	Chloroflexi	Chloroflexi	Chloroflexi	Chloroflexi
Size (bp)	5,727,534	1,469,720	1,395,502	1,341,892	1,413,462
GC%	47.4	48.9	47.0	47.2	47.3
No. of rRNAs operons (16S-23S-5S)	6	1	1	1	1
No. of tRNAs	59	46	46	46	46
Total CDs	5,060	1,580	1,458	1,371	1,442
% coding	84	89	89	89	90
Total RDase genes	2	17	32	11	36
Reference	Nonaka *et al.*, 2006	Seshadri *et al.*, 2005	Kube *et al.*, 2005	McMurdie *et al.*, 2009	McMurdie *et al.*, 2009

In the long term, genomic data will serve as a foundation to develop new phylogenetic and functional marker probes, for detection, and monitoring of *Dehalococcoides* activities in the environment, and for population genetic studies. The whole-genome sequencing of isolated five *Dehalococcoides* strains (195, CBDB1,

BAV1, GT, VS) have all revealed that many RDase genes are located in or near putative integrated mobile genetic elements, providing strong evidence of horizontal gene transfer (Regeard et al., 2005; Krajmalnik-Brown et al., 2007; Taş et al., 2009a). Krajmalnik-Brown et al (2007) conducted the genetic analysis of *tceA* gene and its upstream and downstream regions among 21 TCE-to-ethene dechlorinating enrichment cultures diversified in the USA and found the presence of putative transposable element (PTE DET0076), including phage-like genes and recombinase-like genes adjacent to the *tceA* genes of *Dehalococcoides*. In particular, the PTE DET0076 which also commonly exists in other *Dehalococcoides* strains (e.g. CBDB1), shares 40% identity to the putative site-specific recombinase in several haloalkane-degrading *Rhodococcus* strains, indicating the occurrence of horizontal gene transfer between these two genera.

2.7.4 Microarray analysis

The majority of microorganisms in the environments have not been fully sequenced yet. Consequently, DNA microarray (microchip, biochip, and gene chip) technology allows the parallel analysis of highly complex gene mixtures in a single assay. It used to be largely applied for genome-wide expression analysis and now increasingly used as microbial diagnostic microarrays (MDMs). The MDM consists of nucleic acid probe set, which is designed to be specific for a given strain, subspecies, genus, or higher taxon. The MDMs can also allow the parallel comparisons of genomic DNA from different sources as compared to the model microorganisms, such as the first PCE-dechlorinating *Dehalococcoides ethenogenes* strain 195 (West et al., 2008).

Additionally, functional gene arrays (FGAs) have also been developed recently

to target functional biomarkers, such as nitrogenases, cellulases, reductive dehalogenase genes for more comprehensive analysis on genes or their transcripts to evaluate metabolic potential and microbial activity comparatively (Taş et al., 2009b). For instance, the GeoChip can detect more than 10,000 catabolic genes involved in a broad range of applications, including those dehalorespirators (He et al., 2007b). In 2008, Johnson et al. used temporal transcriptomic microarray analysis on model microorganism (strain 195) during its transition from exponential growth into stationary phase to understand the genes involved with metabolism and translation (Johnson et al., 2008).

Further studies on the development of new functional RDase gene sets will undoubtedly aid in the identification and isolation of novel *Dehalococcoides* spp. and to reveal new and interesting physiology and biochemistry for this unique group of microorganisms.

2.8 Other diagnostics tools

2.8.1 Microscopy

In addition to the biomolecular tools, microscopic examinations have also been applied to the microbial communities in depth, e.g. confocal laser scanning microscope (CLSM), scanning electron microscope (SEM), and atomic force microscope (AFM) (Fang et al., 2000; Li et al., 2007; Mangold et al., 2008).

1) CLSM

Confocal microscopes create images by combining optical microscopy with computer-based image reconstruction techniques. The CLSM is capable of capturing leteral images of stained cells at various depths of specimen. It can be used to obtain

a high-resolution image using the mathematical filtration method. With CLSM, a specific 16S rRNA-targeted oligonucleotide probes (Dhe1259) has been developed for the detection and quantification of *Dehalococcoides* species by fluorescence in situ hybridization (FISH) (Yang and Zeyer, 2003). Dhe1259 is a mixture of two oligomers, Dhe1259t (5'-AGC TCC AGT TC**A** CAC TGT TG-3') and Dhe1259c (5'-AGC TCC AGT TC**G** CAC TGT TG-3') which have one base difference (Yang and Zeyer, 2003). *In situ* hybridization of probe Dhe1259t with *D. ethenogenes* strain 195 and another two enrichment cultures successfully demonstrated the applicability of the probe for monitoring the abundance of active *Dehalococcoides* species in these samples with 30% formamide. The majority of hybridized *Dehalococcoides* cells by Dhe1259t showed irregular cocci shape with a diameter less than 0.5 μm in this study and the relative abundance was estimated over the total count of bacteria using probe Eub338 (5'- GCT GCC TCC CGT AGG AGT-3') (Amann et al., 1995). The probe Dhe1259t was found to be more sensitive than the probe DhEth (5'- ACC TAT TGT TCT GTC CAT T-3') designed in a previous study (Richardson et al., 2002).

2) SEM

It is noted that the diameter commonly observed for *Dehalococcoides* was about 0.5-0.7 μm in previous studies by SEM (Duhamel et al., 2004; He et al., 2005), while the size of *Dehalococcoides* sp. strain GT under SEM was reported ranging from 0.7-1.2 μm in diameter and 200-600 nm in depth (Sung et al., 2006a). The thicker cells of GT were probably in a predivision stage with diameters of 1.1-1.5 μm (Sung et al., 2006a).

3) AFM

AFM has been widely used in biology due to its capability of achieving high resolution images of biological samples even under physiological conditions such as

in fluid. In addition to its powerful imaging abilities, AFM could also provide nanometer scale physicochemical and mechanical characterization of material surfaces.

4) Comparison of the commonly used microscopy methods in dehalogenation

In general, AFM is similar to other electron microscope techniques, SEM and TEM, where proper sample preparation is the key to measuring high quality data. The art of sample preparation is in fact a simple procedure of critical-path steps, where every single step makes a difference. TEM is well known for very time-consuming and complicated sample preparation. SEM samples are "easier (fixed protocol)" but laborious to prepare. Furthermore, the requirement for conductivity adds some difficulty and additional information through the coating steps to the specimen. For SEM, a specimen is normally coated with gold which has a high atomic number and produces high topographic contrast and resolution. The coatings have a thickness of a few nanometers, thick coating about 20 nm (beam: 5-10 kv) for traditional SEM or standard coating about 4-5 nm (beam: 1-5 kv). The 5-20 nm thickness coating of gold typically used in SEM sample preparation often obscures the surface details of the thin specimen (http://www.ecmjournal.org/journal/files/Print-Specimen%20 Preparation-SEM.pdf), especially for the disc-shaped *Dehalococcoides* cells. However, AFM samples do not have to be conductive, which makes sample preparation easier for the user, particularly appropriate for biological samples. There are important criteria to be met in order to do AFM imaging. Tight affinity to the flat substrate is the mandatory requirement for successful AFM imaging. The specific disc-shaped morphology of *Dehalococcoides* cells indicates a natural affinity to the smooth surface of some particular substrate material. For those small disc-shaped

cells, AFM has shown its powerful imaging abilities and advantages since AFM could provide nanometer scale physicochemical and mechanical characterization of material surfaces.

2.8.2 Stable isotope fractionation

In recent years, stable isotope fractionation (SIP) approaches have been increasingly developed for the assessment of *in situ* biodegradation processes (Ewald et al., 2007) (Nijenhuis et al., 2007) (Aeppli et al., 2010). SIP is a method that attempts to incorporate an isotope with low natural abundance (e.g. ^{13}C) into identity of an organism with its metabolic function (McDonald et al., 2008). Dong et al. (2009) assessed the relative importance of abiotic and microbial PCE and TCE reductive dechlorination by stable carbon isotope fractionation (Dong et al., 2009). In some cases, an elegant way for SIP is to "probe" nucleic acids, including those carried out with DNA (DNA-SIP) or RNA (RNA-SIP) (Kittelmann and Friedrich, 2008a). One key advantage of RNA-SIP is the natural amplification of the phylogenetic signature molecule rRNA in active cells. RNA labelled with ^{13}C can proceed much faster than DNA in densely populated bioreactor samples, indicating that RNA-SIP may have greater sensitivity than DNA-SIP. By the RNA-based SIP, Kittelmann and Friedrich (2008) successfully identified a novel PCE-to-*cis*-DCE respiring population which has not been recognized previously (Kittelmann and Friedrich, 2008a).

In addition to those tools, other molecular markers have also been developed for bioremediation studies, including the analysis of lipid fraction, also named as phospholipid fatty acids (PLFA) analyses (White et al., 2005), proteomics (utilized to characterize the proteins) (Chuang et al., 2010). It is reported that *Dehalococcoides* sp. strains FL2 and BAV1 contain high levels of furan fatty acids in the phospholipids

and high levels of high-potential benzoquinones in anaerobic avenues, which are proposed to protect the cells from oxidative pressure (White et al., 2005). When combined with stable isotope labelling approaches, PLFA analyses can offer a convenient means to distinguish the functional microorganisms during biogeochemical transformations (Treonis et al., 2004). For *Dehalococcoides*, these two biomarkers (SIP and PLFA) together with the utilization of acetate as a carbon source indicate that *in situ* exposure of these organisms to ^{13}C-labeled acetate and the subsequent detection of ^{13}C in these biomarker lipids could also provide means for monitoring the growth and putative metabolic activities of these organisms in the field (White et al., 2005). Additionally, multidimensional protein identification technology (MudPIT) is a preferred approach in environmental proteomics studies, but the labor and expense involved could preclude widespread use in bioremediation studies (Wolters et al., 2001).

In summary, every method has its pros and cons. For 16S rRNA gene-based tools, the combination of DGGE and FISH allows the characterization of the predominant microorganisms associated with reductive dehalogenation qualitatively and semi-quantitatively (Yang et al., 2005). DGGE could easily compare the community structures but may not identify new species. T-RFLP could show the comparison of microbial communities clearly and semi-quantitatively, but it may not always identify specific species in particular for novel microorganisms; RFLP analysis and clone library can identify specific species but cannot target all species present; qPCR can give accurate quantification for the chosen targets but appropriate primers/probe need to be carefully chosen. Multiple molecular tools are usually combined to explore the microbial dechlorinating communities for the environmental samples. For instance, Rahm et al. compared three molecular tools, T-RFLP, RFLP

with clone sequencing, quantitative PCR (qPCR), to assess the effectiveness of bioremediation through surveying the microbial differences between two contaminated sites, INEEL, and Seal Beach (Rahm et al., 2006). It is found that the first two methods failed to differentiate the microbial communities for these two sites and *Dehalococcoides* was not targeted for both sites by either T-RFLP or RFLP with sequencing methods. However, qPCR targeting the 16S rRNA gene of *Dehalococcoides* strains which are known for their unique DCE-dechlorinating capacity shows a significant proportion of *Dehalococcoides* in the microbial community of INEEL but no detectable *Dehalococcoides* in the microbial community of Seal Beach. This result could account for the different TCE-dechlorinating activities at bioremediation sites (Rahm et al., 2006).

2.9 Summary

Successful bioremediation of chlorinated ethenes, mainly in the form of bioaugmentation and biostimulation, largely depends on the presence of *Dehalococcoides* sp. which is capable of detoxifying PCE or TCE to ethene in a timely manner. Due to relatively few cultured representatives and limited dechlorinating capacity, there is urgent need to explore novel microorganisms as well as the corresponding RDase genes involved with fast and complete detoxification of all chloroethenes. Isolation of these novel microbes will facilitate more in-depth understanding of unusual dechlorination pathway. These microbes will guide development of efficient bioremediation strategy specifically targeting the chlorinated solvents. More genomic content would also be revealed with the microarray analysis after the new cultures are obtained.

Chapter III A *Dehalococcoides*-Containing Co-Culture That Dechlorinates Tetrachloroethene to *trans*-1,2-Dichloroethene

In the microbial reductive dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE), dechlorinators usually produce *cis*-1,2-dichloroethene (*cis*-DCE) as the predominant product or an intermediate. This chapter shows that dechlorination of PCE and TCE can also lead to the generation of *trans*-1,2-dichloroethene (*trans*-DCE) by a co-culture MB. During its enrichment process, the ratio of *trans*- to *cis*-DCE increased from 1.4 (± 0.1) : 1 to 3.7 (± 0.4) : 1, whereas the TCE reductive dechlorination rate went up from ~ 26.2 to ~ 68.8 μmol l^{-1} day^{-1}. PCR-denaturing gradient gel electrophoresis (PCR-DGGE) revealed that the increased ratio of *trans*- /*cis*-DCE was well correlated with the increased proportions of *Dehalococcoides* and the disappearance of *Desulfuromonas* during the enrichment process. As shown by PCR-DGGE, similar *Dehalococcoides* species were consistently present in another three sediment-free cultures with various *trans*- /*cis*-DCE ratios. The 16S rRNA gene sequence of this *Dehalococcoides* species in co-culture MB is 100% identical (over 1,489 bp) to that of *Dehalococcoides ethenogenes* strain 195 (CP000027), which belongs to the Cornell subgroup of the *Dehalococcoides* cluster. The other bacterium in this co-culture MB was a *Sedimentibacter* species, which showed no PCE or TCE dechlorination activity. Results from this study show that microbial dechlorination of chloroethenes by this particular subgroup of *Dehalococcoides* could result in significant accumulation of *trans*-DCE in the environment if no *trans*-DCE dechlorinators co-exist in the contaminated sites.

3.1 Introduction

Large quantities of chlorinated organic solvents, tetrachloroethene (PCE) and trichloroethene (TCE) have been released into the environment because of their widespread usage (for example, metal degreasing or dry cleaning) (Abelson, 1990). Under anaerobic conditions, PCE and TCE can be dechlorinated to dichloroethenes (DCEs) by a variety of microbes (for example, *Dehalobacter, Desulfuromonas, Sulfurospirillum* and *Dehalococcoides*) (Krumholz, 1997; Maymó-Gatell et al., 1997; Holliger et al., 1998; Luijten et al., 2003; Duhamel et al., 2004; Sung et al., 2006b; Sung et al., 2006a). However, only *Dehalococcoides* species are involved in the complete reductive dechlorination of PCE/TCE beyond DCEs to vinyl chloride (VC) and ethene (Smidt and de Vos, 2004). These dechlorination steps are facilitated by various functional reductive dehalogenase (RDase) genes such as *pceA* (PCE-to-TCE), *tceA* (TCE-to-VC), *bvcA* or *vcrA* (DCEs-to-ethene) genes in *Dehalococcoides* sp. (Magnuson et al., 1998; Krajmalnik-Brown et al., 2004; Müller et al., 2004; Smidt and de Vos, 2004). In particular, *Dehalococcoides* sp. strains BAV1 and FL2 are able to dechlorinate all DCE isomers to VC or ethene (He et al., 2003b; He et al., 2005). Multiple *Dehalococcoides* strains have also been found in mixed cultures that work together to dechlorinate PCE completely to ethene (Hölscher et al., 2004; Waller et al., 2005; Holmes et al., 2006). To date, the characterized PCE and TCE dechlorinators usually produce *cis*-1,2-dichloroethene (*cis*-DCE) predominantly and *trans*-1,2-dichloroethene (*trans*-DCE) negligibly (Smidt and de Vos, 2004). However, *trans*-DCE was found in at least 563 sites of the 1,430 National Priority List Superfund sites identified by the U.S. Environmental Protection Agency (EPA), whereas *cis*-DCE was detected only in 146 locations (ATSDR., 2007). Ratios of *trans*- / *cis*-DCE ranging

from (1 to 7) : 1 have been documented in the TCE-contaminated subsurface of the Naval Air Force Station (Fort Worth, Texas) (http://pubs.usgs.gov/sir/2005/5176/) and in the TCE-contaminated wells of Key West, Florida (SWMU9, 2002). Significant amounts of *trans*-DCE were also detected at concentrations of up to 18 000 μg l^{-1} in groundwater samples taken from the upper tertiary aquifer in Bitterfeld (Germany) (Nijenhuis et al., 2007). The accumulated *trans*-DCE in the chloroethene-contaminated sites tends to persist and disperse in the subsurface (He et al., 2003a; Smidt and de Vos, 2004), hindering the complete removal of chloroethenes.

A few laboratory-scale studies report that certain microorganisms in microcosms or mixed cultures could produce more *trans*- than *cis*- DCE during the reductive dechlorination of PCE/TCE (Löffler et al., 1997b; Griffin et al., 2004; Miller et al., 2005; Kittelmann and Friedrich, 2008b). For instance, a number of uncultured microbes of the *Dehalococcodies* sp. and DF-1 in the *Chloroflexi* cluster were capable of producing *trans*- /*cis*- DCE in various ratios ((1.3-3.5): 1) when fed with PCE/TCE (Futamata et al., 2007). Although certain part of *trans*-DCE may be produced through abiotic processes (Arnold and Roberts, 1998; ATSDR., 2007), a large fraction of accumulated *trans*-DCE at contaminated sites could be a result of microbial reductive dechlorination of PCE and TCE. Enrichment of such dechlorinating bacteria, in particular of the genus *Dehalococcoides,* has been proven to be laborious because of their long doubling time (> 1 day). Therefore, limited information is available for dechlorinating microbes possessing specific RDase(s) involved in *trans*-DCE production and its further detoxification. So far, only *Dehalococcoides* sp. strain BAV1 could completely dechlorinate *trans*-DCE to the benign ethene (He et al., 2003b). Owing to the persistent nature of *trans*-DCE and limited information on *trans*-DCE-dechlorinating bacteria, it would be of interest to

look further into *trans*-DCE detoxification by multiple dechlorinators when *trans*-DCE was formed predominantly at the contaminated sites.

This chapter describes dechlorination of PCE/TCE to various amounts of *trans*-DCE by *Dehalococcoides*-containing microcosms, enrichment, and a co-culture MB. The microbial populations associated with various *trans*- /*cis*-DCE generation ratios were investigated in detail. We have also identified the first *Dehalococcoides* sp. belonging to the Cornell subgroup responsible for *trans*- and *cis*- DCE production in culture MB. In contrast to previous reports linking the *Dehalococcoides* species to VC and ethene generation, in this study, we concluded that the specific *Dehalococcoides* sp. which produced *trans*-DCE predominantly could not dechlorinate DCEs further to VC and ethene. However, complete dechlorination of PCE to ethene through predominant intermediate *trans*-DCE was achieved by co-inoculating the enrichment culture MB and a *trans*-DCE dechlorinating culture 11a that contained multiple *Dehalococcoides* spp. The study of *trans*-DCE producing consortia will provide more information on the diversity of dechlorinators involved in the generation of various dechlorination products (such as *trans*-DCE, VC, or ethene) upon exposure to PCE/TCE. These findings could then be extrapolated to contaminated sites and potentially aid in the determination of the type of bioremediation strategy such as bioaugmentation, with the appropriate dechlorinators.

3.2 Materials and methods

3.2.1 Chemicals

The chlorinated ethenes, ethene, and other chemicals were purchased from Sigma-Aldrich (Saint Louis, MO, USA) with a minimum purity of 99.5%. Hydrogen

was produced from a hydrogen generator (NM-H250, Schmidlin-DBS AG, Neuheim, Switzerland) and used as indicated.

3.2.2 Microcosm preparation

Sediments or slurry used for setting up microcosms were collected from San Francisco Bay Area (CA, U.S.A.), Sungei Buloh Wetland Reserve (Singapore), Wuhan industrial districts (Hubei, China), and Guiyu landfill sites receiving electrical waste (Guangdong, China). Triplicate microcosm studies were conducted in 60-mL serum bottles containing ~ 10 g of sediments or slurry, 25 ml autoclaved, bicarbonate-buffered mineral salts medium reduced with $_{L}$-cysteine (0.2 mM), sodium sulfide (0.2 mM), and $_{DL}$-dithiothreitol (0.5 mM) as previously described (Wolin et al., 1963; Cole et al., 1994; Löffler et al., 1997a; He et al., 2002; He et al., 2003a). The bottles were sealed with black butyl rubber septa (Geo-Microbial Technologies, Inc., Ochelata, OK, USA) and secured with aluminum crimp caps. All microcosms in triplicates were amended with 10 mM of lactate and 55 mM of PCE per bottle. Abiotic controls were carried out by autoclaving another set of microcosm bottles that were set up in the same way as the sample bottles. All bottles were inverted and incubated quiescently in the dark at room temperature.

3.2.3 Culture and growth conditions

After screening the above microcosms, two microcosms MB (San Francisco, CA, U.S.A.) and 11a (Hubei, China) were selected for further enrichment as they showed rapid dechlorination rates. After transferring 20 times in the presence of PCE or TCE, serial dilutions (10%, v/v) were used to enrich the cultures, which were carried out in 20 mL vials filled with 10 mL of mineral salts medium. In addition,

ampicillin (50-300 mg l^{-1}) (Maymó-Gatell et al., 1997; He et al., 2003b) was added to the dilution series spiked with TCE (0.2 mM), acetate (10 mM), and hydrogen (500,000 ppmv). Initially, the cultures from the first three consecutive transfers received 200 mg l^{-1} of ampicillin. Subsequently, three consecutive dilution-to-extinction series and agar shakes were conducted. During these treatments, more than 50 colonies were picked up and re-inoculated back to liquid medium to test their dechlorination activity on exposure to TCE. Dechlorination time course studies were conducted in 160-mL serum bottles containing 100 mL of mineral salts medium amended with TCE or PCE (~ 50 μmoles, nominal concentration 0.5 mM), lactate (10 mM) or acetate (10 mM) and hydrogen (500 000 ppmv), a vitamin solution including 0.05 mg l^{-1} of vitamin B_{12} (He et al., 2007a), and 2 % of inocula. All time-course studies were conducted in triplicates along with an extra abiotic control. All bottles were inverted and incubated quiescently in the dark at 30 ^{o}C. The average dechlorination rate (μmol $L^{-1}day^{-1}$) was calculated from the decrease of substate mass divided by the required reaction time and culture volume used for each bottle, whereas the maximum dechlorination rate indicated the highest concentration change for the substrate.

3.2.4 Analytical methods

Chloroethenes and ethene were measured with a gas chromatograph (GC-6890, Agilent, Wilmington, DE, USA) equipped with a flame ionizing detector and a capillary column (GS-GasPro, 30-m length, 0.32-mm i.d., J&W Sci, Folsom, CA, USA). The oven temperature was initially held at 50 ºC for 2 min, increased at 30 ºC min^{-1} to 220 ºC, and held for 1 min. The supernatants carrying volatile fatty acids (VFAs) were obtained by centrifugation of 1 mL of sample at 14,000×g for 10

minutes at 4 ºC. VFAs were determined on a high performance liquid chromatograph (Agilent 1100 HPLC system, Palo Alto, CA, USA) equipped with a UV detector (set at 210 nm). Separation of VFAs was conducted on an organic acid analysis column Aminex HPX-87H (300×7.8 mm, Bio-Rad, Hercules, CA, USA) at 40 ºC and 5 mM H_2SO_4 was used as the eluent at a flow rate of 0.5 ml min^{-1}.

3.2.5 DNA extraction and PCR amplification

Cells for DNA extraction were collected periodically from 1 mL of culture samples by centrifugation (15 min at 20, 000×g, 4ºC) in DNase/RNase-free microcentrifuge tubes. Cell pellets were stored at -20 ºC until further processing. The genomic DNA was extracted with Qiagen DNeasy Tissue Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. PCR (Eppendorf, Hamburg, Germany) amplification was performed by targeting the genomic DNA with universal Eubacterial primers 8F (Zhou et al., 1995) and 1541R (Lane et al., 1985) to obtain the 16S rRNA genes. Genomic DNA was also screened with the following species-specific primer pairs: *Dehalococcoides*, *Desulfuromonas*, and *Dehalobacter* (Holliger et al., 1998; Löffler et al., 2000; Bunge et al., 2003) as previously described. The primer sequences used in this study were included in the Table 3.1.

The presence of chloroethene RDase genes (*pceA*, *tceA*, *bvcA* and *vcrA*) in the cultures of this study was examined as described previously (Magnuson et al., 1998; Krajmalnik-Brown et al., 2004; Müller et al., 2004; Smidt and de Vos, 2004). The PCR products were subsequently visualized on a Molecular Imager Gel Doc XR System (Bio-Rad, CA, U.S.A.) or on a Bioanalyzer by using a DNA7500 Labchip Kit (Agilent Technologies, Inc, Palo Alto, USA).

Table 3.1 Specific primer sets for the detection of known chloroethene/chloroethane reductive dehalogenase

ID	Name	Primer name	Sequences (5'-3')	Annealing	References
1	*Eubacterial*	8F	AGA GTT TGA TCC TGG CTC AG	45s at 55ºC	Zhou *et al.*, 1995
		1392R	ACG GGC GGT GTG T		
		1541R	AAG GAG GTG ATC CAG CCG CA		
2	*Dehalococcoides*	730F	GCG GTT TTC TAG GTT GTC	45s at 58ºC	Bunge *et al.*, 2003
		1350R	CAC CTT GCT GAT ATG CGG		
3	*Desulfuromonas*	Dsf 205F	AAC CTT CGG GTC CTA CTG TC	45s at 58ºC	Löffler *et al.*, 2000
		Dsf 1020R	GCC GAA CTG ACC CCT ATG TT		
4	*Dehalobacter*	Deb 179F	TGT ATT GTC CGA GAG GCA	45s at 53ºC	Holliger *et al.*, 1998
		Deb 1007R	ACT CCC ATA TCT CTA CGG		
5	*pceA*	pceAF	ATG GTG GAT TTA GTA GCA GCG GTC	60s at 50ºC	Fung et al. 2007
		pceAR	ATC ATC AAG CTC AAG TGC TCC CAC		
6	*tceA*	797F	ACG CCA AAG TGC GAA AAG C	45s at 50ºC	Magnuson *et al.*, 2000
		2490R	TAA TCT ATT CCA TCC TTT CTC		
7	*bvc-A*	bvcAF	TGC CTC AAG TAC AGG TGG T	45s at 52ºC	Krajmalnik-Brown *et al.*, 2004
		bvcAR	ATT GTG GAG GAC CTA CCT		
8	*vcrA*	vcrAF	TGC TGG TGG CGT TGG TGC TCT	60s at 55ºC	Müller *et al.*, 20044
		vcrAR	TGC CCG TCA AAA GTG GTA AAG		

The 16S rRNA genes copies of *Dehalococcoides* sp. in co-culture MB were determined by quantitative real-time PCR (qPCR) (ABI 7500 Fast Real-Time PCR

system, Foster, CA, U.S.A.) as previously described (He et al., 2003a; He et al., 2003b; Sung et al., 2006a). Standard curves spanned a range of 10^2 to 10^7 gene copies per microliter of template DNA with a R^2 linear regression of 99.9%.

3.2.6 PCR-DGGE and T-RFLP

PCR-denaturing gradient gel electrophoresis (DGGE) analyses were carried out for both universal bacterial 16S rRNA gene (with the primer pair 341F-GC and 534R) and *Dehalococcoides*-specific 16S rRNA gene fragments (1F-GC and 259R primer set) with a touch-down thermal program as previously described (Duhamel et al., 2004). PCR-amplified fragments were electrophoresed on an 8% polyacrylamide gel with a 30-60% urea-formamide gradient for 16 h at 120 V and 60ºC.

T-RFLP with the restriction enzyme provides a broad picture of the microbial community composition over time during the enrichment process to estimate the microbial diversity. By using the protocol described previously (Liu et al., 1997), the amplified fragments by universal primer (8F and 1541R, 8F labeled with Cy5) were digested with the restriction endonucleases *Msp* I (NEB, USA) according to the manufacturer's recommendations. The enzyme was deactivated by heating at 65 ºC for 10 min. Analysis of 16S rRNA gene-based T-RFLP was carried out with Beckman Coulter (CEQ 8000 automated sequencer, Beckman Coulter, US). PCR-RFLP products were analyzed by horizontal electrophoresis in 2% agarose or using Agilent Bioanalyzer (Agilent Technologies, US) with DNA 7500 chips (Rahm et al., 2006) and strictly following the manufacturer's protocol.

3.2.7 Clone library

A clone library of 16S rRNA genes of co-culture MB was established by using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, U.S.A.) and all further clone-based experiments were carried out as previously described (Löffler et al., 2000; He et al., 2003a). The clones' purified plasmid DNA concentrations were measured by Nanodrop-1000 (NanoDrop Technologies Inc, Wilmington, DE, USA). The extracted plasmid DNA was subjected to RFLP analysis with the restriction endonucleases *Hha*I and *Msp*I (NEB, Ipswich, MA, USA). The 16S rRNA gene inserts representing groups of distinct enzyme restriction pattern were subsequently sequenced with an ABI 3100 Sequencer (Applied Biosystems, Foster City, CA, U.S.A.) by using primers M13F-20, M13R-24, 533F, 529R, and 907F (http://www.genomics.msu.edu). Sequences were aligned with BioEdit assembly software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and were analyzed with BLASTN (http://www.ncbi.nlm.nih.gov/). The nearly complete *Dehalococcoides*-like 16S rRNA gene sequence (1489 bp) was deposited under GenBank accession number EU073964. The other bacterium was isolated and characterized as being a *Sedimentibacter* sp. under GenBank accession number FJ593657.

3.3 Results

3.3.1 Dechlorination of PCE to predominant *trans*-DCE

From the 23 microcosms established with sediments collected from various locations, three (namely MB, 11a, GY from CA, U.S.A.; Hubei, China; and Guangdong, China; respectively) generated more *trans*-DCE than *cis*-DCE in the

reductive dechlorination of PCE, while SB (Singapore) generated relatively lower *trans*- /*cis*-DCE ratio (Table 3.2). No PCE dechlorination or lactate fermentation was observed in the abiotic control bottles (autoclaved microcosms). Microcosms, MB, SB and GY, generated *trans*-DCE and *cis*-DCE as the final dechlorination products, while 11a could dechlorinate PCE to ethene through intermediates *trans*- and *cis*-DCEs. Among these microcosms, MB exhibited the highest PCE dechlorination rate of 26.2 μmol liter^{-1} day^{-1} with a *trans*- /*cis*- DCE generation ratio of (1.4 ± 0.1) :1 and was selected for further transfers and investigation.

Table 3.2 Summary of microcosms that produced *trans*-DCE and *cis*-DCE from dechlorination of PCE

Source	Final product	*trans*-DCE/ *cis*-DCE ratio
San Francisco Bay Area (CA, USA), MB	*trans*-DCE, *cis*-DCE	1.4 ± 0.1
Wuhan industrial districts (Hubei, China), 11a	Ethene (through *trans*- /*cis*-DCEs)	1.7 ± 0.2
Guiyu landfill sites (Guangdong, China), GY	*trans*-DCE, *cis*-DCE	2.8± 0.3
Sungei Buloh Wetland Reserve (Singapore), SB	*trans*-DCE, *cis*-DCE	0.4 ± 0.06

Note: The presented ratios were from triplicate cultures with standard deviation.

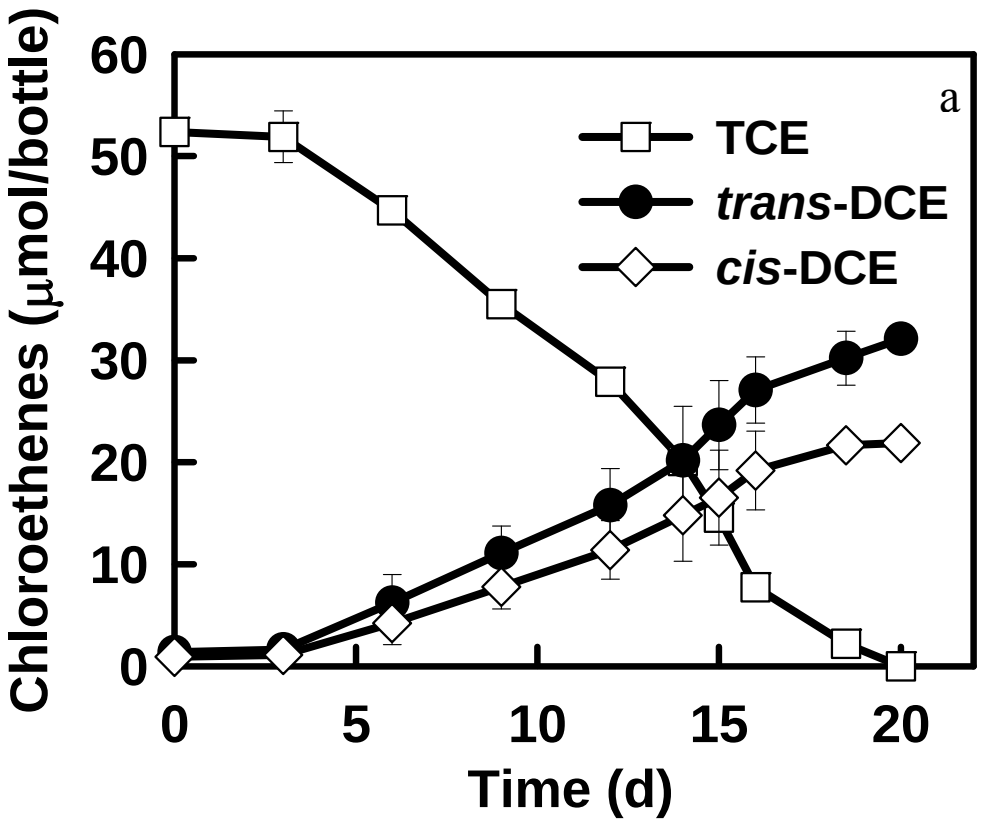
Enrichment culture MB was obtained by sequentially transferring the above PCE-to- *trans/cis*-DCE dechlorinating microcosm. Methane was below detection limit after 10 transfers with PCE as substrate, though it was detected in the microcosms and the previous batches of sediment-free culture. The subsequent 10

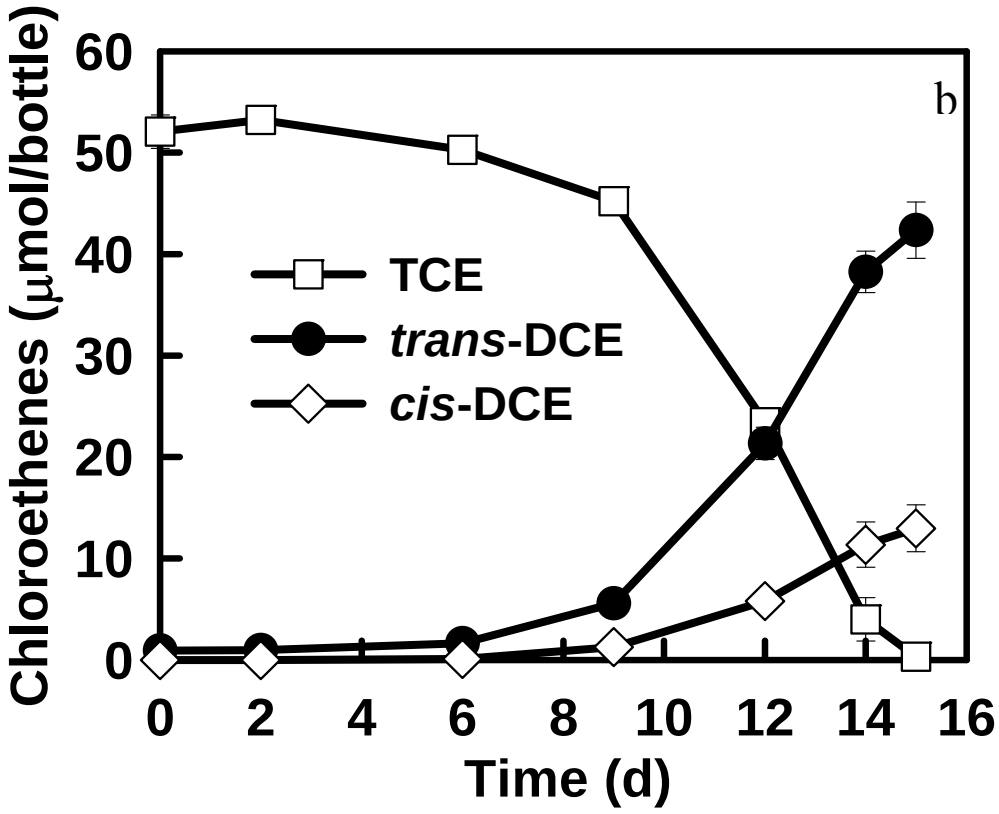
transfers were fed with TCE and the time course studies on the dechlorination of TCE were carried out thereafter. In defined mineral salts medium amended with lactate (10 mM) and TCE (0.2 mM), culture MB dechlorinated ~50 µmoles of TCE completely to *trans*/*cis*- DCEs with a ratio of (1.43 ± 0.04) :1 within 20 days, and a dechlorination rate of 30.6 μmol liter^{-1} day^{-1} (Fig 3.1a). Dechlorination of TCE to *trans*- and *cis*-DCEs (in a ratio of 1.4 : 1) also occurred when culture MB grew in the same mineral salts medium amended with acetate (10 mM) only, or acetate (10 mM) and H_2 (170,000 ppmv). The sole acetate-grown culture (no H_2 supplied) showed the slowest dechlorination rate. Thereafter, the acetate-grown sub-culture was used as inocula for subsequent transfer to fresh acetate and H_2 medium in order to facilitate the enrichment and isolation of the dechlorinators.

When culture MB was treated with ampicillin (50 mg l^{-1}) in the mineral salts medium amended with acetate and H_2, dechlorination of TCE occurred at a slower rate as compared with dechlorination without ampicillin. The treatment of acetate/H_2 cultures with 0 - 300 mg l^{-1} of ampicillin resulted in the variation of *trans*-DCE to *cis*-DCE ratio, ranging from (1.4 to 3.0) : 1. The highest ratio was achieved in the culture receiving 200 mg l^{-1} of ampicillin dosage. After growing in ampicillin-amended medium for two more transfers, the active cultures were then continuously transferred to medium amended with acetate and H_2 only. Consequently, culture MB was capable of dechlorinating ~50 µmoles of TCE to *trans*- and *cis*- DCEs with an increased ratio of 3.7:1 (Fig 3.1b) within 15 days. However, when culture MB was transferred back to the mineral salts medium amended with lactate, an extended lag phase (~13 days) was observed and the dechlorination of TCE (~50 µmoles) took another 17 days in all the triplicate bottles (data not shown). Acetate and propionate, detected as the

fermentation products measured with HPLC, indicated that certain fermentative microbes had yet to be diluted out of the culture.

After three more transfers in the absence of antibiotics, the ampicillin-treated culture MB with acetate and H_2 achieved its highest TCE dechlorination rate (68.8 μmoles $L^{-1}day^{-1}$) but the *trans*-/*cis*- DCE generation ratio remained unchanged (Fig 3.1c). Culture MB was continuously transferred to fresh medium (every 3-4 weeks) over a period of 2 years and showed consistent PCE/TCE dechlorination rates and *trans*- /*cis*- DCE generation ratio. At this stable dechlorination stage, culture MB was observed under light microscopy, which demonstrated the prevalence of small disc-shaped bacteria and a lower abundance of rod-shaped bacteria. The size of the disc-shaped microbes coincides with that of the reported *Dehalococcoides* species (~ 1.0 μm in diameter). Attempts had been made to isolate each of them by both serial dilutions and agar shakes amended with acetate and H_2. However, only the rod-shaped bacteria were able to grow in pure culture successfully. Its 16S rRNA gene sequence was shown to be closely (99% identity over 1504 bp, 5 bp difference) related to *Sedimentibacter* sp. C7, which was reported to grow in co-culture with a *Dehalobacter* species (van Doesburg et al., 2005; He et al., 2007a). This isolated *Sedimentibacter* sp. did not show dechlorination activity on PCE or TCE. Therefore, culture MB should mainly consist of the PCE-dechlorinating *Dehalococcoides* and the *Sedimentibacter* species.


a
TCE
trans-DCE
cis-DCE
Chloroethenes (μmol/bottle)
Time (d)
0
10
20
30
40
50
60
0
5
10
15
20


b
TCE
trans-DCE
cis-DCE
Chloroethenes (μmol/bottle)
Time (d)
0
10
20
30
40
50
60
0
2
4
6
8
10
12
14
16

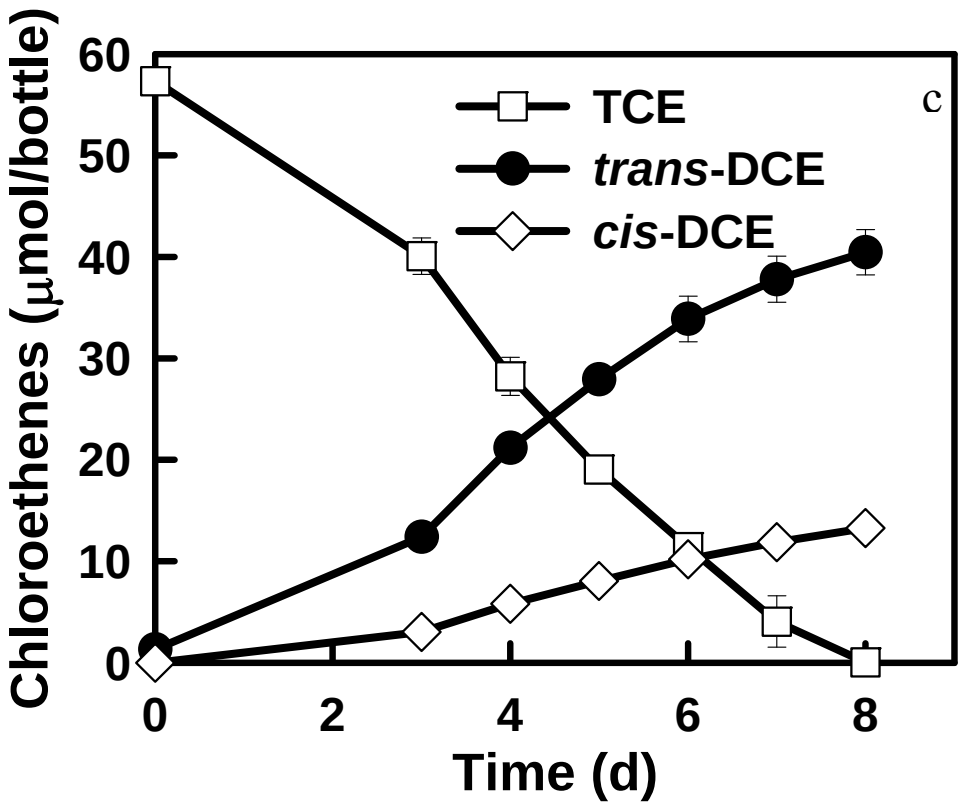


Fig 3.1 Reductive dechlorination of TCE to *trans*-DCE and *cis*-DCE by culture MB at different enrichment phases. (a) Enrichment culture MB after 20 transfers in lactate-amended medium; (b) Further enrichment culture MB grown with acetate (10 mM) and H_2 (260,000 ppmv) after ampicillin treatment; (c) Highly enriched culture MB after three more transfers in acetate- /H_2-amended medium. No VC, ethene, ethane or methane was detected on extended incubation. Datum points were averaged from triplicate cultures. Error bars indicate standard deviation and are not shown when they are smaller than the symbol.

3.3.2 Identification of the *trans*-DCE producing microbes

To identify the dechlorinating microbes in the sediment-free cultures, MB, 11a, GY, and SB, genus-specific primers targeting the16S rRNA genes of *Dehalobacter, Desulfuromonas,* and *Dehalococcoides* were tested on genomic DNA of the above cultures by PCR. When targeted with *Dehalococcoides* specific primers, the PCR amplicons yielded the expected DNA size of 620 bp for all the cultures. When using *Desulfuromonas*- specific primers to amplify the genomic DNA of the above *trans*-

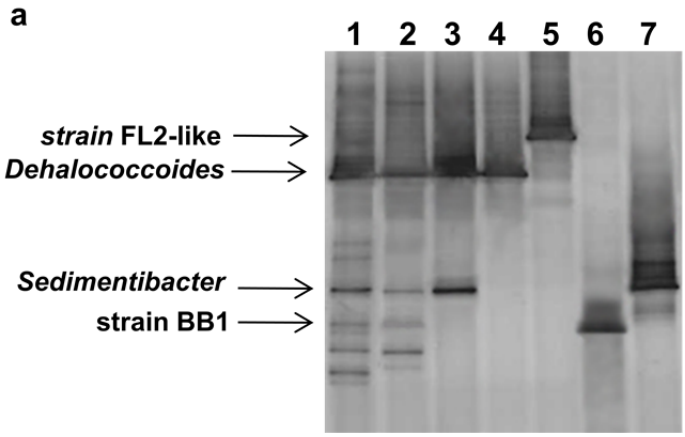
DCE producing cultures, only culture MB exhibited a very weak band of 815 bp. However, the *Desulfuromonas*-like species was no longer detected in MB when the ratio of *trans*- to *cis*- DCE reached ~ 3.7:1. Amplification of the genomic DNA using *Dehalobacter*- specific primers yielded no amplicons for all the cultures. Therefore, the PCR analysis based on 16S rRNA genes suggests that the rod-shaped *Desulfuromonas* or *Dehalobacter* species may not contribute to *trans*-DCE formation, and further confirms the existence of *Dehalococcoides*-like species in the above cultures.

3.3.3 *Dehalococcoides* species diversity versus the ratio of *trans*- to *cis*-DCE

To understand the elevated ratio (1.4 :1 to 3.7:1) of *trans*-/ *cis*-DCE in culture MB, PCR-DGGE was used to track the community structures with universal bacterial primer pair and the diversity of *Dehalococcoides* sp. with genus-specific primer pair by targeting the genomic DNA of culture MB at different enrichment stages. Fig 3.2a shows the microbial community transitions from mixed culture (before ampicillin treatment), highly enriched culture (after one-time ampicillin treatment), to apparent co-culture (after ampicillin treatment), reflected by detecting multiple DGGE bands to two major bands. The band on the top was getting thicker, which turned out to be *Dehalococcoides* species. This was verified by the positive control, plasmid DNA containing the 16S rRNA gene fragment of *Dehalococcoides ethenogenes* strain 195. The second major band in Fig 3.2a corresponded to the minor group in the co-culture, *Sedimentibacter*, which was confirmed by the positive control of this isolate. It is to be noted that the *Desulfuromonas*-like band disappeared in the post ampicillin-treated

MB cultures (Fig 3.2a). Only one band representing the Cornell subgroup of *Dehalococcoides* species (Fig 3.2b) was detected in the *trans*- and *cis*-DCEs producing culture MB. Therefore, the detected *Desulfuromonas*-like species before ampicillin treatment (Fig 3.1b) could have contributed to *cis*-DCE production in the mixed culture MB. In all, the DGGE profiles confirmed that culture MB consisted of the Cornell subgroup of *Dehalococcoides* and *Sedimentibacter* only, while the former was responsible for *trans*-DCE production from PCE/TCE.

Sediment-free cultures obtained from microcosms shown in Table 1 also generated *trans*- and *cis*- DCEs in various ratios. The presence of multiple *Dehalococcoides* sp. bands (e.g., GY and 11a) explained the various ratios of *trans*-/*cis*- DCE as exhibited in this study (Fig 3.2c). Fig 3.2c also shows the stronger the MB-like bands, the higher the *trans*- /*cis*- DCE generation ratios in the sediment-free cultures; conversely, the stronger the FL2-like bands, the lower the *trans*- /*cis*- DCE ratios in these cultures.



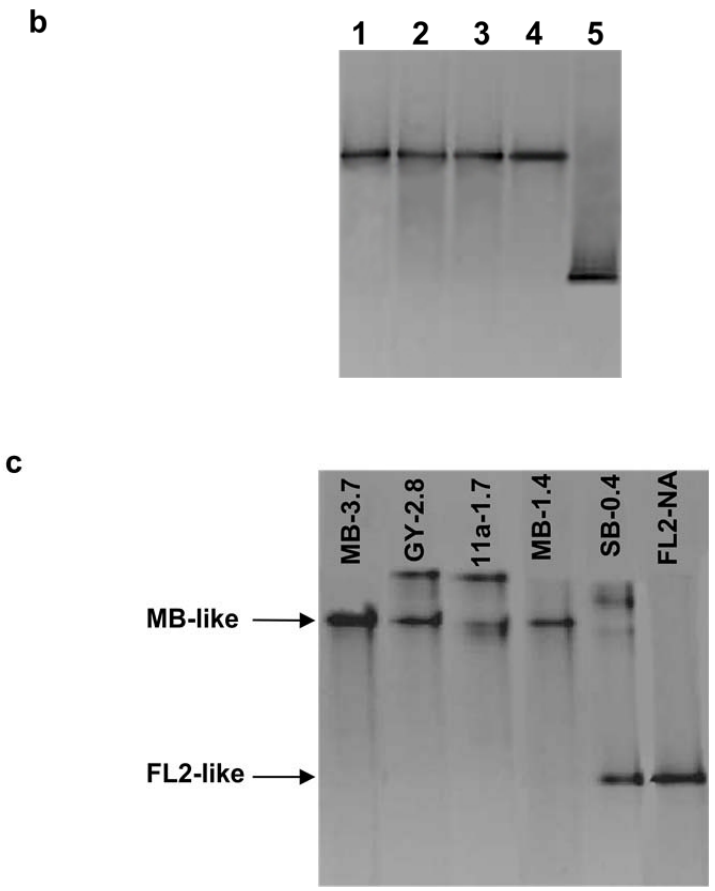


Fig 3.2 PCR-DGGE profiles of *trans*-DCE producing cultures.
(a) DGGE profile of PCR-amplified with a universal bacterial primer pair targeting genomic DNA of culture MB. (b) DGGE profile of PCR-amplified with a *Dehalococcoides*-specific primer pair targeting genomic DNA of culture MB. The template DNA for lane 1, 2 and 3 was extracted from cultures as shown in the time course studies of Fig 1a, 1b and 1c, respectively. Lane 1, culture MB before ampicillin treatment; lane 2, culture MB after one-time ampicillin treatment; lane 3: culture MB post ampicillin treatment; lane 4, positive control of *D. ethenogenes* 195 clone DNA; lane 5, a TCE-to-*cis*-DCE-to-VC culture which shared 100% 16S rRNA gene as *Dehalococcoides* sp. strain FL2; lane 6, *Desulfuromonas* sp. strain BB1; lane 7, an isolate of *Sedimentibacter*. (c). PCR-DGGE profile of *Dehalococcoides* species present in different TCE-dechlorinating cultures. The number on the top of the lane indicates the ratio of *trans*-DCE to *cis*-DCE produced from various microcosms as described in Table 3.1. *Dehalococcoides* sp. strain FL2- like culture serves as a control. NA, no *trans*-DCE was accumulated during the dechlorination of TCE.

During the enrichment process, T-RFLP was also conducted to determine whether *Dehalococcoides* species is the predominant population in culture MB. After utilizing enzyme *Msp*I and *Rsa*I to digest the 16S rRNA genes amplified from the

genomic DNA of highly enriched culture MB, T-RFLP profiles distinguished a peak of *Dehalococcoides* species as shown in Fig 3.3. Comparing the T-RFLP profiles generated from different enrichment phases, the *Dehalococcoides* peak intensity (513 bp for MspI) increased dramatically while other peaks decreased. It indicates that culture MB became highly enriched and the predominance of *Dehalococcoides* populations. Similar *Dehalococcoides* peak changes appeared on the T-RFLP profiles (T-RF of 444 bp) when enzyme *Rsa*I was used to digest the above PCR products. However, no *Dehalococcoides*-like peaks were found in the enzyme *Hha*I digestion profiles, which was similar to that of the isolate *D. ethenogenes* 195 (Maymó-Gatell et al., 1997). The results from T-RFLP show similar trend as PCR-DGGE for the microbial community change of this *trans*-DCE producing culture.

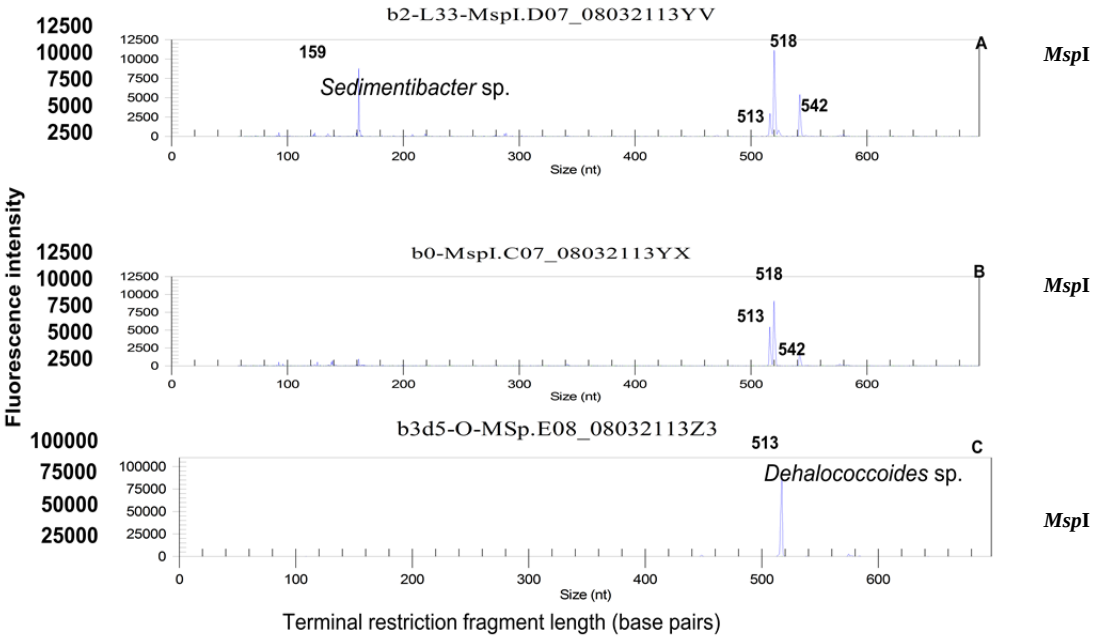


Fig 3.3 T-RFLP profiles digested with *Msp*I for enrichment culture MB at different stages. Note: the template DNA tested for a, b, and c correspond with cultures as shown in the time course studies of Fig 3.1a, 3.1b and 3.1c, respectively. a, culture MB before ampicillin treatment; b, culture MB after one-time ampicillin treatment; c, culture MB post ampicillin treatment.

3.3.4 Clone library and sequence analysis of culture MB

To further determine the phylogeny of the PCE-to-*trans*/*cis*-DCEs dechlorinator(s), a clone library based on 16S rRNA genes was established with the genomic DNA extracted from the apparent co-culture MB fed with acetate and H_2. The restriction fragment length polymorphism analysis of 57 clones revealed two different digestion patterns (56: 1). Twenty-eight of the 56 clones, and the last single clone were sequenced for their 16S rRNA gene inserts in the plasmids. After aligning the sequences with the BioEdit assembly software, the contigs were analyzed using BLASTN, and compared with known existing 16S rRNA gene databases. Results show that the 56 clones possessed identical 16S rRNA gene sequence, which shares 100% identity of the 16S rRNA gene sequence of *D. ethenogenes* strain 195 (accession number CP000027) from its complete genome. The 16S rRNA gene sequence of the uncultured *Dehalococcoides* sp. strain MB (the Cornell subgroup of *Dehalococcoides* sp.) was deposited in GenBank under accession number of EU073964 (1489 bp). In addition to the above *Dehalococcoides* species found in the culture MB, the sequence of the other clone revealed that it belonged to *Sedimentibacter* as isolated in pure culture.

The presence of the known RDase genes was also examined in the *trans*- and *cis*-DCEs producing cultures. The following genes, *pceA* gene, *tce*A gene, *bvcA* gene, or *vcrA* gene were not detected in culture MB when fed with PCE or TCE. The results suggested that a novel RDase gene, instead of *pceA* or *tceA* gene, might be responsible for the production of *trans*-DCE by the *Dehalococcoides* in culture MB. Therefore, on the basis of the above analysis (for example, constant *trans*- /*cis*- DCE ratio, light microscopy, DGGE, clone library, RDase genes), we were able to ascertain

that the culture was further enriched to a co-culture MB consisting only of *Dehalococcoides* species of the Cornell subgroup and *Sedimentibacter* species.

3.3.5 Growth of *Dehalococcoides*-like species in co-culture MB

Quantitative real-time PCR (qPCR) was carried out to further confirm that *Dehalococcoides*-like species was responsible for the generation of *trans*-DCE during the dechlorination of TCE. The *Dehalococcoides* cells were measured by targeting the genomic DNA extracted from co-culture MB. qPCR results demonstrated the increase of *Dehalococcoides* 16S rRNA gene copies with the dechlorination of TCE (Fig 3.4). The total *Dehalococcoides* cell number reached 2.54×10^8 cells/ml from initial 5.91×10^6 cells/ml (~ 43 times increase) in the co-culture, verifying that the increase of the *Dehalococcoides* 16S rRNA gene copies was directly correlated with the dechlorination of TCE to the predominant *trans*-DCE.

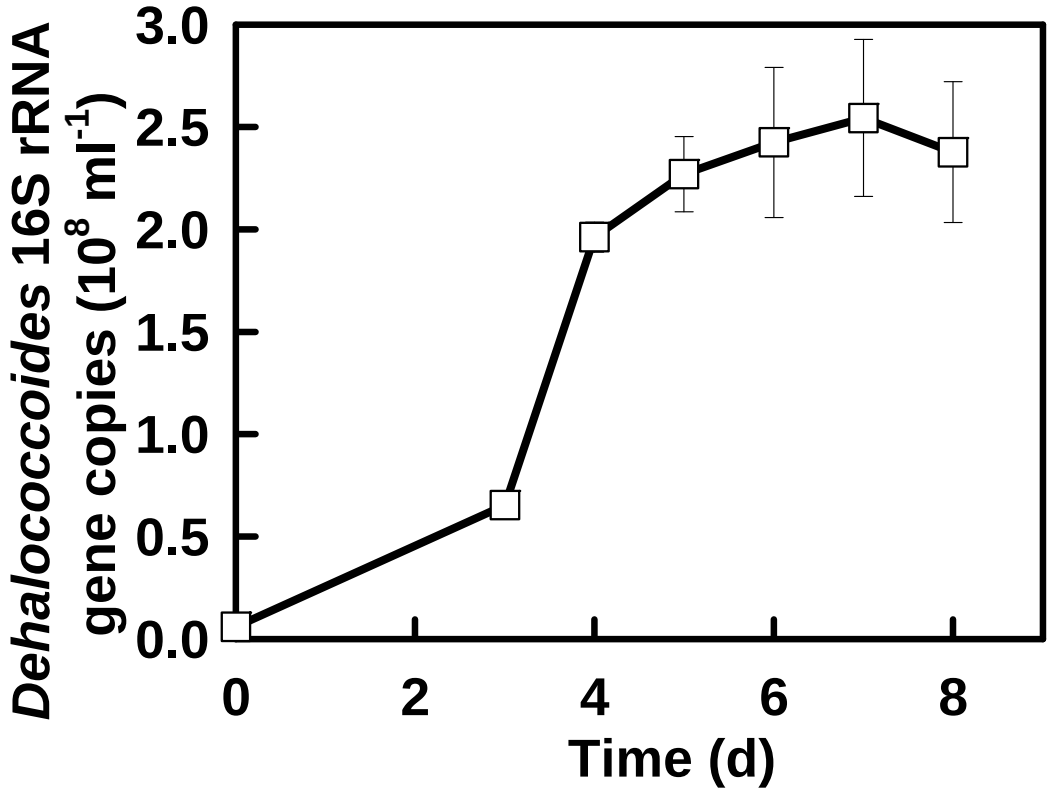


Fig 3.4 The growth of the *Dehalococcoides* species with the dechlorination of TCE to predominant *trans*-DCE by co-culture MB.

3.3.6 Complete dechlorination of TCE to ethene via *trans*- and *cis*-DCEs by culture MB and 11a

Similar to culture MB, the sequential transfers of another microcosm (Hubei, China) generated enrichment culture 11a, which was capable of completely dechlorinating TCE to ethene via intermediates *trans*- and *cis*- DCEs (in a ratio of 1.7: 1) in lactate-amended mineral salts medium (Fig 3.5a). After 40 days, the generated DCEs were completely dechlorinated to ethene. DGGE profile showed that culture 11a contained multiple *Dehalococcoides* species (Fig 3.2c), one of which was MB-like to produce *trans*-DCE whereas another may function to further dechlorinate *trans*-DCE. With the *trans*-DCE dechlorinating mixed culture 11a in hand, it was inoculated together with culture MB to the bottles spiked with TCE to examine the possibility of complete reductive dechlorination of the persistent *trans*-DCE generated biologically by culture MB. As shown in Fig 3.5b, complete dechlorination to ethene via *trans*-DCE occurred within 80 days. The maximum *trans*- to *cis*- DCE ratio reached ~1.75: 1 on day 11 when *cis*-DCE concentration started to decrease. During the reductive dechlorination process, *trans*-DCE was found to be the main intermediate, accumulating up to 18.6 μmoles on day 21, whereas small amounts of *cis*-DCE and VC (about 6.9 and 7.8 μmoles, respectively) appeared and the amount of 1,1-DCE produced was negligible. After day 21, *trans*-DCE started to be dechlorinated to ethene without the accumulation of VC (Fig 3.5b).

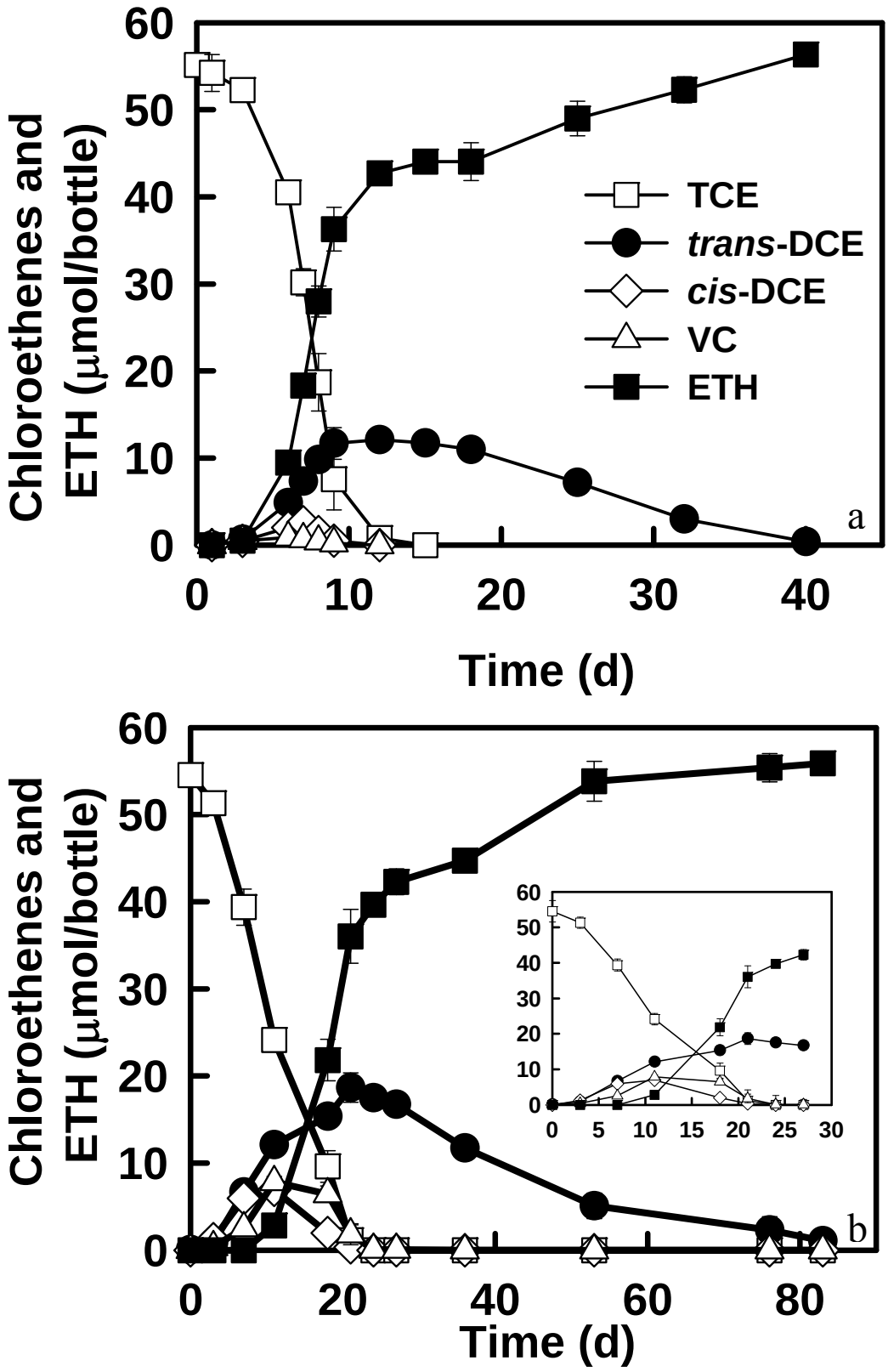


Fig 3.5 Reductive dechlorination of TCE to ethene through *trans*-DCE predominantly. (a) Culture 11a alone amended with lactate; (b) Culture MB and 11a amended with acetate (10 mM) and H_2 (400 000 ppmv). (Datum points were averaged from triplicate cultures. Error bars indicate standard deviation and are not shown when they are smaller than the symbol).

3.4 Discussion and conclusion

In this study, a highly enriched PCE-to-*trans*/*cis*- DCE dechlorinating co-culture MB was characterized and the growth of the newly identified *trans*-DCE producing *Dehalococcoides* species was verified by qPCR. A number of identified *trans*-DCE producing *Dehalococcoides* species, including the Cornell (in this study) and Pinellas (Griffin et al., 2004) subgroups, showed a common inability to dechlorinate PCE/TCE beyond DCEs to VC and ethene. The dechlorination pathway of MB is different from the previously discovered *Dehalococcoides* species linking to VC or ethene production from polychloroethenes, including those that produced predominant 1,1-DCE isomers during the reductive dechlorination of TCE (Zhang et al., 2006). Complete detoxification of PCE/TCE through intermediates *trans*- and *cis*- DCEs also occurred when the required dechlorinating microbes were present in the community, as shown in the mixed culture of 11a and MB. Results from this study indicate that accumulation of *trans*-DCE through microbial reductive dechlorination in contaminated sites could be significant when the *trans*-DCE dechlorinator(s) are absent or are operating at a lower rate than that of *cis*-DCE and VC dechlorinators. The chloroethene distribution in contaminated sites is significantly affected by different types of indigenous dechlorinators (Smidt and de Vos, 2004). The *trans*-DCE generating microbe discovered in this study would enhance the current understanding of the diverse dechlorinating capabilities of the *Dehalococcoides* genus.

During the enrichment process from sediment-free culture to co-culture MB, the ampicillin treatment step significantly increased the culture's TCE-dechlorination rates (from 26.2 to 68.8 μmoles $L^{-1}day^{-1}$) and the ratio of *trans*-DCE to *cis*-DCE (from (1.4 ± 0.1):1 to (3.7 ± 0.4):1). Previous studies showed that sediment-free

cultures generated *trans*-DCE to *cis*-DCE in ratios of (3 ± 0.5):1 (Griffin et al., 2004) and (1.3 ± 0.2):1 (Miller et al., 2005), accompanying with an average TCE dechlorination rate of 4.8 μmoles $L^{-1}day^{-1}$ (Griffin et al., 2004) and 20 μmoles $L^{-1}day^{-1}$ (Miller et al., 2005), respectively. The increased dechlorination rates and *trans*- to *cis*- DCE ratios for culture MB could be explained by that (i) the competitors for electron donor H_2 (e.g., methanogens) were diluted out; (ii) culture MB contained higher number of *trans*-DCE producing *Dehalococcoides* cells, but less or negligible amount of the *cis*-DCE producing *Desulfuromonas*-like cells (revealed by DGGE profiles), compared with previous cultures. This is also supported by the fact that the majority of the clones (56 out of 57) belong to *Dehalococcoides* and the *Desulfuromonas* band disappeared in the co-culture MB. However, culture MB was not a pure culture yet as lactate fermenters (e.g., *Sedimentibacter*) were still present. This is reflected by their recovered dechlorination activity after an extended incubation time when fed back from acetate/H_2- to lactate-amended medium.

By sequencing of the representative clones (established with PCR 16S rRNA gene inserts), *Dehalococcoides* sp. was identified to be responsible for PCE/TCE dechlorination to *trans*- and *cis*- DCEs. On the basis of the 16S rRNA gene sequence identity, *Dehalococcoides* sp. MB belongs to the Cornell subgroup of the *Dehalococcoides* cluster (Hendrickson et al., 2002), which is different from the *trans*- and *cis*- DCEs producing *Dehalococcoides* populations in the Pinellas group (Griffin et al., 2004) (refer to the Table 3.3). Table 3.3 also shows that bacterium DF-1 and TFCC group (T-RF 513bp and T-RF 143 bp), which are distantly related to *Dehalococcoides* sp., contributed to the production of *trans*-DCE. We could not exclude the possibility of other bacteria having a role in *trans*-DCE formation in the environment. However, this study provides conclusive proof that the Cornell

Table 3.3 Comparison of 16S rRNA gene sequences for selected chloroethene-dechlorinating isolates and uncultured clones

Dechlorinating isolates or clones, GenBank accession No	Substrate	End product	Group[a]	No.	No. of base differences (lower left portion of matrix) or identity percentage (upper right portion of matrix)[b]												
					1	2	3	4	5	6	7	8	9	10	11	12	13
Dehalococcoides sp. strain MB, EU073964	PCE, TCE	*trans*/*cis*-DCEs	C	1		100	99	99	98	98	98	98	98	NA	90	88	88
Dehalococcoides ethenogenes strain 195, CP000027	PCE, TCE, *cis*-DCE, *1,1*-DCE	VC, ethene	C	2	0		99	99	98	98	98	98	98	NA	90	88	88
Dehalococcoides enrichment culture clone ANAS, DQ855129	TCE, *cis*-DCE, 1,1-DCE	VC, ethene	C	3	1	1		99	98	98	98	98	98	NA	90	88	88
Dehalococcoides sp. strain VS, AY323233	*1,1*-DCE, VC	ethene	V	4	8	8	9		98	98	98	98	98	NA	90	88	80
Dehalococcoides sp. strain CBDB1, AJ965265	Chlorobenzene; chlorinated dioxins	DCB,TCB; MCDD, DiCDD	P	5	17	17	18	15		100	100	99	99	99[e]	89	88	88
Dehalococcoides sp. strain FL2, AF357918	TCE, *trans*-DCE, *cis*-DCE	VC	P	6	17	17	18	15	0		100	99	99	99[e]	89	88	88
KB-1/VC, AY146779	TCE, *cis*-DCE, VC	ethene	P	7	17	17	18	15	0	0		99	99	99[e]	89	88	88
Dehalococcoides sp. strain BAV1, CP000688	*trans*-DCE, *cis*-DCE, 1,1-DCE, VC	ethene	P	8	18	18	19	16	1	1	1		99	NA	89	88	88
Dehalococcoides sp. strain GT, AY914178	TCE, *cis*-DCE, *1,1*-DCE, VC	ethene	P	9	19	19	20	17	2	2	2	3		NA	89	88	87
Tahquamenon/Perfume River enrichment culture, (no accession number)[c]	PCE, TCE	*trans*/*cis*-DCEs	P	10	NA	NA	NA	NA	NA	NA	NA	NA	NA		89	88	NA
TFCC[d] (T-RF 513 bp), EU362187	PCE	TCE, *trans*/*cis*-DCEs	-	11	85	85	86	90	94	94	94	94	91	NA		88	66
TFC[e] (T-RF 143 bp), EU362197	PCE	TCE, *trans*/*cis*-DCEs	-	12	101	101	101	105	103	103	103	103	101	NA	124		21
Dehalobium chlorocoercia DF-1, AF393781	PCB, chlorobenzenes, PCE and TCE	DCB, *trans*/*cis*-DCEs	-	13	140	140	141	154	142	142	138	142	146	NA	123	97	

a. C, Cornell; V, Victoria; P, Pinellas (Henkrickson et al. 2002)
b. Obtained by using BLASTN program (www.ncbi.nlm.nih.gov).
c. Griffin et al. (2004)
d. Represented by clone TfC20H06 (partial 16S rRNA gene sequence of 885bp), dominant in Tidal Flat Chloroflexi Cluster T-RF 513bp.
e. Represented by clone TfC20H31 (partial 16S rRNA gene sequence of 887bp) in Tidal Flat Chloroflexi Cluster T-RF 143bp.

subgroup of *Dehalococcoides* sp. in co-culture MB grew with the dechlorination of TCE to predominant *trans*-DCE by both qualitative (for example, DGGE and clone library) and quantitative (real-time PCR) molecular tools. On the other hand, owing to the high identity of 16S rRNA gene sequences for the genus *Dehalococcoides*, it is difficult to rule out the presence of other *Dehalococcoides* sp. Nevertheless, the Cornell subgroup (no peak was shown for *Hha*I profile of T-RFLP) of *Dehalococcoides* has never been shown to have this dechlorination ability of producing *trans*-DCE predominantly. The stable *trans*-/*cis*- DCE ratio, two morphologies by light microscopy, and the culture -independent approaches (DGGE, clone sequencing, RDase gene analysis) strongly suggest that co-culture MB contained only one *Dehalococcoides* sp. The interactions between *Dehalococcoides* and *Sedimentibacter* are not clear but may be nutritional in nature. Isolation of this particular strain of *Dehalococcoides* will lead to an improved understanding of the nutritional requirements and physiological characterization of this novel *Dehalococcoides* sp. Therefore, this study and previous reports (Griffin et al., 2004; Miller et al., 2005; Kittelmann and Friedrich, 2008b) suggest that these diverse *trans*-DCE producing dechlorinators belong to the green non-sulfur bacteria, including *Dehalococcoides*, the Tidal Flat Chloroflexi Cluster, and bacterium DF-1.

Despite the high conservation of the 16S rRNA gene among the *Dehalococcoides* species, RDase genes (*pceA*, *tceA, bvcA* and *vcrA*) have been identified to differentiate reductive dechlorination of chloroethenes (Magnuson, 1998; Krajmalnik-Brown, 2004; Müller, 2004). In the enrichment process of culture MB, no *pceA* or *tceA* gene was detected in its genomic DNA. This observation suggests that the *tceA* gene in *D. ethenogenes* 195 is different from the functional *trans*-DCE-producing gene(s) in the *Dehalococcoides* sp. of culture MB. Similarly, bacterium

DF-1 was reported to dechlorinate PCE/TCE to significant amounts of *cis*- and *trans*-DCEs, whereas no *tceA* gene was reported in its genome (Miller et al., 2005). Therefore, the *trans*-DCE producing gene could be quite different from the currently identified RDase genes, which may also be potentially acquired through horizontal gene transfer as suggested for the known RDases in *Dehalococcoides* species (Regeard et al., 2005; Seshadri et al., 2005). PCR-DGGE identified a common band, whose presence may indicate *trans*-DCE production from PCE or TCE. However, the detection of this band in DGGE would not guarantee the generation of *trans*-DCE in the *Dehalococcoides* sp., e.g. *D. ethenogenes* strain 195.

Findings in this and previous studies suggest that diverse *trans*-DCE producing *Dehalococcoides* species are present at chloroethene-contaminated sites and these *Dehalococcoides* species are capable of producing high levels of *trans*-DCE (Löffler et al., 1997b; Griffin et al., 2004; Miller et al., 2005; Futamata et al., 2007; Kittelmann and Friedrich, 2008b). For the first time, we reported that the *trans*-DCE producing *Dehalococcoides* species was unable to generate VC and ethene during the dechlorination process through characterization of the co-culture MB (~ 98% of the bacteria belonging to the Cornell subgroup of *Dehalococcoides* species and ~ 2% affiliated with *Sedimentibacter*), whereas previous *Dehalococcoides* cultures were linked to VC and ethene generation. Hence the large amounts of *trans*-DCE detected in the TCE contaminated sites might be a result of the presence of *trans*-DCE-producing microbes and the lack of *trans*-DCE dechlorinators. Fortunately, culture 11a in this study proves to be a promising candidate in the complete dechlorination of PCE/TCE to harmless ethene through the cooperation of multiple *Dehalococcoides* species. Thus great care must be taken when bioremediation techniques are being applied to such *trans*-DCE formation sites since the *trans*-DCE producing-culture (for

example, MB) is also capable of using the same electron donor as the other *Dehalococcoides* strains such as BAV1. The identification and administration of the microbe(s) responsible for rapid and complete dechlorination at any particular chloroethene-contaminated site may provide a better bioremediation strategy.

Chapter IV Isolation and Characterization of *Dehalococcoides* sp. Strain MB, Which Dechlorinates Tetrachloroethene to *trans*-1, 2-Dichloroethene

In chapter III, the enrichment process of several microcosm studies demonstrated that microorganisms within Cornell subgroup of *Dehalococcoides* could generate more *trans*-1,2-dichloroethene (*trans*-DCE) than *cis*-DCE during the reductive dechlorination of tetrachloroethene (PCE) or trichloroethene (TCE). In an attempt to understand the microorganisms involved in the generation of *trans*-DCE, pure culture *Dehalococcoides* sp. strain MB was isolated from environmental sediments. In contrast to currently known PCE or TCE dechlorinating pure cultures which generate *cis*-DCE as the predominant product, *Dehalococcoides* sp. strain MB reductively dechlorinates PCE to *trans*-DCE and *cis*-DCE at a ratio of 7.3 (± 0.4) : 1. It utilizes H_2 as the sole electron donor and PCE or TCE as the electron acceptor during anaerobic respiration. Strain MB is a disc shaped, nonmotile bacterium. Under an atomic force microscope, the cells appear singly or in pairs and are 1.0 μm in diameter and ~ 150 nm in depth. The purity was confirmed by culture-based approaches and 16S rRNA gene-based analysis and was corroborated further by putative reductive dehalogenase (RDase) gene-based quantitative real-time PCR. Although strain MB shares 100% 16S rRNA gene sequence identity with *Dehalococcoides ethenogenes* strain 195, these two strains possess different dechlorinating pathways. Microarray analysis revealed that 10 putative RDase genes present in strain 195 were also detected in strain MB. Transcriptional analysis of RDases in strain MB grown with PCE shows that one RDase gene, designated *mbrA*,

exhibited 10-fold up-regulation, higher than the rest of RDase genes. Successful cultivation of strain MB indicates that biotic process could contribute significantly to the generation of *trans*-DCE in chloroethene-contaminated sites. The highly expressed RDase gene, *mbrA* gene, may serve as an important biomarker for evaluating, predicting, and elucidating the biological production of *trans*-DCE in the chloroethene-contaminated sites. The results of this chapter enhance our understanding of the evolution of this unusual microbial group, *Dehalococcoides* species.

4.1 Introduction

Dehalorespiring bacteria play an important role in transformation and detoxification of a wide range of halogenated compounds, e.g., chlorophenols, chloroethenes, chlorobenzenes, polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs) (Quensen III et al., 1988; Mohn and Tiedje, 1992; Cole et al., 1994; Gerritse et al., 1996; Sanford et al., 1996; Holliger et al., 1998; Adrian et al., 2000; He et al., 2006; Hiraishi, 2008). Among these compounds, the organic solvents tetrachloroethene (PCE) and trichloroethene (TCE) are suspected carcinogens that are found in soil and groundwater due to their extensive usage and improper disposal (DiStefano et al., 1991). The widespread PCE and TCE in the subsurface environment have driven intensive studies on anaerobic microbes capable of reductive dechlorination of chloroethenes (Smidt and de Vos, 2004). Over the last decade, at least 18 isolates, which belong to the genera of *Desulfitobacterium*, *Sulfurospirillum*, *Desulfomonile*, *Desulfuromonas*, *Geobacter*, *Dehalococcoides*, and *Dehalobacter*, show reductive dechlorination of chlorinated ethenes (Smidt and de Vos, 2004; Hiraishi, 2008). In particular, most of these microbes produce *cis*-1,2-

dichloroethene (*cis*-DCE) as the end product in the chloroethene-contaminated sites, whereas complete detoxification of PCE or TCE to ethene has been restricted only to members of the genus *Dehalococcoides*. Thus, the *Dehalococcoides* species have received considerable attention from the bioremediation community in the past decade.

Several strains of *Dehalococcoides* species (e.g., 195, CBDB1, BAV1, and VS) have been sequenced for their whole genomes (Kube et al., 2005; Seshadri et al., 2005). Their dechlorinating capabilities have also been well addressed through identification and quantification of the known chloroethene reductive dehalogenase (RDase) genes or expression of specific RDase genes (Holmes et al., 2006; Lee et al., 2006; Sung et al., 2006a). In the chloroethene-contaminated sites, the natural activities of single or multiple *Dehalococcoides* strains can lead either to more toxic, mobile intermediates (e.g., *cis*- or *trans*- DCEs and vinyl chloride [VC]) via partial dechlorination of PCE/TCE or to harmless ethene by complete detoxification (He et al., 2003b; Griffin et al., 2004; He et al., 2005; Sung et al., 2006a). Many mixed cultures and pure isolates have been reported to produce *cis*-DCE or VC during PCE/TCE dechlorination processes (Smidt and de Vos, 2004; He et al., 2005; Yoshida et al., 2007). However, *trans*-DCE has been detected in more than one-third of the U.S. Environmental Protection Agency (EPA) superfund sites (Agency for Toxic Substances and Disease Registry in 2007). The source of *trans*-DCE production was thought to be an abiotic process, although recently both *trans*-DCE generation and *cis*- DCE generation was reported to occur via microbial dechlorination.

To date, microbes from either *Dehalococcoides*- or DF-1-containing mixed cultures have been reported to produce more *trans*- than *cis*-DCE with a ratio of 1.2:1 to 3.5:1 in the laboratory-scale studies (Griffin et al., 2004; Miller et al., 2005; Futamata et al., 2007; Kittelmann and Friedrich, 2008b). For example, in a recent

report by Kittelmann and Friedrich, *trans*/*cis*-DCE at a ratio of 3.5:1 was generated in tidal flat sediments-containing microcosms with microbes closely related to *Dehalococcoides* sp. or DF-1-like microbes (Kittelmann and Friedrich, 2008b). Additionally, Griffin et al. (2004) identified *Dehalococcoides* species of the Pinellas subgroup in several enrichment cultures, which dechlorinated TCE (~ 0.25 mM) to *trans*- DCE and *cis*- DCE at a ratio of ~ 3:1 (Griffin et al., 2004). There is no information available on the *Dehalococcoides* isolates that generate *trans*-DCE as the main end product. This also means a lack of information on the genomic contents of *trans*-DCE-producing bacteria. Therefore, finding microorganisms that produce *trans*-DCE in pure culture will be useful for the comprehensive characterization of this group of bacteria.

The aim of this study was to isolate a PCE-to- *trans*-DCE-dechlorinating culture to facilitate the elucidation of *trans*-DCE formation during reductive dechlorination processes. Microarray analysis was conducted to compare the whole-genome contents of the new isolate and the well-characterized *Dehalococcoides ethenogenes strain* 195 (Maymó-Gatell et al., 1997). Expression analysis of the multiple RDase genes of strain MB would help to elucidate how the microbes catalyze dechlorination of PCE/TCE to the predominant *trans*-DCE and thus provide an enzymatic basis of *trans*-DCE production in contaminated sites. In addition, a coculture which consisted of the new isolate and a TCE-to-*cis*-DCE-to-VC-dechlorinating *Dehalococcoides* sp. strain ANAS1 was explored to study the interaction, distribution, and function of the dechlorinators in the dechlorinating process.

4.2 Materials and methods

4.2.1 Chemicals

Unless stated otherwise, chemicals were purchased from Sigma-Aldrich at the highest purity available. The DNA extraction kits were obtained from QIAGEN (Germany), and the GoldTaq DNA polymerase and related PCR reagents were from Applied Biosystems (Foster City, CA). The TOPO-TA cloning kit (Cat. no. K450001) and staining reagents were purchased from Invitrogen (Carlsbad, CA).

4.2.2 Isolation and growth conditions

A *trans*-DCE producing enrichment culture MB that originated from the sediment in San Francisco Bay Area (California) was transferred continuously in 20-ml glass vials containing 10 ml of mineral salt medium. Dilution-to-extinction series and agar shakes were performed to enrich culture MB in acetate (5mM)-H_2 (500, 000 ppm) - amended mineral salt medium spiked with PCE (in a nominal concentration of 2 mM) (He et al., 2003b). During the isolation process, an active culture inoculated from a single colony was subsequently subjected to two repeated dilution-to-extinction series with 100 μg/ml of ampicillin, followed by another seven consecutive transfers amended with neat PCE in liquid medium without any antibiotic.

After obtaining the new *Dehalococcoides* species in pure culture, all subsequent time course experiments were performed in triplicate with 160-ml serum bottles filled with 100 ml growth medium as stated above and with 1-2% inocula. The following halogenated compounds were also tested on the new isolate as electron acceptors: chlorinated ethenes (DCE isomers, VC); 1,1-dichloroethane; 1,2-dichloroethane; chloroform; carbon tetrachloride; PCBs (Aroclor 1260 and CB-155); 2,4,6-

trichlorophenol; pentachlorophenol; and PBDEs (octa-BDE mixture, deca-BDE mixture, and penta-BDE mixture). If the compound was in powder form, it was dissolved in either TCE or the inert solvent nonane before being injected into medium bottles to a final concentration of 0.1 - 0.2 mM. Compounds in liquid or gaseous forms were added to the medium directly to a final concentration of about 0.2 mM. In addition to the above-mentioned halogenated compounds, the following substrates were also tested on this isolate: succinate (10 mM), glucose (10 mM), lactate (5 mM), pyruvate (5 to 10 mM), fumarate (5 to 10 mM), malate (10 mM), glutamate (10 mM), sulfate (5 to 10 mM), sulfite (0.5 to 5 mM), nitrate (5 to 10 mM), and nitrite (1 to 10 mM). The bottles were incubated statically under strict anaerobic conditions in the dark at 30ºC.

By use of similar isolation approaches, another *Dehalococcoides* sp. strain ANAS1 was isolated from a mixed culture ANAS and was characterized for its capability to utilize the above-mentioned compounds (Holmes et al., 2006; West et al., 2008).

4.2.3 Atomic force microscope and sample preparation

A high resolution atomic force microscope (AFM) was chosen to observe the microbes in three dimensions. Bacterial cells (1 ml) were harvested at the mid-exponential phase (~$1x10^8$ cells ml^{-1}) by centrifugation at 8, 000×*g* for 5 min at 4 ºC. The cell pellets were immersed in a phosphate buffer solution (pH 7.2) containing 2.5% glutaldehyde and 2% paraformadehyde for 20 mins and were then rinsed with sterile distilled water twice. The cells were further concentrated to a final volume of 30 μL by centrifugation at 8, 000×*g* for 5 min and a duplicate sample was examined with an upright epifluorescene microscope (CLSM, model LSM Pascal, Carl Zeiss) at a

magnification of ×1,000 to ensure right range of cell density. Prior to AFM imaging, the cell suspension was manually transferred to a clean 5- by 5- by 1-mm silicon wafer with a smooth surface, which had been immersed in 5 M H_2SO_4 and 10% H_2O_2 for 1 h to remove any possible impurity and rinsed with distilled water. The specimen was placed in a sterile petri dish and then dried at 30 ºC for 6 hours in an incubator. The petri dish was sealed with parafilm during the transportation before AFM examination.

Imaging was performed with a Nanoscope III Dimension 3100 AFM (Digital Instruments, Santa Barbara, CA) as described previously (Li et al., 2007). Standard silicon AFM cantilevers with a spring constant of 0.15 N/m and a nominal tip radius of ~ 20 nm were used to image the cells. The samples were imaged in air by use of a contact mode with settings of 512 pixels per line and 1-Hz scan rate. The deflection images were first order flattened and the contrast was enhanced by use of software Nanoscope 5.13 for better resolution.

4.2.4 Analytical methods

Headspace samples of chloroethenes, chloroethanes, and ethene were injected manually with a glass gas-tight, luerlock syringe (Hamilton Co., Nevada) into an Agilent gas chromatography (GC) 6890N equipped with a flame ionization detector and a GS-GasPro column (30m×0.32 mm, J&W Sci, U.S.A), as described previously (Lee et al., 2006), whereas PCBs, chlorophenols, and PBDEs were extracted with isooctane and tested on the same GC but coupled with an electron capture detector (ECD) and an HP-5 column (30 m × 0.32 mm × 0.25 μm). For the GC-ECD test program, the oven temperature was initially held at 170 ^{o}C for 5 min, increased at 5 ^{o}C

min^{-1} to 260 ^{o}C, and held for 5 min. Helium was used as the carrier gas with a column flow of 1.2 ml min^{-1}.

4.2.5 DNA extraction, PCR and sequencing

Total genomic DNA was extracted from 1 ml of cell pellets of the PCE- or TCE-dechlorinating cultures according to the method described previously (Löffler et al., 1997b). The concentration of the nucleic acid was determined by Nanodrop-1000 instrument (NanoDrop Technologies Inc.). Nearly complete 16S rRNA gene sequences were amplified by targeting the genomic DNA with a universal bacterial primer pair (8F and 1541R) while PCR (Eppendorf, Hamburg, Germany) amplification was carried out under conditions described previously (Löffler et al., 2000). A clone library of the 16S rRNA genes was established with the amplified PCR products. A fragment of approximately 1,489 bp of the 16S rRNA gene was cloned, sequenced and aligned as previously described (He et al., 2003a).

4.2.6 Putative RDase gene identification

Genomic DNA of culture MB was used as a template for the amplification of putative RDase genes with degenerate primer pair RRF2 and B1R (Krajmalnik-Brown et al., 2004). Subsequently, construction of clone library, screening of major clone types, and genome walking were conducted as described previously (Krajmalnik-Brown et al., 2004). In brief, 40 clones were selected from the DNA clone library, which showed one major RDase gene clone type, named putative RDase gene *dceA1*. As shown in Table 4.1, a pair of gene specific primers (*dceA1*F and *dceA1*R) targeting the *dceA1* gene was designed, and specificity was confirmed by targeting the genomic

DNA of the *trans*-DCE producing culture MB and the non-*trans*-DCE producing cultures such as *Dehalococcoides* species strains ANAS1 and 195.

Table 4.1 Taqman primers and probes used to quantify cell numbers in this study.

Target gene	Primer/ probe	Sequence	Reference or source
Dehalococcoides 16S rRNA genes	DhcF	5'-GGT AAT ACG TAG GAA GCA AGC G	Holmes et al., 2006
	DhcR	5'-CCG GTT AAG CCG GGA AAT T	
	DhcProbe	5'-VIC-ACA TCC AAC TTG AAA GAC CAC CTA CGC TCA CT-TAMRA	
Bacteria 16S rRNA genes	BacF1	5'-TCC TAC GGG AGG CAG CAG	Holmes et al., 2006
	BacR1	5'-GGA CTA CCA GGG TAT CTA ATC CTG TT	
	BacR2	5'-GGA CTA CCA GAG TAT CTA ATT CTG TT	
	BacProbe	5'-FAM-CGT ATT ACC GCG GCT GCT GGC AC -TAMRA	
tceA gene	tceAF tceAR tceAProbe	5'-ATC CAG ATT ATG ACC CTG GTG AA 5'-GCG GCA TAT ATT AGG GCA TCT T 5'-FAM-TGG GCT ATG GCG ACC GCA GG- TAMRA -3'	Holmes et al., 2006
dceA1 gene	dce*A1*F	5'-GGG TAC ACC CGA AGA AAA TCT G	This study
	dce*A1*R	5'-AGC CGC CAA AAA ACC TGA T	
	dce*A1Probe*	5'-FAM-AAA CCT GCC GTA CTG C-MGB	

Note: MGB, minor groove binder.

4.2.7 Quantitative real-time PCR (qPCR)

qPCR (ABI 7500 Fast Real-Time PCR system, Foster, CA) assay was performed in triplicate for cultures grown with PCE or TCE by using *Bacteria* and *Dehalococcoides* 16S rRNA gene (Holmes et al., 2006), and *dceA1* gene-targeted primers/probes, respectively. The primer and probe sequences used in this study are

shown in Table 4.1. A calibration curve was obtained by using 10-fold serial dilutions of plasmid DNA with cloned *Bacteria* 16S rRNA gene and *dceA1* gene inserts. The standard curves spanned a range of 10^2 to 10^8 gene copies per µl of template DNA.

Amplification of the *Bacteria* 16S rRNA gene, the *Dehalococcoides* 16S rRNA gene, and the *dceA1* gene was done at an annealing temperature of 60 ºC for all assays. The 9600 Emulation PCR cycle parameters were as follows: 2 min at 50 ºC and 10 min at 95 ºC, followed by 40 cycles of 15 sec at 95 ºC and 1 min at 60 ºC. This study utilized sterile, nuclease-free water or plasmid pCR 2.1 DNA without an insert as the negative control for the RDase gene-targeted primer and probe set.

For the cocultures, multiplex qPCR was run with *Dehalococcoides* 16S rRNA gene- and *tceA* gene- targeted primer and probe sets (VIC-TAMRA probe for *Dehalococcoides* and FAM-TAMRA probe for *tceA* [TAMRA is 6-carboxytetrramethylrhodamine and FAM is 6-carboxyfluorescein]). The *tceA* gene copies represent the cell number of common TCE-to-VC dechlorinators for isolate ANAS1 in this study. The cell numbers of the new isolate were calculated by subtracting the number of *tceA* gene copies from the number of total *Dehalococcoides* gene copies, which were double checked by quantifying the number of putative *dceA1* gene copies identified from this new isolate.

4.2.8 Microarray analysis

The genomic DNA of the new isolate was extracted from actively TCE-to-*trans*-DCE dechlorinating cultures and treated with RNase A immediately after cell lysis. Microarray analysis was conducted in triplicate with 1 µg of genomic DNA per array, according to a protocol described previously (Johnson et al., 2008; West et al.,

2008). The statistical analysis of the genomic microarray in this study followed the method that was described previously for genomic analysis of mixed and pure *Dehalococcoides* cultures (Johnson et al., 2008; West et al., 2008). In brief, the Affymetrix GeneChip software and the MAS5 algorithm were used to calculate the hybridization signal intensities from each chip's scan (Affymetrix, 2001; Liu et al., 2002). Prior to comparisons between microarray chips, the data were normalized by scaling the signal intensities of the added positive controls to a target signal intensity of 2,500 (West et al., 2008). A gene was considered "present" in a sample if each triplicate probe set showed signal intensity greater than the highest signal intensity of 129 measured for the negative controls and a P value of less than 0.05 (Liu et al., 2002).

4.2.9 Nucleotide sequence accession number

GenBank accession number EU073964 was assigned to the 16S rRNA gene sequence of the isolate designated *Dehalococcoides* sp. strain MB. The complete sequence of putative RDase gene *dceA1* was also deposited in GenBank under accession number EU625402.

4.2.10 RNA extraction and gene expression study

When pure culture MB was obtained, transcriptional analysis was conducted to find out the functional RDase gene(s) among the seven identified genes (by degenerate primer pairs 1 and 2 in Table 4.2) in strain MB on exposure to PCE (Chow et al., 2010). After starving the MB cells for 72 hrs, 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 4.2).

Firstly, total RNA was extracted from the cell pellet of 1.5 ml pure culture MB by using the RNeasy extraction kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions, except that the tubes holding the cells received 100 µl of 0.1mm 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, Hilden, Germany). First, the corresponding cDNAs were synthesized by incubating 20-50 ng of extracted RNA in a 20 µl reaction mixture at 37ºC for 3 hrs.

Table 4.2 Specific primer sequences designed for culture MB RDase genes

Primer Pair	Primers	5'-3' sequence	Gene targeted	Tm	Size (bp)
1	RR2F*	SHMGBMGWGATTTYATGAARR	RDase genes	48°C	1500
	B1R*	CHADHAGCCAYTCRTACCA			
2	RDH F1C	TTYMVIGAYITIGAYGA	RDase genes	47°C	1200
	RDH R1C	CCIRMRTYIRYIGG			
3	*dceA1*F	CCGTACTGCCATCAGGTTTT	*dceA1*	48°C	556
	*dceA1*R	AAGCCCAAAGGGACAAGAAT			
4	*dceA2*F	AAGAACCCGTGACAACCAAG	*dceA2*	60°C	520
	dceA2R	GCAGTTTCCACCCACAAGTT			
5	*dceA3F*	CTGGTCATCCCCAATGTACC	*dceA3*	60°C	528
	dceA3R	GCACAGGGGCAGATTGTTAT			
6	*dceA4F*	TCTCTCCGGTTTTCCATGAC	*dceA4*	60°C	545
	dceA4R	ATCCTGGGGGTAGAGCATTT			
7	*dceA5F*	TCCGGACAGCTAATGAATCC	*dceA5*	60°C	590
	dceA5R	TGAATTAAGTGCGGGGGTAG			
8	*dceA6F (mbrAF)*	CCTGTAAACGACTCCCCAGA	*dceA6 (mbrA)*	60°C	427
	dceA6R (mbrAR)	GGATTGGATTAGCCAGCGTA			
9	*dceA7F*	GGATATCATGGTCCCACCAG	*dceA7*	60°C	148
	dceA7R	TTCAGCACAACCAGAGATGC			

* Primers from Krajmalnik-Brown *et al.*, 2004.

Secondly, 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, Foster City, CA, USA) by using SYBR green assays and their respective primers (primer pairs 3 to 9) (Table 4.2). 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., 2005a) 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 bounds 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, Hilden, Germany), 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 to 59°C depending on the primer annealing temperature (Table 4.2), 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 individual primers were ligated into the pCR2.1 vector (Invitrogen, Carlsbad, CA, USA), and were transformed into *Escherichia coli* TOP10 chemically competent cells (Invitrogen, Carlsbad, CA, USA). The plasmids were obtained by using the QIAprep Spin Miniprep kit (QIAGEN

GmbH, Hilden, Germany). 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).

4.3 Results

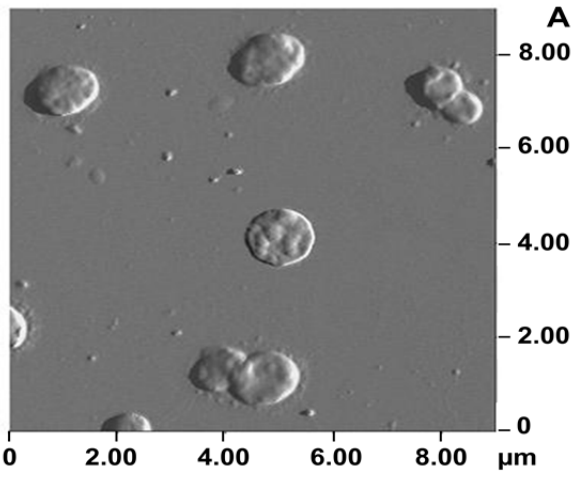
4.3.1 Isolation of *Dehalococcoides* sp. strain MB

A PCE-dechlorinating, mixed-culture MB was initially obtained to be able to generate *trans*- and *cis*- DCEs at a ratio of 1.5:1 in mineral salt medium amended with lactate. *Dehalococcoides* species was detected by species-specific primer pairs and found to be the main PCE-dechlorinator, which usually requires acetate as the carbon source and H_2 as the electron donor. In subsequent serial dilutions, lactate was replaced with acetate (5 mM)-H_2 (500, 000 ppm) in order to enrich the potential *trans*-DCE generating *Dehalococcoides*. In the meantime, ampicillin (50 mg/L) was applied to inhibit the growth of ampicillin-sensitive bacteria while keeping the ampicillin-resistant *Dehalococcoides* species. After 22 transfers, the ratio of *trans*- to *cis*- DCE elevated to 3.7:1, suggesting that there were fewer *cis*-DCE producing microbe(s) in the enrichment. With the dilution-to-extinction strategy, 20 single colonies in agar shakes were picked and then transferred to liquid medium. Three out of the 20 colonies were found to be able to dechlorinate PCE and TCE in the presence of ampicillin. The one that showed the highest ratio of *trans*- to *cis*- DCE (5.3:1) was subjected to the same isolation approach as described above. Eventually, a pure culture, which was capable of generating *trans*- to *cis*- DCE in a ratio of 7.3 (± 0.4) : 1 and dechlorinating PCE twice as fast as the initial microcosms, was obtained. A clone library established with the 16S rRNA genes of culture MB yielded identical

restriction patterns for the 72 clones after digesting with three enzymes, *Hha*I, *Rsa*I and *Msp*I, indicating that the culture became pure. The 16S rRNA genes amplified from genomic DNA of culture MB exhibited a sequence identical to that of *D. ethenogenes* strain 195. The experimental restriction patterns were exactly the same as the predicted restriction patterns based on the 16S rRNA gene sequence. The new isolate was then designated *Dehalococcoides* sp. strain MB.

4.3.2 Morphological characteristics of strain MB

Light microscopy demonstrated that strain MB is a disc-shaped, nonmotile bacterium. Under the AFM, the cells appeared as single or in pairs (Fig 4.1a). The images of a single cell repeatedly show a diameter of 1.0 μm, a maximum height of 158 nm, and a surface area of 1.9 μm^2 (Fig 4.1). The diameter of strain MB (~ 1.0 μm) is slightly larger than that of the previously reported *Dehalococcoides* species (~ 0.5 - 0.7 μm); while the thickness was less (He et al., 2005). Compared to results obtained with scanning electron microscopy, this discrepancy in cell size may be caused by the tight affinity of cells to the silicon wafer used for AFM imaging. The extra material around the surface of the cell may be the macromolecules from the lysed cells.



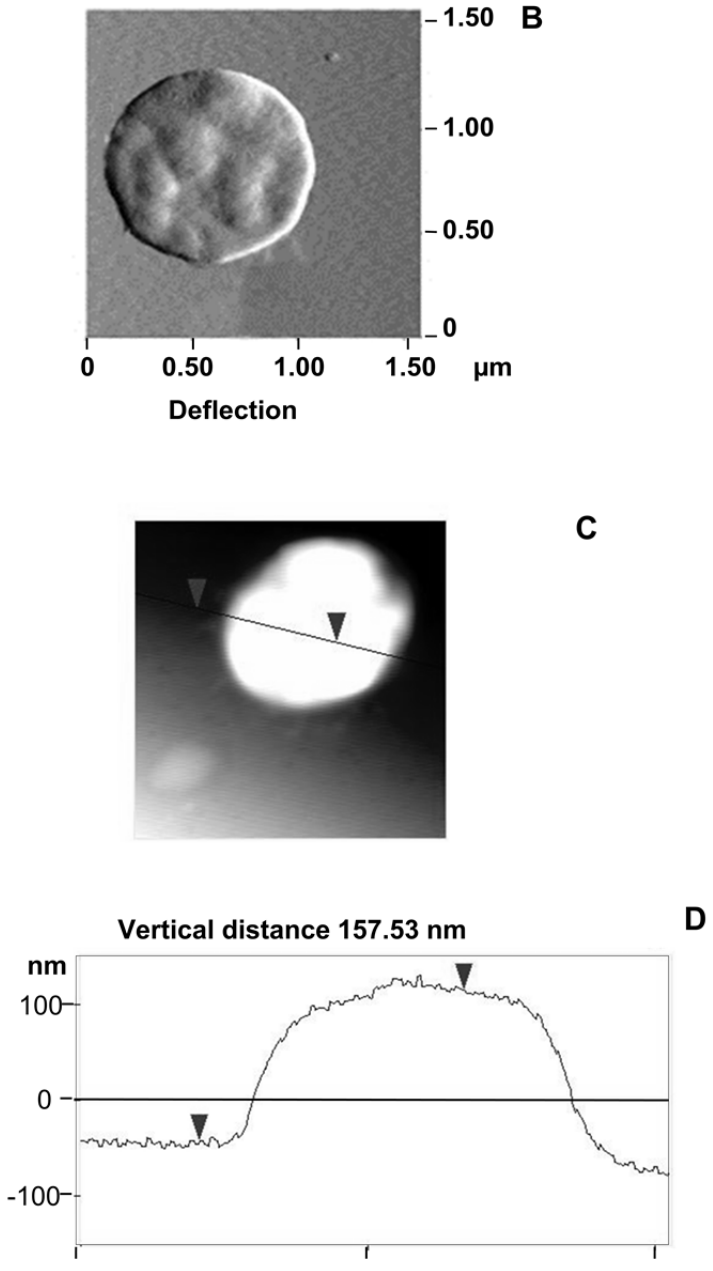


Fig 4.1 AFM examination of *Dehalococcoides* sp. strain MB. (a) Overview of cells; (b) Deflection image of the single cell shown in the middle panel of image (a); (c and d) Section analysis of the cell shown at the top of panel (a).

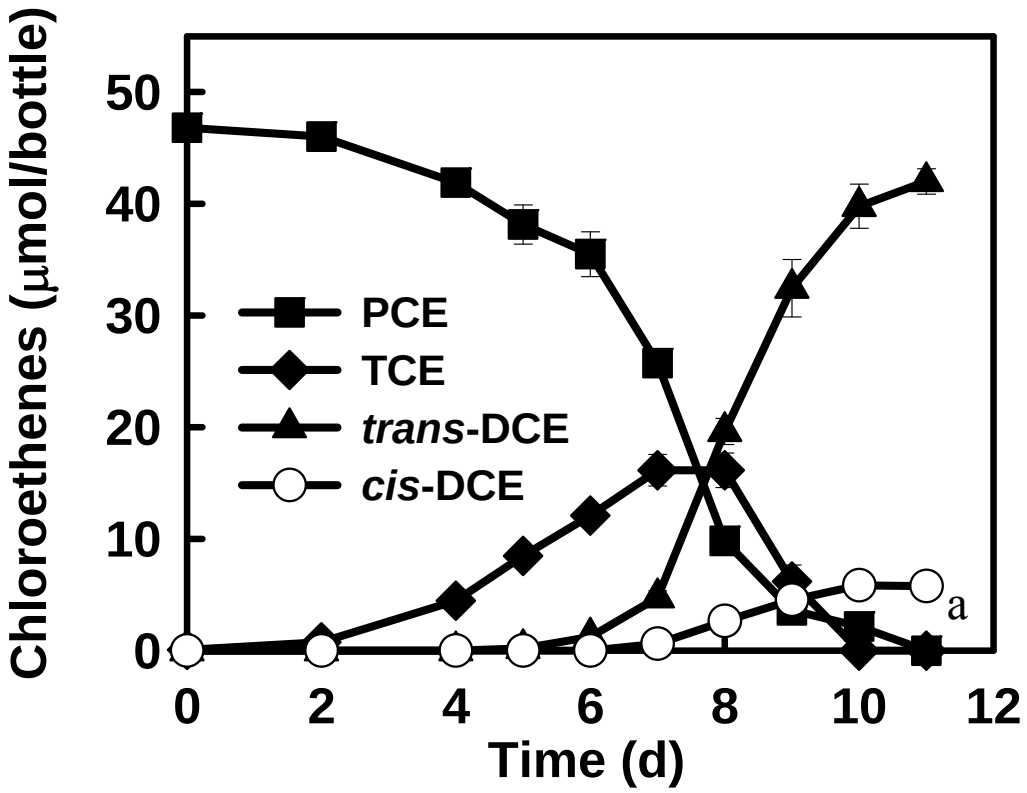
4.3.3 Growth and purity confirmed by qPCR

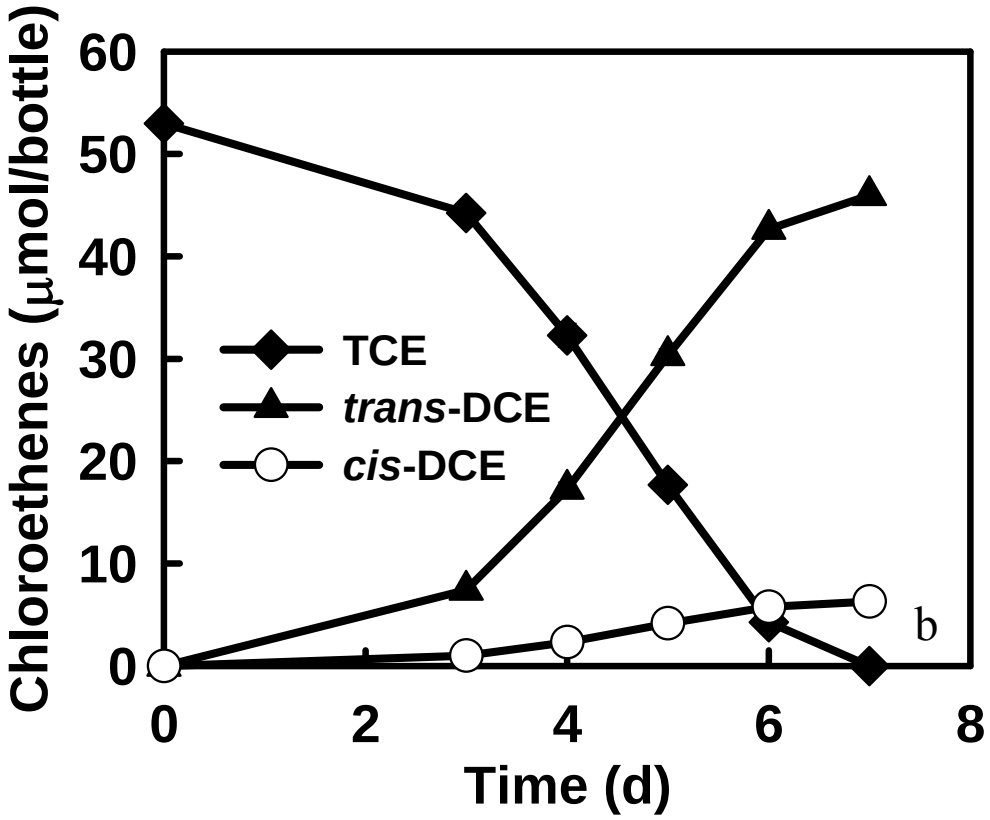
Dehalococcoides sp. strain MB dechlorinated PCE and TCE to predominantly *trans*-DCE with average rates of 44.5 and 78.6 μmol liter^{-1} day^{-1}, respectively (Fig 4. 2a and b). After consuming ~ 50 μmol of PCE (0.5 μmol mL^{-1} of nominal concentration), culture MB reached a density of (10.0±0.16)×10^7 cells mL^{-1} (reflected by the *Dehalococcoides* 16S rRNA gene copies, since one cell contains one 16S rRNA gene copy on the genomic DNA), whereas it increased to (3.09±0.07)×10^7 cells mL^{-1} of 16S rRNA gene when it dechlorinated ~55 μmol of TCE (0.55 μmol mL^{-1} of nominal concentration) (Fig 4.2c and d). The qPCR results showed that MB could capture energy for growth from both PCE-to-TCE and TCE-to-DCE dechlorination steps, suggesting that isolate MB was capable of dechlorinating both PCE and TCE metabolically. There was no obvious growth inhibition on isolate MB when the concentration of PCE increased up to 200 μmol per bottle. No growth was found for isolate MB in the absence of PCE or TCE, and no dechlorination products were detected during abiotic incubation with PCE or TCE within the experimental period.

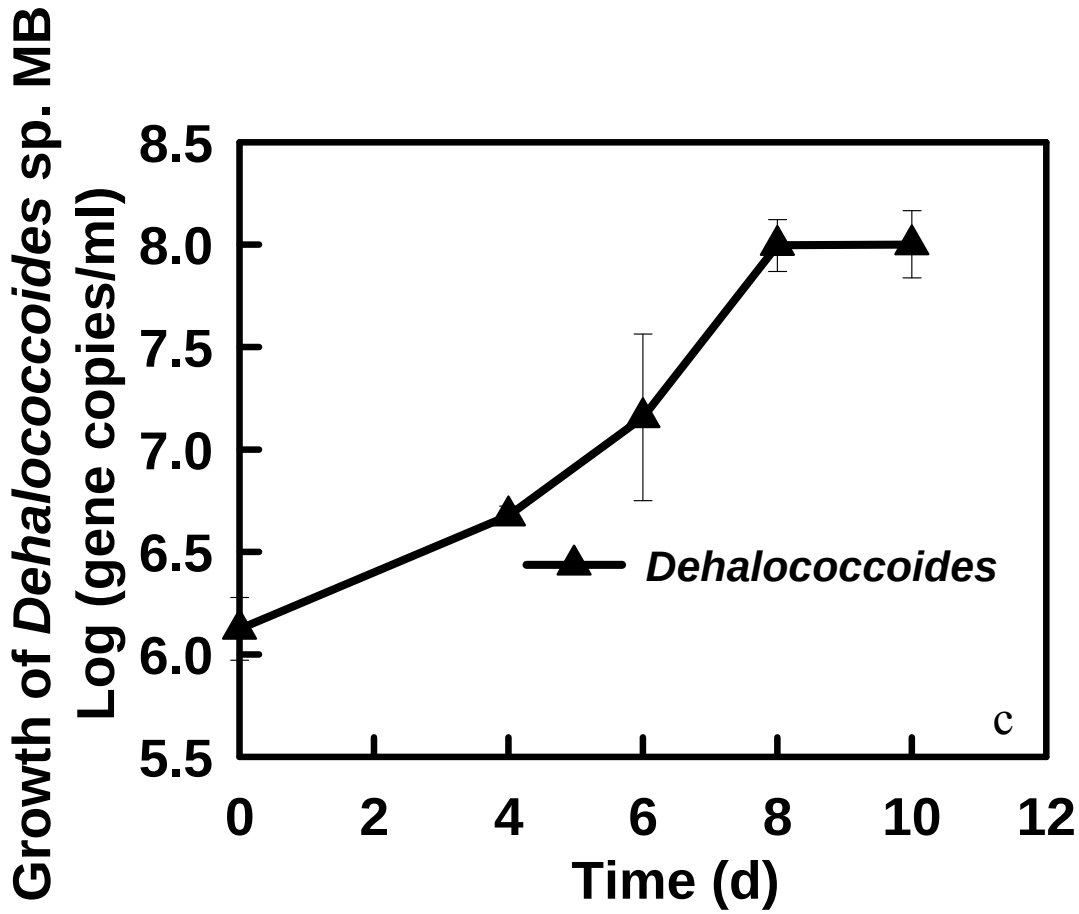
Under optimal anaerobic conditions (pH 7.2, temperature 30 ± 2ºC), strain MB showed a doubling time of ~ 24 hours, which coincides with the growth rates of other known *Dehalococcoides* isolates (He et al., 2007a). Table 4.3 shows a comparison of the growth rates of current known *Dehalococcoides* species. Strain MB yielded 0.12 mg of protein per mol of chloride released, which was comparable to the rates for other *Dehalococcoides* sp. isolates (e.g. strain 195, FL2, and BAV1) but was much lower than the rates for the highly enriched culture VS and mixture culture KB1.

It is noteworthy that isolate MB consistently produced 6.3 μmol of *cis*-DCE after dechlorinating ~ 50 μmol of PCE or TCE. To further corroborate culture MB's

purity, qPCR assays were performed by using three sets of primer and probe pairs to target the genomic DNA of culture MB (Table 4.1). One of the primer and probe sets was designed to target the *dceA1* gene identified in culture MB. The translated amino acid of *dceA1* gene sequence exhibited a 97%, 93%, 64% and 58% identities when compared with the putative RDases of *Dehalococcoides* sp. strains VS, CBDB1, 195, and BAV1, respectively. The specificity of the primer and probe set targeting *dceA1* gene was verified by obtaining a positive response with the *trans*-DCE-producing culture MB but negative responses on the non-*trans*-DCE-producing cultures (such as strains ANAS1 and 195), indicating the uniqueness of the *dceA1* gene primer and probe set. As observed with the qPCR results (Fig 4.2d), the increase in total bacterial cell numbers (measured as *Bacteria* 16S rRNA gene copies) appeared to be the same as that for the *Dehalococcoides* cells (measured as *Dehalococcoides* 16S rRNA genes), suggesting that the community of culture MB consisted of only one genus, *Dehalococcoides*. Additionally, the increase in *dceA1* gene copies identified for culture MB was found to be identical to the increase in *Dehalococcoides* cells, indicating that culture MB consisted of a single strain of the *Dehalococcoides* species. In contrast, the enrichment culture MB (with a *trans*-/*cis*- DCE ratio of 3.7 :1) contained ~ 35% of *Dehalococcoides* sp. strain MB (equal copies of *Dehalococcoides* and *dceA1* gene), indicating that the presence of other genera of dechlorinator(s) may have contributed to the production of *cis*-DCE. In all, we can conclude that a pure culture of *Dehalococcoides* sp. strain MB was obtained to dechlorinate PCE/TCE to predominantly *trans*-DCE.


Chloroethenes (μmol/bottle)
50
40
30
20
10
0
PCE
TCE
trans-DCE
cis-DCE
a
0
2
4
6
8
10
12
Time (d)


Chloroethenes (μmol/bottle)
60
50
40
30
20
10
0
TCE
trans-DCE
cis-DCE
b
0
2
4
6
8
Time (d)


Growth of Dehalococcoides sp. MB
Log (gene copies/ml)
8.5
8.0
7.5
7.0
6.5
6.0
5.5
0
2
4
6
8
10
12
Time (d)
Dehalococcoides
c

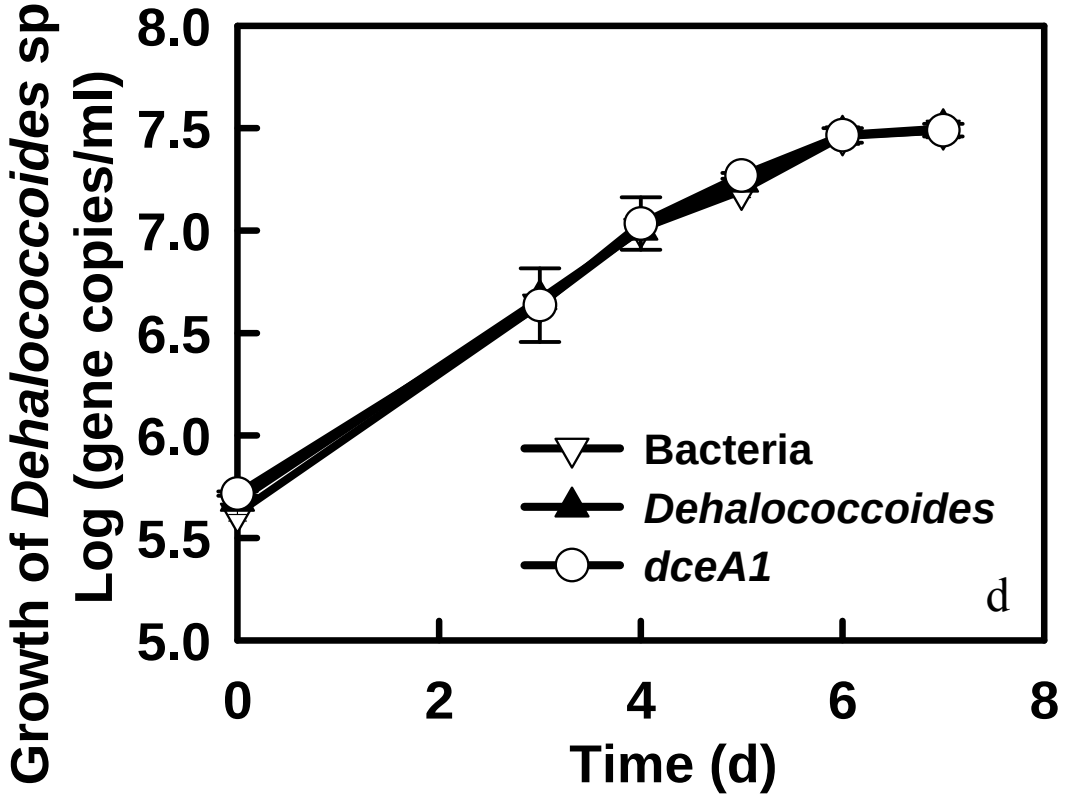

Growth of Dehalococcoides sp. MB
Log (gene copies/ml)
8.0
7.5
7.0
6.5
6.0
5.5
5.0
0
2
4
6
8
Time (d)
Bacteria
Dehalococcoides
dceA1
d

Fig 4.2 Reductive dechlorination of PCE (a) and TCE (b) to *trans*-DCE by *Dehalococcoides* sp. strain MB, and the increase in different gene copies as quantified by qPCR during reductive dechlorination of PCE (c) and TCE (d). Error bars indicate standard deviations. d, days.

Table 4.3 Comparison of the growth rates of known *Dehalococcoides* species

Strain (substrate)	Yield		References
	16S rRNA gene copies/μmol Cl^-	g (dry wt)/ mol of Cl^-	
VS*	$(5.2\pm1.5)\times10^8$	2.2±0.6	Cupples et al. 2003
BAV1	$(1.67\pm0.03)\times10^8$	0.35±0.01	He et al. 2007
KB-1/VC*	$(5.6\pm1.4)\times10^8$	2.4±0.6	Duhamel et al. 2004
GT (TCE)	$(3.1\pm0.2)\times10^8$	0.65±0.05	Sung et al. 2006
FL2 (TCE)	$(7.8\pm0.9)\times10^7$	0.16±0.02	He et al. 2005
CBDB1 (hexachlorobenzene)	-	2.1±0.24	Jayachandran et al. 2003
195 (TCE)	$(1.18\pm0.02)\times10^8$	0.25±0.003	He et al. 2007
MB (TCE)	$(5.62\pm0.13)\times10^7$	0.12±0.01	This study
ANAS1 (TCE)	$(3.18\pm0.04)\times10^8$	0.67±0.03	This study

* mixed culture

4.3.4 Metabolism of *Dehalococcoides* sp. strain MB

Among the potential electron acceptors tested, only PCE and TCE supported the growth of MB within the experimental period. They could not be replaced by DCE isomers; VC; 1,1-dichloroethane; 1,2-dichloroethane; chloroform; carbon tetrachloride; PCBs (Aroclor 1260 and CB-155); 2,4,6-trichlorophenol; pentachlorophenol; PBDEs dissolved in solvent nonane; sulfate; sulfite; nitrate; and nitrite. Similarly to strain 195, strain MB could cometabolize only octa-BDEs (0.2 mM) to penta- and tetra-BDE congeners in the presence of TCE (0.5 mM) (He et al., 2006). Isolate MB could not use fumarate, malate, lactate, pyruvate, glucose, succinate, or glutamate as the carbon source for anaerobic growth. No growth occurred if this culture was transferred to anaerobic medium fed with PCE or TCE but without H_2. Therefore, isolate MB depends strictly on energy from the reductive

dechlorination of PCE/TCE by coupling to electron transport phosphorylation, with acetate as the carbon source, H_2 as the electron donor.

4.3.5 Microarray analysis on genomic DNA of strain MB

Dehalococcoides sp. strain MB shared 100% 16S rRNA gene sequence identity with strain 195. Therefore, a microarray analysis on genomic DNA of strain MB was performed on microarray chips with probes designed to cover >99% of the predicted protein-coding sequences of the strain 195 genome (Seshadri et al., 2005; West et al., 2008).

Of the 1,579 probe sets on the microarray, the genomic DNA of isolate MB hybridized to 1,389 (88.0%), suggesting that genes are highly conserved between strains 195 and MB. Among the 19 RDase genes of strain 195, 10 genes were present in strain MB while 9 genes were designated absent due to the signal intensity criterion (Fig 4.3).

It is not surprising that MB's genomic DNA did not hybridize to probes targeting the *pce*A gene (DET0318) or the *tce*A gene (DET0079), which functions for PCE and TCE dechlorination to *cis*-DCE and VC. In the absence of these two RDase genes (confirmed further by gene-specific primers with PCR), isolate MB has consistently been maintained to dechlorinate both PCE and TCE to *trans*-DCE predominantly, indicating that a novel RDase gene(s) is responsible for the dechlorination process in isolate MB. Additionally, strains MB and CBDB1 showed the closest match with the 19 RDase genes of strain 195, while the other strain of the Pinellas subgroup (e.g., BAV1) matched only 3 of the 19 genes (Fig 4.3). It is apparent that both RDase genes of DET0180 and DET1535 were found to be present among multiple strains of *Dehalococcoides*; however, DET1528 was present in

strains 195 and MB only. The microarray data were highly consistent for triplicate arrays containing DNA from a single sample.

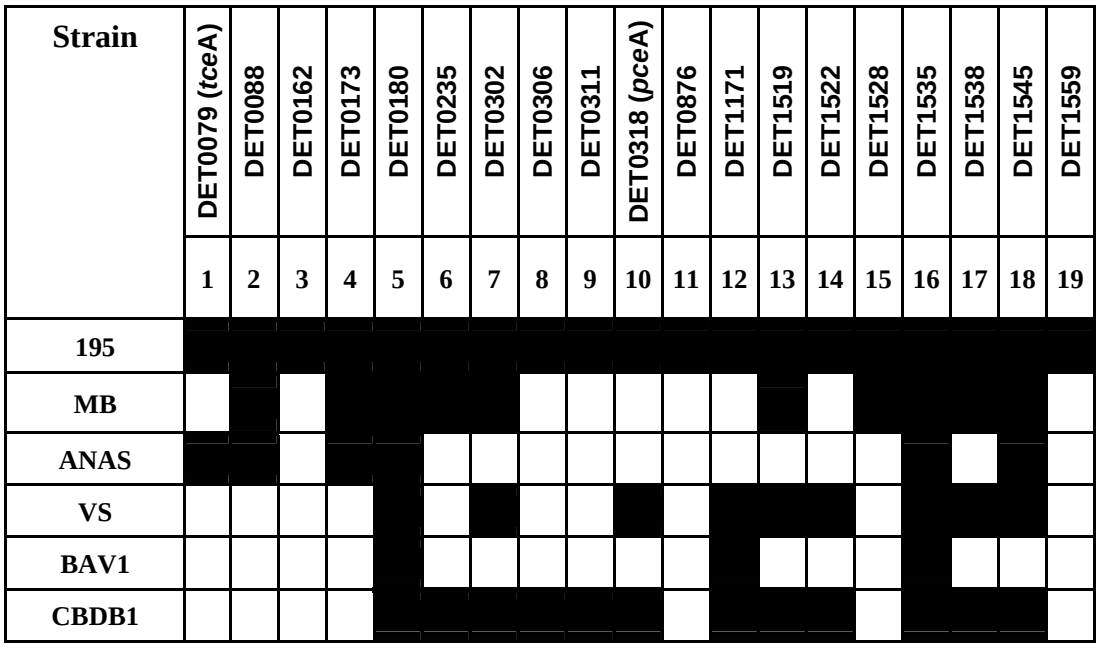


Fig 4.3 Comparison of the RDase genes from *D. ethenogenes* strain 195 with those from different *Dehalococcoides* species. A filled square indicates the presence of the RDase genes, whereas an open square indicates the absence of the gene. The results for ANAS (West et al., 2008) and MB are based on genomic DNA comparison through microarray analysis, while the rest are based on amino acid similarity through BLASTP. The gene is considered present if the relevant amino acid has more than 85% similarity.

Besides the RDase genes, strain MB possesses 26.3% of the ~ 22-kb putative integrated elements (IEs), the mobile genetic elements (MGEs) present in the genome of strain 195 (Regeard et al., 2005; Seshadri et al., 2005) (see Table 4.4). Three entire regions (II, V and the prophage-like region VII) of the IEs were missing from strain MB. The *tceA* gene is located in the first region of IEs of strain 195 but is absent in strain MB, indicating that strain MB may have acquired novel gene(s) to dechlorinate PCE and TCE. Out of the 17 phage-related genes in the whole genome of strain 195, only two (DET0354 and DET0539) were detected in the strain MB genome. As

shown in Table 4.5, these two genes were situated outside of the IEs in the *D. ethenogenes* 195 genome. In addition to the RDase genes and MGEs, strain MB demonstrated a wide-ranging identity with putative housekeeping genes of strain 195. For instance, the microarray results suggested that MB could also have the capability to fix nitrogen by possessing a nitrogenase-encoding operon (*nifHI$_1$I$_2$DKENB*, DET1151 to DET1158) and a distal gene, *nifV* (DET1614), which encodes homocitrate synthetase used in nitrogenase FeMo cofactor biosynthesis. Gene DET1184 encoding a biotin transporter in strain 195, was also found in strain MB, suggesting that these two strains may share similar biotin transport mechanism.

Table 4.4 Detection of integrated elements (IEs) from *Dehalococcoides ethenogenes* strain 195 in isolate MB

IEs	Locus tags	Genes in strain 195	Genes present in strain MB	Presence of genes in MB as a percentage of IE
I	DET0063-0091	29	7	24.1%
II	DET0155-0169	15	0	0.0%
III	DET0251-0272	22	12	54.5%
IV	DET0273-0295	23	13	56.5%
V	DET0875-0883	9	0	0.0%
VI	DET0884-0905	22	12	54.5%
VII	DET1066-1118	53	0	0.0%
VIII	DET1472-1478	7	1	14.3%
IX	DET1552-1561	10	5	50.0%

Note: A gene considered as present has to have a P-value of all triplicates less than 0.05 and an average signal intensity of triplicate higher than 129, which is the highest signal obtained from a negative control. Compared to the 190 genes located in the integrated elements (IEs) of strain 195, about 50 genes (26.3%) were considered to be present in strain MB. Note that there is a triplicate region (III, IV and VI) in the IEs of strain 195.

Table 4.5 Detection of phage-related genes from *D. ethenogenes* strain 195 in *Dehalococcoides* sp. strain MB

No.	IEs	Phage-related genes	Presence[a] of gene in strains		Remark
			195	MB	
1	I	DET0064	+	-	Virulence-related protein
2	I	DET0072	+	-	Phage/plasmid DNA primase
3	II	DET0157	+	-	Site-specific recombinase, phage integrase family
4	III	DET0272	+	-	Site-specific recombinase, phage integrase family
5	IV	DET0295	+	-	Site-specific recombinase, phage integrase family
6	-	DET0323	+	-	Site-specific recombinase, phage integrase family
7	-	DET0354	+	+	Phage domain protein
8	-	DET0539	+	+	dnaD/phage-associated domain protein
9	V	DET0883	+	-	Site-specific recombinase, phage integrase family
10	VI	DET0905	+	-	Site-specific recombinase, phage integrase family
11	VII	DET1067	+	-	Site-specific recombinase, phage integrase family
12	VII	DET1068	+	-	Site-specific recombinase, phage integrase family
13	VII	DET1069	+	-	Site-specific recombinase, phage integrase family
14	VII	DET1089	+	-	Virulence-related protein
15	VII	DET1091	+	-	Virulence-related protein
16	VII	DET1098	+	-	Virulence-associated protein
17	VIII	DET1474	+	-	Site-specific recombinase, phage integrase family

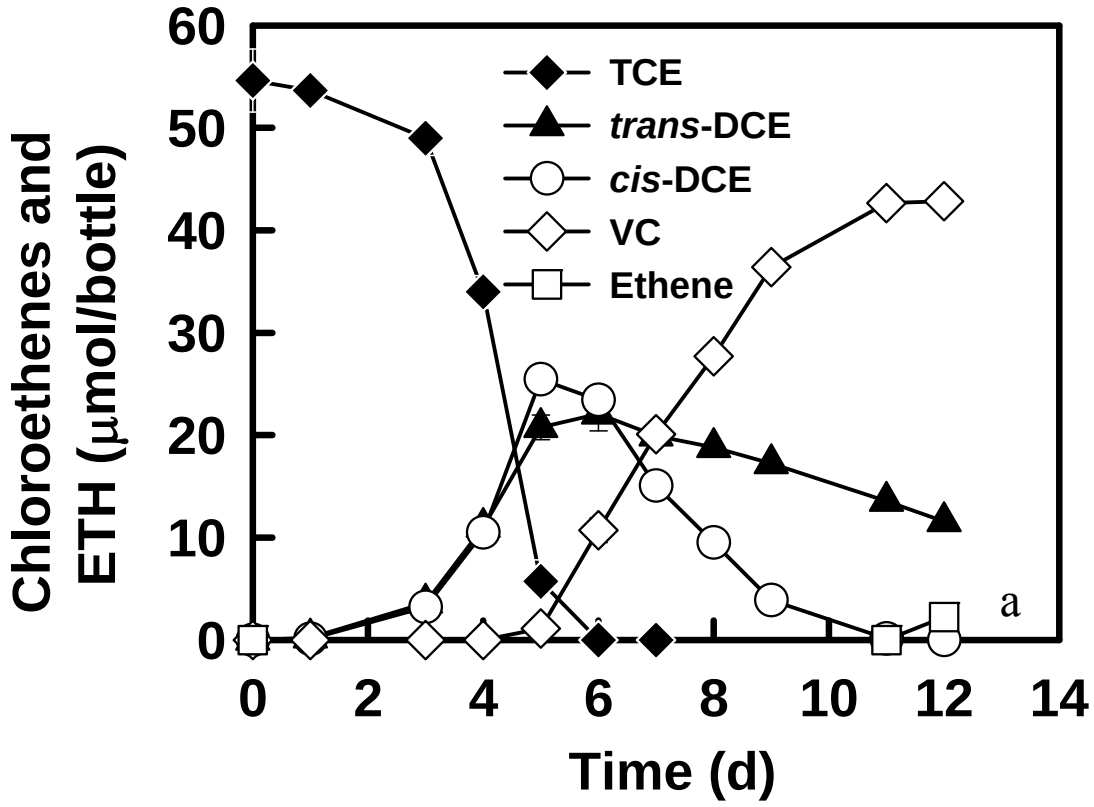
Note: a +, gene was present; -, gene was absent.

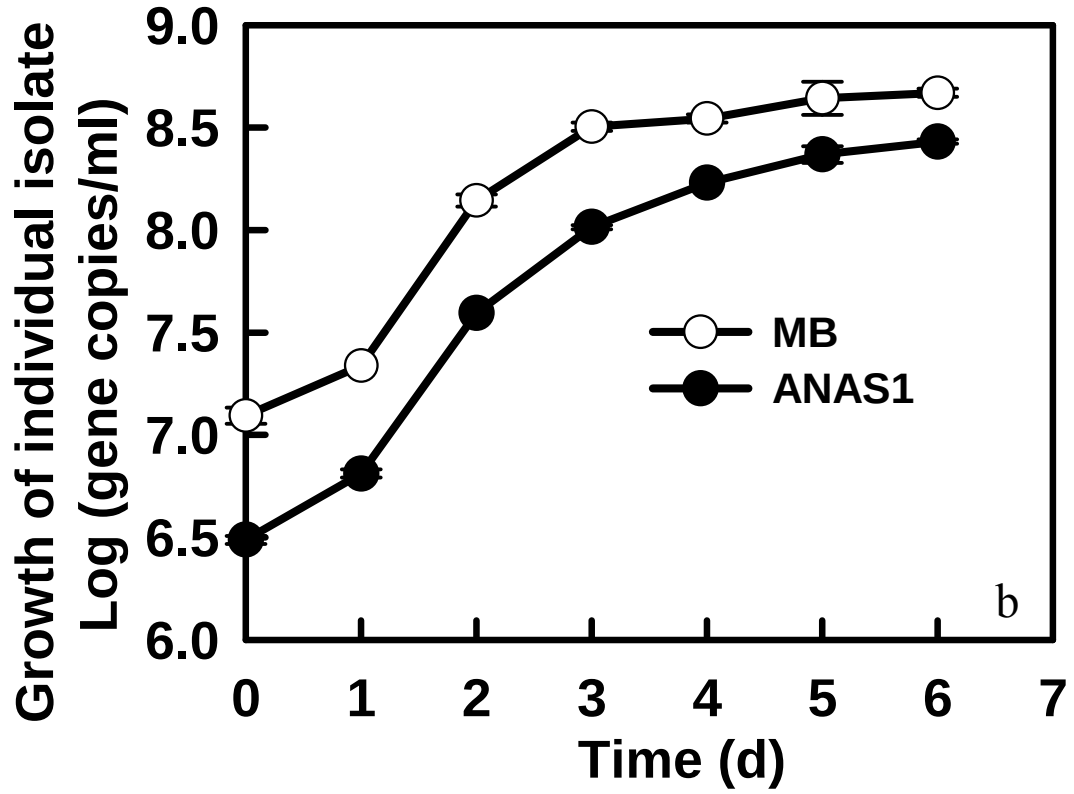
4.3.6 Dechlorination of TCE to *trans*-/*cis*-DCEs by a coculture

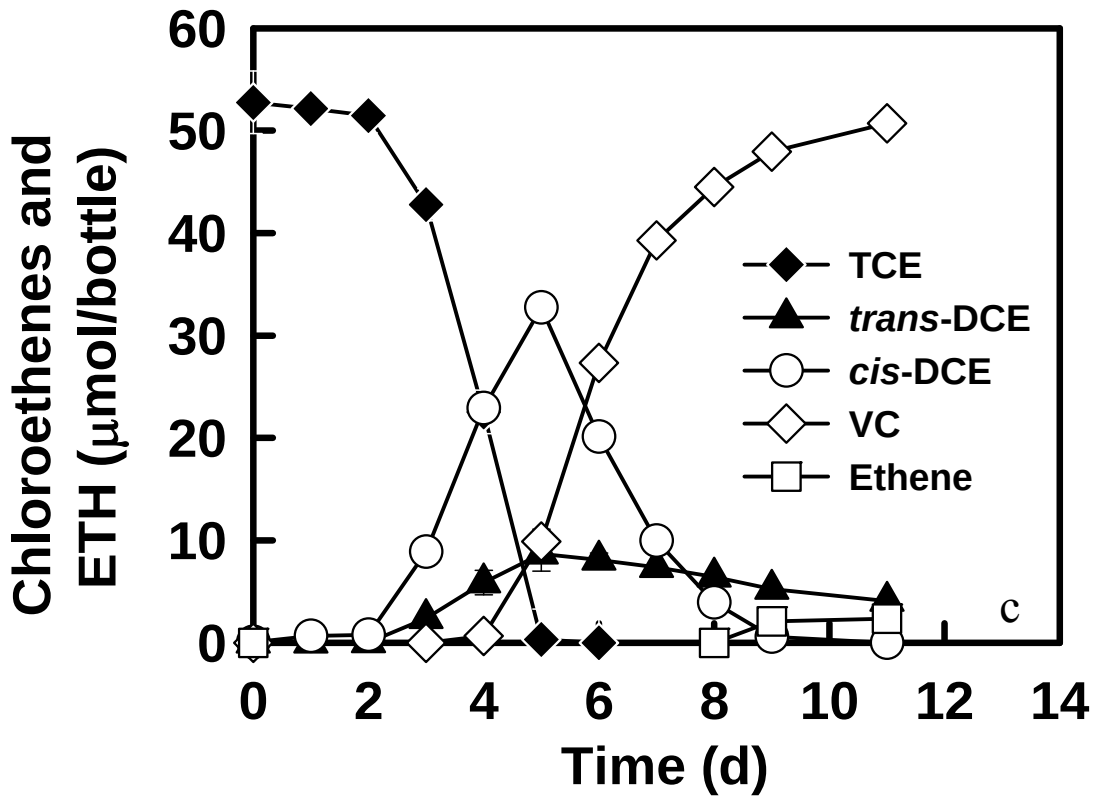
After the first *trans*-DCE producing *Dehalococcoides* sp. strain MB was obtained, it was mixed with a *cis*-DCE producing isolate, *Dehalococcoides* sp. strain ANAS1, to elucidate their interactions in terms of dechlorination products and cell growth, since multiple *Dehalococcoides* species commonly co-exist in chloroethene-

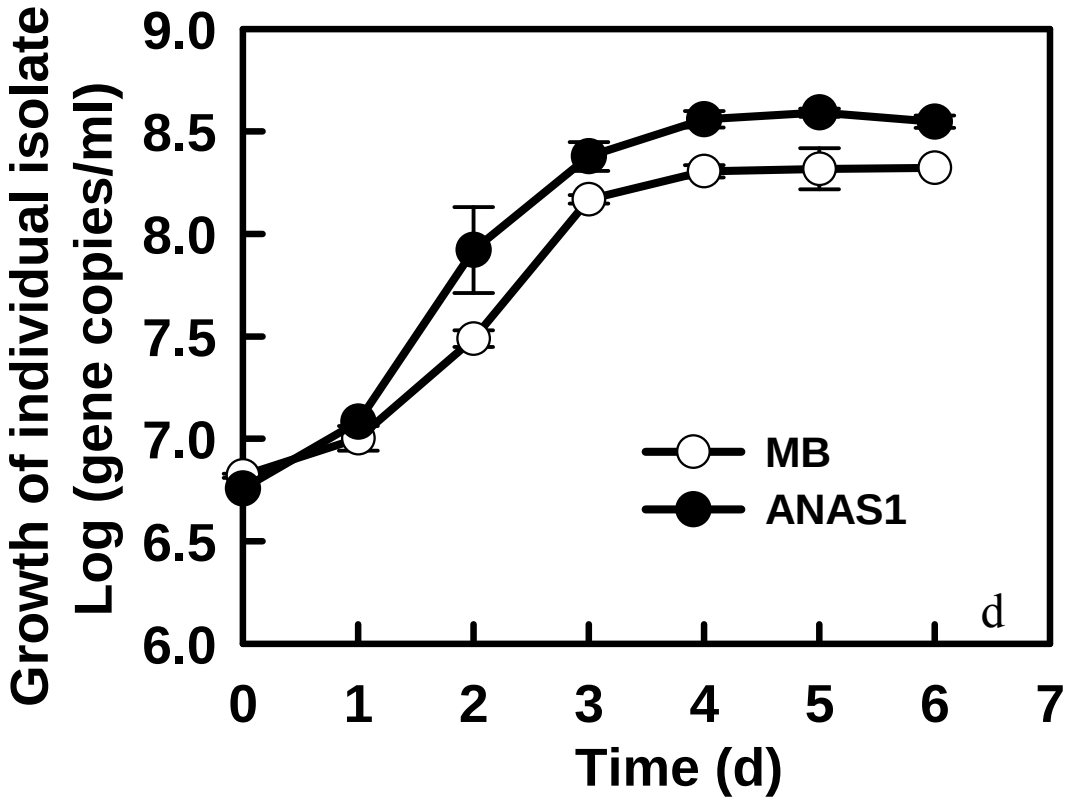
contaminated sites. Similarly to strain MB, isolate ANAS1 utilizes acetate as the carbon source and H_2 as the electron donor, instead of other carbon sources or electron donors, such as pyruvate, lactate, sulfate, or nitrate etc. Among all of the halogenated compounds tested for strain MB, strain ANAS1 was able to dechlorinate TCE, 1,1-DCE, *cis*-DCE to VC but not PCE or *trans*-DCE. The dechlorination of VC by this strain occurred slowly, maybe by a co-metabolic process. Within 7 days, ANAS1 dechlorinated 55 μmols of TCE to VC, accompanied by an increase in cells from 2.6×10^6 to 3.57×10^8 cells ml^{-1}. With different inoculation ratios (MB: ANAS1 at 4:1, 1:1, and 1:4), it is surprising to observe that the formation of *trans*-/*cis*- DCE ratios did not correspond to the same ratios of initial biomass for MB and ANAS1. Although the initial total cell masses were similar among these three sets of experiment, the MB-ANAS1 4:1 coculture produced the largest amount of *trans*-/*cis*-DCE at a ratio of ~ 1:1 (Fig 4.4a), while MB-ANAS1 1:1 coculture showed a 16.7% higher dechlorination rate and a *trans*-/ cis- DCE generation ratio of 1:4 (Fig 4.4c). The dechlorination profile of MB-ANAS1 1:4 coculture was similar to that for the control bottle inoculated with ANAS1 alone, with only negligible amount of *trans*-DCE production (Fig 4.4e), suggesting that culture MB failed to compete with ANAS1 during the dechlorination of TCE.

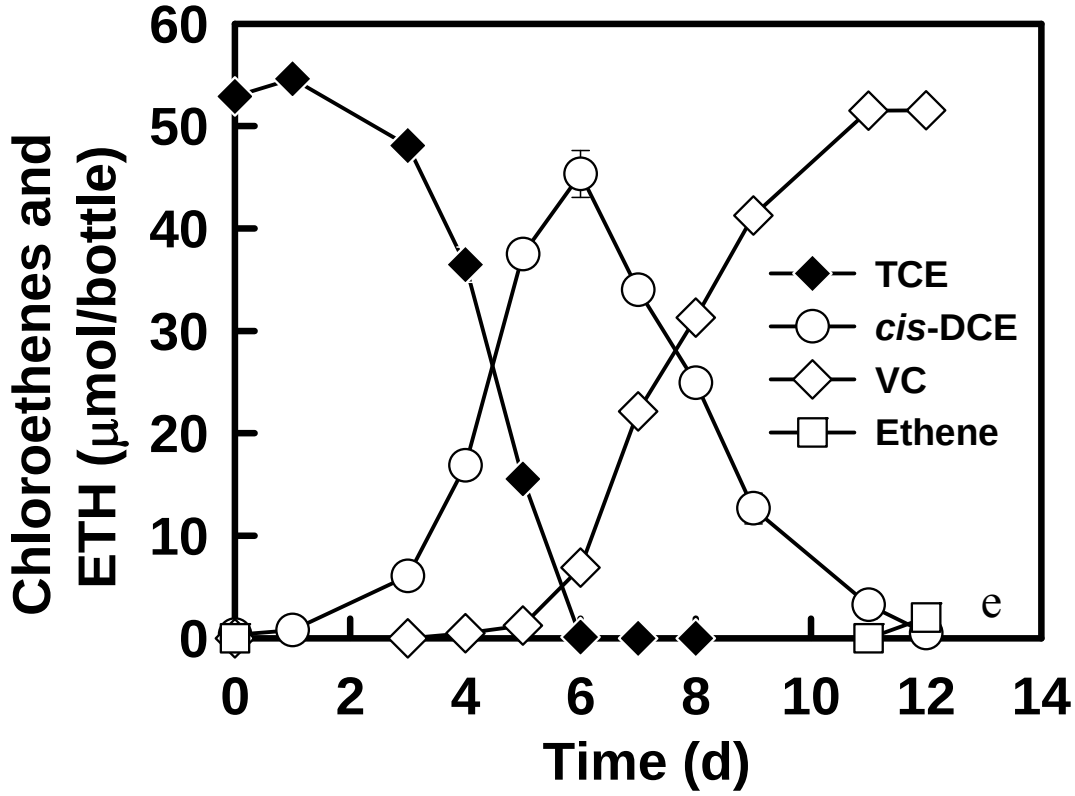
Simultaneously, the cells for each isolate in the coculture were quantified as shown in Fig 4.4b, d, and f. For MB-ANAS1 4:1 coculture, the initial cell numbers for MB and ANAS1 were 1.24×10^7 and 3.1×10^6 cells $\cdot$ ml^{-1}, respectively. On day 6, the cell numbers for MB and ANAS1 increased to 4.67×10^8 and 2.70×10^8 cells$\cdot ml^{-1}$ but with the cell ratio decreased from the initial 4 : 1 to a final ratio of 1.7 : 1. In comparison, the cells in the control bottle inoculated with MB alone (1.08×10^7 cells$\cdot ml^{-1}$) increased to 8.09×10^8 cells$\cdot ml^{-1}$. Similarly, the cells for the MB-ANAS1


Chloroethenes and ETH (μmol/bottle)
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
Time (d)
TCE
trans-DCE
cis-DCE
VC
Ethene
a


Growth of individual isolate Log (gene copies/ml)
9.0
8.5
8.0
7.5
7.0
6.5
6.0
0
1
2
3
4
5
6
7
Time (d)
MB
ANAS1
b


Chloroethenes and ETH (μmol/bottle)
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
Time (d)
TCE
trans-DCE
cis-DCE
VC
Ethene
c


Growth of individual isolate Log (gene copies/ml)
9.0
8.5
8.0
7.5
7.0
6.5
6.0
0
1
2
3
4
5
6
7
Time (d)
MB
ANAS1
d


Chloroethenes and ETH (μmol/bottle)
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
Time (d)
TCE
cis-DCE
VC
Ethene
e

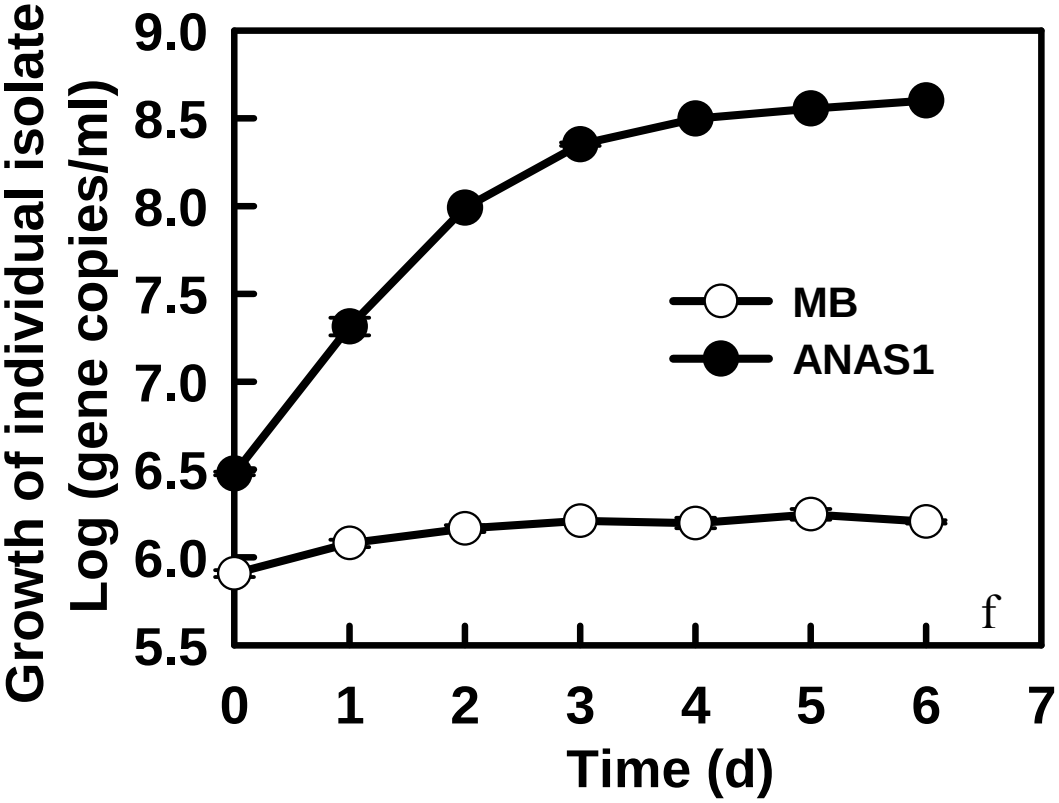

Growth of individual isolate Log (gene copies/ml)
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
0
1
2
3
4
5
6
7
Time (d)
MB
ANAS1
f

Fig 4.4 Dechlorination of TCE and increase in cell growth as determined by qPCR from cocultures consisting of *Dehalococcoides* sp. strain MB and ANAS1. (A and B) MB-ANAS1 4:1; (C and D) MB-ANAS1 1:1; (E and F) MB-ANAS1 1:4. Error bars indicate standard deviations. d, days. ETH, ethene.

1:1 coculture reached the maximal numbers of 2.1×10^8 and 3.9×10^8 cells·ml^{-1} for MB and ANAS1, respectively, on day 6, accompanied by a change in the ratio of MB to ANAS1 cells from the initial ratio of 1:1 to a final ratio of 1:2. Therefore, the growth rate of ANAS1 was at least as twice as that of MB. As for coculture of MB-ANAS1 1:4 coculture, the number of MB cells was increased just slightly from 3.1×10^6 to 6.2×10^6 cells·ml^{-1}. However, the ANAS1 cells measured as *tce*A gene copies increased significantly from 3.0×10^6 to 4.0×10^8 cells·ml^{-1} on day 7.

4.3.7 Reverse transcriptional analysis of RDase genes in strain MB

After isolation of culture MB, Chow et al. (2010) identified seven RDase genes at DNA level in strain MB through degenerate primers but three of them are also identified by microarray analysis. However, the transcription of DNA to copies of mRNA might not occur for all these seven genes. Therefore, transcriptional analysis was conducted to find out which gene among the seven identified ones might be responsible for dechlorinating PCE to *trans*-DCE by the new isolate MB. 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 (Fig 4.5). 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. Furthermore, the extracted RNA samples were not contaminated with genomic DNA, as confirmed by no visible amplicons from amplification of RNA samples with the seven RDase gene-specific primers. Therefore, two (*dceA6* and *dceA7*) out of the seven identified RDase genes are actively expressing in strain MB when fed with PCE. The RDase gene (*dceA6*) with highest expression in strain MB also presented in

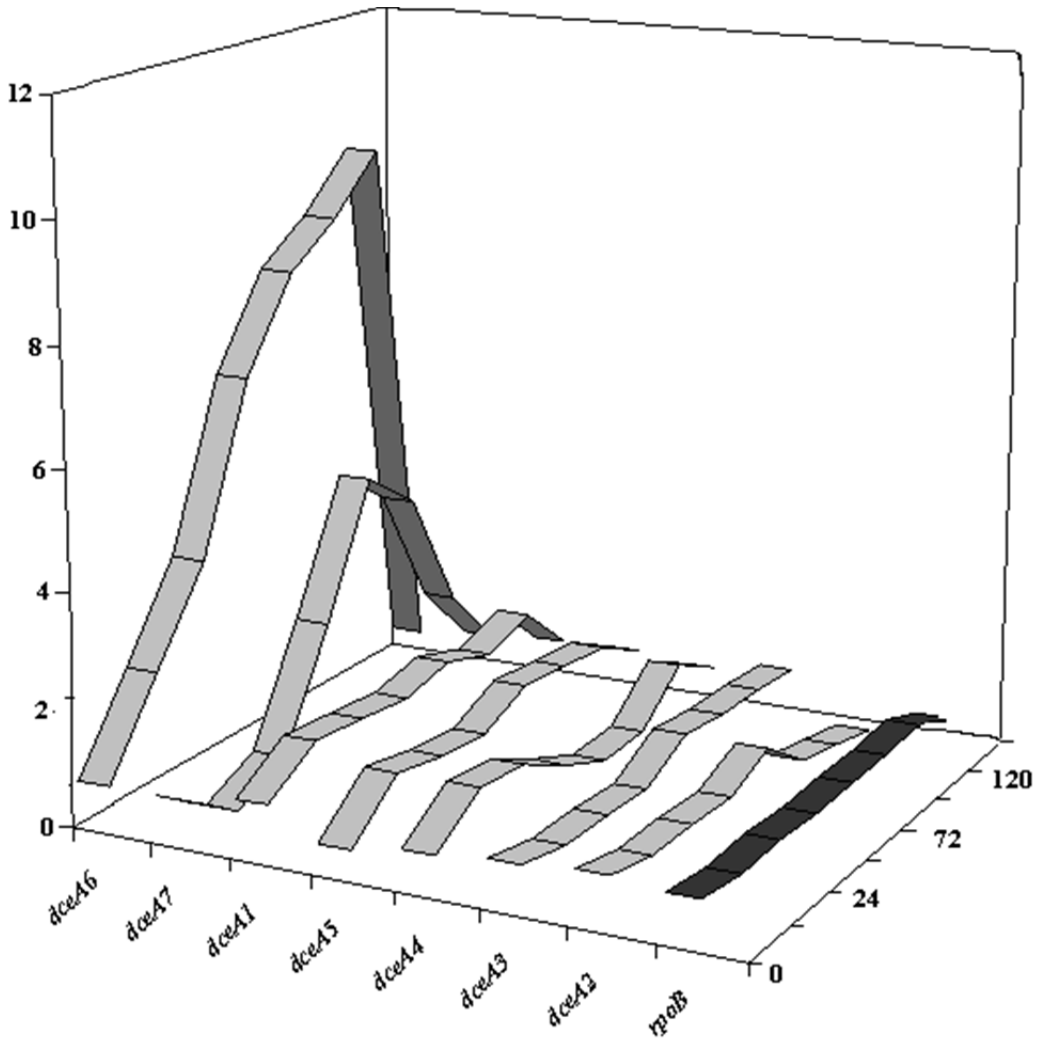


Fig 4.5 Expression profiles of the potential *trans*-DCE-producing RDase genes in *Dehalococcoides* sp. MB when fed with PCE. Transcripts corresponding to *dceA6* and *dceA7* are preferentially expressed.

five other *trans*-DCE-producing microcosms from various locations by gene-specific primer. This result together with the high exression rofile suggests the potential role of *dceA6* in distinguishing *trans*-DCE producing cultures from dechlorinating cultures generating negligible amount of trans-DCE. The RDase gene *dceA6* (also named as *mbrA* gene) may serve as a potential biomarker for *trans*-DCE production at those chloroethene-contaminated sites.

4.4 Discussion and conclusion

Chloroethenes are common organic pollutants that have contaminated the groundwater for over half century. In this study, a novel PCE-to-*trans*-DCE dechlorinating bacterium (*Dehalococcoides* sp. strain MB) was isolated, and it distinguished itself from other dechlorinators by generating predominantly *trans*-DCE. The purity of culture MB was confirmed by (i) a uniform morphology, (ii) a lack of fermentative microbes, and (iii) equal number of cells in the culture measured by targeting 16S rRNA genes and specific putative RDase gene (*dceA1* gene). Prior to the isolation of strain MB, other chloroethene-dechlorinating isolates (Table 4.3) within the genus *Dehalococcoides* were characterized to generate VC or ethene via predominantly *cis*-DCE but negligible amounts of *trans*-DCE (Maymó-Gatell et al., 1997; He et al., 2005; Sung et al., 2006a).

Strain MB belongs to the Cornell subgroup of *Dehalococcoides* as it has the same 16S rRNA gene sequence as strain 195. Other similarities include cell morphology, and a common carbon source (acetate) and electron donor (H_2). These two isolates also demonstrate the capability of debrominating the commercial octa-BDE mixture to penta-/tetra-BDEs in the presence of TCE. The diverse substrate range for *Dehalococcoides* isolates can be explained by their possession of different

RDase genes (e.g., *pceA*, *tceA, bvcA, vcrA* and *cbrA*), which are able to catalyze specific reductive dehalogenation processes (Krajmalnik-Brown et al., 2004; Müller et al., 2004; Smidt and de Vos, 2004; Adrian et al., 2007a). However, the above-identified genes are not responsible for *trans*-DCE production and are not detected in strain MB. Therefore, identification of the key enzymes involved in *trans*-DCE production is desirable for in-depth understanding of the mechanism.

Isolate MB exhibited the highest *trans*-/*cis*-DCE generation ratio ([7.3 ± 0.4] : 1), compared to previous reports on *trans*-DCE producing mixed cultures (Griffin et al., 2004; Miller et al., 2005; Futamata et al., 2007; Kittelmann and Friedrich, 2008b). During the enrichment process, ampicillin played an important role in increasing the ratio of *trans*- to *cis*-DCE by inhibiting other *cis*-DCE producing dechlorinators. The lower ratios of *trans*-DCE to *cis*-DCE commonly observed in the contaminated sites could be a result of the coexistence of MB-like *Dehalococcoides* isolates and other *cis*-DCE generating microbes, e.g. *Desulfuromonas* or other *Dehalococcoides* isolates. This can be seen from the coculture of strain MB and ANAS1. It is interesting to note that the cell growth rate for strain ANAS1 is twice as high as that for strain MB when they coexist, though the specific dechlorination activities of individual cells are the same for both strains. This can be explained by the fact that strain ANAS1 consumes twice the number of electrons from two dechlorination steps as does strain MB from one dechlorination step, though the free energies per electron are nearly the same for both strains. The same explanation may also apply to the difficulty in cultivating *trans*-DCE-producing microbes in pure culture. More MB-like isolates are needed in order to make a conclusive statement that *cis*-DCE-producing microbes outcompete *trans*-DCE producing microbes. Fortunately, the absence of other *cis*-DCE producing *Dehalococcoides* species in the MB mixed culture facilitated the isolation of strain

MB in pure culture, since *trans*-/*cis*-DCEs were the endpoints of the stepwise reductive dechlorination of PCE in mixed-culture MB. In addition, until now, no *Dehalococcoides* species have been found to stop chloroethene dechlorination at only *cis*-DCE. Thus, this study suggests that competition among different dechlorinators determines the fate of chloroethenes at the contaminated sites.

The application of microarrays to query the genome of pure cultures has given us an in-depth understanding of the putative RDase genes of isolate MB. The genomic content derived from a microarray based on strain 195, without complete genome sequencing, provides an insight into this new strain's complex nutrient requirements and its commitment to the dehalorespiratory process. Isolate MB possesses 88% of the genes found in strain 195, indicating their close connection. The close RDase gene match explains the capability of strains 195, MB, and CBDB1 to dehalogenate aromatic halogenated compounds. To date, more than 90 RDase genes have been identified in the five *Dehalococcoides* isolates, many of which exist in more than one *Dehalococcoides* sp. strain (Morris et al., 2007). For example, both RDase genes DET0180 and DET1535 are present among multiple strains of *Dehalococcoides*; however, DET1528 is present in strains 195 and MB only. Correspondingly, the inability of strain MB to dechlorinate DCEs might be due to the absence of the four genes, DET0079, DET0162, DET0876 and DET1559 (located in IEs I, II, V and IX, respectively), which may define an organism's ability to dechlorinate TCE past DCEs, based on strain 195 (Seshadri et al., 2005). Additionally, isolate MB shows a lack of large proportions of genes found in *D. ethenogenes* strain 195, such as IEs (73.7% absent) and the phage-related genes, indicating a high level of variation in the MGEs. This is consistent with the microarray study conducted for the enrichment culture ANAS (88% absent) (West et

al., 2008). Additional sequence information about strain MB is needed for a better understanding the dynamics of MGE acquisition and maintenance, as well as the related plasmid or phage families. Isolation of strain MB will fill the gap regarding *trans*-DCE-producing genes and add new information to the RDase gene library. The *mbrA* RDase gene (*dceA6*) highly expressed on exposure to PCE is suggested to be involved in the reductive dechlorination of PCE/TCE to the major end product *trans*-DCE, corroborated by its wide distribution in other *trans*-DCE producing cultures. However, more in-depth study through proteomic analysis may be needed to enhace our understanding about its direct role in generating trans-DCE by MB-like culture.

In all, a novel *Dehalococcoides* species strain MB was isolated and grown in a defined medium by dechlorinating PCE to *trans*-DCE predominantly. Obtaining this new isolate could provide insights into the source of *trans*-DCE and increase our understanding of the diversity, specificity, and evolution of the genus *Dehalococcoides*, which will contribute to the implementation of a bioremediation strategy.

Chapter V Rapid Detoxification of Trichloroethene by a New Isolate *Dehalococcoides* Species Strain 11a and Its Potential Application to Remediate Chloroethene-Contaminated Groundwater

Efforts to remediate chloroethenes, such as tetrachloroethene (PCE) and trichloroethene (TCE) often result in the accumulation of dichloroethenes (DCEs) or vinyl chloride (VC) in the anoxic subsurface. Although *Dehalococcoides* spp. can convert TCE, 1,1-DCE, *cis*-DCE, and VC to ethene, those that can detoxify both TCE and *trans*-DCE completely to ethene still remain elusive. Finding an effective and energy-conserving technology that helps to dechlorinate all the DCEs and VC is thus an important research discipline in environmental engineering. In this study, two novel strains were obtained from industrial sludge. The first isolate, *Dehalococcoides* sp. strain 11a5 could only transform TCE and all three DCE isomers to VC metabolically. The second isolate, *Dehalococcoides* sp. strain 11a, rapidly dechlorinated TCE, 1,1-DCE, *trans*-DCE, *cis*-DCE, VC, and 1,2-DCA metabolically to ethene with an average dechlorination rate of 53.1, 22.5, 21.6, 24.8, 86.5, and 16.7 μmol L^{-1} day^{-1}, respectively. Strain 11a can also co-metabolically dechlorinate PCE to TCE, which is subsequently converted to ethene via a metabolic process. Strain 11a shares 100% 16S rRNA gene sequence identity with the first VC-dechlorinating isolate *Dehalococcoides* sp. strain BAV1, but differs in the reductive dehalogenases used (VcrA for 11a and BvcA for BAV1). The *vcrA* gene of culture 11a also shows a difference of nine and ten base pairs (bp) from strain ANAS2 and VS, respectively.

As compared with previous dechlorinators (e.g., strain GT and ANAS2), strain 11a is the fastest one till date, particularly 3-folds higher for VC dechlorination rates, catalyzed by the newly discovered VcrA RDase. Most importantly, complete detoxification of PCE to ethene in contaminated groundwater was achieved within 10 days by co-culturing strain 11a with another PCE-to-*cis*-DCE dechlorinating bacteria, *Sulfurospirillum multivorans*. This study indicates that there are other *vcrA*-containing *Dehalococcoides* strains that have not been successfully targeted previously but were actively involved in remediation of TCE-contaminated sites. It also shows strain 11a's potential application in industries where bioremediation of chloroethene-contaminated sites is needed.

5.1 Introduction

Chloroethenes, such as tetrachloroethene (PCE) and trichloroethene (TCE) and their transformed products remain a global threat to groundwater due to their potential carcinogenic effect and persistence in the environment (Smidt and de Vos, 2004; Bhatt et al., 2007). Natural attenuation of PCE and TCE typically results in partial dechlorination, leading to the accumulation of *cis*-1,2-dichloroethene (*cis*-DCE) or vinyl chloride (VC, a proven carcinogen) in the groundwater (Taş et al., 2009a). Dehalogenating anaerobes at these chloroethene-contaminated sites have therefore been well characterized and identified (Maymó-Gatell et al., 1997; Cupples et al., 2003, 2004; Duhamel et al., 2004; Smidt and de Vos, 2004; Bhatt et al., 2007; Fung et al., 2007; Futamata et al., 2007; Behrens et al., 2008; Lee et al., 2008; Taş et al., 2009a). However, the production of 1,1-DCE (Zhang et al., 2006), and the prevalence of *trans*-DCE (ATSDR., 2007) have also been observed at several chloroethene-

contaminated sites as the main intermediates. Toxic levels of 1,1-DCE (2,000 μg L^{-1}) and *trans*-DCE (18,000 μg L^{-1}) are also detected at several chloroethene-contaminated sites, possibly generated through the microbial reductive dechlorination process (e.g., *Dehalococcoides,* bacteria capable of reductive dechlorination of PCE and TCE) (Benson, 2003; Löffler et al., 1997b; Griffin et al., 2004; Miller et al., 2005; Zhang et al., 2006; ATSDR, 2007; Futamata et al., 2007; Kittelmann and Friedrich, 2008a, b; Cheng and He, 2009; Cheng et al., 2010; Chow et al., 2010; Macro-Urrea et al., 2011). For instance, strains MB and CBDB1 belonging to genus of *Dehalococcoides* have been reported to be able to dechlorinate PCE and TCE to *trans*- and *cis*-DCEs with a ratio of 7.3 (±0.4):1 and 3.4 (±0.2):1, respectively (Cheng and He, 2009; Marco-Urrea et al., 2011). Due to the frequent detection of DCEs at federal and state hazardous waste sites, the toxicity of *trans*- and *cis*- DCEs has been re-evaluated by the integrated risk information system (IRIS) program within U.S. EPA in 2010 (Galizia, 2010). It has been found that the most striking and unique biological consequence of DCEs is their ability to inhibit hepatic cytochrome P450 (CYP), which is associated with competitive inhibition of the metabolism of other CYP substrates (e.g., volatile organic compounds) in rat liver (Freundt, 1978). Due to the accumulation of DCEs and VC, complete reductive dechlorination of PCE or TCE cannot be assured in the natural environment despite the local abundance of certain *Dehalococcoides*.

Various strains beloning to genus of *Dehalococcoides* and only seven functionally diverse members have been isolated with specific halogenated compounds as the growth substrates, namely strains 195, CBDB1, BAV1, FL2, GT, DCMB5, and MB (as summarized in Table 5.1). While each of these individual strains was found to be capable of dechlorinating their favoured halogenated compounds, complete and rapid detoxification of a mixture of PCE, TCE and all DCE

isomers to harmless ethene was not achievable by a single strain (He at al., 2005; ITRC, 2005; Cheng et al., 2009; Taş et al., 2009a). For instance, a VC-dechlorinating

Table 5.1 *Dehalococcoides* species and their metabolic substrates

Name of the culture	Halogenated compounds reduced	End-products	References
Dehalococcoides ethenogenes strain 195	PCE, TCE, *cis*-DCE, 1,1-DCE	VC (ethene)	Maymó-Gatell et al., 1997
	1,2-DCA,	Ethene	Maymó-Gatell et al., 1999
	1,2,3,4-tetrachlorodibenzo-*p*-dioxin	1,2,4-trichlorodibenzo-p-dioxin, 1,3-dichlorodibenzo-*p*-dioxin	Fennell et al., 2004
	2,3,4,5,6-pentachlorophenyl	2,3,4,6-, or 2,3,5,6-tetra-chlorobiphenyl, 2,4,6-tri-chlorobiphenyl	
	Hexachlorobenzene (HCB)	1,2,3,5-tetrachlorobenzene, 1,3,5-trichlorobenzene	
	2,3-DCP, 2,3,4-TCP, 2,3,6-TCP	Lower chlorinated phenols (ortho chlorine removed)	Adrian et al., 2007b
Dehalococcoides sp. strain BAV1	*trans*-DCE, *cis*-DCE, 1,1-DCE, VC, 1,2-DCA	Ethene	He et al., 2003b
Dehalococcoides sp. strain CBDB1	HCB	1,3-DCB, 1,4-DCB, and 1,3,5-TB	Adrian etal., 2000
	2,3-DCP, all six TCPs, all three triCPs and penta-CP	Lower chlorinated phenols	Adrian et al., 2007b
	Polychlorinated dioxins	Dichloro-dioxins	Bunge et al., 2003
	Polychlorinated biphenyls (Aroclor 1260)	various	Adrian et al., 2009
Dehalococcoides sp. strain FL2	TCE, *trans*-DCE, *cis*-DCE, 1,1-DCE	VC (ethene)	He et al., 2005
Dehalococcoides sp. strain GT	TCE, *cis*-DCE, 1,1-DCE, VC	Ethene	Sung et al., 2006a
Dehalococcoides sp. strain DCMB5	1,2,4-Trichlorodienzo-*p*-dioxin	2-Monochlorodizenbo-*p*-dioxin	Bunge et al., 2008
	1,2,3-TCB	1,3-DCB	
Dehalococcoides sp. strain MB	TCE, PCE	*trans*-DCE, *cis*-DCE	Cheng and He, 2009
Dehalococcoides sp. strain ANAS1	TCE, *cis*-DCE, 1,1-DCE	VC (ethene)	Cheng and He, 2009
Dehalococcoides sp. ANAS2	TCE, *cis*-DCE, 1,1-DCE, VC	Ethene	Lee et al., 2011
Dehalococcoides spp. (VS, mixed culture)	TCE(slow), *cis*-DCE, 1,1-DCE, VC	Ethene	Cupples et al., 2003
Dehalococcoides spp. (KB-1, mixed culture)	TCE, *cis*-DCE, VC	Ethene	Duhamel et al., 2004

pure culture, *Dehalococcoides* sp. strain GT, cannot respire *trans*-DCE and requires longer time (about two weeks) to initiate TCE-dechlorination activity (Sung et al., 2006a). Strain 195 shows the capability of dechlorinating PCE, but the dechlorination of VC to ethene is rate-limiting (McMurdie et al., 2009).

Several other genera, such as *Sulfurospirillum, Desulfitobacterium*, *Dehalobacter* are also able to dechlorinate PCE or TCE but result in the accumulation of *cis*-DCE (Smidt et al., 2004). Therefore, it is suggested that complete dechlorination of PCE to ethene requires the cooperation of multiple dehalogenating strains unless novel dechlorinators are to be discovered (ITRC 2005, 2007). In fact, a number of *Dehalococcoides* (one or multiple strains)-containing enrichment cultures have also been reported to dechlorinate TCE, *cis*-DCE, 1,1-DCE, and VC to ethene such as cultures VS (Cupples et al., 2003), KB-1 (Duhamel et al., 2004) and ANAS (Holmes et al., 2006).

Present in either pure or mixed cultures, the dechlorinators catalyze reductive dechlorination of chloroethenes by the reductive dehalogenase (RDase), which could be expressed from genes including *tceA*, *pceA*, *bvcA*, as well as *vcrA* (Maphosa et al., 2010). The role of VcrA in the dechlorination of *cis*-DCE, 1,1-DCE or VC has been proven previously, whereas the discrepancy occurs for *trans*-DCE (Müller et al., 2004; Holmes et al., 2006). Although the purified enzyme VcrA from culture VS showed dechlorination ability for *trans*-DCE, the *vcrA* gene-containing cultures failed to use this substrate, e.g. (Duhamel et al., 2004; Holmes et al., 2006; Sung et al., 2006a). Very little has been reported on the role of *vcrA* gene during the dechlorination of *trans*-DCE and thus uncovering their association can be insightful for further development of the dechlorination process.

The aim of this study is to cultivate a rapid trichloroethene-to-ethene-detoxifying culture and to investigate the microbe's catalyzing ability (e.g., the *vcrA* gene) during the dechlorination of TCE, *trans*-DCE, and VC to ethene. The application of this novel culture will provide a cost-effective and environmentally-friendly microbiological application that helps to completely detoxify PCE-/TCE-contaminated sites with minimum accumulation of intermediate toxic products.

5.2 Materials and methods

5.2.1 Chemicals

All chemicals were supplied from Sigma-Aldrich (U.S.A.) with more than 97% purity unless stated otherwise. Genomic DNA, plasmid DNA and RNA extraction kits were purchased from Qiagen (QIAGEN GmbH, Hilden, Germany). PCR reagents including the GoldTaq DNA polymerase were obtained from Applied Biosystems (Foster City, CA, U.S.A.).

5.2.2 Isolation and cultivation conditions

Isolation of functional microbes in a TCE-dechlorinating microcosm was carried out by series dilution in 20-ml glass vial containing 10-ml of liquid medium spiked with either TCE or VC as electron acceptor to enrich diverse dechlorinators within this culture. Three consecutive treatments with ampicillin (100 mg l^{-1}) were applied to two active sub-cultures, the TCE-fed and VC-fed cultures of the dilution series, with acetate as carbon source and hydrogen as electron donor. Subsequent dilution batches were free of any antibiotics. The TCE-fed culture was serially diluted again and transferred to two subcultures from the dilution series according to

the observed predominant dechlorination product (VC or ethene). Thereafter, dilution-to-extinction procedure was carried out on these three sub-cultures, TCE-to-ethene culture (named 11a4), TCE-to-VC culture (named 11a5), and VC-to-ethene culture (11a). All cultures were grown in mineral salt medium reduced with $_{L}$-cysteine (0.2 mM), sodium sulfide (0.2 mM) and DL-dithiothreitol (0.5 mM) in liquid medium and agar shakes as described previously (He et al 2003b). Active dechlorinating culture was observed periodically under light microscope, Nikon Eclipse E200 (Nikon, Melville, N.Y., U.S.A.) until the culture became morphologically uniform.

After obtained the new *Dehalococcoides* species in pure culture, they were also tested on other chloroethenes, like PCE, TCE (or VC), 1,1-DCE, *trans*-DCE, *cis*-DCE (about 2 mM each in the liquid phase). The dechlorination time course studies were carried out in 160-ml serum bottles in triplicates under the same medium conditions as described above with 2% (v/v) inocula. One ml of cells was kept periodically for downstream nucleic acid studies.

In addition to these chloroethenes, the following halogenated compounds were also tested on the new isolates as electron acceptors: 1,1,1-trichloroethane (TCA), 1,1-dichloroethane (1,1-DCA), 1,2-dichloroethane (1,2-DCA), chloroform, carbon tetrachloride, PCBs (Aroclor 1260 and CB-155), 2,4,6-trichlorophenol (TCP) and pentachlorophenol (PCP), and PBDEs (octa-BDE mixture, deca-BDE mixture, and penta-BDE mixture). If the compound is in powder form, it was dissolved in either TCE or inert solvent nonane before being injected into medium bottles with a final concentration of 0.1 - 0.2 mM. Compounds in liquid or gaseous forms were added to the medium directly with a final concentration of 0.2 mM. Other substrates tested on the new isolates include succinate (10 mM), succinate (10 mM), glucose (10 mM), lactate (10 mM), pyruvate (10 mM), propionate (10 mM), fumarate (10 mM), malate

(10 mM), glutamate (10 mM), sulfate (5 mM), sulfite (5 mM), nitrate (10 mM), and nitrite (5 mM). The bottles were incubated statically under strict anaerobic conditions in the dark at 30ºC.

5.2.3 Analytical procedures

The dehalogenation activity of chloroethenes and chloroethanes was closely monitored by injecting 100 µl of headspace sample manually to Agilent gas chromatograph (GC) 6890N equipped with flame ionization detector (Agilent, Wilminton, DE, USA) as described previously (Cheng et al., 2010). PCBs, chlorophenols, and PBDEs were tested by GC coupled with an electron capture detector (ECD) as described previously (Cheng and He, 2009).

5.2.4 DNA extraction, PCR and sequencing

Total genomic DNA was extracted from one ml of cell pellets of the active dechlorinating cultures with Qiagen DNeasy tissue kit according to the method described previously (Löffler et al., 1997b). The concentration of the nucleic acid was determined by Nanodrop-1000 (NanoDrop Technologies Inc., U.S.A.). The 16S rRNA gene sequences were amplified by targeting the genomic DNA with the universal bacterial primer pair (8F and 1392R) while PCR (Eppendorf, Hamburg, Germany) amplification was carried out under conditions as described previously (Löffler et al., 2000). A clone library of the 16S rRNA gene was established with the amplified PCR products. A fragment of approximately 1311 bp of the 16S rRNA gene was cloned, sequenced and aligned as previously described (He et al., 2003a). PCR-DGGE was carried out to confirm the purity of the new isolates as previously described in chapter III (Cheng et al., 2010).

Sequences were compared to other published sequences with the BLASTN search tool provided in the National Center for Biotechnology Information. Deduced amino acid sequences were obtained with the TRANSLATE program (http://us.espasy.org/tools/dna.html) and aligned with the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) as described previously (Chow et al., 2010)

5.2.5 Amplification of the putative RDase genes

PCR was used to check the presence of known RDase genes, such as *tceA*, *bvcA*, *pceA*, *mbrA*, and *vcrA* genes in the genomic DNA by following the method described previously (Holmes et al., 2006; Chow et al., 2010). For the unknown putative RDase genes, clone library was constructed with two degenerate primer sets, RRF2 and B1R (Krajmalnik-Brown et al., 2004), or RDH F1C (5'-TTY MVI GAY ITI GAY GA-3') and RDH R1C (3'-CCI RMR TYI RYI GG-3') as described previously (Chow et al., 2010) to target RDase genes from the genomic DNA of culture 11a grown with TCE. PCR mixtures (20 µl) contained about 10 ng of template DNA (genomic DNA from strain 11a), 0.5 µM of each primer, 2.5 mM $MgCl_2$, 0.25 mM each deoxynucleoide, 0.13 mg of bovine serum albumin ml^{-1}, and 0.5 U of Taq DNA polymerase (Applied Biosystems). The PCR was run with the following parameters: 130 s at 95 °C; 40 cycles of 30 s at 95 °C, 45 s at 49 °C, and 130 s at 72 °C; and a final extension of 6 min at 72°C. A total of 250 µl of amplified PCR products were purified with a QIAquick PCR purification kit and the purified PCR products were subsequently cloned with the pCR2.1-TOPO TA cloning kit (Invitrogen, Carlsbad, CA, U.S.A.) according to manufactures' instructions. Positive clones were screened with restriction enzyme *Hha*I and *Msp*I (New England Biolabs, Ipswich, MA, U.S.A.)

digestion. Plasmids containing insert with different restriction patterns were extracted from the respective *E. coli* clones with a Qiaprep Spin Miniprep kit (QIAGEN) and sent for sequencing with primer M13F and M13R.

5.2.6 Gene expression studies for culture 11a with different substrates

To investigate the role of *vcrA* gene in the pure culture, TCE or VC were supplied as individual substrate in respective culture bottles for culture 11a which had been starved with chloroethenes for about three days. Briefly, two weeks were required for the single dose of TCE (5 μL, about 55 μmol) completely consumed by 11a. After 72-hour starvation, two ml of 11a cells were inoculated to two parallel batches of 100-ml medium amended with TCE (5 μL, 55.6 μmol) or VC (1.5 mL, 61.3 μmol) in triplicate, respectively; the control bottles were set up exactly the same way but without the addition of any chloroethene. RDase gene expression was performed on these chloroethene-fed subcultures and the control bottles as described previously (Chow et al., 2010). The growth conditions for 11a have been described previously in multiple 160-ml serum bottles (100 ml of medium, 2% inoculation). Cells were intensively collected before dechlorination activity was observed for the transcription studies, including the control bottle.

Cells for DNA and RNA extraction were collected periodically from 1-ml and 1.5-ml of culture samples by centrifugation (5 min at 20, 000× g, 4ºC) in RNase-free microcentrifuge tubes. The cell pellets were stored at -20 ºC or -80 ºC, respectively until DNA and RNA extraction. Measurements of *vcrA* gene transcription levels were tracked over 24h or one week under the aforementioned dechlorination activity.

5.2.7 Cell lysis and RNA extraction

Cell lysis was enhanced by 45 μL of proteinase K (25 mg mL^{-1}), 20 μL of Lysozyme (100 mg mL^{-1}), and 10 μL achromopeptidase (7,500 U mL^{-1}) (He et al., 2003a).

Total RNA was isolated using RNeasy extraction kit (Qiagen, Germany) according to the manufacturer's instructions with some modifications described previously (Krajmalnik-Brown et al., 2004; Johnson et al., 2005b; Lee et al., 2008). 2 μL of 10^7 transcripts μL^{-1} of luciferase control RNA (reference mRNA) was added to the cell suspension prior to the addition of lysozyme digestion buffer. The cell lysate was transferred to a tube with 100 μL of 0.1mm zirconia-silica beads, which has a density of 3.7g cm^{-3} (BioSpec, Bartlesville, OK, USA) and followed by bead beating. Reverse transcription PCR was conducted with the two-step RT-PCR Sensiscript kit (Qiagen). Removal of contaminating DNA was performed after RNA isolation using Ambion DNase I (Ambion, Inc, Austin, TX, U.S.A.) following the manufacturers' instructions.

For reverse transcription PCR (RT-PCR), each 20-μL reaction mixture contained 1 mM random hexamer primers (Promega, Madison, USA) and 5-50 ng of RNA from samples or serially diluted reference luciferase RNA standard and was incubated for 3 hrs at 37 ºC. Triplicate quantitative PCR (qPCR) was applied to analyze both gene copy numbers of the Eubacteria, genus of *Dehalococcoides* or the RDase genes, and the reverse transcripts of the RDases. Duplicate qPCR was also applied to each RNA sample which was subjected to similar RT reaction but without any reverse transcriptase to examine the presence of contaminating DNA.

5.2.8 PCR-DGGE

Genomic DNA was PCR amplified with a *Dehalococcoides*-specific primer pair (1F-GC and 259R) as described previously (Duhamel et al., 2004). The PCR fragments were resolved on an 8% polyacrylamide gel with 30-60% urea-formamide gradient for 16 h at 115 V and 60 °C.

5.2.9 Quantitative real-time PCR (qPCR)

Total numbers of Eubacterial- and *Dehalococcoides*-targeted 16S rRNA genes, *vcrA* gene as well as the *tceA* gene were determined by ABI 7500 FAST Real-Time PCR system instrument with v1.4 software (Foster, CA, USA), as described previously (Holmes et al., 2006). The Taqman-based primers and probe for *vcrA* gene were designed using the Primer Express software package suite based on the nearly complete condons of the VC dehalogenase gene of strain 11a. In brief, the *vcrA* gene (vcrAB operon) over 1482 bp was firstly amplified using the following forward and reverse primers: 5'-CTA TGA AGG CCC TCC AGA TGC-3' (21nt) and 5'-GTA ACA GCC CCA ATA TGC CAA GTA-3' (24nt) (Müller et al., 2004). The sequencing result of *vcrA* gene from culture 11a was compared with that of culture VS and ANAS2 (unpublished result) as shown in Fig 5.1.

A new set of Taqman primers and probe was designed to quantify *vcrA* gene: forward primer vcrA11F (5'-GTA TGG TCC GCC ACA TGA TTC-3'), reverse primer vcrA11R (5'-TCT TCT GGA GTA CCC TCC CAT TT-3'), and probe vcrA11P (5'-FAM-CGC CAC CTG ATG GGA GCG TAC C-TAMRA-3'). This new set of Taqman-based primers and probe was designed to target various kinds of *vcrA* gene in order to cover a number of other VC-dechlorinating microbes, e.g. KB-1 like culture, ANAS enrichment culture, VS and GT.

The specificity of the selected primers and probe were examined with the plasmid containing a cloned *vcrA* gene from strain 11a under 9600 Emulation mode. A calibration curve was obtained using 10-fold serial dilutions or pure culture plasmid DNA carrying either a cloned Eubacterial 16S rRNA gene (including genes specific for *Dehalococcoides* 16S rRNA), or *vcr*A gene cloned from reductive dehalogenase genes from strain 11a, respectively. Standard curves spanned a range of 100 to 10^7 gene copies per µl of template DNA.

Fig 5.1 ClustalW2 alignment of the sequence of *vcrA* gene from three different *vcrA*-containing *Dehalococcoides* sp. strains VS, ANAS2, and 11a.

5.2.10 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The PAGE gel was run for the cell extracts of active cultures fed with *trans*-DCE and TCE, respectively. The gel was stained with PlusOne Silver Staining Kit

(GE Healthcare) as previously described (Chow et al., 2010).

5.2.11 Application of culture 11a in contaminated water samples

Microcosm study was conducted to evaluate the amendments needed to enhance complete reductive dechlorination. The groundwater sample was collected from a leather cleaning facility located in Conyers, Georgia. The initial analysis of the groundwater samples revealed the presence of PCE (6,700 μg L^{-1}), TCE (690 μg L^{-1}), *trans*-DCE (11 μg L^{-1}) and *cis*-DCE (4,100 μg L^{-1}), but VC was not detected. Field investigations revealed the presence of a source of chloroethenes at the drainfield of the facility. For demonstration, additional 20 μmols of PCE was supplied per 40-ml of groundwater to evaluate the potential application of the new culture in the bioremediation site. The surface aquifer was under aerobic condition with pH ranging from 4.15 to 5.36. Enhanced reductive dechlorination (ERD) was conducted in this study with the new TCE-dechlorinating isolate 11a and another PCE dechlorinating isolate, *Sulfurospirillum multivorans* comb. nov. (DSM 12446, known as the fastest culture converting PCE to *cis*-DCE (Luijten et al., 2003)). Microcosms in duplicates were set up with medium comprising of contaminated groundwater or defined medium, and 20 μmoles of PCE (liquid concentration ~ 57,965 μg L^{-1}) or TCE. All sample bottles and controls were inverted and incubated in the dark at 30°C statically.

5.2.12 Nucleotide sequence accession number

GenBank accession numbers FJ593658 and HM138520 were assigned to the 16S rRNA gene sequence of two isolates, designated as *Dehalococcoides* sp. strain 11a and 11a5, respectively. GenBank accession numbers HM138513-HM138519 were assigned to the RDase genes 1-7 of the new isolate 11a.

5.3 Results

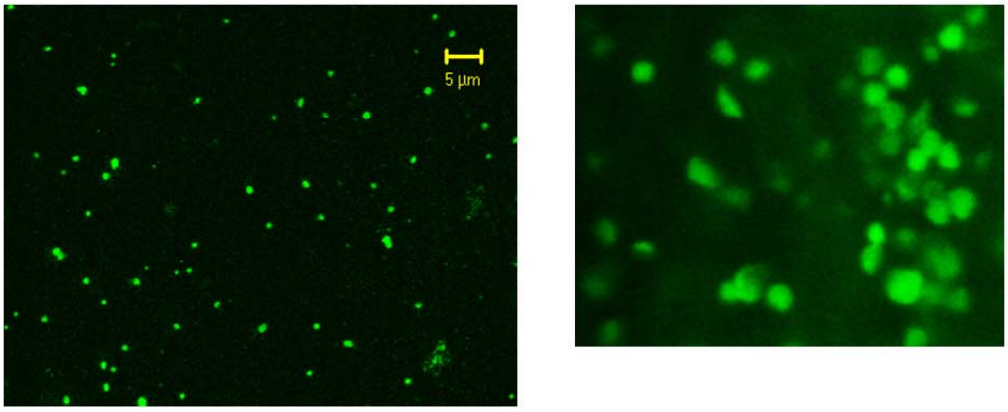
5.3.1 Isolation of *Dehalococcoides* sp. strain 11a and strain 11a5.

A TCE-dechlorinating microcosm was originated from the outlet of an industrial wastewater treatment station of Gehua Group in Wuhan, Hubei, China. It used to produce measurable amount of *trans*-DCE for the first three times' transfer during its dechlorination of TCE to ethene in defined mineral salts medium buffered with bicarbonate at 30 ºC(Cheng et al., 2010). However, further transfer of this culture resulted in the loss of its capability to produce *trans*-DCE but still maintained its role in actively dechlorinating both TCE and VC to ethene.

A TCE-dechlorinating mixed culture (named 11a) was enriched and was reported to be able to dechlorinate TCE to ethene through *trans*- and *cis*-DCEs under strict anaerobic condition in mineral salts medium amended with acetate and hydrogen (Cheng et al., 2010). *Dehalococcoides* was detected by genus-specific primer pairs and was found to be the main TCE-dechlorinators, which usually required acetate as carbon source and H_2 as electron donor. Ampicillin was then applied to inhibit the growth of methanogens or acetogens present in this culture. After two consecutive treatments with ampicillin (100 mg L^{-1}), the dilution series amended with TCE showed different dechlorination end products. The first four vials (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) in the dilution series showed ethene as the main product similar to the initial microcosm, whereas the next one (10^{-5}) showed significant accumulation of VC. The different dechlorination end products indicate the presence of at least two different strains of *Dehalococcoides* in this microcosm and the fifth vial may lose one *Dehalococcoides* strain responsible for VC-dechlorinating. Therefore, two subcultures, VC-generating culture (vial 10^{-5}, named 11a5) and ethene-generating

batch (vial 10^{-4}, named 11a) were selected as the main target for isolation. They were maintained, serial diluted, and transferred with respective substrates TCE and VC in liquid medium. After 23rd and 25th transfers respectively, both light microscopy and epifluorescence microscopy revealed morphologically homogenous disc-shaped bacteria (about 1 µm in diameter as shown in Fig 5.2) in culture 11a5 and 11a.

(a)



(b)

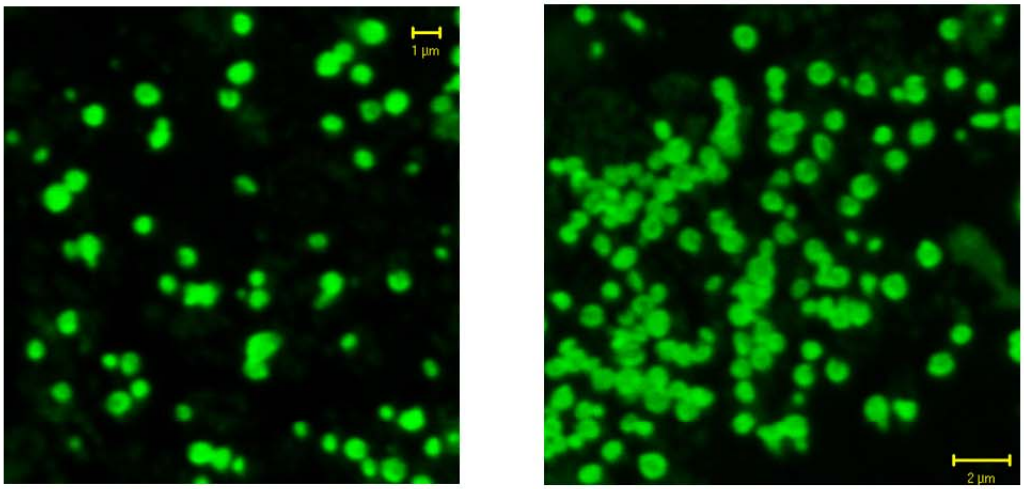


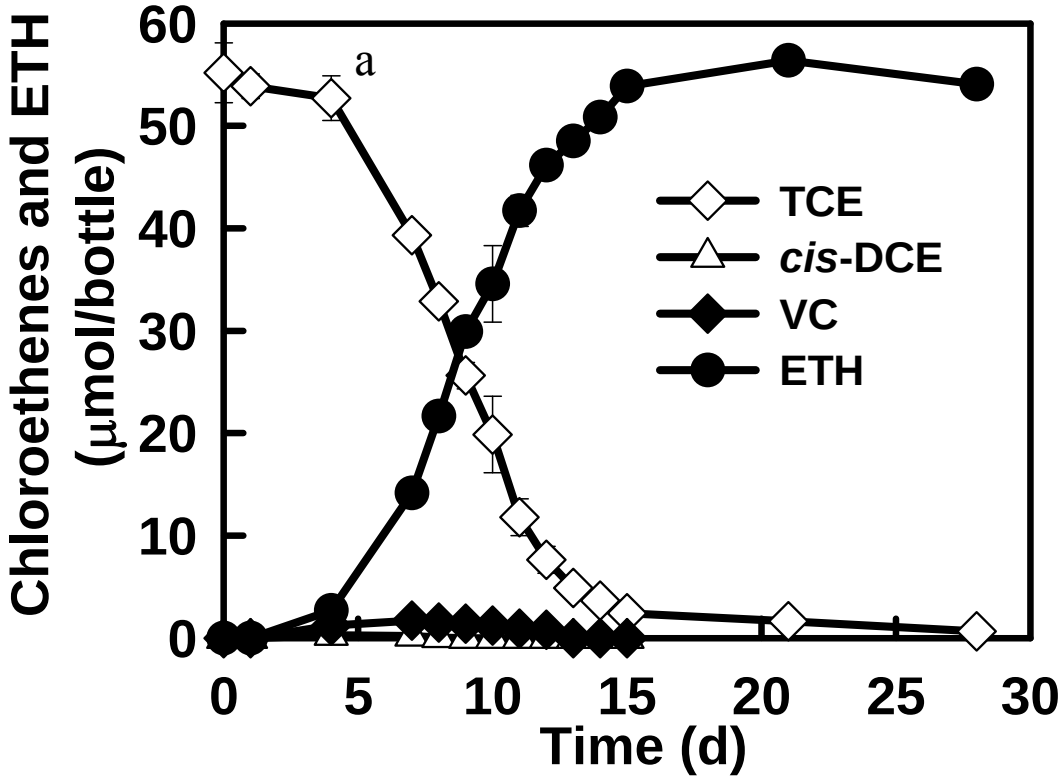
Fig 5.2 Morphology of (a) *Dehalococcoides* sp. strains 11a and (b) 11a5 as observed with confocal laser scanning microscopy (Confocal LSM 5 Pascal). Cells were stained with the fluorescent dye Syto-9 (5 µM) and viewed under 1000× magnification through the Carl Zeiss scope lens. Both strains (11a and 11a5) show similar disc-shape with a diameter of 1.0 µm.

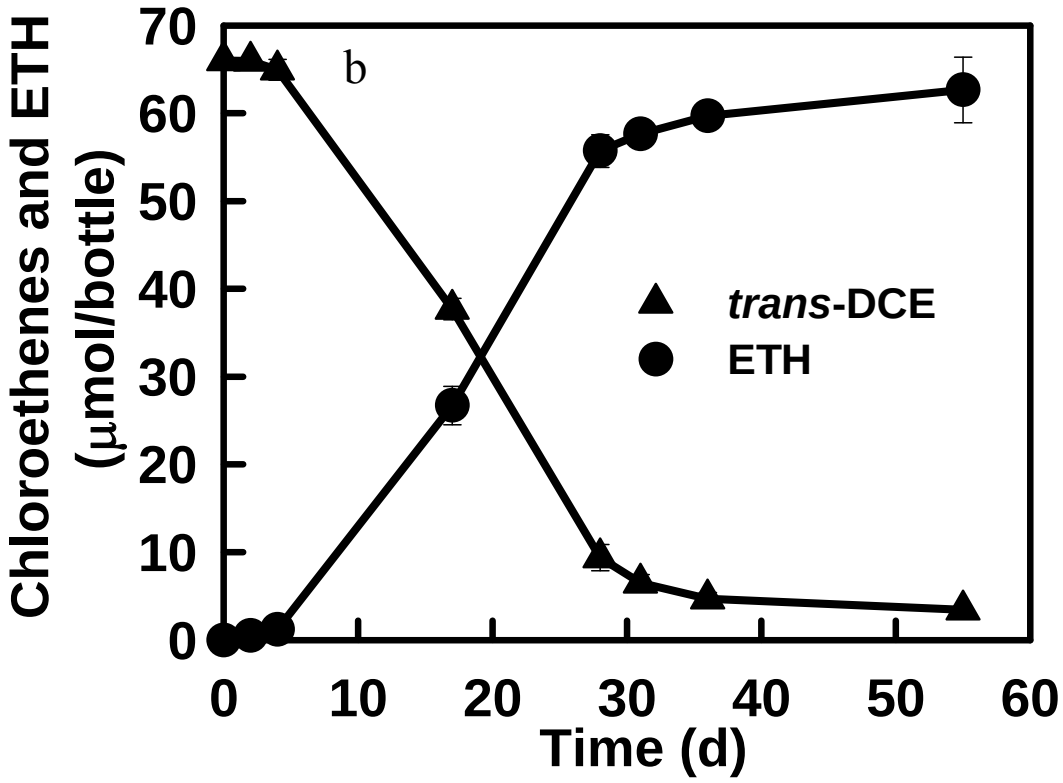
The 16S rRNA gene amplified from genomic DNA of culture 11a showed a sequence identical to that of *Dehalococcoides* sp. strain BAV1, whereas culture 11a5 showed 100% 16S rRNA gene sequence identical to that of *Dehalococcoides* sp.

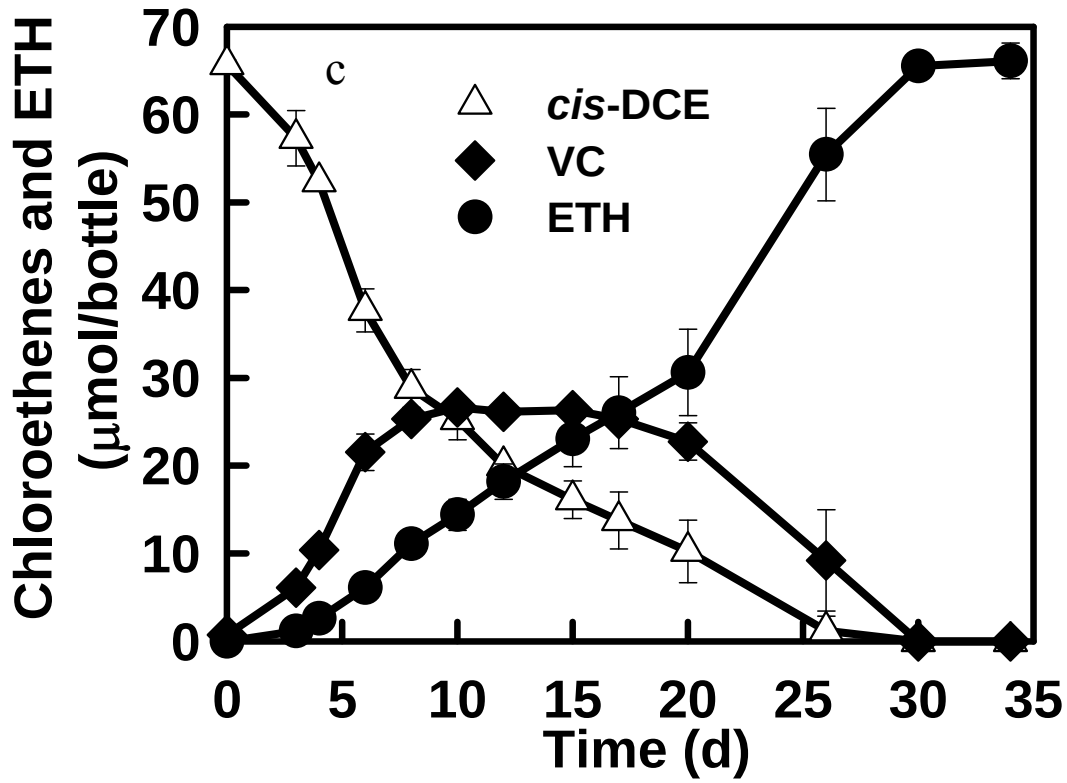
strain FL2. In summary, serial dilution resulted in the separation and isolation of two functionally different *Dehalococcoides* species.

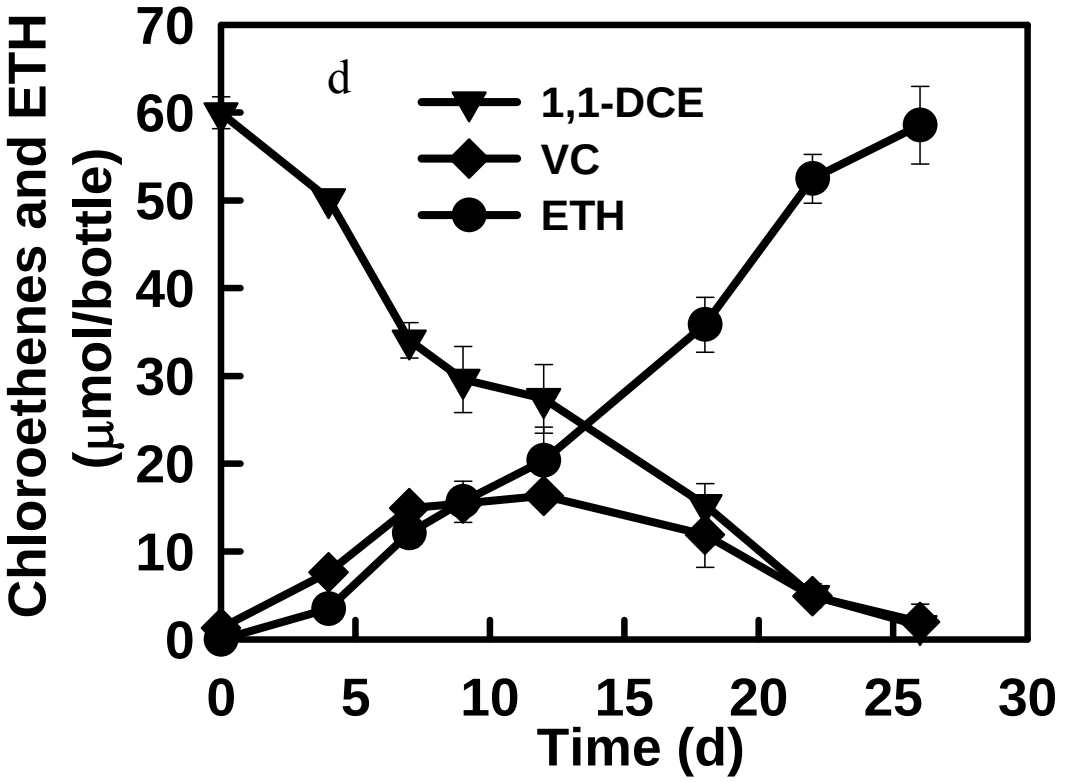
5.3.2 Substrate utilization by culture 11a and 11a5

Two *Dehalococcoides* sp. strains 11a and 11a5 were found to couple reductive dechlorination of TCE, three DCE isomers with growth, while only strain 11a could dechlorinate VC and 1,2-DCA to ethene with growth. Strain 11a differs from strain BAV1 from physiological aspects, such as metabolizing versus co-metabolizing TCE and fast VC dechlorination rate. Fig 5.3a demonstrates that the amount of TCE (52.7 μmol) in the medium spiked with *Dehalococcoides* sp. **s**train 11a decreased at an average rate of 80.8 (±0.2) μmol L^{-1} day^{-1} from day 4 to day 13. In addition to TCE dechlorination, 1,1-DCE, *trans*-DCE, *cis*-DCE, VC, and 1,2-DCA could also be completely dechlorinated to ethene, at rates reaching 53.7 (±0.7), 68.8 (±0.1), 73.2 (±1.2), 407.1 (±2.3), 25.3 (±0.8) μmol L^{-1} day^{-1}, respectively (Fig 5.3). Noteworthy, culture 11a could dechlorinate TCE (Fig 5.3a) within two weeks with extremely low level of VC accumulation (1.72 (±0.02) μmol per bottle), while none of the previous dechlorinators showed similar phenomenon (Fig 5.3). The fast TCE-dechlorination process indicates its promising application for bioremediation. Additionaly, similar to strain 195 (He et al., 2006), strain 11a could cometabolize octa-PBDEs (0.2 mM) to penta-BDE congeners in the presence of TCE (0.5 mM). It could dechlorinate PCE cometabolically in the presence of TCE but could not dechlorinate 1,1-DCAor 1,1,1-TCA even in the presence of TCE or VC.


a
Chloroethenes and ETH (μmol/bottle)
Time (d)
TCE
cis-DCE
VC
ETH
0
10
20
30
40
50
60
0
5
10
15
20
25
30


b
Chloroethenes and ETH (μmol/bottle)
Time (d)
trans-DCE
ETH
0
10
20
30
40
50
60
70
0
10
20
30
40
50
60


c
cis-DCE
VC
ETH
Chloroethenes and ETH (μmol/bottle)
Time (d)
0
10
20
30
40
50
60
70
0
5
10
15
20
25
30
35


d
1,1-DCE
VC
ETH
Chloroethenes and ETH (μmol/bottle)
Time (d)
0
10
20
30
40
50
60
70
0
5
10
15
20
25
30

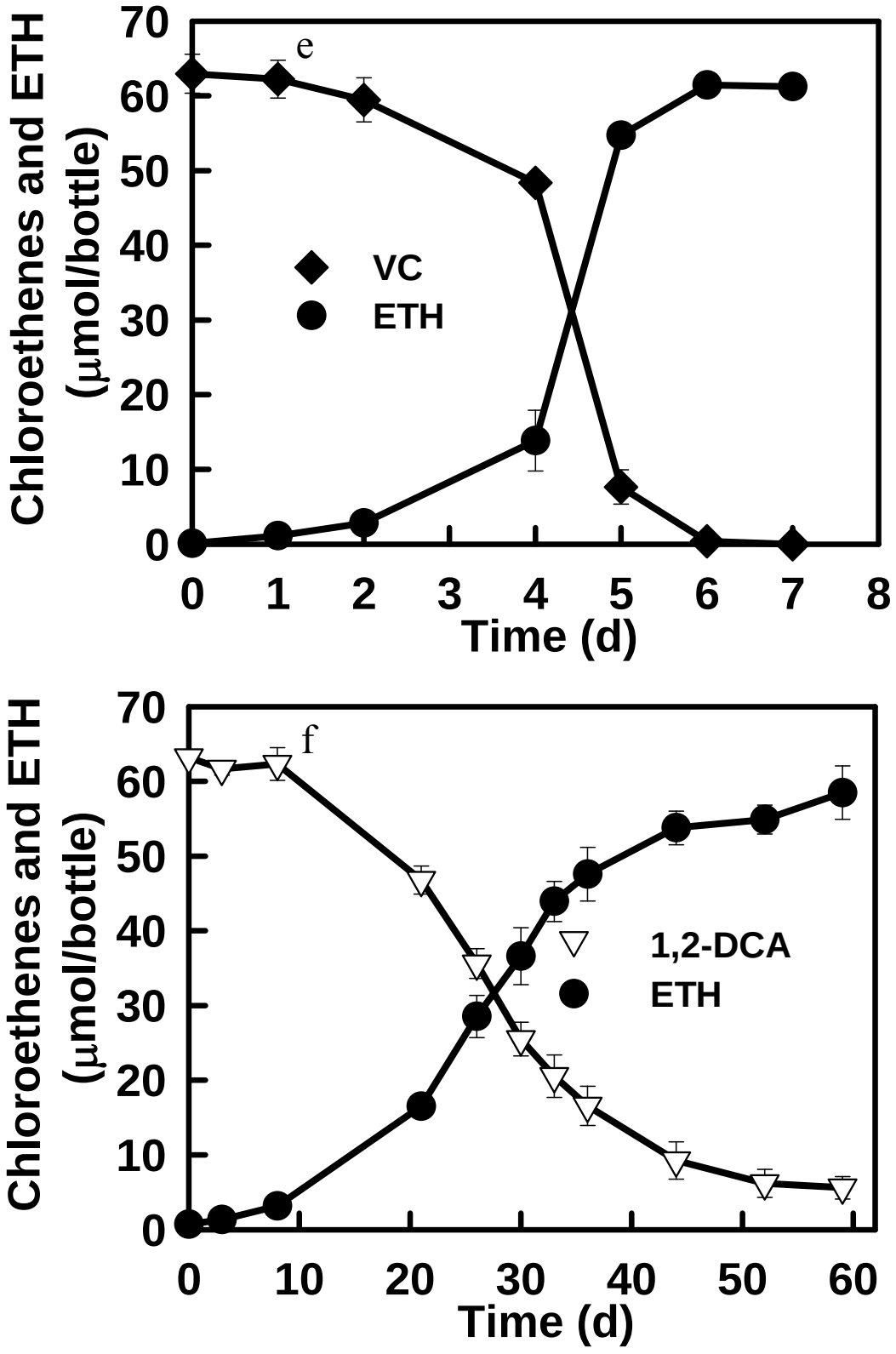


Fig 5.3 Reductive dechlorination of halogenated compounds by *Dehalococcoides* sp. strain 11a. (a) TCE, (b) *trans*-DCE, (c) *cis*-DCE, (d) 1,1-DCE, (e) VC, (f) 1,2-DCA. Data points were averaged from triplicate cultures, and error bars represent standard deviations.

In the process of dechlorination, strain 11a utilized acetate as the carbon source and H_2 as the electron donor. Some of the other common substrates, e.g. lactate, pyruvate, propionate, glucose, succinate, or glutamate were not feasible for anaerobic culturing of strain 11a. Since strain 11a showed advantages in dechlorinating VC than strain 11a5, the performance of strain 11a was thus of greater interest to our application and was studied in details on a biomolecular level.

5.3.3 Functional gene of strain 11a

Previously reported gene-specific primers (for *tceA*, *pceA*, *mbrA, vcrA* or *bvcA*) were used to target the genomic DNA from culture 11a to find out more about the genes that transcribed the RDase. The PCR results suggest that only *vcrA* gene was present in strain 11a, the sequence of which was comparable to those identified from cultures VS, GT, ANAS2, or KB-1. The *vcrA* gene (1482 bp) from culture 11a was found to have 10 bp difference from VS, 9 bp from that of GT, 9 bp with ANAS2, and 8 bp with KB1, up to 99% identity over 1482 bps. There was one-bp mismatch in the reverse primers of the previously published two sets of Taqman primers and probes (Holmes et al., 2006; Ritalahti et al., 2006) as shown in Fig 5.1. These two sets of primers and probe failed to target *vcrA* gene in current culture 11a by qPCR or PCR. Thus a new set of primers and probe (highlight in the square of Fig 5.1) was designed in this study to quantify all the *vcrA*-containing cultures. The specificity was confirmed through BLASTN program and PCR.

5.3.4 Growth and purity confirmation

PCR-DGGE with *Dehalococcoides*-specific primers was conducted to further check the subgroup of *Dehalococcoides* for the isolated cultures (11a and 11a5).

Three bands were observed for the initial mixed cultures after 3 consecutive ampicillin treatments, indicating the existence of at least 3 subgroups of *Dehalococcoides*. In contrast, a single band was observed for culture 11a and 11a5 respectively, suggesting that culture 11a or 11a5 consisted of a single *Dehalococcoides* species as shown in Fig 5.4. It also agrees well with the sequencing results that 11a shared an identical 16S rRNA gene sequence with strain BAV1, while the other isolate 11a5 shared the same sequence with strain FL2. The single band in PCR-DGGE indicates that cultures 11a and 11a5 are likely to be pure cultures.

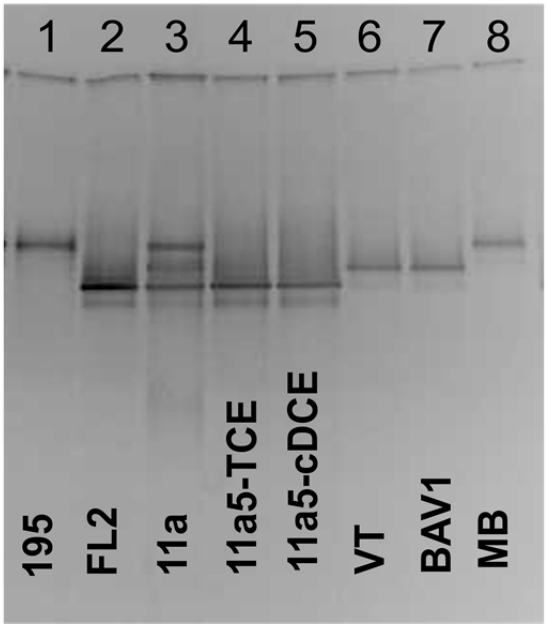


Fig 5.4 Confirmation of the purity for two isolate, *Dehalococcoides* sp. strains 11a and 11a5 by PCR-DGGE. Lane 1, *Dehalococcoides ethenogenes* strain 195; lane 2, *Dehalococcoides* sp. strain FL2; lane 3, TCE-dechlorinating mixed culture 11a after ampicillin treatment; lane 4, 5, *Dehalococcoides* sp. strain 11a5 grown with TCE and *cis*-DCE, respectively; lane 6, *Dehalococcoides* sp. strain 11a grown with TCE; lane 7, *Dehalococcoides* sp. strain BAV1; lane 8, a *trans*-DCE producing pure culture strain MB (positive control).

To further corroborate culture 11a's purity, Taqman qPCR assays were performed to quantify three genes in the genome of culture 11a, i.e., Eubacterial 16S rRNA gene, *Dehalococcoides* 16S rRNA gene, and *vcrA* gene (with newly designed primers and probe mentioned above). After dechlorinating about 50 μmol of TCE at

day 15, the cells of 11a reached a density of $(8.95\pm0.02)\times10^8$ copies ml^{-1} for 16S rRNA gene, and showed an increase of $(8.92\pm0.01)\times10^8$ copies ml^{-1} of *Dehalococcoides* 16S rRNA gene and $(8.94\pm0.03)\times10^8$ copies ml^{-1} of *vcrA* gene (Fig 5.5). The almost identical trend of the three curves indicates that culture 11a consisted of only a single strain of the *Dehalococcoides* species. Additionally, the *bvcA* gene previously identified in culture BAV1 was not found in culture 11a by PCR or qPCR, ruling out the existence of BAV1-like culture.

During the dechlorination of three DCE isomers - 1,1-DCE (60.0 μmol per bottle), *trans*-DCE (66.0 μmol per bottle), and *cis*-DCE (65.7 μmol per bottle), the cells of culture 11a increased 29, 41, and 37 folds to a cell density of $(3.79\pm0.19)\times10^8$, $(4.60\pm0.40)\times10^8$, and $(4.65\pm0.32)\times10^8$ cells ml^{-1}, respectively measured by the *vcrA* gene copies. Rapid dechlorination of the three DCE isomers (up to 0.14, 0.15, and 0.16 μmol $cell^{-1}$ d^{-1} for 1,1-DCE, *trans*-DCE, and *cis*-DCE, respectively) further confirmed that culture 11a, unlike other *vcrA*-gene containing cultures, was able to utilize chloroethenes for growth, including the recalcitrant *trans*-DCE.

No growth was observed for isolate 11a in the absence of chloroethenes, and no dechlorination products were detected during abiotic incubation with the above mentioned substrates (chloroethenes or 1,2-DCA) within the experimental period. Similar studies that were conducted on the isolate 11a5 showed that *tceA* gene instead of *vcrA* gene was involved in the dechlorination of chloroethenes to VC by this isolate (data not shown). No *tceA* gene was detected in pure culture 11a by PCR. In contrast, the earlier mixed culture that included strains 11a and 11a5 showed the presence of four RDase genes, *pceA*, *tceA*, *bvcA*, and *vcrA* gene, indicating the presence of multiple dechlorinator(s) or multiple *Dehalococcoides* spp. In all, a pure culture of

Dehalococcoides sp. strain 11a was obtained to dechlorinate TCE to ethene, and the dechlorination process is encoded by *vcrA* gene.

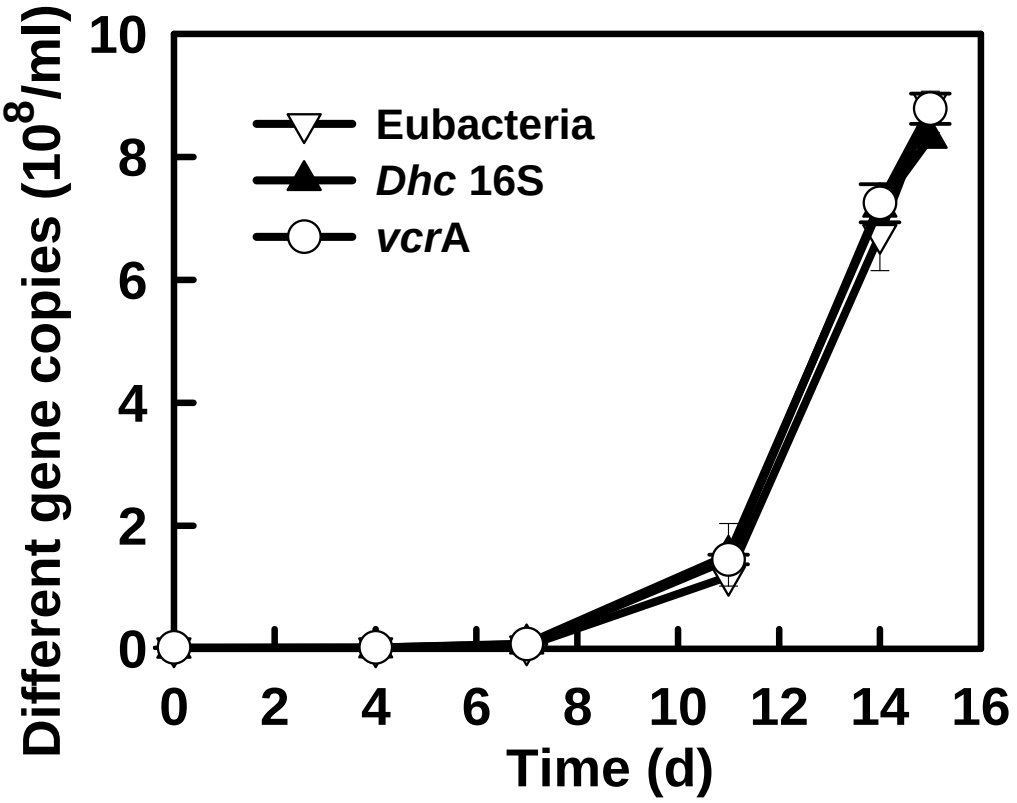


Fig 5.5 Cell growth of pure culture *Dehalococcoides* sp. strain 11a during reductive dechlorination of TCE to ethene as quantified by qPCR.

Isolate 11a showed a doubling time of 1.3 days with TCE as substrate, which is shorter than that of strain GT (2 to 2.5 days) (Sung et al., 2006a) or BAV1 (2.2 days) (He et al., 2003b). During VC dechlorination, the growth yield of culture 11a (2.27[±0.01] g dry wt per mol of Cl^-) was comparable with that of culture VS, (2.2±0.6) g dry wt per mol of Cl^- (*24*); however, the maximum dechlorination rate of culture 11a (407 μmol L^{-1} d^{-1}) was at least 3 times faster than that of VS (127 μmol L^{-1} d^{-1}) (Table 5.2). This accelerated dechlorination rate of VC may be resulted from the reduced doubling time and thus the faster growth rate of strain 11a.

Table 5.2 Comparison of the growth yields and maximum daily dechlorination rates on selected *vcrA* gene-containing *Dehalococcoides* isolates

Substrate	Growth yields (g (dry wt)/mol of Cl^-)		Maximum daily dechlorination rates (μmol L^{-1} d^{-1})		
	11a	ANAS2[A]	11a	GT[B]	ANAS2[A]
TCE	1.16±0.03	0.52±0.04	81	40	47
1,1-DCE	0.65±0.03	0.78±0.07	54	62	53
cis-DCE	0.74±0.01	0.58±0.01	73	41	55
trans-DCE	0.71±0.06	NA	69	NA	NA
VC	2.27±0.01	0.57±0.02	407	127	89
1,2-DCA	ND	NA	25	NA	NA

Note: A, referred to Lee et al. (2011). B, referred to Sung et al. (2006). NA, no activity; ND, not detected.

5.3.5 Putative RDase genes identified from culture 11a

Table 5.3 shows that seven RDase genes named as 11aRdh 1-7 were found in strain 11a and were deposited in GenBank under the accession numbers (HM138513-HM138519), 5 of which are similar to RDase genes in strain CBDB1 (cbdbA187 (or DehaBAV1_173), cbdbA1618, cbdbA1624, cbdbA1627, cbdbA1638) and the other two similar to the RDase genes in culture VS (VSorf1196-*vcrA* and VSorf1137). The sequence analysis of 11a shows that RDase genes 11aRdh1, 11aRdh4, 11aRdh5, 11aRdh7 sharing 99% identity with that from strain BAV1, GT, CBDB1, and VS, while 11aRdh2 and 11aRdh6 share 98% identity with that from strain FL2 and VS respectively, whereas 11aRdh3 only shares about 94% with strains GT, FL2, and CBDB1. No RDase genes of strain 195 were found in 11a by PCR. The diversity of RDase genes in this new isolate provides another evidence for the occurrence of horizontal gene transfer.

Table 5.3 Putative RDase genes identified from isolate *Dehalococcoides* sp. strains 11a and specific primers designed

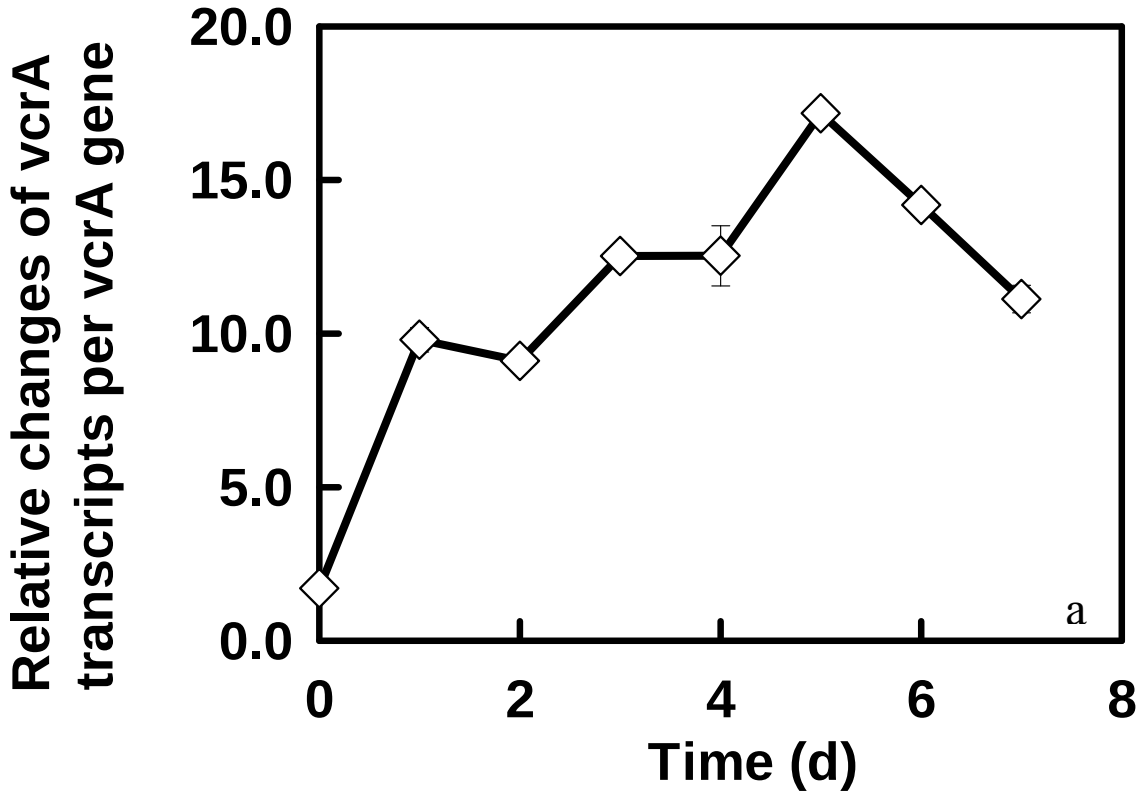
ID	RDase genes	Primer	Sequences (5'-3')	Amplicon	Annealing	Base pair differences
11aRdh 1	DehaBAV1-0173 (cbdbA0187)	Forward	ACC AGA GGC CCG GTA AGT GCA T	736 bp	60 ºC	3 (BAV1), 3 (CBDB1)
		Reverse	AAG GTG CAG GAC TTG CAT CGG C			
11aRdh 2	cbdb_A1618	Forward	TTT TGG GTG CGG AGG AAG GTG C	684 bp	60 ºC	8 (FL2), 13 (CBDB1), 14 (KB1)
		Reverse	CGC AAG GAC CAA ATT CCG GGC T			
11aRdh 3	cbdb_A1624	Forward	ACG TCA GGA CTA CCT CAC TCT G	789 bp	55 ºC	40 (FL2), 40 (CBDB1), 41 (KB1)
		Reverse	TAA GCC CGA AAG GAC AAG CAT C			
11aRdh 4	cbdb_A1627	Forward	CCG CTC TTA ACG CAG GGG CAA A	629 bp	60 ºC	0 (CBDB1), 3 (KB1), 4 (FL2)
		Reverse	AGC CGG TGA AGG ACA AAT GCC G			
11aRdh 5	cbdb_A1638	Forward	CGC TGC CGA ACT GGC TGA AAG A	672 bp	60 ºC	0 (FL2), 13 (KB1), 17 (CBDB1)
		Reverse	AGT TTC TGG CGG CTG GCT TCA C			
11aRdh 6	VSORF1137	Forward	CAG CGT TAA AGC ATG CTG GTG	857 bp	55 ºC	12 (VS)
		Reverse	ACA GAT ACC ACA GGT CTC GCA			
11aRdh 7	VSORF1196 (*vcrA*)	Forward	CTA TGA AGG CCC TCC AGA TGC	1482 bp	50 ºC	10 (VS), 9 (ANAS2)
		Reverse	GTA ACA GCC CCA ATA TGC CAA GTA			

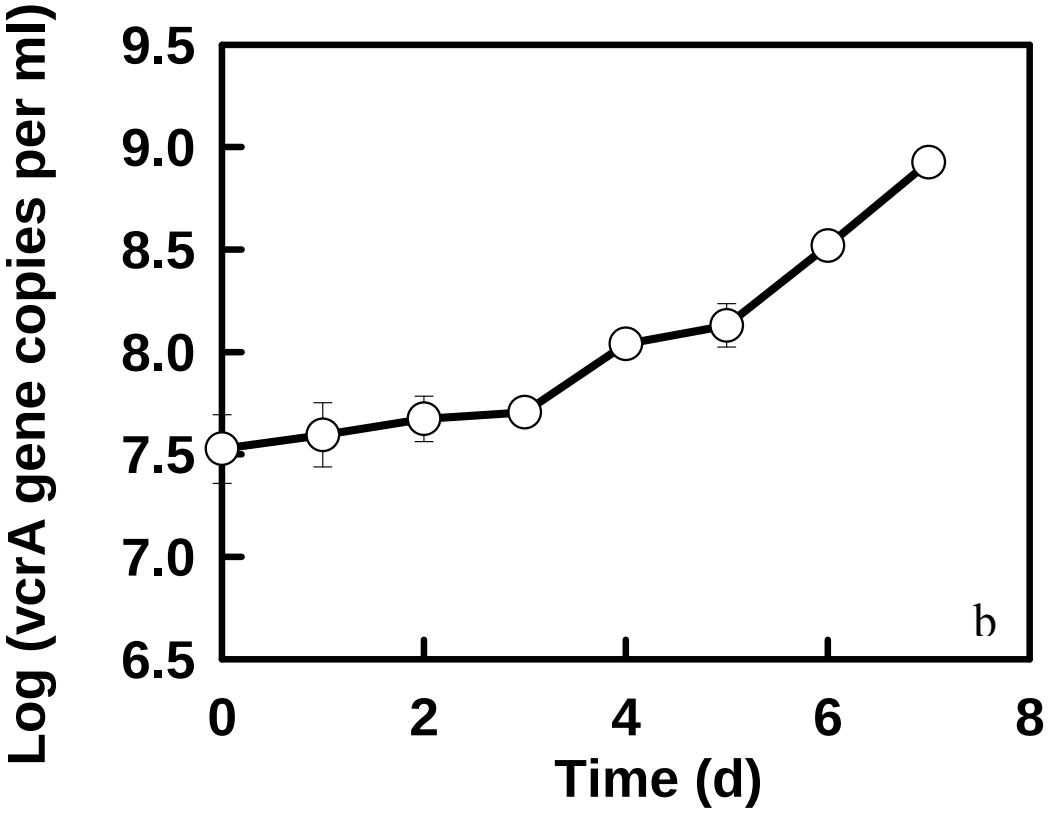
Note: *, reference Müller et al (2004).

5.3.6 The role of VcrA in strain 11a during dechlorination of TCE, *trans*-DCE and VC

It was hypothesized that the *vcrA* gene could be related to the rapid dechlorination of VC and TCE. The expression of *vcrA* gene was conducted for starved cells of culture 11a when exposed with 61.3 μmol of VC (Fig 5.3e). During the complete dechlorination of VC (Fig 5.3e), the maximum expression (17 folds) of *vcrA* gene was found at day 5 (Fig 5.6a), close to the end of the exponential growth phase (Fig 5.6b). After day 5, a slight decrease was observed for the transcription level of *vcrA*, probably due to the faster increase in the DNA copies (intensive growth of culture 11a with VC) when dechlorination of VC was nearly completed.

To further understand the role of *vcrA* gene in TCE dechlorination, *vcrA* gene expression profile within the first 24 h was captured. The expression of *vcrA* in pure culture 11a was up-regulated about 40 folds during the first 12 hours' exposure to TCE. The observed up-regulation of the *vcrA* gene (about 40-fold) of strain 11a on TCE is comparable with that of strain 195 on TCE (about 30-fold) (Lee et al., 2006). The transcript levels of *vcrA* gene exposed to TCE were found to be higher than that of VC (Fig 5.6c), indicating that this *vcrA* gene could be functionally important for the respiration of both TCE and VC. Additionally, an up-regulation of *vcrA* gene (about 30 fold) was also observed in the presence of *trans*-DCE (~ 68 μmol) after 24 hrs. Different from culture VS, strain 11a shows unique capability of coupling *trans*-DCE with growth (Table 5.2) and *vcrA* gene expression.


Relative changes of vcrA transcripts per vcrA gene
20.0
15.0
10.0
5.0
0.0
0
2
4
6
8
Time (d)
a


Log (vcrA gene copies per ml)
9.5
9.0
8.5
8.0
7.5
7.0
6.5
0
2
4
6
8
Time (d)
b

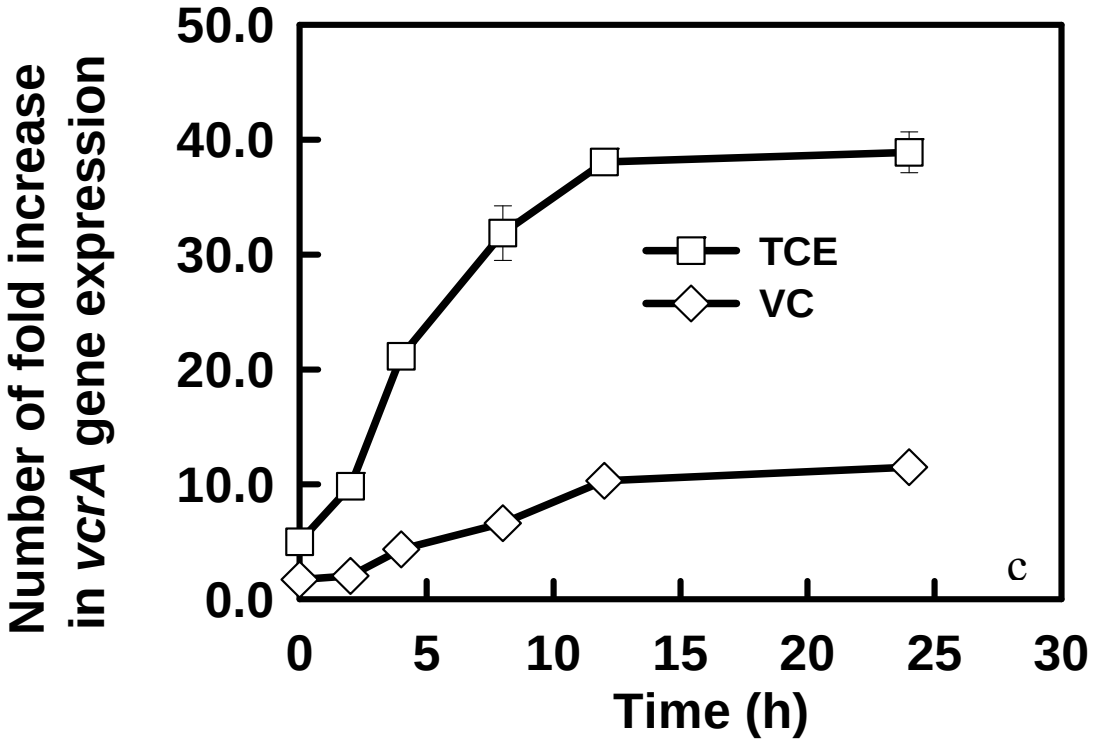


Fig 5.6 The role of vcrA gene during reductive dechlorination of TCE and VC by *Dehalococcoides* sp. strain 11a. (a) Gene expression of *vcrA* gene with TCE and VC, (b) change of transcript numbers of *vcrA* for strain 11a, (c) growth of culture 11a during 7-day dechlorination of VC. Data points were averaged from triplicate runs, and error bars represent standard deviations.

The role of VcrA in dechlorination of *cis*-DCE or 1,1-DCE was proven previously (Müller et al., 2004), whereas the discrepancy occurs for *trans*-DCE. The purified enzyme VcrA showed dechlorination ability for *trans*-DCE, however the previously found *vcrA*-containing active cultures failed to use this substrate including culture VS, KB-1, GT or ANAS. The SDS-PAGE was conducted to check the role of VcrA in the active *trans*-DCE dechlorinating culture 11a as compared to TCE. Fig 5.7 shows that the same major band (~ 62 kDa) was found to have highest expression during the reductive dechlorination of TCE and *trans*-DCE. The position of this expressed 62-kDa band agrees well with the VC-dechlorinating enzyme, VcrA found in culture VS (Müller et al., 2004), suggesting the functional role of VcrA of strain 11a in dechlorination of not only VC, but also TCE and *trans*-DCE. This versatile

dechlorinating activity was consistent with the activity of in vitro assay of VcrA purified from mixed culture VS (Müller et al., 2004) and agreed well with the cell growth measured by qPCR during the dechlorination of *trans*-DCE by 11a (Table 5.2). The results of this study showed that the highly expressed RDase (VcrA) in culture 11a has broader substrate range than that of culture VS or GT in that neither of these active cultures showed metabolic activity for *trans*-DCE. In other words, the SDS-PAGE results together with 11a's physiology and qPCR results confirm that the VcrA in culture 11a encodes the dechlorination of VC, all DCEs isomers and TCE to ethene.

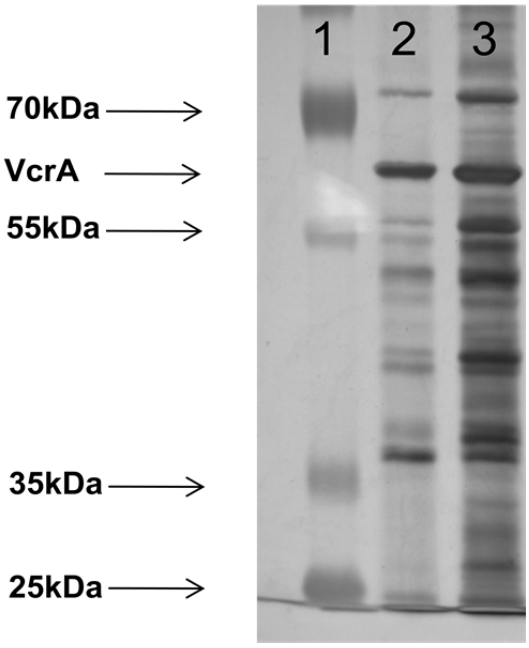


Fig 5.7 SDS-polyacrylamide gel of VC-reductive dehalogenase of *Dehalococcoides* sp. strain 11a. Lane 1, prestained pageruler protein ladder; lane 2 and 3, active cell extract of *trans*-DCE and TCE, respectively (after gel filtration with sizes of major peptides).

The variability of culture 11a's *vcrA* gene sequences as compared with other TCE-to-ethene cultures resulted in amino acid substitutions at the C terminus of the protein (Fig 5.8), suggesting their close relationship. As shown in Fig 5.8, the active

site of VcrA RDase could be represented with two motifs of iron-sulfur clusters-$CX_2CX_2CX_3CP$ (Müller et al., 2004) identified in the previous *vcrA* gene-containing *Dehalococcoides* species (e.g., culture ANAS2, GT, VS, or KB-1). The first motif of strain 11a contains a histidine (H) after the second cysteine (C) instead of tyrosine (Y), while the second motif displays a threonine (T) before the fourth cysteine (C) instead of serine (S). These amino acid substitutions might have accounted for the differences in the biocatalytic response to chloroethenes. The relationship between the RDase structure changes and the activity of the protein will be rationalized in the discussion section.

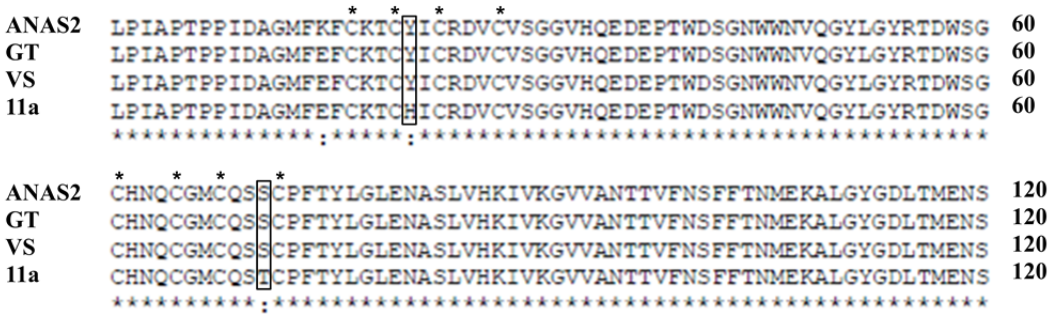


Fig 5.8 ClustalW alignment of the VcrA sequence of *Dehalococcoides* sp. strain VS (AY322364), ANAS2 (HM241732), GT (CP001924) and 11a (HM138519). *, conserved cysteines in the two iron-sulfur clusters binding motifs ($CX_2CX_2CX_3CP$). The varied regions are boxed.

5.3.7 Dechlorination of PCE to ethene by a coculture

The microcosm study was performed to evaluate the bioremediation of chloroethenes-contaminated groundwater carrying some sediment by bioaugmenting the newly isolated *Dehalococcoides* sp. strain 11a. It is found that *Dehalococcoides* sp strain 11a alone did not show any activity for chloroethenes (e.g., PCE- or TCE-contaminated groundwater) without the amendment of nutrients or reductants. After

amendment of lactate and vitamins, TCE was completely dechlorinated to ethene within 9 days by strain 11a. However, PCE dechlorination did not occurred after 60 days incubation. Therefore, a PCE-to-*cis*-DCE dechlorinating culture - *Sulfurospirillum multivorans* (Fig 5.9) and culture 11a was co-inoculated to the PCE-spiked microcosm. Surprisingly, this coculture was able to completely detoxify the source contaminant PCE to ethene in both of the collected contaminated groundwater samples within 10 days (Fig 5.10a) and the defined medium (control) (Fig 5.10b) within 16 days. The bioaugmention for groundwater was found to proceed relatively faster than the defined medium, probably due to the presence of unknown trace amounts of nutrients. Either culture 11a or *S. multivorans* alone would not generate ethene regardless of the nutrients added.

Fig 5.9 shows the growth of pure culture of *S. multivorans* during reductive dechlorination of about 50 µmols of PCE to *cis*-DCE within 2 days in defined medium, as revealed by 40-times increase in 16S rRNA gene copies by qPCR. Fig 5.10b shows complete detoxification of PCE by the same co-culture in defined medium. As a robust indicator for *cis*-DCE dechlorination, the generation of ethene was found to be correlated to the increased percentage of cells of culture 11a (as measured by *vcrA* gene copies) over the total co-culture cell numbers from 1% to 65% by qPCR during microcosm incubation (Fig 5.11). No activity was detected for the control bottles. It is concluded that bioaugmentation with co-culture (11a and SM) could successfully remediate chloroethene-contaminated groundwater.

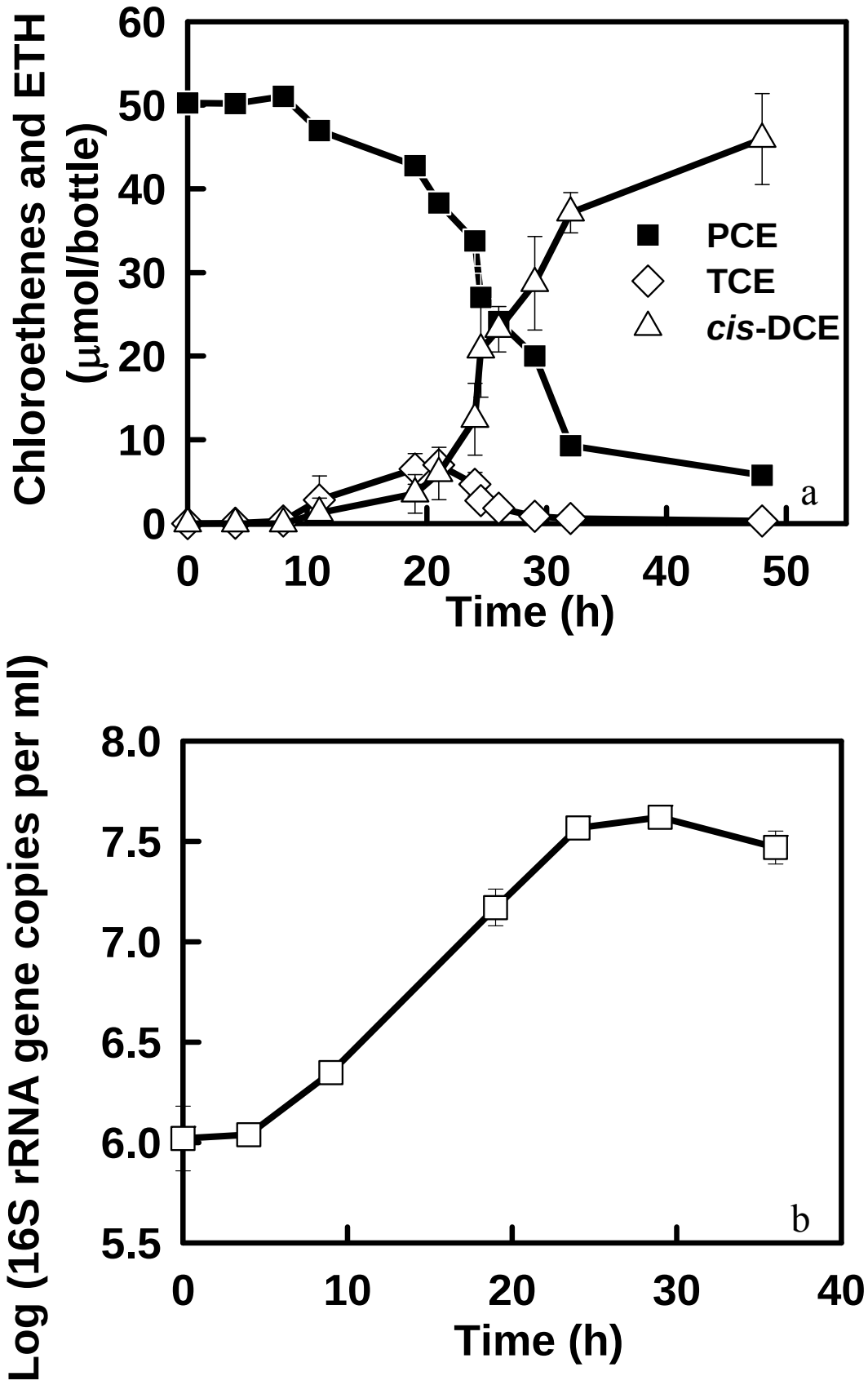


Fig 5.9 Dechlorination of PCE (a) by *Sulfurospirillum multivorans* only and its growth (b) in defined medium.

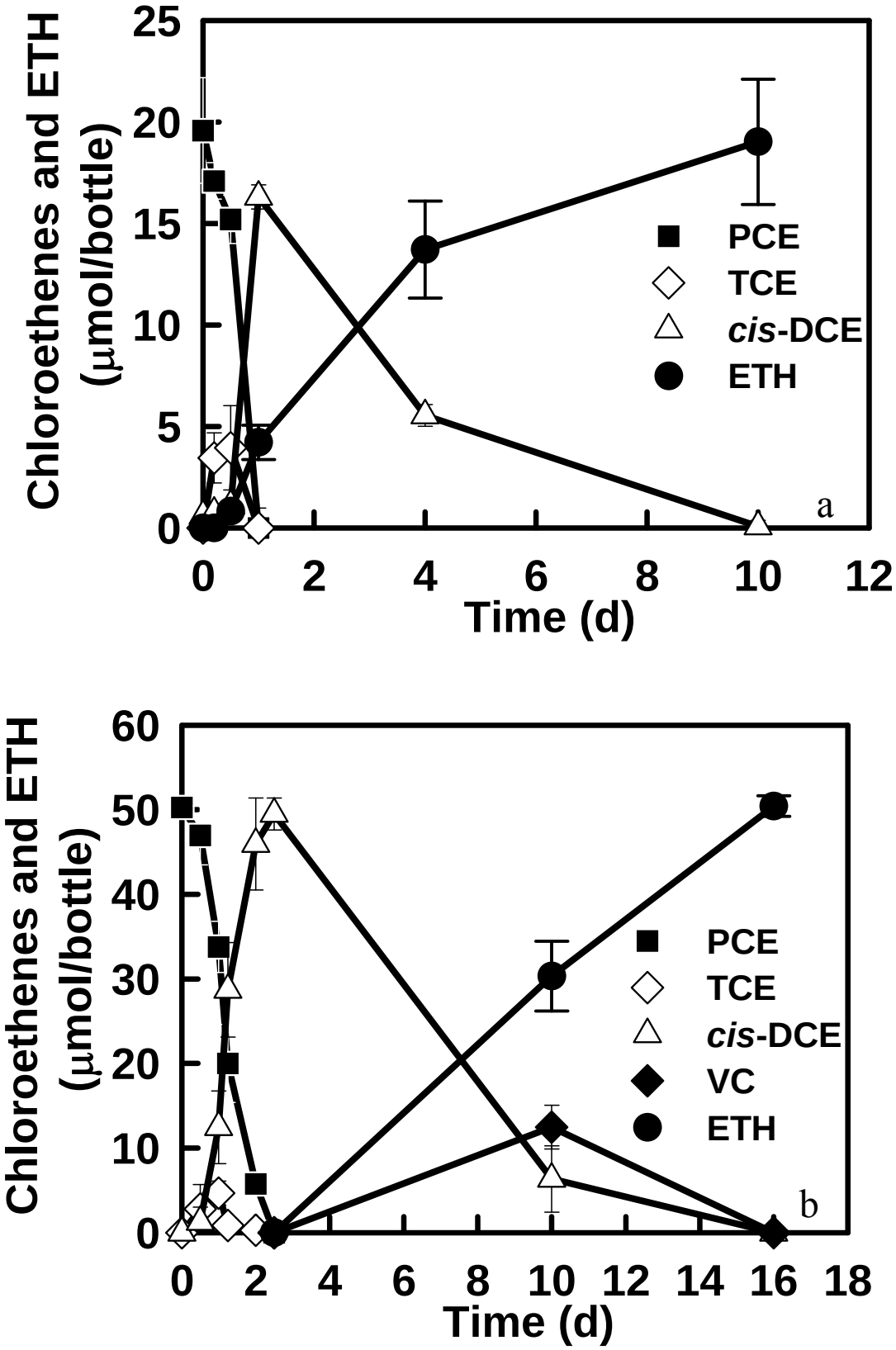


Fig 5.10 Dechlorination of PCE by a coculture containing *Sulfurospirillum multivorans* and *Dehalococcoides* sp. 11a. (a), in chloroethene- contaminated groundwater; (b), in defined medium.

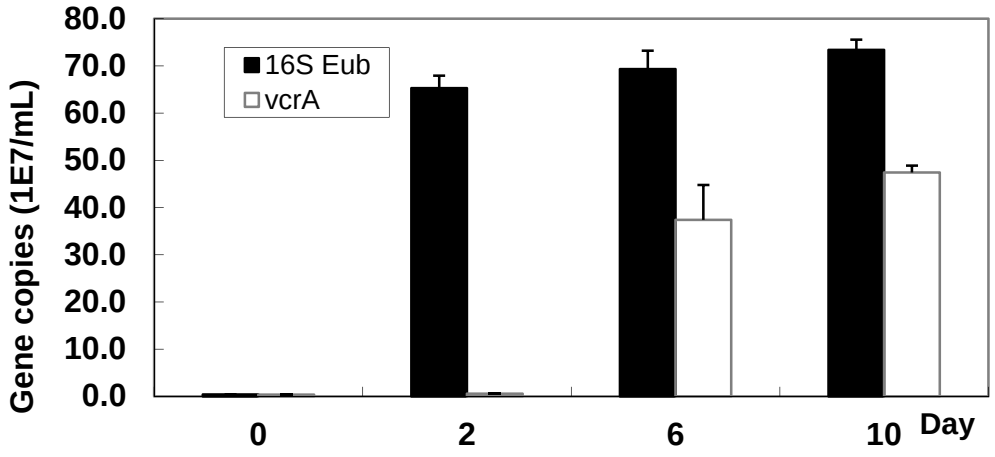


Fig 5.11 Relative abundance of *Dehalococcoides* sp. 11a in the co-culture (with *Sulfurospirillum multivorans*) during the remediation of contaminated groundwater as revealed by qPCR. Note, the dechlorination process is shown in Fig 5.10a.

5.4 Discussion and conclusion

The newly identified *Dehalococcoides* sp. strain 11a is capable of rapid detoxification of chloroethenes to ethene. Apart from the ability to dechlorinate 1,1-DCE, *cis*-DCE and VC (i.e. a common trait among *Dehalococcoides* sp.), the VcrA RDase in strain 11a demonstrated an essential role in dechlorinating *trans*-DCE and TCE to ethene. The rapid dechlorination capability of strain 11a is reflected by the minimal VC accumulation (<2 μmol) during the dechlorination of *trans*-DCE and TCE (50-60 μmol) in the 100-ml defined medium, while strains BAV1 and FL2 usually show 35 μmol of accumulated VC intermediate during the dechlorination of similar amount of *trans*-DCE under similar conditions (He et al., 2003a; He et al., 2005). Strain 11a is also able to grow with 1,2-DCA, which has never been reported for other *vcrA* gene-containing strains. Additionally, strain 11a could transform PCE cometabolically in the presence of TCE, which also distinguishes 11a from all other VC-dechlorinating isolates, such as BAV1 (from DCEs to ethene) (He et al., 2003b),

ANAS2 (from TCE, *cis*- and 1,1-DCEs to ethene) (Lee et al. 2011), VS (from TCE, *cis*- and 1,1-DCEs to ethene) (Müller et al., 2004) or GT (from TCE, *cis*- and 1,1-DCEs to ethene) (Sung et al., 2006). The maximum daily VC dechlorination rate by 11a was found to be 3.2 times higher than that of GT as revealed by the dechlorination time course study. The average VC-dechlorination rate (86 μmol L^{-1} day^{-1}) by 11a was 4 times higher than isolate BAV1 (20 μmol L^{-1} day^{-1}) (He et al., 2007). Therefore, the fast dechlorination rate of culture 11a shows most promising application in various chloroethene-impacted sites, including those sites with *trans*-DCE accumulation.

Previous studies have suggested that the expression level of RDase genes is intimately related to the corresponding substrates that the dechlorinating culture utilizes. Lee et al (2006) reported that the expression level of *tceA* gene was up-regulated to ~ 30-fold during 12-h exposure to TCE for *Dehalococcoides etheneogenes* strain 195 (Lee et al., 2006), while Chow et al (2010) found that the *mbrA* gene was up-regulated about eight-fold after 72-h exposure to PCE for *Dehalococcoides* sp. strain MB (Chow et al., 2010). The elevated transcription levels (up to 40-fold) of *vcrA* gene to TCE, *trans*-DCE and VC for strain 11a was also closely related to its metabolism of chloroethenes and indicated that the *vcrA* gene in strain 11a could be functionally important for the respiration of these chloroethenes. It also suggests that strain 11a had a broader substrate range than culture VS, GT or ANAS2, given that none of them could metabolize *trans*-DCE. Essentially, these results together with 11a's physiology and qPCR results suggest that the VcrA in culture 11a encodes the dechlorination of VC, all DCEs isomers and TCE to ethene.

While the *vcrA* gene is commonly present among several *Dehalococcoides* species, plasticity in the protein expressed from the *vcrA* gene, particularly the active

iron-sulfur clusters, could potentially lead to differences among various *Dehalococcoides* strains in their biocatalytic responses to chloroethenes. This is because changes to the primary sequence, particularly when occurring near or at the active sites, might alter the conformation of the sites and lead to changes in the protein activity and substrate specificity (Yoshikuni et al., 2006). In the case of strain 11a, amino acid substitutions were found in the iron-sulfur clusters (Fe_4S_4) - important structures in mediating electron transfer (Müller et al., 2004; Ciurli and Musiani, 2005), therefore, these changes might be related to the enhanced rate of reductive dechlorination and the broader substrate diversity. The substitution of "H" in strain 11a for "Y" in strain VS introduced a basic imidazole group (i.e. pK_A ~7.0) to strain 11a. Since histidine (H) is suspected to be involved in electron donation and acceptance particularly for dehalogenation (Marsh and Ferguson, 1997; Smidt et al., 2000; Hölscher et al., 2004) and the incorporation of histidine amino acid substitutions in the active site of cytochrome P450 enzymes has resulted in the most significant rate enhancement for the His 96 mutant in the protonated state (Manchester and Ornstein, 1996). This imidazole group, while protonated, might develop transient electrostatic interactions between the active site of VcrA and nucleophiles (e.g. hydride ions). Such interactions could have potentially helped to mediate electron transfers that were essential to reductive dechlorination, thus directly contributing to a broader substrate utilization range and consequently a faster dechlorination for strain 11a. On the other hand, the substitution of "S" in VS to "T" in 11a added one side chain ($-CH_3$). Since the substitutions by bulkier side chains has been found to weaken the binding of chloroethane but increase the dehalogenation rate (Walsh et al., 2000), this substitution might reinforce the stability of VcrA with faster dechlorination rate. However, the precise function of this evolution remains to

be further elucidated. Future analysis of the active binding site of VcrA, by applying the homology models and X-ray crystallography (Damborský and Koča, 1999) (both theoretical and experimental) will be necessary to fully understand the unique activity of the enzyme system for strain 11a, which is also important for developing an enhanced bioremediation system.

Bioaugmention is considered as a technically feasible and cost-effective remedy for the high concentrations of multiple halogenated contaminants in groundwater (GeoSyntecConsultants., 2005; Ritalahti et al., 2005). The fast metabolism of isolate 11a on TCE, all the DCEs isomers, VC, and 1,2-DCA has proven its practical advantages in terms of efficient and cost-effective bioaugmentation in chlorinated ethene- or chlorinated ethane- contaminated sites. This fast metabolism also helped to minimize the competitive inhibition of higher chlorinated ethenes on the lesser chlorinated ethenes (Yu et al., 2005). The additional amendment of nutrients (e.g., lactate or vitamins) ensured the success of immediate bioremediation in the presence of strain 11a-like *Dehalococcoides* and *S. multivorans*. At contaminated sites, it would be cost-effective to treat the entire contaminant plume over a short period; the rapid detoxification of chloroethenes (with the concentration comparable to the level of field sites) by culture 11a would thus significantly reduce both the time and the cost for bioremediation. In all, culture 11a is able to effectively dechlorinate a wide variety of chlorinated compounds including TCE and *trans*-DCE to ethene. The variation of VcrA sequences suggests that culture 11a may have been adapted to the environment under intense evolutionary pressure. The proposed bio-inocula could be encouraging for the clean-up of numerous chloroethene-contaminated sites as the complete detoxification of PCE is readily achievable without accumulating the carcinogenic VC.

Chapter VI Conclusion and Recommendations

6.1 Conclusion

This thesis work was originated from the novel cultures involved in both partial dechlorination of PCE or TCE to various amounts of *trans*-DCE and complete dechlorination of PCE to ethene via predominant intermediate *trans*-DCE. Three *Dehalococcoides* isolates, named MB, 11a and 11a5 were obtained and characterized with respect to specific dehalogenating capacities. The key conclusions made in this study are listed below.

A number of microcosms containing Cornell subgroup of *Dehalococcoides* species could produce various amounts of *trans*-DCE through the enrichment process. These screened cultures showed different capacity in dechlorinating PCE or TCE as compared with *Dehalococcoides ethenogenes* strain 195, the first isolate of Cornell subgroup. The screened microcosms, MB, SB and GY, originally generated *trans*-DCE and *cis*-DCE as the final dechlorination products in a ratio of (1.4±0.1):1, (0.4±0.06):1, and (2.8±0.3):1, respectively. The ratio of *trans*-DCE to *cis*-DCE in the final products has been found to be positively correlated with the proportion of specific Cornell subgroup of *Dehalococcoides* over the total community. The identified co-culture MB and other *trans*-DCE producing cultures show an inability to dechlorinate PCE/TCE beyond DCEs to VC and ethene. This process was found to be mainly catalyzed by a novel RDase gene, instead of those commonly found *pceA* gene, *tceA* gene, *bvcA* gene, or *vcrA*. It also complimented the current understanding about the generation of *trans*-DCE, not only by those Pinellas subgroup of *Dehalococcoides* spp., but also new members of Cornell subgroup of *Dehalococcoides* spp.

To understand the generation of *trans*-DCE, pure culture *Dehalococcoides* sp. strain MB was eventually isolated from environmental sediments. Strain MB reductively dechlorinates PCE to *trans*-DCE and *cis*-DCE in a ratio of 7.3 (± 0.4) : 1, which is found to be the highest *trans*-/*cis*-DCE ratio as compared to previous reports on *trans*-DCE producing mixed cultures. Different ratio of MB and ANAS1 (unpublished information, a TCE-to-*cis*-DCE-to-VC-dechlorinating culture) resulted in different profiles of *trans*-DCE and *cis*-DCE in the dechlorination products. It has been found that competition among different dechlorinators would determine the fate of chloroethenes at contaminated sites.

Fourteen putative RDase genes were identified from isolate *Dehalococcoides* sp. strain MB by using comparative genomic study (10 from microarray analysis on the basis of strain 195 genome) and biomolecular tools (7, but 3 of them are found by both method). Of the 1,579 probe sets on microarray targeting genes from *D. ethenogenes* strain 195, the genomic DNA of isolate MB hybridized to 1389 (88.0%), indicating that genes are highly conserved between strains 195 and MB. New strain MB also demonstrates a lack of large proportions of genes found in strain 195, such as integrated elements (73.7% absent) and the phage-related genes by microarray analysis. This study has provided a direct evidence for the accumulation of *trans*-DCE that MB-like culture is present or novel RDase gene *mbrA* is highly expressed in PCE/TCE-contaminated sites. The RDase gene, *mbrA* gene responsible for dechlorinating PCE to *trans*-DCE is found to be 98% similar to the putative RDase gene (cbdbA80) of *Dehalococcoides* sp. strain CBDB1. This novel *mbrA* gene can be served to quantify *trans*-DCE producing cultures. In a mixed community, more *trans*-DCE than *cis*- DCE could be produced when *mbrA* gene-containing culture is at least four times more than the *tceA* gene-containing culture. The obtained dechlorinating

pure culture MB could give detailed information about the diversity of microorganisms in the natural environments and also provide fundamental understanding of the diverse mechanisms involved. Both of the factors studied here may be of great importance to investigate the correlation of the novel *mbrA* gene with the relevant microbes in order to understand the presence of other *trans*-DCE-producing cultures in the contaminated sites.

Dehalococcoides sp. strain 11a was obtained to be able to rapidly dechlorinate TCE, all DCE isomers, VC, and 1,2-DCA metabolically to ethene, catalyzed by VcrA. An ERD solution employing co-culture of *Dehalococcoides* sp. strain 11a and *Sulfurospirillum multivorans* demonstrated the complete detoxification for PCE-contaminated groundwater without any accumulation of chloroethene. Isolate 11a can be successfully applied in rapid bioremediation of chloroethene-contaminated sites for complete detoxification.

The enrichment culture 11a (including pure *Dehalococcoides* sp. strain 11a) screened in this study proves to be a promising candidate in the complete dechlorination of PCE/TCE via predominantly *trans*-DCE to ethene through the cooperation of multiple *Dehalococcoides* species. The highest ratio of *trans*-DCE and *cis*-DCE was found to be 1.7:1 in lactate-amended medium by culture 11a. It also demonstrated that the metabolism of this novel strain (11a) of *Dehalococcoides* would be of great importance for the bioremediation of chloroethene-contaminated sites with significant accumulation of *trans*-DCE or PCE.

In all, pure culture MB has facilitated detailed studies on the genomic contents of new species prior to the whole genome sequencing by comparative genomics. The origins of *trans*-DCE in natural environments were postulated through the identified functional gene studies (*mbrA* gene) as compared with the commonly detected *tceA*

gene involved with reductive dechlorination of TCE. The isolation of strain 11a confirmed that TCE, *trans*-DCE, 1,1-DCE and 1,2-DCA could also serve as electron acceptors besides those commonly detected compounds, *cis*-DCE and VC as summarized in Table 6.1. Complete detoxification of PCE to ethene in the contaminated site was achieved with the co-culture of strain 11a and a PCE-dechlorinating isolate *Sulfurospirillum multivorans*. Complete detoxification of PCE via predominantly *trans*-DCE could also be carried out by two pure cultures isolated therein, the novel *trans*-DCE producing culture(s) *Dehalococcoides* sp strain MB and the *trans*-DCE dehalogenating culture *Dehalococcoides* sp strain 11a. Results from this study would significantly improve the treatment efficiency towards halogenated compounds both in the engineered systems and *in situ* bioremediation sites.

Table 6.1 Summary of chloroethene utility profile by current *Dehalococcoides* isolates.

Substrate	195	CBDB1	BAV1	FL2	VS	GT	MB	ANAS1[b]	ANAS2[b]	DCMB5	11a	11a5
PCE	+	-	-[a]	-[a]	-	-	+	-	-	NA	Co	-
TCE	+	-	-[a]	+	Co	+	+	+	+	NA	+	+
1,1-DCE	+	-	+	+	+	+	-	+	+	NA	+	+
trans-DCE	Co	-	+	+	NA	-	-	-	-	NA	+	+
cis-DCE	+	-	+	+	+	+	-	+	+	NA	+	+
1,2-DCA	+	-	+	-	NA	-	-	-	NA	NA	+	NA
VC	Co	-	+	Co	+	+	-	-	+	NA	+	-

Notes: "Co" refers to co-metabolic process and "NA" means information not available. a, Cometabolic reaction takes place in cultures with another growth supporting electron acceptor. b, unpublished information.

6.2 Recommendations

The pure culture *Dehalococcoides* sp. strain MB obtained in this work provides scientific basis for understanding the production of *trans*-DCE during reductive dechlorination of PCE and TCE. Also, *Dehalococcoides* sp. strain 11a has

demonstrated its metabolic capacity towards all chloroethenes and 1,2-DCA with rapid dechlorination rates. A novel dechlorination pathway from PCE to ethene via *trans*- and *cis*-DCEs can thus be elaborated. However, several knowledge gaps still exist in this body of thesis and there are four main areas in which we feel further research are needed.

Firstly, further studies of other ubiquitously distributed halogenated compounds, e.g. ethylene dibromide (McConnel, 1984; Henderson et al., 2008), 1,1,1-TCA, chlorinated benzenes, and polychlorinated dibenzo-*p*-dioxins, could also be helpful in elucidating the potential role of strain MB, 11a, and 11a5 in bioremediation. Bacteria of the genus of *Dehalococcoides* are known to be extraordinarily specialized in detoxifying halogenated compounds. Several *trans*-DCE producing culture have shown their capability of dehalogenating PCBs, chlorobenzenes, dioxins etc (Futamata et al., 2007; Miller et al., 2005; Yoshida et al., 2005). This study also found that strain MB was able to debrominate one of the commercial PBDE mixtures (octa-BDE) to penta-BDE and finally stopped at tetra-BDE cometabolically with the dechlorination of TCE. It is also documented from the sequence of the putative RDase gene *dceA1*, which showed a classical aromatizing enzyme for dehalogenating complicated organic compounds. However, we have found no obvious expression of this gene when exposed to PCE or TCE. Thus it is desirable to further explore other potential substrate for this *trans*-DCE producing culture MB. In addition, through the comparison of the key RDase, VcrA, culture 11a has shown a unique adaptation of metabolic capacity towards various chlorinated ethenes and ethanes (like 1,2-DCA) as compared with other VcrA-related culture. It is also of importance to investigate its potential role in TCA-contaminated groundwater through the given pressure of adaption as limited information is available for the complete detoxification of TCA by

Dehalococcoides. Last but not the least, the collected groundwater was used as a demonstration of bioaugmentation study to estimate the site dechlorination rate by the proposed coculture containing strain 11a, which may not reveal the real conditions for the contaminated sites. Therefore, it is desirable to conduct more site investigation and *in situ* bioremediation in large scale for better illustration of its real application.

Secondly, sequencing of *Dehalococcoides sp.* strains MB and 11a genome will provide additional insight into these two novel strains and allow more in-depth understanding on the role of other putative RDase genes, in addition to *mbrA* and *vcrA*, respectively. The comparison of genome sequences between strain CBDB1 and 195 shows that CBDB1 has a greater potential as a dehalogenator than strain 195, probably due to higher number of RDase genes in strain CBDB1 than strain 195 (Kube et al., 2005; Seshadri et al., 2005). *Dehalococcoides* seems particularly well adapted to carry out respiratory reductive dehalogenation. Genome sequencing could give us a simpler but more direct and more powerful way to study the evolutionary relationships among different isolates within this unique genus of *Dehalococcoides*. However, the genomic contents of novel cultures, particularly for strain 11a, are largely unknown in current study. Potential novel RDase genes, phage-related genes, and comparison of genomic contents of all *vcrA*-containing isolates should hence be evaluated and particularly addressed. Transcriptional analysis of all the identified RDase genes and sequencing of the most expressed enzyme shall also be done for the new *Dehalococcoides* isolates, MB, 11a5, and 11a.

Thirdly, the unique disc-shaped morphology of *Dehalococcoides* spp. indicates the potential attachment of *Dehalococcoides* cells onto a flat surface, especially those highly used nanomaterials. This may greatly increase the chance of stabilizing *Dehalococcoides* with the indigenous microorganisms thus promoting the attachment

of cells with the substrates available in the contaminated sites. It may significantly reduce the reaction time as well as the treatment cost for efficient bioremediation strategy.

Lastly, there is great necessity in reducing the cost used for cultivating *Dehalococcoides* in large-scale at the bioremediation sites. It is known *Dehalococcoides* spp. are difficult to grow as they are strictly anaerobic cultures. Addition of extra vitamin B_{12} could result in at least two times higher density than that without addition of vitamin B_{12} (He et al., 2007a). Therefore, investigation of microorganisms capable of producing vitamin B_{12} for *Dehalococcoides* and collaborating with *Dehalococcoides* would be of scientific and commercial interest to bioremediation community. On the other hand, *Dehalococcoides* needs acetate as the sole carbon source for growth, hydrogen as electron donor for reductive dehalogenation, and high salt solution in the medium. Both acetate and hydrogen can be biologically supplied from the side products during the bioconversion of tropical waste into renewable energy sources, such as bioethanol or biobutanol, mainly by the solvent-producing bacteria, e.g. *Chlostridium acetobuylicum* (Calam, 1980). The cultivation of dehalogenating bacteria with these solvent-producing bacteria could not only supply nutrients for large amounts of *Dehalococcoides*, but also provide an alternative for handling the wastewater from the bioconversion process of solid waste. In addition, the abundant seawater in Singapore also provides a natural source for supplying the high-salt concentration solution to the medium needed for growing *Dehalococcoides*. Further research into an eco-friendly bioremediation approach by these novel cultures would be desirable to save the cost for limited energy resources in today's society.

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Appendix

1. List of accession number for the sequences submitted to GenBank

1) 16S rRNA gene sequence of *Dehalococcoides* sp. strain MB (EU073964)

2) 16S rRNA gene sequence of *Sedimentibacter* sp. strain WS2 (FJ593657)

3) 16S rRNA gene sequence of *Dehalococcoides* sp. strain 11a (FJ593658)

4) 16S rRNA gene sequence of *Dehalococcoides* sp. strain 11a5 (HM138520)

5) 16S rRNA gene sequence of *Dehalococcoides* sp. strain ANAS1 (HM241729)

6) 16S rRNA gene sequence of *Dehalococcoides* sp. strain ANAS2 (HM241730)

7) RDase gene, *mbrA* sequence of *Dehalococcoides* sp. strain MB (GU120391)

8) RDase gene sequence (11aRdh 1-7) of *Dehalococcoides* sp. strain 11a (HM138513-HM138519)

2. Papers generated from this study

Cheng, D., Chow, W.L., and He, J. (2010) A *Dehalococcoides*-containing co-culture that dechlorinates tetrachloroethene to *trans*-1,2-dichloroethene. *ISME J* **4**: 88-97.

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Cheng, D., and He, J. (2010) Isolation of a *Dehalococcoides* sp. strain 11a for rapid detoxification of chloroethenes to ethene in groundwater. (*In preparation*)

Lee, P.K.H., Cheng, D., Hu, P., West, K.A., Dick, G.J., Brodie, E.L. et al. (2011) Comparative genomics of two newly isolated *Dehalococcoides* strains and an enrichment using a genus microarray. *ISME J*.

Lee, P.K.H., Cheng, D., Hu, P., West, K.A., Brodie, E.L., Andersen, G.L. et al. Querying the genomes of new un-sequenced *Dehalococcoides* isolates via a microarray targeting the *Dehalococcoides* genus. (*In preparation*)

3. Alignment of the 16S rRNA gene sequences of several known *Dehalococcoides* spp.

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CLUSTAL 2.0.10 multiple sequence alignment


CBDB1           ------------------GATGAACGCTAGCGGCGTGCCTTATGCATGCAAGTCGAAC 40
KB1-VC          ------------------GATGAACGCTAGCGGCGTGCCTTATGCATGCAAGTCGAAC 40
FL2             ------------------GATGAACGCTAGCGGCGTGCCTTATGCATGCAAGTCGAAC 40
BAV1            ----------------------------CGGCGTGCCTTATGCATGCAAGTCGAAC 28
195             AGAGTTTGATCCTGGCTCAGGATGAACGCTAGCGGCGTGCCTTATGCATGCAAGTCGAAC 60
VS              ------------------GATGAACGCTAGCGGCGTGCCTTATGCATGCAAGTCGAAC 40
                                   ****************************

CBDB1           GGTCTTAAGCAATTAAGATAGTGGCGAACGGGTGAGTAACGCGTAAGTAACCTACCTCTA 100
KB1-VC          GGTCTTAAGCAATTAAGATAGTGGCGAACGGGTGAGTAACGCGTAAGTAACCTACCTCTA 100
FL2             GGTCTTAAGCAATTAAGATAGTGGCGAACGGGTGAGTAACGCGTAAGTAACCTACCTCTA 100
BAV1            GGTCTTAAGCAATTAAGATAGTGGCGAACGGGTGAGTAACGCGTAAGTAACCTACCTCTA 88
195             GGTCTTAAGCAATTAAGATAGTGGCAAACGGGTGAGTAACGCGTAAGTAACCTACCTCTA 120
VS              GGTCTTAAGCAATTAAGATAGTGGCAAACGGGTGAGTAACGCGTAAGTAACCTACCTCTA 100
                ************************ ***********************************

CBDB1           AGTGGGGGATAGCTTCGGGAAACTGAAGGTAATACCGCATGTGGTGGGCCGACATATGTT 160
KB1-VC          AGTGGGGGATAGCTTCGGGAAACTGAAGGTAATACCGCATGTGGTGGGCCGACATATGTT 160
FL2             AGTGGGGGATAGCTTCGGGAAACTGAAGGTAATACCGCATGTGGTGGGCCGACATATGTT 160
BAV1            AGTGGGGGATAGCTTCGGGAAACTGAAGGTAATACCGCATGTGGTGGACCGACATATGTT 148
195             AGTGGGGGATAGCTTCGGGAAACTGAAGGTAATACCGCATGTGATGGGCTGACATAAGTC 180
VS              AGTGGGGGATAGCTTCGGGAAACTGAAGGTAATACCGCATGTGGTGGGCCGACATAAGTT 160
                ****************************************** *** * ****** **

CBDB1           GGTTCACTAAAGCCGTAAGGCGCTTGGTGAGGGGCTTGCGTCCGATTAGCTAGTTGGTGG 220
KB1-VC          GGTTCACTAAAGCCGTAAGGCGCTTGGTGAGGGGCTTGCGTCCGATTAGCTAGTTGGTGG 220
FL2             GGTTCACTAAAGCCGTAAGGCGCTTGGTGAGGGGCTTGCGTCCGATTAGCTAGTTGGTGG 220
BAV1            GGTTCACTAAAGCCGTAAGGCGCTTGGTGAGGGGCTTGCGTCCGATTAGCTAGTTGGTGG 208
195             GGTTCATTAAAGCCGCAAGGTGCTTGGTGAGGGGCTTGCGTCCGATTAGCTAGTTGGTGG 240
VS              GGTTCACTAAAGCCGTAAGGTGCTTGGTGAGGGGCTTGCGTCCGATTAGCTAGTTGGTGG 220
                ****** ******** **** ****************************************

CBDB1           GGTAATGGCCTACCAAGGCTTCGATCGGTAGCTGGTCTGAGAGGATGATCAGCCACACTG 280
KB1-VC          GGTAATGGCCTACCAAGGCTTCGATCGGTAGCTGGTCTGAGAGGATGATCAGCCACACTG 280
FL2             GGTAATGGCCTACCAAGGCTTCGATCGGTAGCTGGTCTGAGAGGATGATCAGCCACACTG 280
BAV1            GGTAATGGCCTACCAAGGCTTCGATCGGTAGCTGGTCTGAGAGGATGATCAGCCACACTG 268
195             GGTAATGGCCTACCAAGGCTTCGATCGGTAGCTGGTCTGAGAGGATGATCAGCCACACTG 300
VS              GGTAACGGCCTACCAAGGCTTCGATCGGTAGCTGGTCTGAGAGGATGATCAGCCACACTG 280
                ***** ****************************************************

CBDB1           GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGCAAGGAATCTTGGGCAATGGG 340
KB1-VC          GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGCAAGGAATCTTGGGCAATGGG 340
FL2             GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGCAAGGAATCTTGGGCAATGGG 340
BAV1            GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGCAAGGAATCTTGGGCAATGGG 328
195             GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGCAAGGAATCTTGGGCAATGGG 360
VS              GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGCAAGGAATCTTGGGCAATGGG 340
                ************************************************************

CBDB1           CGAAAGCCTGACCCAGCAACGCCGCGTGAGGGATGAAGGCTTTCGGGTTGTAAACCTCTT 400
KB1-VC          CGAAAGCCTGACCCAGCAACGCCGCGTGAGGGATGAAGGCTTTCGGGTTGTAAACCTCTT 400
FL2             CGAAAGCCTGACCCAGCAACGCCGCGTGAGGGATGAAGGCTTTCGGGTTGTAAACCTCTT 400
BAV1            CGAAAGCCTGACCCAGCAACGCCGCGTGAGGGATGAAGGCTTTCGGGTTGTAAACCTCTT 388
195             CGAAAGCCTGACCCAGCAACGCCGCGTGAGGGATGAAGGCTTTCGGGTTGTAAACCTCTT 420
VS              CGAAAGCCTGACCCAGCAACGCCGCGTGAGGGATGAAGGCTCTCGGGTTGTAAACCTCTT 400
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CBDB1           TTCATAGGGAAGAATAATGACGGTACCTGTGGAATAAGCTTCGGCTAACTACGTGCCAGC 460
KB1-VC          TTCATAGGGAAGAATAATGACGGTACCTGTGGAATAAGCTTCGGCTAACTACGTGCCAGC 460
FL2             TTCATAGGGAAGAATAATGACGGTACCTGTGGAATAAGCTTCGGCTAACTACGTGCCAGC 460
BAV1            TTCATAGGGAAGAATAATGACGGTACCTGTGGAATAAGCTTCGGCTAACTACGTGCCAGC 448
195             TTCACAGGGAAGAATAATGACGGTACCTGTGGAATAAGCTTCGGCTAACTACGTGCCAGC 480
VS              TTCACAGGGAAGAATAATGACGGTACCTGTGGAATAAGCTTCGGCTAACTACGTGCCAGC 460
                **** ************************************************** 

CBDB1           AGCCGCGGTAATACGTAGGAAGCAAGCGTTATCCGGATTTATTGGGCGTAAAGTGAGCGT 520
KB1-VC          AGCCGCGGTAATACGTAGGAAGCAAGCGTTATCCGGATTTATTGGGCGTAAAGTGAGCGT 520
FL2             AGCCGCGGTAATACGTAGGAAGCAAGCGTTATCCGGATTTATTGGGCGTAAAGTGAGCGT 520
BAV1            AGCCGCGGTAATACGTAGGAAGCAAGCGTTATCCGGATTTATTGGGCGTAAAGTGAGCGT 508
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VS              AGCCGCGGTAATACGTAGGAAGCAAGCGTTATCCGGATTTATTGGGCGTAAAGTGAGCGT 520
                ************************************************************ 

CBDB1           AGGTGGTCTTTCAAGTTGGATGTGAAATTTCCCGGCTTAACCGGGACGAGTCATTCAATA 580
KB1-VC          AGGTGGTCTTTCAAGTTGGATGTGAAATTTCCCGGCTTAACCGGGACGAGTCATTCAATA 580
FL2             AGGTGGTCTTTCAAGTTGGATGTGAAATTTCCCGGCTTAACCGGGACGAGTCATTCAATA 580
BAV1            AGGTGGTCTTTCAAGTTGGATGTGAAATTTCCCGGCTTAACCGGGACGAGTCATTCAATA 568
195             AGGTGGTCTTTCAAGTTGGATGTGAAATTTCCCGGCTTAACCGGGACGTGTCATTCAATA 600
VS              AGGTGGTCTTTCAAGTTGGATGTGAAATTTCCCGGCTTAACCGGGACGTGTCATTCAATA 580
                ********************************************** ************ 

CBDB1           CTGTTGGACTAGAGTACAGCAGGAGAAAACGGAATTCCCGGTGTAGTGGTAAAATGCGTA 640
KB1-VC          CTGTTGGACTAGAGTACAGCAGGAGAAAACGGAATTCCCGGTGTAGTGGTAAAATGCGTA 640
FL2             CTGTTGGACTAGAGTACAGCAGGAGAAAACGGAATTCCCGGTGTAGTGGTAAAATGCGTA 640
BAV1            CTGTTGGACTAGAGTACAGCAGGAGAAAACGGAATTCCCGGTGTAGTGGTAAAATGCGTA 628
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VS              CTGTTGGACTAGAGTACAGCAGGAGAAAACGGAATTCCCGGTGTAGTGGTAAAATGCGTA 640
                ************************************************************ 

CBDB1           GATATCGGGAGGAACACCAGAGGCGAAGGCGGTTTTCTAGGTTGTCACTGACACTGAGGC 700
KB1-VC          GATATCGGGAGGAACACCAGAGGCGAAGGCGGTTTTCTAGGTTGTCACTGACACTGAGGC 700
FL2             GATATCGGGAGGAACACCAGAGGCGAAGGCGGTTTTCTAGGTTGTCACTGACACTGAGGC 700
BAV1            GATATCGGGAGGAACACCAGAGGCGAAGGCGGTTTTCTAGGTTGTCACTGACACTGAGGC 688
195             GATATCGGGAGGAACACCAGAGGCGAAGGCGGTTTTCTAGGTTGTCACTGACACTGAGGC 720
VS              GATATCGGGAGGAACACCAGAGGCGAAGGCGGTTTTCTAGGTTGTCACTGACACTGAGGC 700
                ************************************************************ 

CBDB1           TCGAAAGCGTGGGGAGCGAACAGAATTAGATACTCTGGTAGTCCACGCCTTAAACTATGG 760
KB1-VC          TCGAAAGCGTGGGGAGCGAACAGAATTAGATACTCTGGTAGTCCACGCCTTAAACTATGG 760
FL2             TCGAAAGCGTGGGGAGCGAACAGAATTAGATACTCTGGTAGTCCACGCCTTAAACTATGG 760
BAV1            TCGAAAGCGTGGGGAGCGAACAGAATTAGATACTCTGGTAGTCCACGCCTTAAACTATGG 748
195             TCGAAAGCGTGGGGAGCGAACAGAATTAGATACTCTGGTAGTCCACGCCTTAAACTATGG 780
VS              TCGAAAGCGTGGGGAGCGAACAGAATTAGATACTCTGGTAGTCCACGCCTTAAACTATGG 760
                ************************************************************ 

CBDB1           ACACTAGGTATAGGGAGTATCGACCCTCTCTGTGCCGAAGCTAACGCTTTAAGTGTCCCG 820
KB1-VC          ACACTAGGTATAGGGAGTATCGACCCTCTCTGTGCCGAAGCTAACGCTTTAAGTGTCCCG 820
FL2             ACACTAGGTATAGGGAGTATCGACCCTCTCTGTGCCGAAGCTAACGCTTTAAGTGTCCCG 820
BAV1            ACACTAGGTATAGGGAGTATCGACCCTCTCTGTGCCGAAGCTAACGCTTTAAGTGTCCCG 808
195             ACACTAGGTATAGGGAGTATCGACCCTCTCTGTGCCGAAGCTAACGCTTTAAGTGTCCCG 840
VS              ACACTAGGTATAGGGAGTATCGACCCTCTCTGTGCCGAAGCTAACGCTTTAAGTGTCCCG 820
                ************************************************************ 

CBDB1           CCTGGGGAGTACGGTCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCA 880
KB1-VC          CCTGGGGAGTACGGTCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCA 880
FL2             CCTGGGGAGTACGGTCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCA 880
BAV1            CCTGGGGAGTACGGTCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCA 868
195             CCTGGGGAGTACGGTCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCA 900
VS              CCTGGGGAGTACGGTCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCA 880
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CBDB1           GCGGAGCGTGTGGTTTAATTCGATGCTACACGAAGAACCTTACCAAGATTTGACATGCAT 940
KB1-VC          GCGGAGCGTGTGGTTTAATTCGATGCTACACGAAGAACCTTACCAAGATTTGACATGCAT 940
FL2             GCGGAGCGTGTGGTTTAATTCGATGCTACACGAAGAACCTTACCAAGATTTGACATGCAT 940
BAV1            GCGGAGCGTGTGGTTTAATTCGATGCTACACGAAGAACCTTACCAAGATTTGACATGCAT 928
195             GCGGAGCGTGTGGTTTAATTCGATGCTACACGAAGAACCTTACCAAGATTTGACATGCAT 960
VS              GCGGAGCGTGTGGTTTAATTCGATGCTACACGAAGAACCTTACCAAGATTTGACATGCAT 940
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CBDB1           GTAGTAGTGAACTGAAAGGGGAACGACCTGTTAAGTCAGGAACTTGCACAGGTGCTGCAT 1000
KB1-VC          GTAGTAGTGAACTGAAAGGGGAACGACCTGTTAAGTCAGGAACTTGCACAGGTGCTGCAT 1000
FL2             GTAGTAGTGAACTGAAAGGGGAACGACCTGTTAAGTCAGGAACTTGCACAGGTGCTGCAT 1000
BAV1            GTAGTAGTGAACTGAAAGGGGAACGACCTGTTAAGTCAGGAACTTGCACAGGTGCTGCAT 988
195             GAAGTAGTGAACCGAAAGGGAAACGACCTGTTAAGTCAGGAGTTTGCACAGGTGCTGCAT 1020
VS              GAAGTAGTGAACCGAAAGGGAAACGACCTGTTAAGTCAGGAGTTTGCACAGGTGCTGCAT 1000
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CBDB1           GGCTGTCGTCAGCTCGTGCCGTGAGGTGTTTGGTTAAGTCCTGCAACGAGCGCAACCCTT 1060
KB1-VC          GGCTGTCGTCAGCTCGTGCCGTGAGGTGTTTGGTTAAGTCCTGCAACGAGCGCAACCCTT 1060
FL2             GGCTGTCGTCAGCTCGTGCCGTGAGGTGTTTGGTTAAGTCCTGCAACGAGCGCAACCCTT 1060
BAV1            GGCTGTCGTCAGCTCGTGCCGTGAGGTGTTTGGTTAAGTCCTGCAACGAGCGCAACCCTT 1048
195             GGCTGTCGTCAGCTCGTGCCGTGAGGTGTTTGGTTAAGTCCTGCAACGAGCGCAACCCTT 1080
VS              GGCTGTCGTCAGCTCGTGCCGTGAGGTGTTTGGTTAAGTCCTGCAACGAGCGCAACCCTT 1060
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CBDB1           GTTGCTAGTTAAATTTTCTAGCGAGACTGCCCCGCGAAACGGGGAGGAAGGTGGGGATGA 1120
KB1-VC          GTTGCTAGTTAAATTTTCTAGCGAGACTGCCCCGCGAAACGGGGAGGAAGGTGGGGATGA 1120
FL2             GTTGCTAGTTAAATTTTCTAGCGAGACTGCCCCGCGAAACGGGGAGGAAGGTGGGGATGA 1120
BAV1            GTTGCTAGTTAAATTTTCTAGCGAGACTGCCCCGCGAAACGGGGAGGAAGGTGGGGATGA 1108
195             GTTGCTAGTTAAATTTTCTAGCGAGACTGCCCCGCGAAACGGGGAGGAAGGTGGGGATGA 1140
VS              GTTGCTAGTTAAATTTTCTAGCGAGACTGCCCCGCGAAACGGGGAGGAAGGTGGGGATGA 1120
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CBDB1           CGTCAAGTCAGCATGGCCTTTATATCTTGGGCTACACACACGCTACAATGGACAGAACAA 1180
KB1-VC          CGTCAAGTCAGCATGGCCTTTATATCTTGGGCTACACACACGCTACAATGGACAGAACAA 1180
FL2             CGTCAAGTCAGCATGGCCTTTATATCTTGGGCTACACACACGCTACAATGGACAGAACAA 1180
BAV1            CGTCAAGTCAGCATGGCCTTTATATCTTGGGCTACACACACGCTACAATGGACAGAACAA 1168
195             CGTCAAGTCAGCATGGCCTTTATATCTTGGGCTACACACACGCTACAATGGACAGAACAA 1200
VS              CGTCAAGTCAGCATGGCCTTTATATCTTGGGCTACACACACGCTACAATGGACAGAACAA 1180
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CBDB1           TAGGTTGCAACAGTGCGAACTGGAGCTAATCCCCAAAGCTGTCCTCAGTTCGGATTGCAG 1240
KB1-VC          TAGGTTGCAACAGTGCGAACTGGAGCTAATCCCCAAAGCTGTCCTCAGTTCGGATTGCAG 1240
FL2             TAGGTTGCAACAGTGCGAACTGGAGCTAATCCCCAAAGCTGTCCTCAGTTCGGATTGCAG 1240
BAV1            TAGGTTGCAACAGTGCGAACTGGAGCTAATCCCCAAAGCTGTCCTCAGTTCGGATTGCAG 1228
195             TAGGTTGCAACAGTGTGAACTGGAGCTAATCCCCAAAGCTGTCCTCAGTTCGGATTGCAG 1260
VS              TAGGTTGCAACAGTGTGAACTGGAGCTAATCCTCAAAGCTGTCCTCAGTTCGGATTGCAG 1240
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CBDB1           GCTGAAACCCGCCTGCATGAAGTTGGAGTTGCTAGTAACCGCATATCAGCATGGTGCGGT 1300
KB1-VC          GCTGAAACCCGCCTGCATGAAGTTGGAGTTGCTAGTAACCGCATATCAGCATGGTGCGGT 1300
FL2             GCTGAAACCCGCCTGCATGAAGTTGGAGTTGCTAGTAACCGCATATCAGCATGGTGCGGT 1300
BAV1            GCTGAAACCCGCCTGCATGAAGTTGGAGTTGCTAGTAACCGCATATCAGCATGGTGCGGT 1288
195             GCTGAAACCCGCCTGCATGAAGTTGGAGTTGCTAGTAACCGCATATCAGCAAGGTGCGGT 1320
VS              GCTGAAACCCGCCTGCATGAAGTTGGAGTTGCTAGTAACCGCATATCAGCAAGGTGCGGT 1300
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CBDB1           GAATACGTTCTCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGCCGGTAACACTTG 1360
KB1-VC          GAATACGTTCTCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGCCGGTAACACTTG 1360
FL2             GAATACGTTCTCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGCCGGTAACACTTG 1360
BAV1            GAATACGTTCTCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGCCGGTAACACTTG 1348
195             GAATACGTTCTCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGCCGGTAACACTTG 1380
VS              GAATACGTTCTCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGCCGGTAACACTTG 1360
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CBDB1           AAGTCGATGTGCCAACCGCAAGGAGGCAGTCGCCGAGGGTGGGACTGGTAATTGGGACGA 1420
KB1-VC          AAGTCGATGTGCCAACCGCAAGGAGG---------------------------------- 1386
FL2             AAGTCGATGTGCCAACCGCAAGGAGGCAGTCGCCGAGGGTGGGACTGGTAATTGGGACGA 1420
BAV1            AAGTCGATGTGCCAACCGCAAGGAGGCAGTCGCCGAGGGTGGGACTGGTAATTGGGACGA 1408
195             AAGTCGATGTGCCAACCGCAAGGAGGCAGTCGCCGAGGGTGGGACTGGTAATTGGGACGA 1440
VS              AAGTCGATGTGCCAACCGCAAGGAGGCAGTCGCCGAGGGTGGGACTGGTAATTGGGACGA 1420
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CBDB1           A---------------------------------------------------------- 1421
KB1-VC          ---------------------------------------------------------- 
FL2             AGTCGTAACAAGGTA---------------------------------------------- 1435
BAV1            AGTCGTAACAAG------------------------------------------------ 1420
195             AGTCGTAACAAGGTAGCCGTAGCGGAAGCTGCGGCTGGATCACCTCCTT 1489
VS              AGTCGTAACAAGGTA---------------------------------------------- 1435
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