MICROBIAL REDUCTIVE DECHLORINATION OF TRICHLOROETHANE/CHLOROFORM AND GENOMIC CHARACTERIZATION OF THE DECHLORINATORS

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety.

I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has not been submitted for any degree in any university previously.

ZHAO SIYAN

17AUG2015

I dedicate this thesis to

My parents,

Who never stop loving me and supporting me

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SUMMARY

With extensive utilization and impropriate disposal, anthropogenic halogenated organic compounds have been widely distributed in the environment and pose adverse effects to both environment and human beings. Bioremediation through reductive dehalogenation by bacteria is an effective technology to detoxify halogenated compounds. However, some halogenated compounds are still recalcitrant to degradation or satisfactory performance cannot be achieved by available dehalorespiring bacteria. These halogenated compounds include 1,1,2-trichloroethane, tetrachloroethene and the compounds with the trihalomethyl group (-CX_3) such as chloroform ($CHCl_3$) and 1,1,1-TCA (CH_3CCl_3). Microorganisms that can efficiently remove these organic pollutants are thus promising for bioremediation *in situ*. By intensive screening of microbial cultures from various locations in Singapore, China, Malaysia and the United States, this doctoral study successfully characterized bacterial cultures, *Desulfitobacterium* sp. strain PR, *Dehalococcoides mccartyi* strain 11a5 and *Dehalobacter*-containing co-culture SN, that exhibited special dehalogenating performance on these specific organic pollutants.

This doctoral study reported three bacterial strains which are promising candidates in bioremediation. Firstly, *Desulfitobacterium* sp. strain PR was discovered to reductively dechlorinate 1,1,2-trichloroethane to predominately to 1,2-dichloroethane and chloroethane. This novel pathway avoids production of the carcinogenic intermediate dechlorination product vinyl chloride, providing insights of more environmentally friendly strategy to treat 1,1,2-TCA. By co-culturing strain PR with *Dehalococcoides mccartyi* strain 11a (capable of detoxifying TCE and 1,2-DCA), complete detoxification of co-

contaminants, TCE and 1,1,2-TCA, was achieved. Secondly, *Dehalococcoides* sp. strain 11a5 was found to reductively dechlorinate PCE to VC and ethene with TCE as the intermediate, but negligible DCEs. Additionally, a *Dehalobacter*-containing co-culture SN was able to dehalogenate chloroform, 1,1,1-TCA, and for the first time bromoform.

Mechanisms involved in reductive dehalogenation of specific halogenated compounds were investigated. novel reductive dehalogenase genes (*rdhA*s), *ctrA* for dechlorination of 1,1,2-TCA to 1,2-DCA, pteA for dechlorination of PCE to TCE, and *thmA* for dehalogenation of chloroform, 1,1,1-TCA and bromoform, were identified. Therefore, the biomarkers can be designed to target, predict and monitor reductive dechlorination of specific halogenated organic compounds *in situ*. During the characterization of the reductive dehalogenases, the understanding of specificity of these catalytic enzymes were enlarged from single substrate to substrates of similar functional groups. These findings can be helpful to look for potential application of the organohalide respiring bacteria.

Phylogenetic, biochemical, evolutionary and ecological features of one of the representative organohide respiring bacteria, strain 11a5, were further explored through genomic analysis to better guide its application in contaminated sites. The complete genome of *Dehalococcoides mccartyi* strain 11a5 was sequenced and the discovery of the plasmid and prophage in strain 11a5 may provide a useful insight to investigate how reductive dehalogenases are transferred and carried by dehalorespiring anaerobes.

To summarize, the findings of the three cultures should provide useful information on the bioremediation strategies dealing with halogenated compounds.

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ABBREVIATIONS

aa	amino acid
ANI	average nucleotide identity
ATSDR	Agency for Toxic Substances and Disease Registry
BLAST	basic local alignment search tool
bp	base pair
BGI	Beijing Genomic Institution
CA	monochloroethane
CF	chloroform
CDS	coding sequence
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
DCA	dichloroethane
DCE	dichloroethene
DCM	dichloromethane
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DR	direct repeat
GC	gas chromatography
gDNA	genomic DNA
GI	genome island
GMMC	geometric mean maximum concentration
HPLC	high performance liquid chromatography
HPR	high plasticity region
kDa	kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
K_{ow}	octanol-water partition coefficient
MALDI-TOF-MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MCL	maximum contaminant level
MCM	monochloromethane

mRNA	messenger RNA
MS	mass spectrometry
NCBI	National Center for Biotechnology Information
ncRNA	non-coding RNA
OHRB	organohalide respiring bacteria
ORF	open reading frame
ori	origin of replication
PBDE	polybrominated diphenyl ether
PCB	polychlorinated biphenyl
PCE	tetrachloroethene
PCP	pentachlorophenol
PCR	polymerase chain reaction
POPs	persistent organic pollutants
qPCR	quantitative real-time PCR
rdhA	reductive dehalogenase homologue subunit A
rdhB	reductive dehalogenase homologue subunit B
RNA	ribonucleic acid
rRNA	ribosomal RNA
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	1,1,1-trichloroethane
TCE	trichloroethene
TCP	trichlorophenol
TeCP	tetrachlorophenol
TES	2-[tris(hydroxymethyl)methylamino]-1-ethanesulfonic acid
tmRNA	transfer-messenger RNA
T-RFLP	terminal restriction fragment length polymorphism
tRNA	transfer RNA
USEPA	United States Environmental Protection Agency
UV	ultraviolet
VC	vinyl chloride
VFA	volatile fatty acid

1 Chapter I Introduction

In recent years, growing environmental concerns has been gained for sites and groundwater contaminated by halogenated compounds. As a result of public awareness, research efforts exploring the potential carcinogenic effects of halogenated compounds have intensified, resulting in the development of environmental regulations, safe handling procedures and clean-up technologies. The origins of halogenated compounds pollution and current treatment strategies for remediating contaminated systems, followed by the rationale and aims of this thesis study are presented in this chapter.

1.1. Environmental contamination by halogenated organic compounds

Halogenated organic compounds are a group of the most widely distributed anthropogenic environmental contaminants, many of which are toxic, persistent, and bioaccumulative (Holliger, et al., 2003). This category of chemicals comprises molecules in which one or more carbon atoms are linked with one or more halogen atoms through covalent bonds, and includes chloroethenes, chloroethanes, chlorobenzenes, polychlorinated phenyls (PCBs), polychlorinated-dibenzo-p-dioxins (PCDDs) and polybrominated diphenyl ethers (PBDEs), etc. (Figure 1.1).

Widespread utilization of halogenated organic compounds in manufacture began during World War II. However, due to inappropriate handling in usage, transport and disposal, large quantities of halogenated organic compounds have been released into the environment, thereby contaminating soils and aquatic systems. Most of the halogenated solvents are synthesized and are recalcitrant in the environment, with half-lives ranging from months to years in soils and groundwater (Susarla, et al., 1998). When

these halogenated organic compounds are released into the environment, they tend to accumulate as dense non-aqueous phase liquids (DNAPL) because of their low solubility in water and relatively high octanol-water partition coefficients (Markowitz, et al.) (Abeleson, 1990). Aggregation as DNAPLs also often results in long term secondary contaminant source for groundwater.

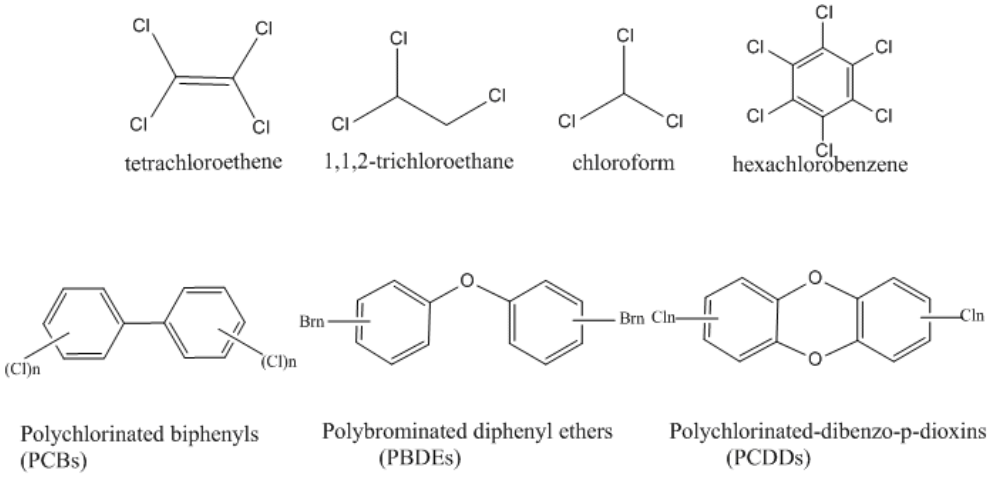


Figure 1.1 Chemical structures of typical halogenated organic contaminants

The environmental toxicity and persistency of halogenated organic compounds have drawn a great deal of public attention, so that all of the original "dirty dozen" restricted or banned substances at the original Stockholm Convention in 1997 were halogenated organic pollutants (UNEP, 1997). Recently, several studies have linked exposure to halogenated organic compounds to human diseases in liver, kidneys, nervous and immune systems (IARC, 2010). In an attempt to mitigate the risks posed by halogenated organic compounds, intensive research efforts have been made in the development and refining of methods for environmental remediation and clean-up technologies.

1.2. Remediation of halogenated organic compounds in contaminated sites

Environmental remediation deals with the removal of contaminants from environmental media such as soil, sediment or surface water (Fortuna, et al., 2011). There are multiple technologies including physical, chemical and biological treatments employed to remove these halogenated organic contaminants and achieve remediation. Physical remediation includes soil washing and soil vapour extraction, and chemical remediation involves the use of chemicals to extract contaminants from contaminated media, including solvent extraction and chemical oxidation (Reddy, 2008). Biological remediation, or bioremediation, involves the use of organisms to remove pollutants from contaminated sites (Gillespie and Philp, 2013). Although physical and chemical treatments can effectively remove halogenated organic contaminants to some extent, these approaches tend to be energy and cost intensive, and are often ineffective at low contaminant concentrations. Additionally, such treatments often irreversibly damage the chemical properties of remediated sites. Bioremediation, on the other hand, has advantages in cost and efficiency compared to physical and chemical remediation, and typically has less severe negative impacts on the remediated ecosystem (Wenning, et al., 2006).

1.2.1. Bioremediation technologies

Bioremediation processes rely on a broad range of terrestrial and aquatic organisms that are capable of assimilating, degrading or metabolizing halogenated organic contaminants (Megharaj, et al., 2011). Although a variety of biological agents can degrade and/or transform halogenated organic compounds, bacterial remediation releasing less toxic forms as by-products of

metabolic function has proven to be an effective strategy for almost all types of halogenated contaminants (Timmis and Pieper, 1999). The comprehensive characterization of indigenous microbial communities of a contaminated site is usually the first task in initial determination of appropriate bioremediation strategies for a specific site (Tiehm and Schmidt, 2011). When an indigenous microbial community is capable of detoxifying relevant halogenated organic contaminants, bioremediation efforts tend to focus on modifying the environment to stimulate existing bacteria potential for bioremediation via the addition of various forms of rate limiting nutrients, a process termed biostimulation (Tyagi, et al., 2011). However, in cases where the necessary populations capable of detoxifying targeted halogenated contaminants are absent or in too low abundance to facilitate bioremediation, bioaugmentation, e.g., the introduction of specific bacterial populations, is necessary (Vogel, 1996). Different from biostimulation and bioaugmentation, monitored natural attenuation (MNA) is an approach involving no human intervention other than long-term monitoring to demonstrate that contaminant concentrations continue to decrease at a rate sufficient to remain below regulatory limits and not become a health threat (Adamson, et al., 2015).

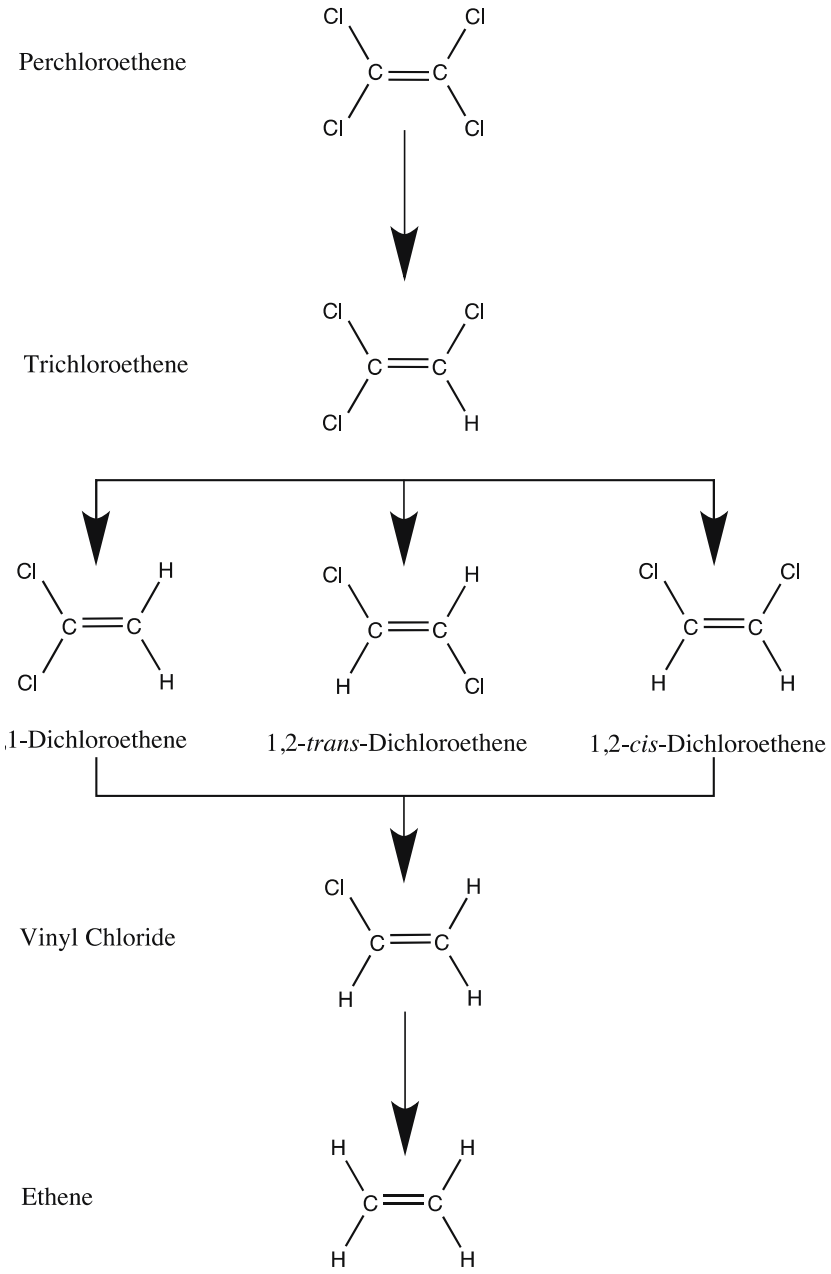
There are two different types of methodologies, *ex situ* and *in situ* that can be employed in bioremediation. *Ex situ* bioremediation involves the excavation of soil or groundwater, which is then placed in above-ground treatment areas to enhance the degradation of organic contaminants (Eldho, 2003). However, this type of operation tends to be highly energy intensive, particularly when anaerobic bacteria are used to treat the excavated soil and water. *In situ* bioremediation is the application of bioremediation on site, i.e.

in unsaturated/vadoze zones or in saturated soils and groundwater (Tyagi, et al., 2011). Compared with *ex situ* treatment, *in situ* bioremediation is less disruptive to the environment at the contaminated site and generally poses a lower risk of human exposure to contaminated media.

1.2.2. Reductive dehalogenation of halogenated organic compounds

The only documented microbial process that can degrade highly halogenated organic compounds, e.g. PCE, PCB, PBDEs, is termed reductive dehalogenation and occurs only under anaerobic conditions (Tiedje, 1998, Lee and He, 2010, Wang, et al., 2014a). Reductive dehalogenation is an anaerobic respiratory process that removes halogen substituents from halogenated organic compounds while simultaneously adding electrons to the molecule (Mohn and Tiedje, 1992). This dehalogenation process can be broadly classified as metabolic or co-metabolic in nature. Metabolic reductive dehalogenation utilizes specific catalytic enzymes to couple dehalogenation with microbial growth, where the co-metabolic process is described as specific dehalogenation by enzymes expressed in other metabolic processes that does not result in microbial growth (Tiehm and Schmidt, 2011, Futagami, et al., 2014). The reductive dehalogenation can proceed via two pathways: hydrogenolysis, the replacement of the halogen atom with a hydrogen atom, and dihaloelimination, the removal of two adjacent halogen atoms on the same alkane or alkene molecule and subsequent formation of an additional carbon-carbon bond (Figure 1.2) (Smidt, et al., 2000).

(A) Hydrogenolysis



(B) dihaloelimination

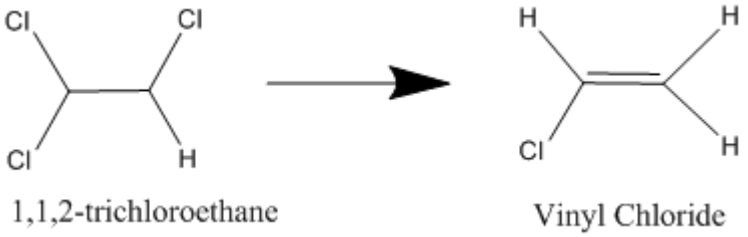


Figure 1.2 Reductive dehalogenation pathways

The replacement of a halogen atom with hydrogen in reductive dehalogenation is mediated by a class of enzymes termed reductive dehalogenases (RDases) (Holliger, et al., 1999). Most of the known RDases dehalogenate halogenated aliphatic or aromatic compounds and have a specific, narrow substrate range. The genes encoding RDases are termed reductive dehalogenase homologous genes (*rdhA*s), however, only limited numbers of *rdhA*s have been functionally characterized (Hug, et al., 2013). Functional identification of *rdhAs* is of great importance because these genes can be utilized as biomarkers to indicate dehalogenating capabilities in the evaluation, monitoring and modification of bioremediation efforts at contaminated sites (Morris, et al., 2007, Futagami, et al., 2009, Werner, et al., 2009).

The organohalide respiring bacteria (OHRB) are a group of bacteria couple growth with ATP synthesis and reduction of halogenated organic compounds (Löffler, et al., 1996). OHRB have been identified from a variety of different phyla, including Proteobacteria, Chloroflexi and Firmicutes (Figure 1.3) and can be grouped as either obligate or non-obligate (facultative) OHRB (Hug, et al., 2013). Proteobacteria, including *Geobacter*, *Desulfuromonas*, *Anaeromyxobacter* and *Sulfurospirillum*, are all non-obligate OHRB that make use of a variety of electron acceptors other than halogenated compounds, such as other organic compounds and metals (Sung, et al., 2003, Sung, et al., 2006a, van der Zaan, et al., 2009). Contrarily, bacteria of the phylum Chloroflexi, e.g., *Dehalococcoides* and *Dehalogenimonas*, are all obligate OHRB whose respiratory capacity are restricted in halogenated compounds. Firmicutes include both obligate, e.g., *Dehalobacter*, and non-

obligate, e.g., *Desulfitobacterium*, OHRB. While the discovery of the first OHRB, *Desulfomonil tiedjei* strain DCB-1, in 1984 (Shelton and Tiedje, 1984) sparked the origin of the concept of bioremediation to remove halogenated contaminants. Successful bioremediation using this technique has only been achieved by some commercial bioaugmentation inocula, e.g. KB-1 (SiREM) and Bio-Dechlor INOCULUM Plus (Regenesis). Conventional bioremediation research is restricted by a lack of microorganisms which can efficiently dehalogenate halogenated contaminants (Megharaj, et al., 2011).

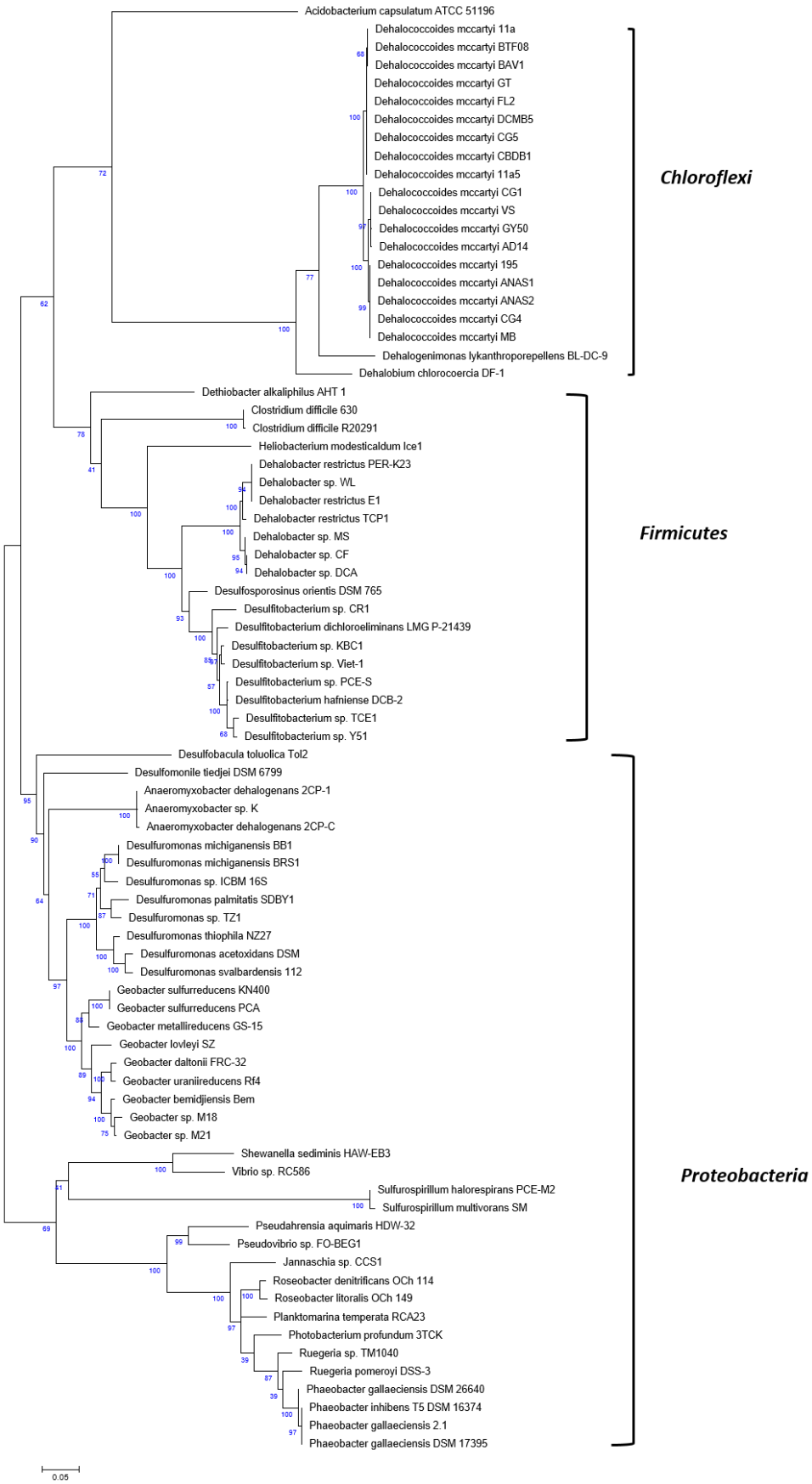


Figure 1.3 Molecular Phylogenetic analysis of known OHRB which contain at least one *rdhA* by Maximum Likelihood method. The evolutionary history was inferred by

using the Maximum Likelihood method based on the General Time Reversible model (Nei and Kumar, 2000). The tree with the highest log likelihood (-14795.2630) is shown. The percentage of trees in which the associated taxa clustered together is shown below the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.4373)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 37.3363% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 80 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1237 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura, et al., 2013).

The genome information of some OHRB has been investigated to explore in-depth features of this interesting group of organisms. The genomes of numerous OHRB have revealed both similarities and differences, at the genetic level, among the diverse bacteria capable of organohalide respiration and will provide some insight into the different evolutionary paths that have resulted in the variety of bacteria occupying this niche.

1.3. Rationale and aims

Although mixed dehalogenating cultures may sometimes provide information about dehalogenation processes, it is essential to isolate dehalogenating bacterial strains to gain insights into their characteristics. Without knowledge of the physiological properties of specific bacteria, planning, monitoring and optimizing bioremediation strategies are greatly hindered by the lack of information about bacterial nutritional requirements and responses in different environments. Specific halogenated compounds, 1,1,2-TCA, 1,1,1-TCA, PCE and chloroform, are given high priority in this study due to their high toxicity, ubiquity and recalcitrance. The overall objective of this study is to facilitate *in situ* bioremediation of halogenated solvents at contaminated sites. The specific aims of this study are:

- To look for 1,1,2-TCA, 1,1,1-TCA, PCE and chloroform dehalogenating cultures with satisfactory performance, e.g., with high removal efficiency, representing a different dehalogenating pathway, or belonging to different phylogenetic groups, through intensively screening bacterial isolations and microcosms.
- To phylogenetically, physiochemically, or genetically characterize 1,1,2-TCA, 1,1,1-TCA, PCE and chloroform dehalogenating bacteria, which can be potential candidates for in situ bioremediation.
- To identify novel distinct 1,1,2-TCA-,1,1,1-TCA, PCE- and chloroform-RDases and characterize their phylogenetic and physiochemical properties, which can be used as biomarkers for in situ bioremediation
- To treat multiple halogenated compounds (co-contaminated with TCE) by establishing robust cultures containing identified microbes as described above.
- To explore evolutionary features of OHRB through genomic analyses.

The results of this study will broaden our understanding of OHRB and provide feasible solutions for halogenated organic contaminants treatment. The contents of this thesis are arranged as follows:

- Chapter I introduces background of bioremediation.
- Chapter II summarizes relevant literature.
- Chapter III describes characterization of a 1,1,2-TCA dechlorinating culture and identification of functional reductive dehalogenases.
- Chapter IV reports characterization and genome features of an unusual plasmid-containing Dehalococcoides mccartyi strain 11a5.

- Chapter V describes characterization of a chloroform, 1,1,1-TCA and bromoform dehalogenating co-culture.
- Chapter VI summarizes this study and makes recommendations for future studies.

2 Chapter II Literature Review

This chapter provides a comprehensive background of reductive dehalogenation mediated by OHRB. This review summarizes bacterial cultures which can reductively dechlorinate specific chlorinated compounds, e.g. 1,1,2-TCA, chloroform, 1,1,1-TCA and PCE. Additionally, the reductive dehalogenation process, including molecular and genetic mechanisms, is discussed.

2.1 Specific chlorinated compounds

1,1,2-TCA, 1,1,1-TCA, chloroform and PCE are usually used as organic solvents or as precursors in synthetic industries (Abeleson, 1990). These chlorinated solvents are toxic to humans; inhalation may cause dizziness, drowsiness, headache, nausea, shortness of breath, unconsciousness, or in some cases, death (NIH, 2009). Particularly chloroform is lethal to humans, having an estimated mean oral dose of 45 g for an adult (Watts, et al., 2004). Apart from posing a health threat, these contaminants also cause severe environmental issues. For instance 1,1,1-TCA is ozone-depleting and chloroform severely inhibits methanogenesis and bioremediation processes (ATSDR, 2006a).

1,1,2-TCA, 1,1,1-TCA, chloroform and PCE are common, recalcitrant soil and groundwater contaminants (Table 2.1) that tend to aggregate as DNAPLs in soils and groundwater thereby becoming long term secondary contaminant source for groundwater. The detected geometric mean maximum concentrations (GMMC) of these solvents can reach milligram per kilogram levels in soils and groundwater, up to several hundred times higher than the

safe drinking water maximum contaminant level (MCL) set by the USEPA (USEPA, 2009, ATSDR, 2011b).

Table 2.1 Disposal, environmental fate and regulations of specific chlorinated solvents.

	Total disposal in 2013 (pounds)	NPL frequency (%)	GMMC		MCL in drinking water (mg/L)
			Ground water(mg/L)	Soils (mg/kg)	
Chloroform	484,579	59.2	3E-02	3E-01	0.07
1,1,1-TCA	110,012	59.9	1E-01	2E-00	0.2
1,1,2-TCA	3,811	19.9	8E-02	2E-00	0.005
PCE	1,053,991	71.4	5E2	9E-00	0.005
VC	447,049	44.8	5.77E-02	9.62E-01	0.005

NPL: National priorities list; this rank is based on toxicity, frequency and potential to human exposure at NPL sites. GMMC: Geometric mean maximum concentration; MCL: Maximum contaminant level.

1,1,2-TCA, 1,1,1-TCA, chloroform and PCE are common, recalcitrant soil and groundwater contaminants (Table 2.1) that tend to aggregate as DNAPLs in soils and groundwater thereby becoming long term secondary contaminant source for groundwater. The detected geometric mean maximum concentrations (GMMC) of these solvents can reach milligram per kilogram levels in soils and groundwater, up to several hundred times higher than the safe drinking water maximum contaminant level (MCL) set by the USEPA (USEPA, 2009, ATSDR, 2011b).

Due to this potential for toxicity and widespread distribution of environmental contaminants, it is of great urgency to remove such chlorinated solvents from the environment. However, the lack of bacterial cultures with satisfactory removal performance, e.g., faster dehalogenating processes, more environmental friendly dehalogenating pathways and less residue of halogenated compounds left after treatment, limits the applicability of bioremediation processes for removal of these compounds.

2.2 Bacterial cultures mediating reductive dechlorination of specific chlorinated compounds

A variety of OHRB which are capable of dehalogenating different types of halogenated compounds have been enriched and characterized. However, the applicability of OHRB to remediation of several types of chlorinated compounds is limited by a lack of suitable dechlorinating bacteria. Most of the cases, multiple chlorinated compounds co-exist in contaminated sites, which leads to more complicated circumstances and requires more thorough study of OHRB. These environmentally recalcitrant chlorinated compounds include chlorinated alkanes and chlorinated alkenes.

2.2.1 1,1,2-TCA-dechlorinating bacterial cultures

Though biodegradation of 1,1,2-TCA in aerobic systems does not occur or too slow to be detected, 1,1,2-TCA can be degraded either metabolically or co-metabolically by a range of bacteria phyla in anaerobic systems through reductive dechlorination to 1,2-dichloroethane (1,2-DCA), chloroethane (CA) and/or VC (Table 2.2).

Desulfomonile tiedjei strain DCB-1(Fathepure and Tiedje, 1994), *Methanobacterium thermoautotrophicum* (Castro, et al., 1994) and *Clostridium bifermentans* DPH-1 (Chang, et al., 2001) have all been reported to incompletely co-metabolically dechlorinate 1,1,2-TCA. However, this dechlorination is usually incomplete and occurs in the presence of low concentration of 1,1,2-TCA. For example, *Desulfomonile tiedjei* strain DCB-1 converts 37% of 1,1,2-TCA from an initial concentration of ~10.7 µM.

Table 2.2 Mixed cultures and isolates capable of anaerobically dechlorinating 1,1,2-TCA

Sample ID	Initial conc. (μM)	Products	Sample nature	Metabolic ?[c]	Ref.
Desulfomonile tiediei strain DCB-1	10.7[a]	1,2-DCA (Major, et al.)	Isolate	No	(Fathepure and Tiedje, 1994)
Unknown	16.2	VC(80%);1,2-DCA (20%); Ethane & ethane (trace amount)	Mixed culture	N/A[b]	(Chen, et al., 1996)
Methanobacterium thermoautotrophicum	N/A	VC(89%); 1,2-DCA(10%); Ethene (trace amount)	Isolate	No	(Castro, et al., 1994)
Desulfitobacterium dichloroeliminans strain DCA1	N/A	VC	Isolate	Yes	(De Wildeman, et al., 2003)
Unknown	N/A	VC(Major, et al.)	Mixed culture	N/A	(Aulenta, et al., 2006)
Dehalobacter sp.	~670	VC	Mixed culture	Yes	(Grostern and Edwards, 2006b)
Dehalogenimonas alkenigignens strain IP3-3	~2420	VC	Isolate	Yes	(Maness, et al., 2012)
Dehalogenimonas lykanthroporepellens strain BL-DC-9	~1650	VC	Isolate	Yes	(Moe, et al., 2009)
Dehalogenimonas alkeniginens strain SBP-1	N/A	VC	Isolate	Yes	(Maness, et al., 2012)
Desulfitobacterium sp. strain PR	~1400	1,2-DCA, CA, VC & Ethene (trace amount)	Isolate	Yes	(Ding, et al., 2014)

[a] 1,1,2-TCA was not completely dechlorinated (only 37% conversion).

[b]Not available.

[c]Metabolic reductive dehalogenation.

The unsatisfactory 1,1,2-TCA dechlorination that can be achieved co-metabolically makes such processes unsuitable for bioremediation. Metabolic 1,1,2-TCA dechlorination was first reported in *Desulfitobacterium dichloroeliminans* strain DCA1. This strain couples microbial growth with dihaloelimination of 1,1,2-TCA, resulting in production and accumulation of VC (De Wildeman, et al., 2003), which is an identified carcinogenic

compound and ranks 4th on the U.S. EPA National Priorities List (ATSDR, 2013). Compared with co-metabolic 1,1,2-TCA dechlorination, strain DCA1 can tolerate high concentrations of 1,1,2-TCA, as high as ~500 μM, and is therefore be applicable at contaminated sites. Later, other genera including *Dehalobacter* and *Dehalogenimonas* were also found to be involved in metabolic reductive dehalogenation of 1,1,2-TCA via dihaloelimination (De Wildeman, et al., 2003, Grostern and Edwards, 2006b, Maness, et al., 2012, Bowman, et al., 2013). Similar to incomplete co-metabolic dechlorination of 1,1,2-TCA, dihaloelimination of 1,1,2-TCA, which results in the accumulation of carcinogenic VC as an end product, limits the application of these bacteria in bioremediation efforts.

In summary, co-metabolic dechlorination of 1,1,2-TCA is in low initial concentrations and the dechlorination is usually incomplete, and the only described metabolic pathway for TCA degradation leads to the accumulation of carcinogenic compound, VC. As a result, bioremediation of 1,1,2-TCA requires discovery of alternative degradation pathways that both circumvent the production of VC and completely dechlorinate the parent compound.

2.2.2 Chloroform/1,1,1-TCA-dechlorinating bacterial cultures

Since chloroform and 1,1,1-TCA share a similar structure and both contain a trihalomethyl group, they are often dechlorinated to less substituted molecules by the same mechanisms (Grostern, et al., 2010, Ding, et al., 2014).

Chloroform and 1,1,1-TCA can be both degraded either via co-metabolic or metabolic processes (Table 2.3 & Table 2.4). Co-metabolic degradation usually takes place in methanogenic or sulfate reducing conditions in anaerobic environments. Several strains belonging to the genera

Methylosinus, *Methanosarcina*, *Desulfobacterium* and *Pseudomonas* have been reported to co-metabolically degrade chloroform or 1,1,1-TCA. The degradation products include less chlorine substituted molecules (DCM from chloroform and 1,1-DCA, CA, ethane and 1,1-DCE from 1,1,1-TCA), carbon dioxide, carbon monoxide and organic acids. For example, *Methylosinus trichosporium* strain OB3b can degrade both chloroform or 1,1,1-TCA to carbon dioxide. However, co-metabolic degradation requires the addition of other nutrients for growth. Strains DHM-1B and DHM-1T are excellent examples of this phenomenon, both of which can degrade chloroform in concentrations as high as 3700 μM, yet the cultures need to be pregrown with methane prior to exposure to chloroform. Additionally, application of the co-metabolic degradation of 1,1,1-TCA and chloroform by methanogens and sulfate reducing cultures is restricted because the carbon cycling pathways can be severely inhibited by even very low initial chloroform or 1,1,1-TCA concentrations. Metabolic degradation can overcome the low tolerance to chloroform and 1,1,1-TCA as the initial concentrations can be a hundred times higher than is inhibitory to co-metabolic processes. The metabolic degradation of chloroform and 1,1,1-TCA can be achieved through stepwise reductive dechlorination, resulting in the production of DCM from chloroform and 1,1-DCA and/or CA from 1,1,1-TCA, respectively. Only a few bacterial cultures, belonging to *Dehalobacter* and *Desulfitobacterium* genera, have been reported to be involved in such stepwise reductive dechlorination. So far, only two strains, *Desulfitobacterium* sp. strain PR, dechlorinating both chloroform and 1,1,1-TCA, and *Dehalobacter* sp. strain TCA1, dechlorinating 1,1,1-TCA, have been isolated and characterized.

Table 2.3 Mixed cultures and isolates capable of anaerobically dechlorinating chloroform

Sample ID	Initial Conc. (µM)	Product	Type	Metabolic [c]?	Reference
Desulfobacterium autotrophicum	2.0	DCM	isolate	No	(Egli, et al., 1987)
Methylosinus trichosporium strain OB3b	0.2-750 [a]	CO_2	isolate	No	(Oldenhuis, et al., 1991)
(methanogenic culture)	27.3	DCM, CO_2	mixed	No	(Bagley and Gossett, 1995)
Methanosarcina barkeri strain 227	4.8	DCM, CO_2	isolate	No	(Bagley and Gossett, 1995)
(toluene-oxidizing *Pseudomonas* strains)	20	CO_2	isolate	No	(McClay, et al., 1996)
(butane-oxidizing strains)	38.6	CO_2	isolate	No	(Hamamura, et al., 1997)
(methanogenic culture)	8.5	DCM	mixed	No	(Weathers and Parkin, 2000)
(butane-oxidizing microcosms)	2.7-73	CO_2	mixed	No	(Frascari, et al., 2005)
(butane-oxidizing microcosms)	8.4-84	CO_2	mixed	No	(Frascari, et al., 2007)
Dehalobacter-containing culture Dhb-CF	490	DCM	mixed	Yes	(Grostern, et al., 2010)
culture DHM-1	4188 [b]	CO, CO_2, organic acids	mixed	No	(Shan, et al., 2010)
Pantoea strain DHM-1B	3700	Fatty acids	isolate	No	(Shan, et al., 2010)
Pantoea strain DHM-1T	3700	Fatty acids	isolate	No	(Shan, et al., 2010)
Dehalobacter-containing culture CFEVO	300	DCM	mixed	Yes	(Lee, et al., 2012a)
Desulfitobacterium strain PR	1200	DCM,MCM	isolate	Yes	(Ding, et al., 2014)

[a] Degradation of chloroform was measured with cell suspensions pregrown with methane in a fermenter.

[b] Culture DHM-1 grown in corn syrup underwent acclimation to increasing levels of chloroform before being exposed to this chloroform concentration.

[c]Metabolic reductive dehalogenation.

Table 2.4 Mixed cultures and isolates capable of anaerobically dechlorinating 1,1,1-TCA

Sample ID	Initial Conc. (μM)	Product	Type	Metabolic[b]?	Reference
(mud microcosm)	32	1,1-DCA and CA	mixed	N/A	(Parsons and Lage, 1985)
Desulfobacterium autotrophicum	2.0	1,1-DCA	isolate	N/A	(Egli, et al., 1987)
(methanogenic culture)	<0.7	1,1-DCA, CA, CO_2, 1,1-DCE, acetic acid	mixed	No	(Vogel and McCarty, 1987)
Methylosinus trichosporium strain OB3b	0.2-750 [a]	CO_2	isolate	No	(Oldenhuis, et al., 1991)
(methanogenic culture)	16	1,1-DCA and CA	mixed	No	(Ahlert and Enzminger, 1992)
(acidic / methanogenic culture)	(N/A)	1,1-DCA and CA	mixed	No	(Deipser and Stegmann, 1997)
(methanogenic, sulfate-reducing culture)	10	1,1-DCA and CA	mixed	No	(deBest, et al., 1997)
(wastewater sludge)	38	1,1-DCA, CA, ethane, 1,1-DCE	mixed	N/A	(Chen, et al., 1999)
Dehalobacter strain TCA1	450	1,1-DCA, CA	isolate	Yes	(Sun, et al., 2002)
Dehalobacter-containing culture	190	1,1-DCA, CA	mixed	Yes	(Grostern and Edwards, 2006a)
Desulfitobacterium strain PR	1200	CA	isolate	Yes	(Ding, et al., 2014)

[a] Degradation of chloroform was measured with cell suspensions pregrown with methane in a fermenter.
[b] Metabolic reductive dehalogenation.

Limited knowledge about chloroform and 1,1,1-TCA dechlorinating microbes hinders practical bioremediation strategies of these compounds. Further exploration of chloroform and 1,1,1-TCA dechlorinating cultures could help characterize their properties.

2.2.3 PCE-dechlorinating bacteria

PCE is persistent in aerobic conditions but can be reduced in anaerobic environments via reductive dechlorination. Members of genera *Desulfitobacterium, Dehalobacter, Sulfurospirillum, Desulfuromonas,*

Desulfomonile, *Geobacter* and *Dehalococcoides* have all been reported to anaerobically dechlorinate PCE to less chlorinated ethenes (Table 2.5).

Table 2.5 Isolates capable of anaerobically dechlorinating PCE

Sample ID	Product	Metabolic?	Reference
Desulfitobacterium PCE1	TCE	Yes	(Gerritse, et al., 1996)
Desulfitobacterium Viet1	TCE	Yes	(Tront, et al., 2006)
Sulfurospirillum SM	TCE	Yes	(Neumann, et al., 1998)
Dehalococcoides CG1	TCE,*trans*,*cis*-DCE	Yes	(Wang, et al., 2014a)
Dehalococcoides CG4	TCE,*trans*,*cis*-DCE	Yes	(Wang, et al., 2014a)
Dehalococcoides CG5	TCE,*trans*,*cis*-DCE	Yes	(Wang, et al., 2014a)
Dehalobacter PER-K23	*cis*-DCE	Yes	(Holliger, et al., 1998)
Dehalococcoides CBDB1	*cis*-DCE	Yes	(Marco-Urrea, et al., 2011)
Desulfitobacterium TCE1	*cis*-DCE	Yes	(Prat, et al., 2011)
Sulfurospirillum PCE_M2	*cis*-DCE	Yes	(Luijten, et al., 2003)
Geobacter SZ	*cis*-DCE	Yes	(Sung, et al., 2006a)
Desulfuromonas BB1	*cis*-DCE	Yes	(Sung, et al., 2003)
Desulfuromonas BRS1	*cis*-DCE	Yes	(Sung, et al., 2003)
Dehalococcoides MB	*trans*,*cis*-DCE	Yes	(Cheng and He, 2009)
Dehalococcoides 195	VC, ethene	Yes	(Maymó-Gatell, et al., 1997)
Dehalococcoides FL2	VC, ethene	No	(He, 2003, He, et al., 2005)
Dehalococcoides BAV1	ethene	No	(He, 2003, He, et al., 2005)
Dehalococcoides BTF08	ethene	Yes	(Poritz, et al., 2013)

The most dominant pathway for PCE dechlorination is stepwise dehalogenation from PCE to *cis*-DCE with TCE produced as an intermediate product. This pathway was first discovered in an enriched *Dehalobacter*-containing culture capable of dechlorinating PCE to *cis*-DCE in 1993 (Holliger, et al., 1993). *Dehalobacter restrictus* PER-K23, which was ultimately isolated in 1998 from this culture, was found to couple growth with PCE dechlorination (Holliger, et al., 1998). Later, isolates belonging to the genera *Desulfitobacterium*, *Geobacter, Sulfurospirillum,* and *Desulfuromonas* were also found to be capable of dechlorinating PCE to *cis*-DCE (Miller et al., 1997)(Luijten, et al., 2003, Sung, et al., 2003, Sung, et al., 2006a). Besides *cis*-DCE, its stereoisomer *trans*-DCE was also observed as an end product in some rare cases, suggesting an alternative mechanism for PCE dechlorination This alternative dechlorination pathway was first characterized in the *trans*-

DCE producing, enriched, *Dehalococcoides mccartyi*-containing culture MB, which was found to produce *trans*-DCE and *cis*-DCE in a ratio of 3.4:1 as the end products of PCE dechlorination (Cheng and He, 2009).

Compared with DCEs, TCE is less commonly observed as the end product of PCE dechlorination. Some *Desulfitobacterium* strains PCE1 and Viet1 (Gerritse, et al., 1996) as well as *Sulfurospirillum multivorans* strain SM (Neumann, et al., 1998) predominantly dechlorinate PCE via substitution of one chlorine to TCE.

Until now, the only bacteria shown to dechlorinate PCE beyond DCEs to VC and ethene are members of the genus *Dehalococcoides*. *Dehalococcoides mccartyi* strain 195, the first *Dehalococcoides* isolate obtained, was found to be capable of dechlorinating PCE to ethene (Maymó-Gatell, et al., 1997). Though this strain dechlorinates PCE to ethene, the predominant product is VC, which is then dechlorinated to ethene co-metabolically. Later, two other *Dehalococcoides mccartyi* strains, BAV1 able to dechlorinate all types of DCEs and VC and FL2 able to dechlorinate TCE, *tans-/cis*-DCE to VC and ethene, were reported. Although BAV1 and FL2 did not grow with PCE, but were able to co-metabolically dechlorinate PCE when other growth-supporting chloroethenes are present (He, 2003, He, et al., 2005). Until now, the only bacterium reported to metabolically dechlorinate PCE to ethene is *Dehalococcoides mccartyi* strain BTF08 which was identified in a highly enriched culture. Strain BTF08 can dechlorinate different types of chloroethenes and exhibits great potential for treating chloroethenes contaminated sites (Cichocka, et al., 2010, Poritz, et al., 2013).

PCE bioremediation aims to achieve complete detoxification from PCE to ethene. Although many different bacteria have the ability to dechlorinate PCE, most produce DCEs as final products and only one *Dehalococcoides* strain completely dechlorinates PCE past DCEs to ethene. Limited information is currently available to provide a clear insight into the characteristics of PCE detoxifying strains. Such insight would be helpful in development of bioremediation strategies for treating PCE contaminated sites.

2.3 *Dehalococcoide*s *mccartyi* strains and their genomic characteristics

Dehalococcoides mccartyi is the only genus known to be involved in complete detoxification of chloroethenes, including PCE, TCE, DCEs and VC. *Dehalococcoides mccartyi* strains also have versatile dehalogenating capabilities, utilizing numerous types of halogenated compounds, e.g. PCBs, PBDEs, chlorobenzenes and chlorophenols.

2.3.1 *Dehalococcoides mccartyi* strains

Dehalococcoides are obligate OHRB. The cells are non-motile, disc-shaped with a diameter of 0.3 - 1 μm and a thickness of 0.1 - 0.2 μm, and indented on opposite sides of the cell. Although a member of the Bacteria, *Dehalococcoides* strains have an Archaea-like cell walls comprising of a S-layer, rather than having a cell wall composed of peptidoglycan as is typical of bacterial cell walls (Hug, et al., 2013). *Dehalococcoides* are extremely fastidious, requiring acetate as a carbon source, hydrogen as an electron donor, specific halogenated compounds as electron acceptors, and vitamin B12 as growth cofactor (He, et al., 2007). *Dehalococcoides* are also strict anaerobes; oxygen exposure can irreversibly inhibit growth and viability (Amos, et al., 2007). This sensitivity to redox potential restricts the distribution of

Dehalococcoides to a narrow range of niches they usually exist and are isolated from aquifers containing halogenated compounds, contaminated or un-contaminated river sediments or anaerobic digester sludge.

The 16S rRNA genes of the *Dehalococcoides* strains share more than 98% identity and are all affiliated with the phylum *Chloroflexi*, forming a deeply branched lineage within the domain *Bacteria* and distinguishing with other described isolates by more than 10% sequence divergence (Hug, et al., 2013). *Dehalococcoides* are categorised into three phylogenetic subgroups; Cornell, Victoria and Pinellas, based on sequence differences in the hypervariable regions (V2 and V6) in the 16S rRNA gene. The Pinellas subgroup contains more members than Cornell and Victoria subgroups do (Table 2.6). The members of the Pinellas subgroup share almost identical (0-3 base differences) 16S rRNA gene sequences with each other and are different from Victoria and Cornell subgroups with 14-16 base and 23-24 base differences, respectively. Although *Dehalococcoides* can be differentiated by 16S rRNA genes, subgroup affiliation is not associated with geographic distributions and the range of halogenated compounds that can be respired by a particular strain.

Since the isolation and metabolic characterization of *Dehalococcoides* strain 195, more *Dehalococcoides* have been identified, widening the array of halogenated compounds known to be dehalogenated by this unique genus to other aliphatic chlorinated compounds and aromatic chlorinated compounds. Notably, the *Dehalococcoides* can dechlorinate aliphatic chlorinated compounds to less or non-chlorinated molecules, whereas aromatic

chlorinated compounds, such as chlorophenols, chlorobenzenes and PCBs, can only be dechlorinated to less chlorinated molecules thus far (Table 2.6).

2.3.2 Genomic features of the genus *Dehalococcoides*

Since the release of the complete genomes of *Dehalococcoides mccartyi* strains 195 and CBDB1 in 2005, complete genome sequences of 11 different *Dehalococcoides* strains have been made available online in the National Center for Biotechnology Information (NCBI) database (Table 2.7). Genomic characterization can help gain a better insight of physicological, biochemical, evolutionary and ecological characteristics of *Dehalococcoides* strains.

Dehalococcoides strains have smaller genomes, within 1.34 and 1.48 Mb and tend to contain *rdhAs*, ranging from 11 to 37, compared to non-obligate OHRBs which typically have genome sizes of about 5 Mb and harbour less than six *rdhA*s. The small genome size characteristic of *Dehalococcoides* results in fewer predicted open reading proteins (ORFs) and may be the reason for the restricted versatile metabolic activities of *Dehalococcoides*. The large number of *rdhA*s may increase the niche-specific adaptability of *Dehalococcoides*.

Table 2.6 *Dehalococcoides mccartyi* strains and substrate spectra

Subgroup	ID	Substrate	Metabolites	Ref.
Cornell	195	PCE, TCE, *cis*-DCE, 1,1-DCE	VC(ethene)	(Maymó-Gatell, et al.,
		1,2-DCA	Ethene, trace amounts of VC	1997, Maymó-Gatell, et al.,
		1,2,3,4-TeCDD	1,2,4-CDD, 1,3-CDD	1999, Fennell, et al., 2004)
		Hexachlorobenzene	1,2,3,4-tetrachlorobenzene, 1,3,5-TCB	
		2,3,4,5-chlorobiphenyl	2,3,4,6-CB,2,3,5,6-CB, 2,4,6-CB	
		2,3-DCP	Ortho chlorine removal	
	MB	PCE,TCE	*trans*-DCE, *cis*-DCE	(Cheng and He, 2009)
	ANAS1	TCE, *cis*-DCE, 1,1-DCE	VC(ethene)	(Lee, et al., 2011)
	ANAS2	TCE, *cis*-DCE, 1,1-DCE,VC	Ethene	(Lee, et al., 2011)
	CG4	PCE	TCE, *trans*-DCE, *cis*-DCE	(Wang, et al., 2014a)
		PCB (Aroclor 1260)	Various products	
Pinellas	CBDB1	PCE,TCE	*trans*-DCE, *cis*-DCE	(Adrian, et al., 2007,
		Hexachlorobezene, pentaCB, 1,2,3,5-CB	1,3-DCB,1,4-DCB,1,3,5-TCB	Wagner, et al., 2009,
		2,3-DCP, TCPs, TeCPs and pentaCPs	Lower chlorinated phenols	Schiffmann, et al., 2014)
		Polychlorinated dioxins	Dichloro-dioxins	
		PCBs (Aroclor 1260)	Various products	
	BAV1	DCEs, VC, 1,2-DCA	Ethene	(He, 2003)
	FL2	TCE, DCEs	VC(ethene)	(He, et al., 2005)
	GT	TCE, *cis*-DCE, 1,1-DCE, VC	Ethene	(Sung, et al., 2006b)
	DCMB5	1,2,4-TrCDD	2-MCDD	(Bunge, et al., 2003, Bunge,
		1,2,3-trichlorobezene	1,3-DCB	et al., 2008)
	11a	TCE, DCEs, VC, 1,2-DCA	Ethene	(Lee, et al., 2012b)
	11a5	TCE, DCEs	VC(ethene)	(Lee, et al., 2012b)
	BTF08	PCE,TCE,*cis*-DCE,1,1-DCE,VC	Ethene	(Poritz, et al., 2013)
	CG5	PCE	TCE, *trans*-DCE, *cis*-DCE	(Wang, et al., 2014a)
		PCB (Aroclor 1260)	Various products	
	SG1	PCE	TCE, *trans*-DCE, *cis*-DCE	(Wang, et al., 2014b)
		PCB (Aroclor 1260)	Various products	
	JNA	Pentachlorophenol	3,5-DCP	(Fricker, et al., 2014)
		2,3,4,6-TeCP	2,4-DCP,3,4-DCP	
		2,3,6-TCP	3-CP	
		2,3,4-TCP	3,4-DCP,2,4-DCP	
		2,3-DCP	3-CP	
		PCB (Aroclor 1260)	Various products	
Victoria	VS	TCE, *cis*-DCE, 1,1-DCE, VC	Ethene	(Müller, et al., 2004)
	CG1	PCE	TCE, *trans*-DCE, *cis*-DCE	(Wang, et al., 2014a)
		PCB (Aroclor 1260)	Various products	

Table 2.7 Genomic characteristics of OHRB

Taxon ID*	Strain ID	Phylum	Genome Size (Mbp)	ORFs	GC%	# *rdh*
640427111	***Dehalococcoides mccartyi* BAV1**	**Chloroflexi**	**1.342**	**1443**	**47**	**11**
2540341061	***Dehalococcoides mccartyi* BTF08**	**Chloroflexi**	**1.452**	**1580**	**47**	**20**
637000090	***Dehalococcoides mccartyi* CBDB1**	**Chloroflexi**	**1.396**	**1517**	**47**	**32**
2540341062	***Dehalococcoides mccartyi* DCMB5**	**Chloroflexi**	**1.432**	**1526**	**47**	**23**
646564526	***Dehalococcoides mccartyi* GT**	**Chloroflexi**	**1.360**	**1483**	**47**	**20**
2554235449	***Dehalococcoides mccartyi* GY50**	**Chloroflexi**	**1.407**	**1591**	**47**	**25**
646311919	***Dehalococcoides mccartyi* VS**	**Chloroflexi**	**1.413**	**1489**	**47**	**37**
670941901	***Dehalococcoides mccartyi* CG1**	**Chloroflexi**	**1.487**	**1557**	**47**	**35**
670943471	***Dehalococcoides mccartyi* CG4**	**Chloroflexi**	**1.382**	**1421**	**49**	**15**
670944970	***Dehalococcoides mccartyi CG5***	**Chloroflexi**	**1.362**	**1413**	**47**	**26**
648028022	***Dehalogenimonas lykanthroporepellens BL-DC-9***	**Chloroflexi**	**1.687**	**1771**	**55**	**21**
2510065016	***Dehalobacter restrictus DSM 9455***	**Firmicutes**	**2.943**	**2908**	**45**	**23**
2519899776	***Dehalobacter sp. DCA***	**Firmicutes**	**3.070**	**3038**	**45**	**18**
2519899539	***Dehalobacter sp. CF***	**Firmicutes**	**3.092**	**3040**	**44**	**18**
2507262031	*Desulfitobacterium dehalogenans JW/IU-DC1*	Firmicutes	4.322	4252	45	6
2507149019	*Desulfitobacterium dichloroeliminans LMG P-21439*	Firmicutes	3.624	3537	44	1
643348537	*Desulfitobacterium hafniense DCB-2*	Firmicutes	5.279	5042	48	7
637000093	*Desulfitobacterium hafniense Y51*	Firmicutes	5.728	5208	47	1
2508501136	*Desulfosporosinus orientis Singapore I, DSM 765*	Firmicutes	5.863	5638	43	1
643886183	*Dethiobacter alkaliphilus AHT1*	Firmicutes	3.117	3213	48	1
641522632	*Heliobacterium modesticaldum Ice1*	Firmicutes	3.075	3142	57	1
637000007	*Anaeromyxobacter dehalogenans 2CP-C*	Proteobacteria	5.013	4421	75	1
2524023220	*Desulfobacula toluolica Tol2*	Proteobacteria	5.198	4435	41	2
2509601019	*Desulfomonile tiedjei DCB-1, DSM 6799*	Proteobacteria	6.527	5664	50	3
642555130	*Geobacter lovleyi SZ*	Proteobacteria	3.995	3777	55	2
637000137	*Jannaschia sp. CCS1*	Proteobacteria	4.404	4339	62	2
2510065028	*Phaeobacter gallaeciensis 2.10*	Proteobacteria	4.161	3798	60	1

2510065029	*Phaeobacter gallaeciensis DSM 17395*	Proteobacteria	4.227	3960	60	1
2558309061	*Phaeobacter gallaeciensis DSM 26640*	Proteobacteria	4.540	4437	59	1
2574179718	*Phaeobacter inhibens T5, DSM 16374*	Proteobacteria	4.159	3976	60	1
2548877138	*Planktomarina temperata RCA23, DSM 22400 (RCA23)*	Proteobacteria	3.288	3101	54	2
2511231065	*Pseudovibrio sp. FO-BEG1*	Proteobacteria	5.917	5560	52	1
639633056	*Roseobacter denitrificans OCh 114*	Proteobacteria	4.331	4201	59	1
2510065042	*Roseobacter litoralis Och 149*	Proteobacteria	4.745	4668	57	1
637000267	*Ruegeria pomeroyi DSS-3*	Proteobacteria	4.601	4355	64	2
637000268	*Ruegeria sp. TM1040*	Proteobacteria	4.154	3964	60	1
640753050	*Shewanella sediminis HAW-EB3*	Proteobacteria	5.518	4666	46	5
2558860208	*Sulfurospirillum multivorans DSM 12446*	Proteobacteria	3.176	3285	41	2

*Genome information mined from the NCBI database (Markowitz, et al., 2012). Obligate OHRB indicated in bold.

Each *Dehalococcoides* genome comprises three characteristic features, the core genome region and two high plasticity regions (HPRs) (McMurdie, et al., 2009). The core genome is highly conserved among *Dehalococcoides* strains and accounts for 68%-77% of the whole genome (Löffler, et al., 2013). Genes in this region determine core features such as metabolic activities including, DNA replication, transcription and translation, amino acid synthesis and acetyl-CoA for acetate uptake. Analyses of the core genome corroborate morphological and physicological observations of *Dehalococcoides*. Consistent with morphological characteristics, genes involved in motility, sporulation and peptidoglycan biosynthesis are also absent (Maymó-Gatell, et al., 1997). Different from conserved regions, gene synteny in the HPRs is predominately disrupted, containing numerous genomic islands, insertion sequences and repeated elements, as well as *rdhAB*s (McMurdie, et al., 2009). The HPRs may indicate an unusual biological solution by *Dehalococcoides* exposed in evolutionary pressures for genome streamlining and respiratory diversification. The HPRs are variable in length (up to 200 kbp) and also include ~100 kbp of core genes which are occasionally interrupted in order. Many of these conserved HPR genes are thought to be involved in core metabolic functions, encoding different types of hydrogenases which function in electron transport.

The average nucleotide identities between different *Dehalococcoides* strains reveal >98.7 % similarity of core genes within subgroups and 86-87% similarity among different subgroups (Hug, et al., 2013). The strain-specific genes, reflecting the degree of evolutionary divergence, are restricted to the *rdh* operons, which are mostly located in HPRs. The presence or absence of

specific *rdhA* genes can be used to differentiate *Dehalococcoides* strains. For example, although strains ANAS1 and ANAS2 have identical 16S rRNA genes, they harbour different *rdhA*s; ANAS1 has *tceA* catalysing transformation of TCE to VC whereas ANAS2 has *vcrA* catalysing TCE to ethene (Lee, et al., 2012b).

2.4 Reductive dehalogenation process

2.4.1 Reductive dehalogenase

The characteristic reductive dehalogenation of OHRB is mediated by a class of highly conserved proteins (RDase). Theses enzymes were first biochemically characterized in *Desulfomonile tiedjei* strain DCB-1, which catalyses the reductive dechlorination of 3-chlorobenzoate (Cole, et al., 1995). Later, more RDases were identified from an archaeal genus, *Ferroglobus,* and from a wide variety of anaerobic bacteria, including *Anaeromyxobacter Sulfurospirillum, Desulfitobacterium, Dehalobacter* and *Dehalococcoides* (Table 2.8). RDases are membrane-anchored, oxygen-sensitive proteins which exhibit several highly conserved features, particularly a twin-arginine signal motif RRXFX, which is associated with translocating folded proteins to or across membranes, , and two iron-sulfur cluster binding motifs $CX_3CX_2CX_2CX$, which mediate electron transfer, (Magnuson, et al., 2000).

The characterized RDases catalyse halogen removal from a wide range of halogenated substrates. Though originally thought to be highly specific for single substrates, recent studies suggest that RDases are capable of catalytic dehalogenation of halogenated compounds bearing similar structures or functional groups

(Grostern, et al., 2010, Ding, et al., 2014). Other evidence seems to suggest that a single RDase can even catalyse dehalogenation of highly structurally dissimilar halogenated compounds, such as PCBs and PCE (Wang, et al., 2014a).

The first crystal structure of an RDase, PceA from *Sulfurospirillum multivorans,* was solved soon after the first heterologous production of a functional PceA, derived from *Desulfitobacterium hafniense* (Bommer, et al., 2014, Mac Nelly, et al., 2014, Payne, et al., 2014). The crystal structure revealed that the overall structure of PceA is dimeric with the catalytic site of the RDase deeply buried within the enzyme. Before the substrate can be recognized by the selective layer of the catalytic site, it must pass through two stereoselective channels formed by a structurally variable region leading to the catalytic site. The tightly packed amino acid side chains leave little conformational latitude for binding of non-target substrates, which explains the substrate specificity of the RDase. The crystal structure has provided both insights into many features of these enzymes, including two-electron transfer step in reductive dehalogenation, and a tantalizing clue to the relationship between amino acid sequence and substrate preference.

2.4.2 Reductive dehalogenase homologous genes

OHRBs may contain multiple reductive dehalogenase homologous genes, e.g. *Dehalococcoides* strains harbour 11-32 such genes. *rdh* genes tend to exist as operons comprising an *rdhA,* encoding the catalytic subunit, and an *rdhB,* encoding a membrane-anchoring protein. Sometimes additional genetic elements such as transcriptional regulators, trigger factors and corrinoid cofactors are also collocated with *rdhAB* gene clusters (Figure 2.1). The

regulatory mechanisms of *rdhA* expression remain unclear, however indirect evidence from transcriptional studies suggests a positive correlation between *rdhA* expression and dehalogenation activity (Holmes, et al., 2006). This would imply that an *rdhA* should be upregulated by the presence of a specific halogenated compound that serves as a substrate for the enzyme encoded by the *rdhA*.

Although the function of several *rdhAs* have been identified through N-terminal amino acid sequencing of purified enzymes, low biomass yields of OHRB have impeded large-scale purification and biochemical characterization and only a few among several hundred *rdhA*s have been functionally characterized (Table 2.8). Slow progress have made towards heterologous production of functional *rdhA* enzymes to facilitate analysis of the substrate spectra of different *rdhA* over nearly a decade (Suyama, et al., 2001, John, et al., 2006), however the successful recent heterologous expression of PceA from *Desulfitobacterium hafniense* in a non-dechlorinating host, *Shimwella blattae*, was successfully achieved (Mac Nelly, et al., 2014). This success was achieved by a co-production of a trigger factor, PceT, and amendment with specific precursors for RDase co-factors. The ability to heterologously express *rdhA* provides a new avenue which may be helpful for functional analysis of other *rdhAs.*

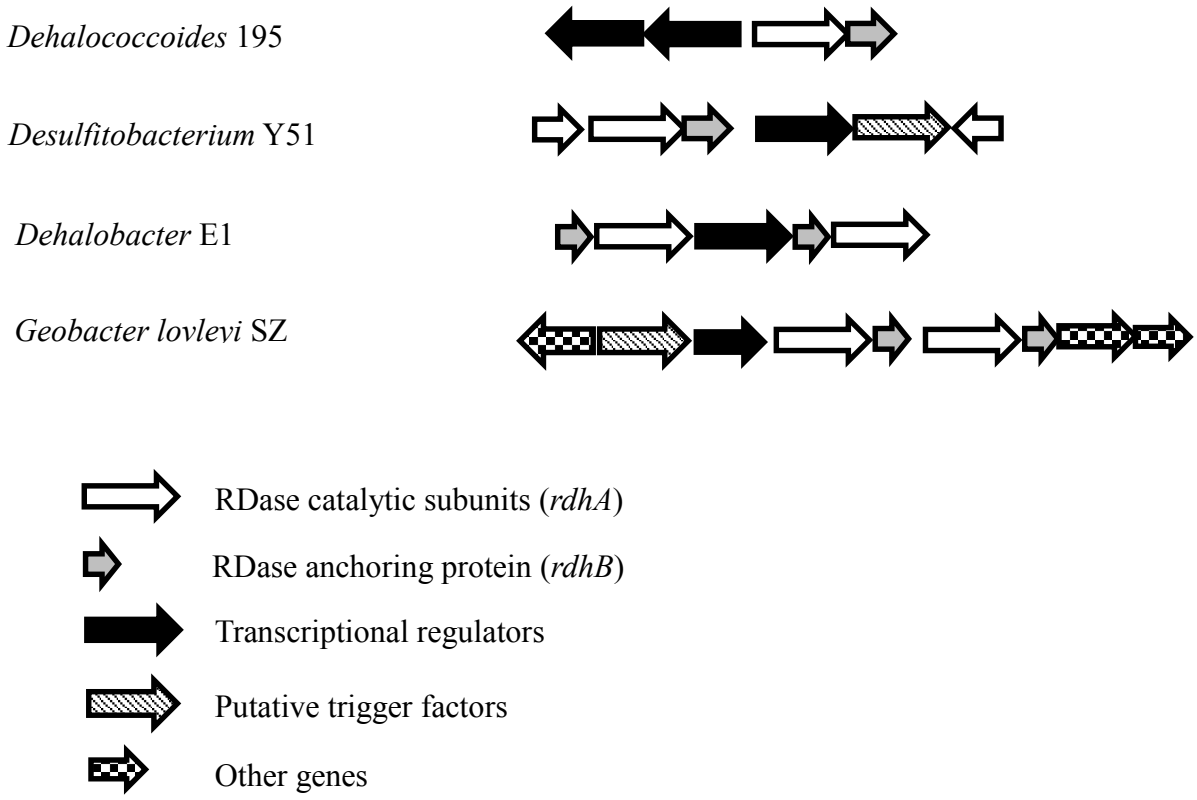


Figure 2.1 Functional *rdh* gene clusters in OHRB

*rdhA*s have a high substrate specificity, such that the substrate spectrum may vary with only 2% base pair differences between *rdhA* sequences. As such, the presence or absence of specific *rdhA* has been used to predict dehalogenation potential of existing communities at contaminated sites and these genes are extensively used as biomarkers for monitoring active and passive bioremediation efforts.

Table 2.8 Identified reductive dehalogenase genes in dehalogenators

rdhA	Microorganism	Catalysed Substrate	Products	Reference
PceA	*Sulfurospirillum multivorans*	PCE, TCE,	*cis*-DCE	(Neumann, et al., 1996, Neumann, et al., 1998, Ye, et al., 2010)
PceA	*Dehalobacter restrictus*	PCE, TCE	*cis*-DCE	(Schumacher, et al., 1997, Maillard, et al., 2003)
PceA	*Dehalococcoides mccartyi* strain 195	PCE	TCE	(Magnuson, et al., 1998)
PceA	*Desulfitobacterium hafniense* strain PCE-S	PCE, TCE, *cis*-/*trans*-DBE,	*cis*-DCE	(Miller, et al., 1998, Ye, et al., 2010)

PceA	*Desulfitobacterium* sp. strain PCE1	PCE	*cis*-DCE	(van de Pas, et al., 2001)
PceA	*Desulfitobacterium hafniense* strain TCE1	PCE, TCE	*cis*-DCE	(van de Pas, et al., 2001)
PceA	*Desulfitobacterium* sp. strain Y51	PCE, TCE	*cis*-DCE	(Suyama, et al., 2002)
Ssed_3769 (PceA)	*Shewanella sediminis*	PCE	TCE	(Lohner and Spormann, 2013)
TceA	*Dehalococcoides mccartyi* strain 195	TCE	VC	(Magnuson, et al., 1998, Magnuson, et al., 2000)
VcrA	*Dehalococcoides mccartyi* strain VS	VC	Ethene	(Müller, et al., 2004)
BvcA	*Dehalococcoides mccartyi* strain BAV1	VC	Ethene	(Krajmalnik-Brown, et al., 2004)
MbrA	*Dehalococcoides mccartyi* strain MB	TCE	*trans*-DCE, *cis*-DCE	(Chow, et al., 2010)
DcaA	*Desulfitobacterium dichloroeliminans* strain DCA1	1,2-DCA	VC	(Marzorati, et al., 2007)
WL RdhA1	*Dehalobacter* sp.	1,2-DCA	VC	(Grostern and Edwards, 2009)
CfrA	*Dehalobacter* sp. strain CF50	chloroform, 1,1,1-TCA	DCM 1,1-DCA	(Tang and Edwards, 2013)
DcrA	*Dehalobacter* sp. strain DCA	1,1-DCA	CA	(Tang and Edwards, 2013)
CtrA	*Desulfitobacterium sp.strain PR*	1,1,1-TCA chloroform	CA DCM	(Ding, et al., 2014)
CprA	*Desulfitobacterium* sp. strain PCE1	Cl-OH-phenylacetate	OH-phenylacetate	(van de Pas, et al., 2001)
CprA	*Desulfitobacterium hafniense* strain DCB-2	3-chloro-4-hydroxypheny lacetate	4-hydroxyphenyla cetate	(Christiansen, et al., 1998)
CprA	*Desulfitobacterium dehalogenans*	A number of *ortho*-chlorinated phenols	Less chlorinated phenols	(van de Pas, et al., 1999)
CprA	*Desulfitobacterium chlororespirans* strain Co23	3-chloro-4-hydroxybenzo ate, chlorinated phenols	4-hydroxybenzoat e; less chlorinated phenols	(Löffler, et al., 1996, Krasotkina, et al., 2001)
CrdA	*Desulfitobacterium hafniense* strain PCP-1	2,4,6-TCP, PCP	Ortho chlorine removal	(Boyer, et al., 2003)
CprA5	*Desulfitobacterium hafniense* strain PCP-1	3,5-DCP	*meta* and *para-chlorine removal*	(Thibodeau, et al., 2004)
CprA3	*Desulfitobacterium hafniense* strain PCP-1	PCP, TeCP, TCP	*Ortho* chlorine removal	(Bisaillon, et al., 2010)
CBDB A187	*Dehalococcoides mccartyi* strain CBDB1	1,2,3-TCB	1,3-DCB	(Wagner, et al., 2009)
CBDB A1624	*Dehalococcoides mccartyi* strain CBDB1	1,2,4-TCB	1,4-DCB	(Wagner, et al., 2009)
CbrA	*Dehalococcoides mccartyi* strain CBDB1	Chlorinated benzenes	Less chlorinated bezenes	(Adrian, et al., 2007, Wagner, et al., 2009)
CBDB A1453	*Dehalococcoides mccartyi* strain CBDB1	1,2,3-TCB	1,3-DCB	(Wagner, et al., 2009)

(four to six RdhAs)	*Dehalococcoides mccartyi* strain CBDB1	brominated benzenes	Less brominated benzenes	(Wagner, et al., 2012a)
3-ClBA–RD	*Desulfomonile tiedjei* strain DCB-1	3-chlorobenzoate	chlorobenzoate	(Ni, et al., 1995)
PcbA1	*Dehalococcoides mccartyi* strain CG1	PCB (Aroclor 1260); PCE	Less chlorinated PCBs; TCE, *trans*-,*cis*-DCEs	(Wang, et al., 2014a)
PcbA4	*Dehalococcoides mccartyi* strain CG4	PCB (Aroclor 1260); PCE	Less chlorinated PCBs; TCE, *trans*-,*cis*-DCEs	(Wang, et al., 2014a)
PcbA5	*Dehalococcoides mccartyi* strain CG5	PCB (Aroclor 1260); PCE	Less chlorinated PCBs; TCE, *trans*-,*cis*-DCEs	(Wang, et al., 2014a)

2.4.3 Horizontal acquisition and transfer of reductive dehalogenase genes

Similarities in the organization of *rdh* operons and in the protein sequences of RDases among phylogenetically distinct OHRB have raised questions about the evolutionary origin of *rdh* . The several hundred known RDases can be classified into at least four phylogenetically distinct groups suggesting that these enzymes may have evolved independently from unconnected ancestors. It has been hypothesized that duplication, multiplication and transfer of genetic elements, may have allowed OHRB to adapt to environments containing a rapidly expanding quantity and range of xenobiotic halogenated organic compounds (Richardson, 2013).

Though the mechanisms of *rdh* gene flow remain unknown, genomic analyses of *Dehalococcoides* have shed some light on potential acquisition mechanisms. Firstly, in strain VS, the VC RDase encoding gene, *vcrAB*, is embedded in genomic islands (Prat, et al.,2011) that is flanked by site specific recombinase A (*ssrA*) and its 20 bp direct repeats. Later, this type of *ssrA*-specific genomic islands (*ssrA*-GIs) were characterized as a common feature among *Dehalococcoides* genomes (McMurdie, et al., 2009). McMurdie *et al*.

(2011) found 31 *ssrA*-GIs in *Dehalococcoides* strains harbouring 41 *rdhA* genes, further implicating *ssrA*-specific integration as a mechanism of *rdhA* acquisition in *Dehalococcoides* (McMurdie, et al., 2011). Additionally, the HPR1 of strain BAV1 contains four repeated 3' fragments of tRNA-Ala-1 located closely to a putative *rdhA*, two similar fragments were also identified in HPR1 of strain CBDB1(Kube, et al., 2005). tRNA-Ala was also found to be located at each boundary of HPR1 and at the downstream boundary of HPR2. These findings suggest another mechanism of acquisition of genetic elements with the tRNA-Ala gene apparently a recombination site. Finally, rearrangement could act as another mechanism for acquisition of genetic elements through observation of multiple *rdhA* gene clusters presenting in a single chromosome. For example, the flanking insertion elements (repeats) were found in *pceA* operon from *Desulfitobacterium hafniense* strain TCE1 which contains identical *pceA* operon in both chromosome and the transposable element, Tn-Dha1 (Maillard, et al., 2005).

In this chapter, the previous studies relating to OHRB mediating dechlorination of specific chlorinated compounds including 1,1,2-TCA, 1,1,1-TCA, chloroform and PCE, and the mechanisms of this reductive dehalogenation process as well as the physical, phylogenetic and genomic characteristics of *Dehalococcoides* which is one of the representative OHRB have been reviewed. Although several bacterial cultures have been enriched to remediate different types of chlorinated compounds, bacteria with satisfactory performance to deal with these chlorinated compounds or co-contaminates are still limited. The mechanisms, that the RDase genes catalysing these reductive dehalogenation process, need to be further explored.

3 Chapter III Detoxification of 1,1,2-Trichloroethane to Ethene by *Desulfitobacterium* and Identification of its Functional Reductase Gene

3.1 Introduction

1,1,2-trichloroethane (1,1,2-TCA), molecular formula $CHCl_2$-CH_2Cl, has been widespread in the environment by extensive usage as a degreasing agent, through improper storage and disposal, and from incomplete dechlorination of the solvent 1,1,2,2-tetrachloroethane. 1,1,2-TCA is very persistent in the environment, with an estimated half-life of 136-360 days in soil and 136-720 days in groundwater (ATSDR, 2010). It is found in at least 262 out of 1293 sites in the United States on the National Priorities List (NPL) identified by the U.S. Environmental Protection Agency (ATSDR, 2013). The geometric mean maximum concentrations of 1,1,2-TCA is 0.57 μM in water and 2.14 mg/kg in soil. Pollution caused by 1,1,2-TCA has raised concerns because of its potential for adverse effects on the liver, the kidneys, and the nervous and immune systems (ATSDR, 2010). Detoxification of 1,1,2-TCA has historically been focused on chemical oxidation or physical adsorption (Coons, et al., 2000, Plagentz, et al., 2006). However, both of these approaches are limited by high energy and material costs, as well as the potential for incomplete degradation of 1,1,2-TCA.

Since the first reports of anaerobic dechlorination of 1,1,2-TCA by a chlorobenzoate enriched bioreactor containing *Desulfomonile tiedjei* strain DCB-1 in 1994 (Fathepure and Tiedje, 1994), the promised potential of microorganisms for treatment of this environmental contaminant has been demonstrated (Table 2.2). Although the enriched culture containing strain

DCB-1 exhibited the ability to dechlorinate 1,1,2-TCA, it failed to convert 63% of an initial concentration of ~10.7 μM 1,1,2-TCA to 1,2-DCA. Thus far, both uncharacterized mixed cultures (Chen, et al., 1996, Aulenta, et al., 2006) and other reported strains belonging to the *Desulfitobacterium*, *Dehalobacter*, and *Dehalogenimonas* genera (De Wildeman, et al., 2003, Grostern and Edwards, 2006b, Moe, et al., 2009, Maness, et al., 2012, Bowman, et al., 2013) have been shown to dechlorinate 1,1,2-TCA through dihaloelimination, resulting in the accumulation of vinyl chloride as the end product. Vinyl chloride is an identified carcinogenic compound and ranks 4th on the U.S. EPA National Priorities List (ATSDR, 2006b, 2013). Therefore, it is essential to look for alternative pathways that circumvent the production of vinyl chloride so as to achieve complete detoxification of 1,1,2-TCA. For 1,2-DCA, another metabolic intermediate of 1,1,2-TCA, is also a groundwater pollutant found in 582 of 1293 NPL sites in the United States identified by the U.S. Environmental Protection Agency (ATSDR, 2013). Although 1,2-DCA is much less toxic than vinyl chloride, its removal is still of importance.

Desulfitobacterium sp. strain PR was isolated from a bioreactor performing reductive dechlorination of chloroethenes and chloroethanes (Ding, et al., 2014). This strain has been shown to reductively dechlorinate 1,1,1-TCA and chloroform, and harbours two reductive dehalogenase (RDases) genes, *ctrA* and *prdhA*. CtrA has been identified to reductively dechlorinate 1,1,1-TCA and chloroform, but the function of *prdhA* remains unknown. In this study, the dechlorination capability of strain PR was further explored on 1,1,2-TCA and the responsible 1,1,2-TCA RDase gene was identified by both gene expression studies and proteomics tools. Due to the

accumulation of 1,2-DCA from dechlorination of 1,1,2-TCA by strain PR, a *Dehalococcoides*-containing mixed culture GEO, which was enriched from the same source as strain PR and dechlorinates 1,2-DCA rapidly, was co-cultivated with strain PR to explore potential for complete detoxification of 1,1,2-TCA. Additionally, to achieve complete detoxification of 1,1,2-TCA and to address the problems posed by co-contamination of trichloroethene (TCE) and 1,1,2-TCA, a co-culture consisting of strain PR and *Dehalococcoides mccartyi* strain 11a was established. *Dehalococcoides mccartyi* strain 11a was chosen to address co-contamination caused by TCE and 1,1,2-TCA since it can reductively dechlorinate both TCE and 1,2-DCA (Lee, et al., 2013).

3.2 Materials and Methods

3.2.1 Chemicals and bacterial growth conditions

All chemical reagents including components in DCB1 medium, chlorinated chemicals and reagents used in *in vitro* assays were purchased from Sigma-Aldrich (St. Louis, MO) or Merck (Darmstadt, Germany) at the highest purity available. Cultures were grown in an anaerobic bicarbonate-buffered defined DCB1 medium. Strain PR and culture GEO were spiked with filter-sterilized pyruvate (10 mM) as the carbon source/electron donor and 1,1,2-TCA, 1,1,1-TCA or chloroform as the electron acceptor. *Dehalococcoides mccartyi* strain 11a was spiked with sodium acetate (10 mM) as the carbon source, hydrogen as the electron donor (0.33 atm) and TCE as the electron acceptor as previously described (He, et al., 2007). For all dechlorination kinetics studies and cell collections for proteomic analysis, strain PR was grown in 160-mL serum bottles containing 100 mL liquid

medium at 30 °C. TCE ranging from 0.002 mM to 1.12 mM were added to cultures provided with 0.30 mM 1,1,2-TCA to explore TCE inhibitory effects on 1,1,2-TCA dechlorination. Cell density of cultures amended with 1,1,2-TCA ranging from 0.10 mM to 1.20 mM at day 4 was used to assess net growth yield per moles of 1,1,2-TCA. Two sets of experiments were conducted to investigate the cometabolic dechlorination of 1,2-DCA to chloroethane. In the first set, 0.92 mM 1,2-DCA was added as the sole electron acceptor. In the second set, 0.30 mM 1,1,2-TCA was spiked initially, and another 0.30 mM 1,1,2-TCA was spiked on day 8. When exploring dechlorinating preference of strain PR on different substrates, 0.51 mM 1,1,2-TCA and 0.59 mM chloroform was amended simultaneously. All kinetics studies were performed in triplicate with abiotic controls (without bacterial inocula or with autoclaved cultures).

3.2.2 Chemical analytical procedures

Headspace samples of chloromethanes, chloroethanes, and chloroethenes were analyzed with an Agilent gas chromatograph (GC7890) equipped with a flame ionization detector and a GS-GasPro column (30 m by 0.32 mm; J&W Scientific). The oven temperature was initially held at 50 ºC for 2 min, increased to 220 ºC at 30 ºC /min and held for 1min. Headspace samples were manually injected to the GC using a gas-tight glass syringe (Model Gastight #1725, Hamilton Co., Reno, Nevada).

3.2.3 Molecular analyses

Cells for DNA extraction were collected from 1 mL culture samples by centrifugation (10 min at 10,000 g, 4 ºC). Cell pellets were stored at -20 ºC until further processing. Genomic DNA was extracted using the Qiagen

DNeasy Blood and Tissue Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was conducted on an ABI 7500 Fast System using SYBR green (Lo-ROX, SensiFASTTM SYBR, BIOLINE) as described previously (Lee and He, 2010). The thermocycling program was as follows: an initial step of 3 min at 95 ºC, followed by 40 cycles of 5 s at 95 ºC and 30s at 60 ºC. Construction of plasmids carrying targeted genes to serve as qPCR standards was performed as described previously (Chow, et al., 2010). *Desulfitobacterium*-genus specific primers targeting 16S rRNA gene (Dsb1299F and Dsb1448R) were utilized to monitor cell growth. The cell numbers of strain PR equal to the gene copies of 16 S rRNA gene considering that strain PR contains single-copy 16 S rRNA gene. *ctrA* gene primers (ctrAF and ctrAR), and *prdhA* gene primers (prdhAF and prdhAR) were used to monitor gene expression levels in strain PR (Ding, et al., 2014). To identify the 1,1,2-TCA RDase genes, transcription of *ctrA* and *prdhA* were monitored during dechlorination of 1,1,2-TCA. 5% (v) inoculum (starved for 3 days) was transferred to fresh medium with or without 1,1,2-TCA. Duplicate cell samples were collected at 12 hour intervals for DNA (1 mL of culture) and RNA (1.5 mL of culture) extractions (10 min at 10,000 g, 4 ºC). The RNA extraction and reverse transcription were conducted as described previously (Ding, et al., 2014). That is, Trizol was used for cell lysis and RNA stabilization. The QIAGEN RNeasy mini kit was used for RNA extraction (QIAGEN GmbH) according to the manufacturer's instructions. Luciferase control RNA (Promega, Fitchburg, WI, USA) was added before RNA extraction as an internal standard to evaluate mRNA loss during RNA extraction, reverse transcription and quantification. Reverse

transcription was conducted directly after RNA elution using the QIAGEN Sensiscript kit according to the manufacturer's instructions with random hexa primer (Promega) and RNase inhibitor (Promega).

3.2.4 Enzyme assays

To characterize the localization of 1,1,2-TCA RDase, membrane and crude proteins each collected from 100 mL cultures were used for *in vitro* activity assays. Cell pellets were collected by centrifugation at 10,000 g for 20 min at 4 ºC and re-suspended in 0.1 mL fresh DCB1 medium. To prepare crude proteins, cells were broken by ultrasonication using a VCX130 sonicator (Vibra-Cell, SONICS) (130W; 20% duty cycle; for 3 min). The lysate was centrifuged at 10,000 g for 30 min and the supernatant containing the crude protein was harvested. To obtain membrane proteins, the crude protein extract was ultracentrifuged at 120,000 g for 1 h at 4 ºC. The pellets containing membrane proteins were solubilized in 10 mM CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) and concentrated by centrifugation through a membrane filter with a molecular cutoff of 5 kDa (Vivaspin2, Sartorius Stedium Biotech). The *in vitro* activity assay was carried out in 10-mL vials containing 2 mL assay solution (2 mM methyl viologen; 1.5 mM titanium (III) citrate; 100 mM Tris-HCl buffer (pH 7.0)) spiked with 300 µM 1,1,2-TCA inside an anaerobic chamber as described previously (Adrian, et al., 2007). The mixtures were incubated at 30 ºC for 72 h prior to GC analysis.

1,1,2-TCA RDase substrate range characterization was performed with 0.1 mM of each substrate (1,1,2-TCA, 1,1,1-TCA, 1,2-DCA, 1,1-DCA or chloroform) added to duplicate 4-mL vials containing the assay solution.

Crude extracts were obtained by disrupting harvested cells with a VCX 130 sonicator (Vibra-Cell, SONICS) (130W; 20% duty cycle; for 3 min). Crude extracts obtained from cultures grown with 1,1,2-TCA were added to 2 mL assay solution. The mixtures were incubated at 30 ºC for 24 h prior to headspace analysis. The effect of substrate concentration on the rate of dechlorination for each substrate (1,1,1-TCA, 1,1,2-TCA, or chloroform) was assayed. For each substrate, crude cell extracts from cultures grown on the corresponding substrate was incubated at 30ºC for 2 h with six concentrations of substrates ranging from 0.05 mM to 2.50 mM. Reactions were quenched by adding 0.5 mL of hydrochloric acid solution (0.012 mM) and analyzed by GC. All enzyme assays were performed with abiotic controls without addition of crude cell extract.

3.2.5 Proteomic analyses

Native polyacrylamide gels (native-PAGE) with a 10% resolving gel and a 5% stacking gel were prepared as described previously (U.K.Laemmli, 1970), except that the experiment was carried out in an ice box and sodium dodecyl sulfate (SDS) was removed from all buffers to avoid protein denaturation.

Native PAGE analysis using a modified staining protocol was conducted with crude cell proteins (Adrian, et al., 2007). Briefly, the same protein samples were added on adjacent gel lanes. Gel electrophoresis was run at 160 V for 2 h. After electrophoresis, one lane was silver stained using the PlusOne Silver Staining kit according to manufacturer's instructions, while the other lane was left unstained. Bands in the unstained lane were excised for RDase activity assays with 0.05 mM of substrate (1,1,2-TCA, 1,1,1-TCA, or

chloroform). After 24 h of incubation at 30 ºC, headspace of each sample was analyzed by GC.

Prominent bands in the stained lanes corresponding to gel regions exhibiting 1,1,2-TCA dechlorinating activity in non-stained bands were cut and analyzed by MALDI-TOF-MS at the Protein and Proteomics Centre, National University of Singapore. Gel bands were trypsin digested and loaded onto an ABI Voyager STR MALDI-TOF mass spectrometer. Output signals were searched for probable protein hits in the Mascot database, which was constructed based on the National Center for Biotechnology Information Database.

3.3 Results

3.3.1 Growth coupling dechlorination of 1,1,2-TCA

In contrast to bacteria that dechlorinate 1,1,2-TCA to vinyl chloride via dihaloelimination, strain PR completely dechlorinated 1,1,2-TCA (~1.12 mM) to 1,2-DCA and chloroethane at a 1: 2 ratio within 20 days via reductive dechlorination (Figure 3.1). The concentration of 1,2-DCA reached the highest point (0.92 mM) on day 6 and dropped to 0.37 mM at the end of the dechlorination process. Chloroethane started to accumulate on day 4, reaching a final concentration of 0.75 mM on day 20. Only trace amounts (~0.001 mM) of vinyl chloride and ethene were produced. No 1,1,2-TCA dechlorination was observed in the abiotic controls (neither in cultures without inocula nor in autoclaved cultures). Strain PR dechlorinated 1.12 mM of 1,1,2-TCA (CH_2Cl-$CHCl_2$) within 10 days (from day 0 to day 10) at an average dechlorination rate of 0.11 mM/day, while 0.55 mM of 1,2-DCA (CH_2Cl-CH_2Cl) was dechlorinated to chloroethane within 14 days (from day 6

to day 20) at an average dechlorination rate of 0.028 mM/day. The average dechlorination rate of the dihalomethyl (-CCl_2) group is almost four times higher than that of the monohalomethyl (-CCl) group. During dechlorination of 1,1,2-TCA to 1,2-DCA, cell growth was closely coupled to the consumed amount of 1,1,2-TCA. Regression between cell numbers and the amount of chlorine removal shows a linear correlation, indicating a growth yield of ~3.72 $\times 10^{13}$ cells / mole chlorine removed (Figure 3.2). Together, these phenomena implicate 1,1,2-TCA as a growth-supporting electron acceptor for strain PR; no further cell growth was observed after 1,1,2-TCA was depleted on day 8. However, later experiments showed that cell growth occurred upon supplementary addition of 1,1,2-TCA after the initial 1,1,2-TCA had been depleted (Figure 3.3). Additionally, no 1,2-DCA dechlorination was observed when 1,2-DCA was fed to strain PR as the sole electron acceptor (Figure 3.4). These results indicate that dechlorination of 1,2-DCA to chloroethane is a cometabolic process.

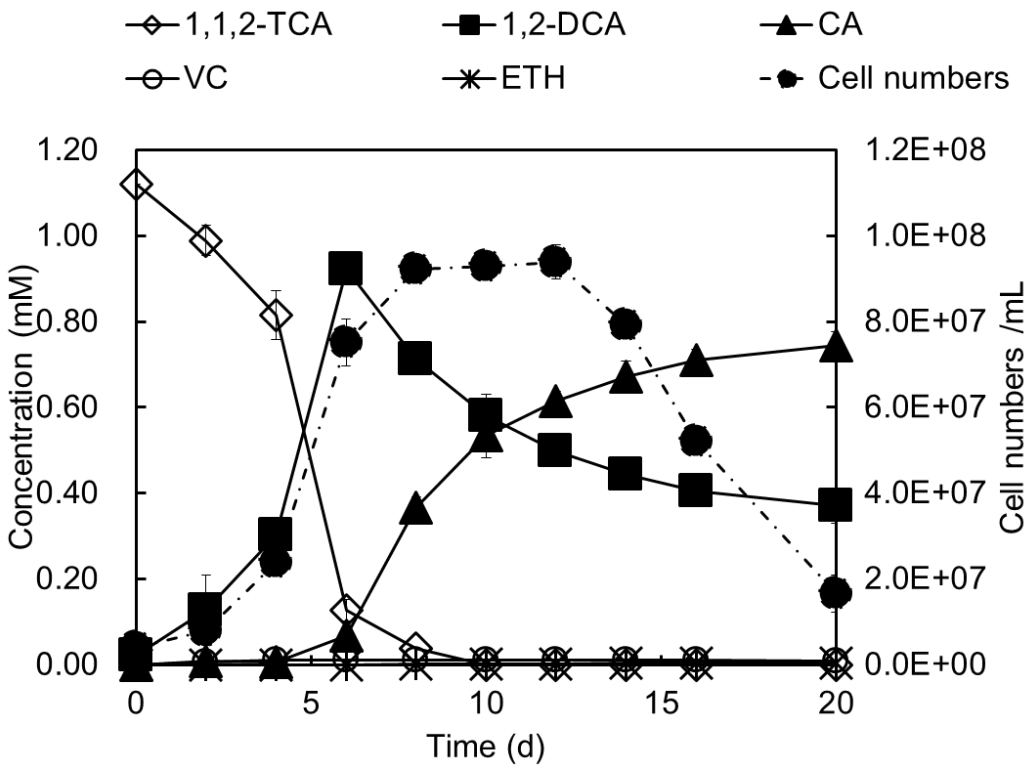


Figure 3.1 Dechlorination of 1,1,2-TCA by *Desulfitobacterium* sp. strain PR.
Note: CA, chloroethane; VC, vinyl chloride; ETH, ethene.

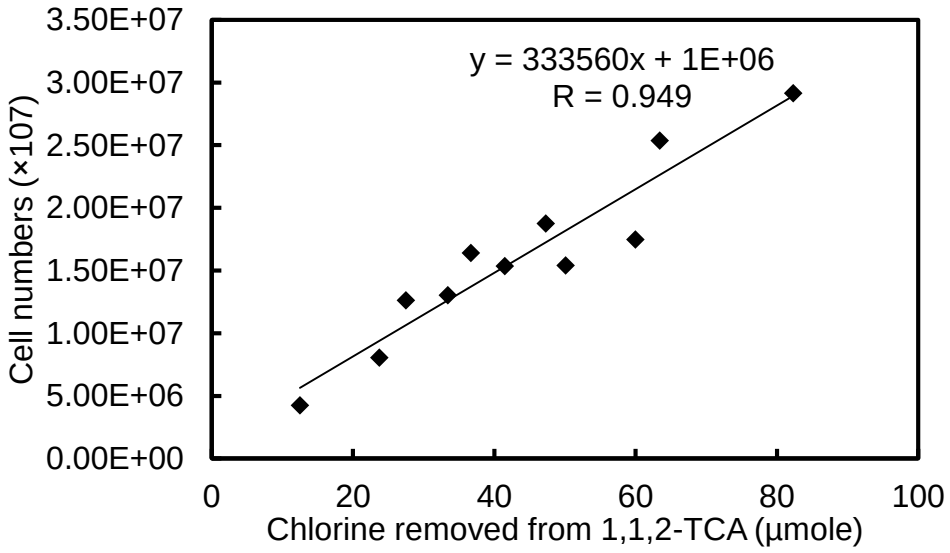


Figure 3.2 Linear correlation between cell number of strain PR and chlorine removed from 1,1,2-trichloroethane on day 4.

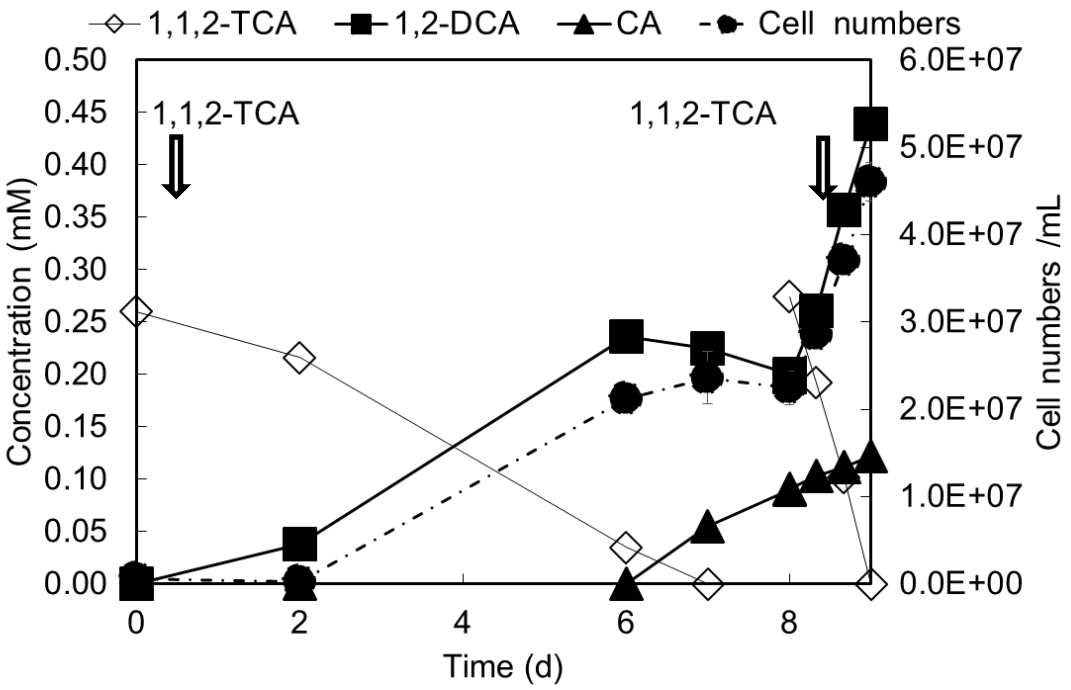


Figure 3.3 Dechlorination of 1,1,2-TCA by *Desulfitobacterium* sp. strain PR via stepwise spiking 1,1,2-TCA. Note: CA, chloroethane.

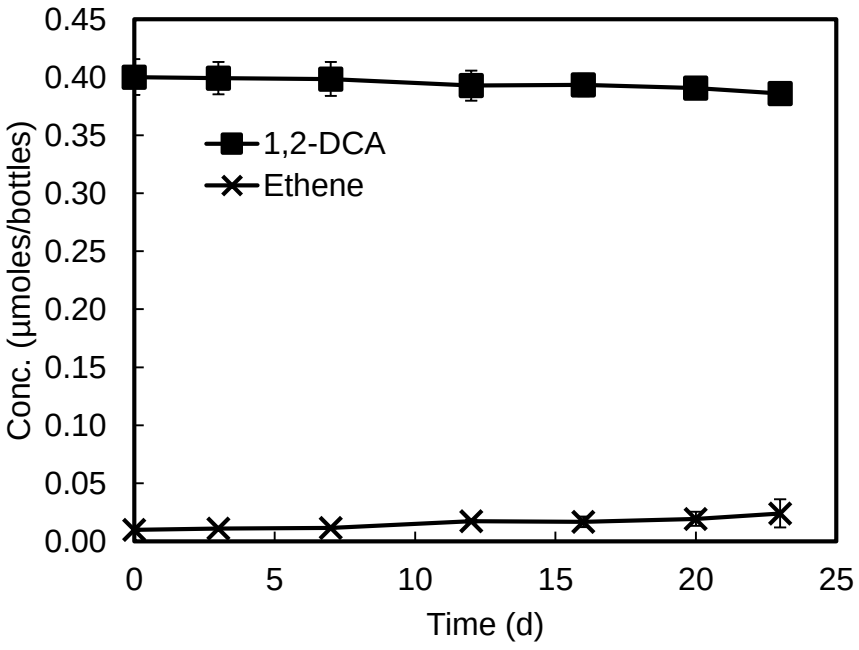


Figure 3.4 Dechlorination of 1,2-DCA by *Desulfitobacterium* sp. strain PR.

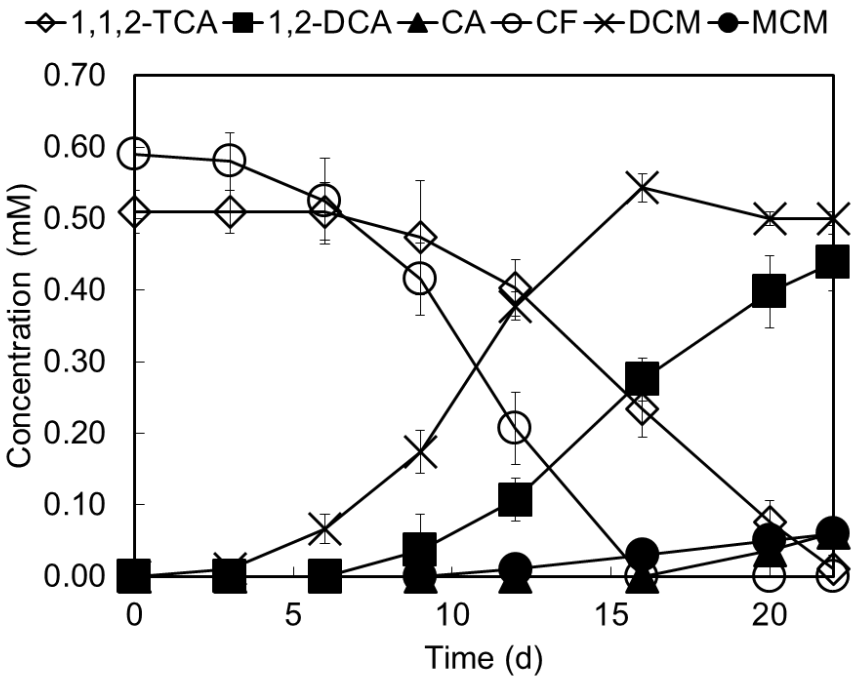


Figure 3.5 Dechlorination of 1,1,2-TCA and chloroform simultaneously by strain PR. Note: CA, chloroethane; DCM, dichloromethane; MCM, chloromethane.

To understand the substrate preference of strain PR on 1,1,2-TCA and chloroform, dechlorinating profile was monitored in cultures amended with 1,1,2-TCA and chloroform simultaneously. Strain PR exhibited a longer lag phase to dechlorinate 1,1,2-TCA as compared to chloroform (Figure 3.5). Dechlorination of 1,1,2-TCA occurred more slowly than that of chloroform, an average rate of 0.036 mM per day (day 6 to day 22) versus 0.045 mM per day (day 3 to day 16), and did not commence until ~16% of the chloroform had been converted to dichloromethane.

3.3.2 Identification of 1,1,2-TCA RDase gene ctrA in strain PR

Strain PR harbours both a functionally characterized RDases gene, *ctrA*, and an as-yet uncharacterized RDase gene, *prdhA* (Ding, et al., 2014)(Figure 3.6). In order to determine whether these genes are responsible for 1,1,2-TCA dechlorination in strain PR, transcripts of *ctrA* and *prdhA* were

monitored during exposure to 1,1,2-TCA. As shown in Figure 3.7, transcription of the *ctrA* gene increased to 8.53 transcripts/cell prior to the onset of 1,1,2-TCA dechlorination, while the *prdhA* gene showed negligible transcription.

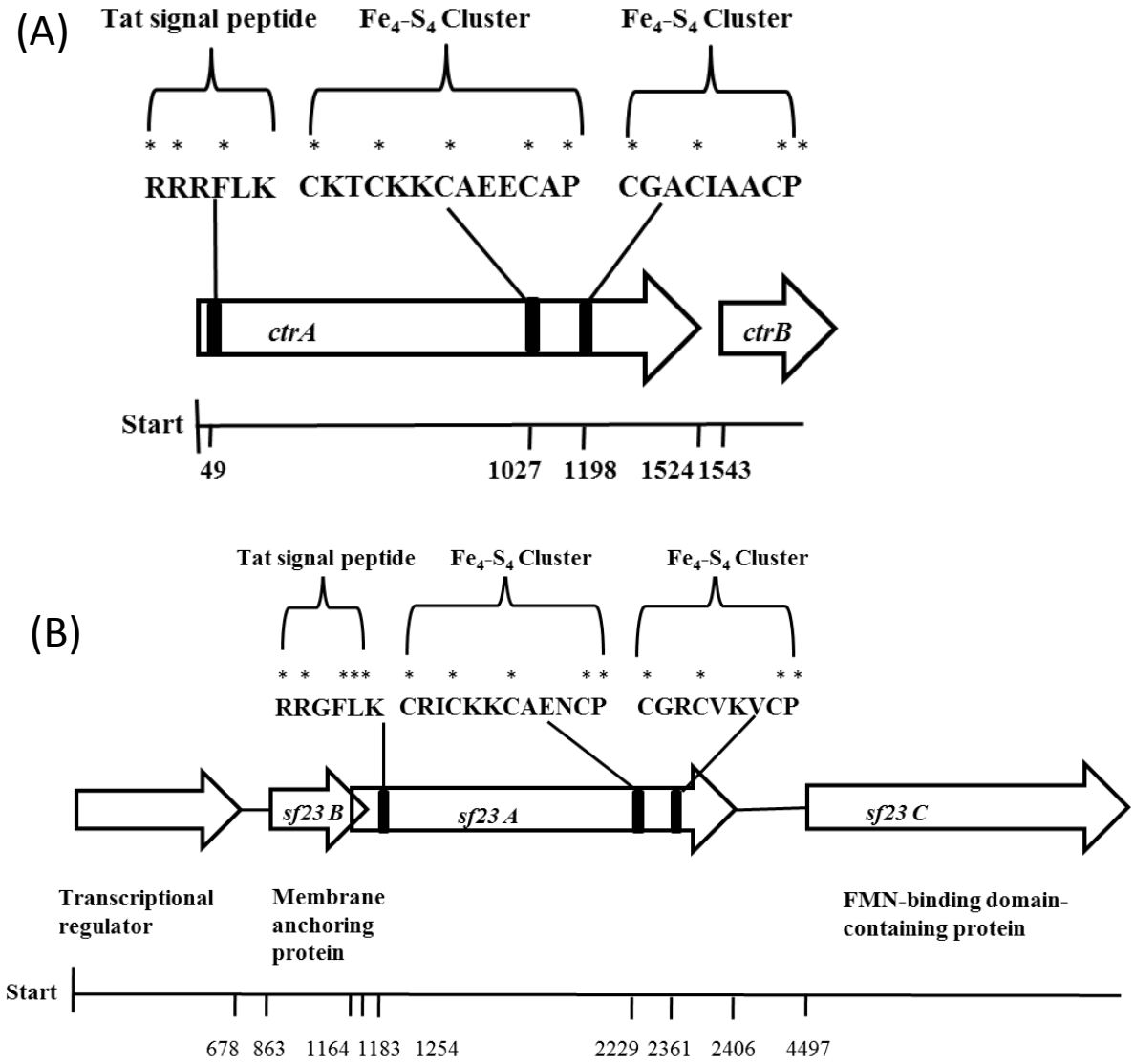


Figure 3.6 Physical map of *ctrA* (A) and *prdhA* (B).

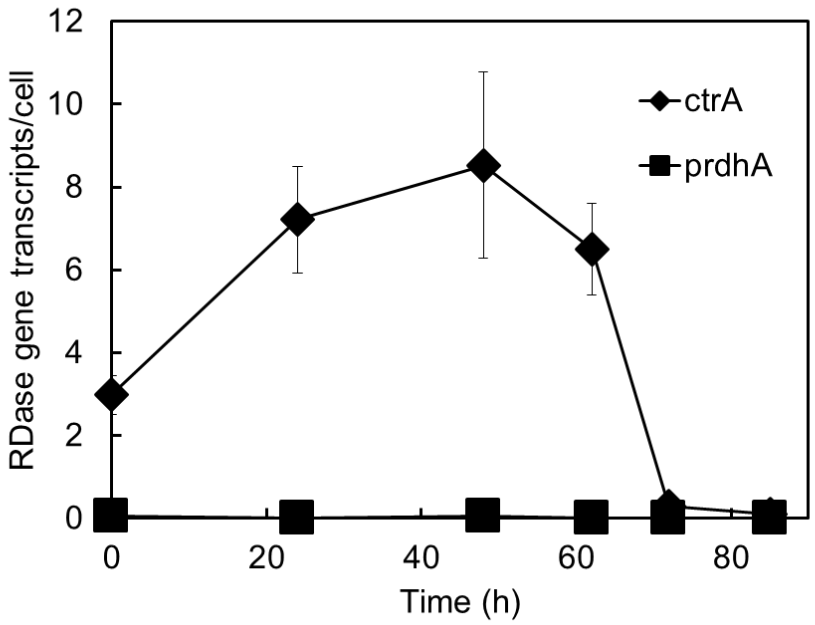


Figure 3.7 Transcription of *ctrA* and *prdhA* genes in *Desulfitobacterium* sp. strain PR when fed 1,1,2-TCA.

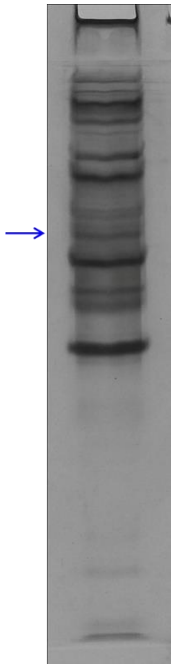


Figure 3.8 Native-PAGE gel profile of crude cell proteins of strain PR fed with 1,1,2-TCA

The role of CtrA in 1,1,2-TCA dechlorination was further confirmed by native-PAGE. Crude cell extracts taken from strain PR during exponential growth phase were separated on a native PAGE gel and dechlorinating activity

was recovered directly from native gels. The corresponding dechlorination products (1,2-DCA, 1,1-DCA, or dichloromethane) from 1,1,2-TCA, 1,1,1-TCA and chloroform were detected from the same single band, while other gel fragments failed to show dechlorinating activity. This indicates that the proteins in this band are responsible for dechlorination of the above-mentioned chlorinated compounds. The silver stained counterpart of the gel fragment showing dechlorination activity was analyzed by MALDI-TOF-MS (Figure 3.8). Results from MALDI-TOF-MS analysis identified CtrA as the only detected reductive dehalogenase in the active gel fragment (Mascot score of 447 with 41.8% sequence coverage). Together, these analyses strongly implicate the *ctrA* gene as responsible for dechlorinating 1,1,2-TCA as well as 1,1,1-TCA and chloroform in strain PR.

3.3.3 Characterization of CtrA gene in strain PR

Both crude proteins and membrane proteins from strain PR showed identical dechlorination profiles for 1,1,2-TCA. This suggests that 1,1,2-TCA dechlorination was mediated by a membrane-associated protein. Substrate range characterization revealed that the CtrA in strain PR produced the metabolites 1,2-DCA, 1,1-DCA, dichloromethane and chloroethane from 1,1,2-TCA, 1,1,1-TCA, chloroform and DCAs, respectively. Similar to dechlorination performance in active cultures of strain PR, dechlorination of 1,2-DCA to chloroethane was slow and only a small amount of 1,2-DCA had been dechlorinated when most TCAs were dechlorinated to DCAs. Induction of the *ctrA* gene by 1,1,1-TCA and chloroform yielded identical *in vitro* enzyme kinetics.

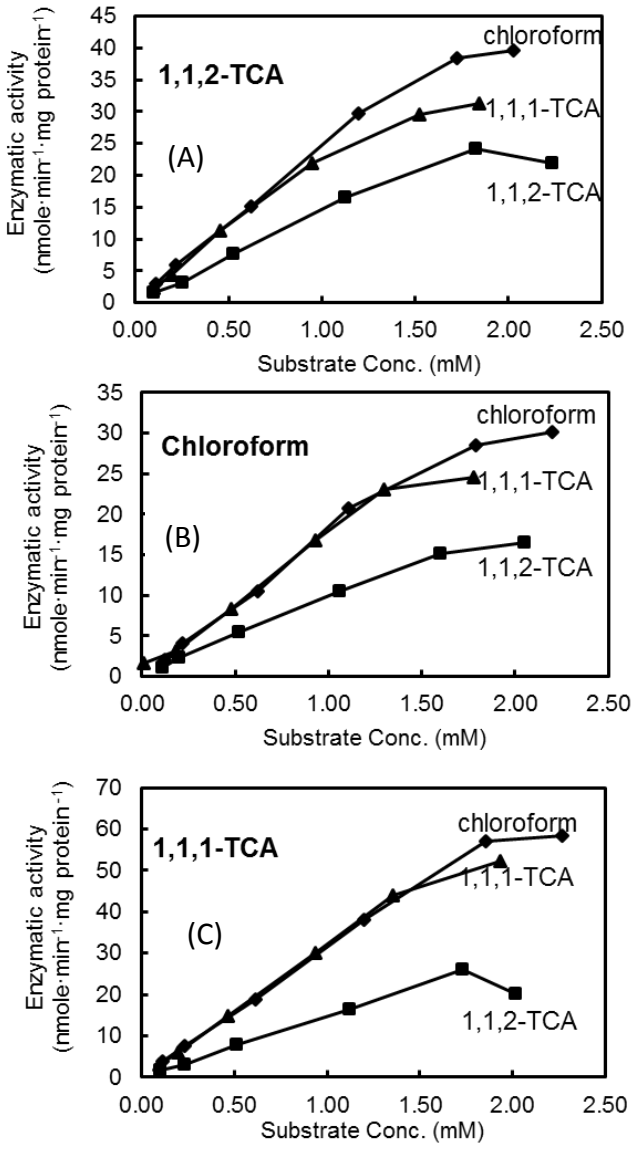


Figure 3.9 Dechlorination rates of crude cell extracts upon exposure to various concentrations of 1,1,2-TCA,1,1,1-TCA, or chloroform. The cell extracts were obtained from culture PR fed with (a) 1,1,2-TCA, (b) 1,1,1-TCA, or (c) chloroform

Figure 3.9 shows the effect of substrate concentration on the rate of dechlorination for each substrate (1,1,1-TCA, 1,1,2-TCA, or chloroform) by using crude cell extracts. The dechlorination rates increased linearly as the initial electron acceptor concentrations increased, and the dechlorinate rates ceased to increase as electron acceptor concentrations reached around 2.00 mM. The maximum reaction rates, $v_{max,}$ for each substrate are in the same

order of magnitude ranging from 15 to 60 nmoles substrate dechlorinated per min per mg protein. Interestingly, the dechlorination rates of 1,1,1-TCA and chloroform were ~1.5-2.0 times that of 1,1,2-TCA.

3.3.4 Establishment of co-cultures for complete dechlorination of 1,1,2-TCA

In order to achieve complete dechlorination of 1,1,2-TCA, a co-culture was established with strain PR and a highly enriched mixed culture, GEO, that is capable of dechlorinating 1,2-DCA to ethene via dihaloelimination. When 1,1,2-TCA (~ 0.62 mM) was spiked into the medium, this co-culture sequentially dechlorinated 1,1,2-TCA to 1,2-DCA and chloroethane on day 11 (Figure 3.10). Most of the 1,2-DCA produced was rapidly dechlorinated to ethene, however, a fraction of 1,2-DCA was still dechlorinated to chloroethane co-metabolically by strain PR. By day 25, all of the 1,1,2-TCA had been converted to chloroethane (~ 0.35 mM) and ethene (~ 0.26 mM).

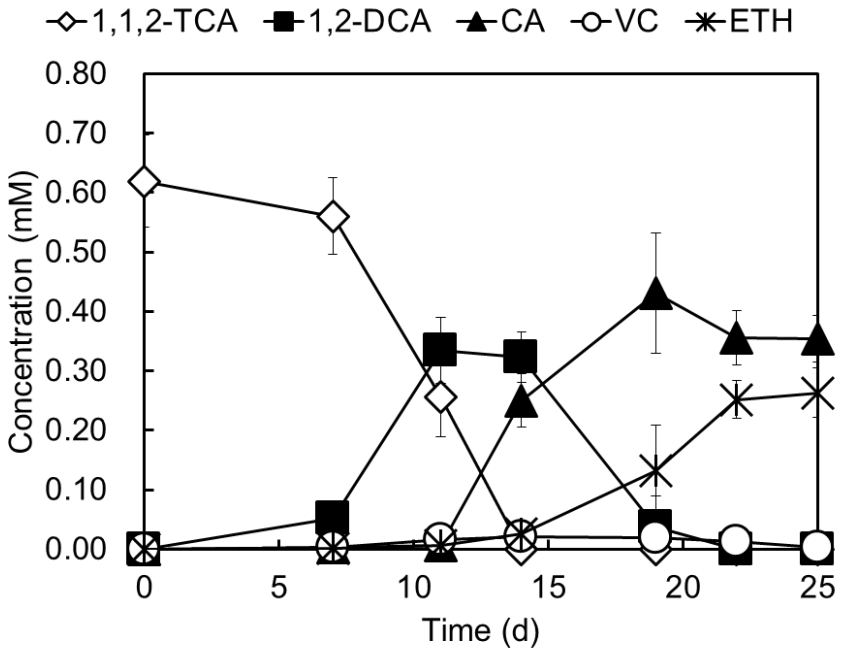


Figure 3.10 Dechlorination of 1,1,2-TCA by a co-culture consisting of strain PR and a mixed culture GEO. Note: CA, chloroethane; VC, vinyl chloride; ETH, ethene

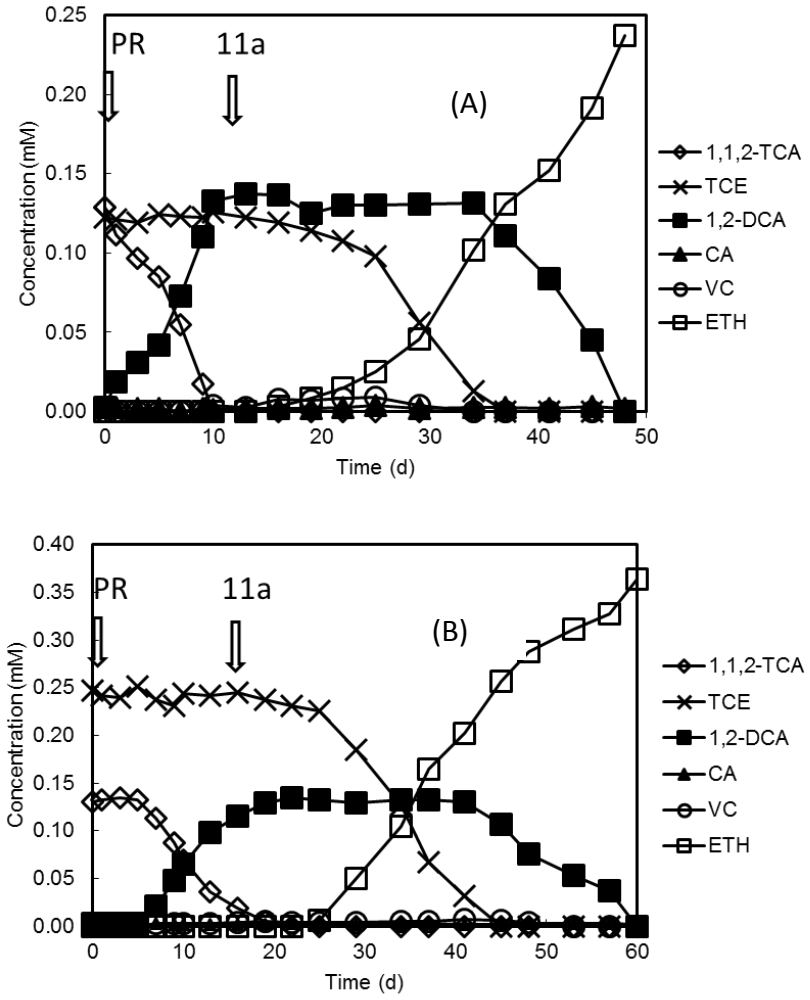


Figure 3.11 Inhibition effects of TCE (A) 0.12 mM and (B) 0.24 mM on 1,1,2-TCA dechlorination activity by strain PR. Co-culture was cultivated in 30 mL serum bottles, amended with acetate and pyruvate (10 mM each) together with H2 (10 mL). Strain PR was inoculated on day 0, while strain 11a was inoculated on day 13. Note: CA, chloroethane; VC, vinyl chloride; ETH, ethene.

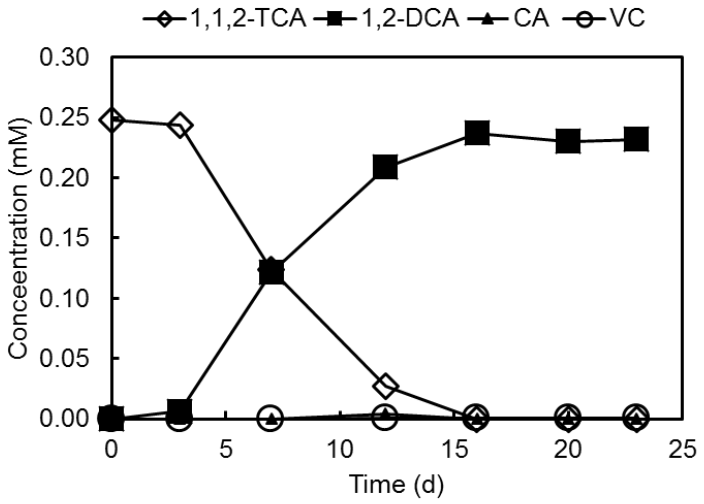


Figure 3.12 Inhibitory effects of 0.05 mM TCE on 1,1,2-TCA dechlorination by strain PR

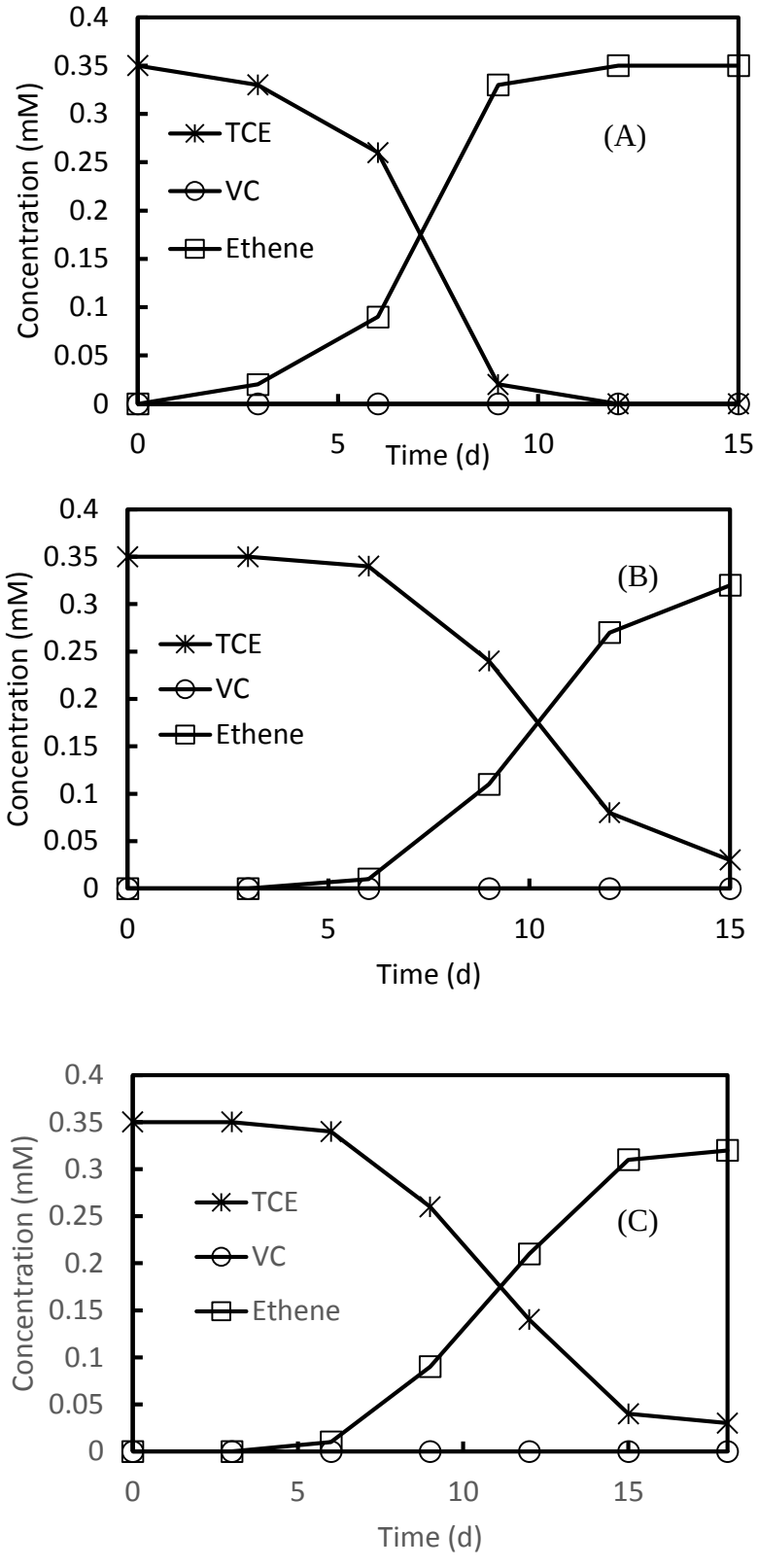


Figure 3.13 1,1,2-TCA effects on dechlorination of TCE by strain 11a. (A) 0 mM TCE (B) 0.34 mM TCE (C) 0.71 mM TCE

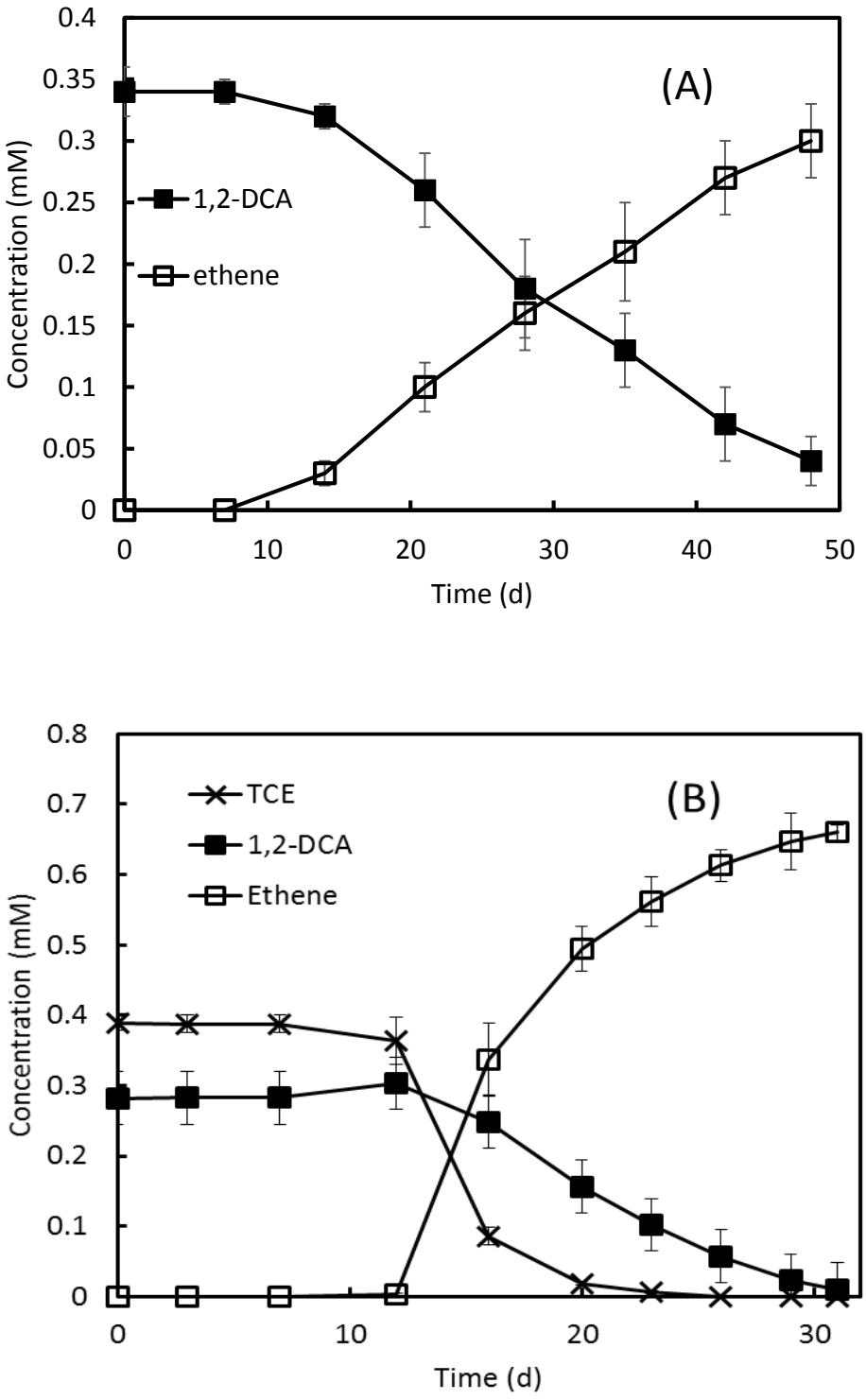


Figure 3.14. Effects of TCE on dechlorination of 1,2-DCA by strain 11a. (A) Dechlorination of 1,2-DCA by strain 11a. (B) Dechlorination of co-contaminants TCE and 1,2-DCA by strain 11a

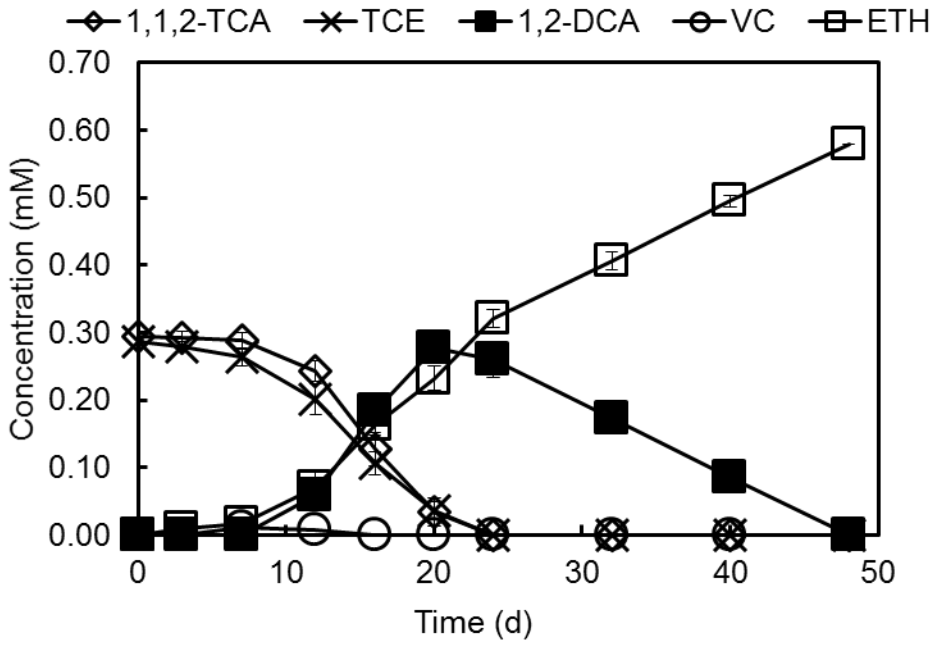


Figure 3.15 Dechlorination of 1,1,2-TCA and TCE by a co-culture consisting of strains PR and 11a. Note: CA, chloroethane; VC, vinyl chloride; ETH, ethene.

As a co-contaminant with 1,1,2-TCA, TCE ranging from 0.01 mM to 1.12 mM has no inhibitory effect on dechlorination of 1,1,2-TCA to 1,2-DCA by strain PR and Figure 3.11 shows dechlorination profile at a representative low (0.12 mM) and high (0.24 mM) TCE concentration. Moreover, production of chloroethane by strain PR is inhibited by as little as 0.05 mM TCE in the medium, resulting in the production of only 1,2-DCA from the dechlorination of 1,1,2-TCA (Figure 3.12). However, when the TCE concentration increased to 0.63 mM, dechlorination of 1,1,2-TCA by strain PR was ceased. In order to achieve complete dechlorination of 1,1,2-TCA without accumulation of either 1,2-DCA or chloroethane in the presence of TCE/1,1,2-TCA co-contaminants, *Dehalococcoides mccartyi* strain 11a which can dechlorinate both TCE and 1,2-DCA was introduced. The inhibitory effects of TCE on dechlorination of 1,1,2-TCA by strain PR and 1,2-DCA by strain 11a was first investigated. In fact, the dechlorination of 1,1,2-TCA by

strain PR were not affected too much by TCE that the dechlorination rate declined within two times even the TCE concentration was as high as 0.71 Mm (Figure 3.13). In contrast to inhibition, the presence of TCE actually enhanced the dechlorination of 1,2-DCA (Figure 3.14). Therefore, the co-culture was established with strain PR and *Dehalococcoides mccartyi* strain 11a. After spiking TCE (0.29 mM) and 1,1,2-TCA (0.29 mM) to the co-culture, 1,1,2-TCA and TCE were dechlorinated at similar rates to 1,2-DCA (by strain PR) and ethene (by strain 11a), within 24 days (Figure 3.15). The amount of 1,2-DCA peaked (0.28 mM) on day 20, after which it was completely dechlorinated to ethene by strain 11a. Throughout the experiment, the concentration of CA was below detection limit (0.006 mM). A trace amount of VC (0.012 mM) was generated on day 7, but disappeared after 9 days. Overall, the co-culture of strains PR and 11a was able to achieve complete detoxification of 1,1,2-TCA and TCE to ethene.

3.4 Discussion

Anaerobic bacterial dechlorination of 1,1,2-TCA by dihaloelimination, which results in the accumulation of the carcinogenic compound vinyl chloride, has been known since the 1990s (Castro, et al., 1994, Chen, et al., 1996, De Wildeman, et al., 2003, Aulenta, et al., 2006, Grostern and Edwards, 2006b, Maness, et al., 2012, Bowman, et al., 2013). In this study, we show that *Desulfitobacterium* sp. strain PR reductively dechlorinates 1,1,2-TCA to 1,2-DCA and chloroethane via a stepwise reductive dechlorination process. Strain PR metabolically dechlorinates a much higher concentration (1.12 mM versus 0.011mM) of 1,1,2-TCA than the previously reported cometabolic reductive dechlorination of 1,1,2-TCA to DCA by *Desulfomonile tiedjei* strain

DCB-1 (Fathepure and Tiedje, 1994). The growth yield of strain PR from dechlorination of 1,1,2-TCA is 3.72×10^{13} cells per mole chlorine, which is comparable to the growth yields from dechlorination of chloroform or 1,1,1-TCA by strain PR and of chloroethanes by other organohalide respiring bacteria (Grostern and Edwards, 2006a, Ding, et al., 2014). When strain PR was amended with different substrates, the enzymatic activities on dechlorination of each substrate exhibit similar trend that the enzymatic activities increase as the substrate concentrations increase and stabilize to some extent as the substrate concentrations reach around 2.00 mM. This could possibly because RDase have a tolerance for the substrate and may be inhibited by high concentrations of substrates. This inhibition of high concentrations of substrates on RDase is consistent with the phenomenon that the dechlorination of substrates by organohalide respiring bacteria is also inhibited when the substrates concentrations are high.

Strain PR exhibits a broad substrate range of chlorinated ethanes/methanes, including 1,1,2-TCA, 1,1,1-TCA and chloroform. Although structurally distinct, 1,1,2-TCA (-CCl_2), chloroform (-CCl_3), and 1,1,1-TCA (-CCl_3) share similar functional groups (tri- or dichloromethyl groups). Unlike many other organohalide respiring bacteria that harbor multiple functional RDase genes which catalyze various substrates, such as *Dehalococcoides mccartyi* strains 195 and CBDB1 (Regeard, et al., 2005, Schiffmann, et al., 2014), the *ctrA* gene in strain PR is responsible for dechlorination of 1,1,2-TCA, chloroform and 1,1,1-TCA. This finding suggests that organohalide respiring bacteria are able to reductively dechlorinate multiple substrates with similar functional groups by using a

single RDase, which suggests that structural analogs should be considered during the exploration of potential substrate range in novel RDases. The two-fold difference in dechlorination rates exhibited by CtrA in enzyme assays in catalysis of the trihalomethyl (-CCl3) and the dihalomethyl (-CCl2-) groups suggests that a higher number of chlorine substitutes could lead to higher dechlorination rates for CtrA in strain PR. Similarly, when 1,1,2-TCA and chloroform co-exist, strain PR exhibits a faster rate of dechlorination for chloroform than for 1,1,2-TCA. These findings agree with previous reports indicating a relationship between chlorine number and dechlorination rate (Magnuson, et al., 2000).

Enzyme CtrA shows a slight substrate binding preference to chloroform in the presence of chloroform and 1,1,2-TCA. Also, the cometabolic dechlorination from 1,2-DCA to chloroethane is not affected by the presence of 1,1,2-TCA or chloroform. TCE inhibits the conversion of 1,2-DCA to chloroethane, but not that of 1,1,2-DCA to 1,2-DCA. The complete dechlorination from 1,1,2-TCA to 1,2-DCA shows that TCE does not inhibit CtrA. Therefore, the cessation of 1,2-DCA dechlorination might result from competitive inhibition by TCE over 1,2-DCA. In all, CtrA demonstrates a substrate binding preference as: chloroform (CCl3-H) > 1,1,2-TCA (H-CCl2-CCl-H2) >> TCE (CCl2=CCl2) >> 1,2-DCA (H2-CCl-CCl-H2).

Chloroethane was accumulated in the co-culture comprising strain PR and the mixed culture GEO amended solely with 1,1,2-TCA. Although the same dechlorinating capability (1,2-DCA to ethene) is shared by mixed culture GEO and strain 11a, complete detoxification of TCE/1,1,2-TCA by co-culturing strain PR and 11a was achieved after the additional amendment of

TCE. TCE inhibited the dechlorination of 1,2-DCA to CA, effectively eliminating the accumulation of chloroethane. In the presence of TCE/1,1,2-TCA and co-cultivated with strain PR, strain 11a started to dechlorinate 1,2-DCA when the initial TCE was depleted, indicating that strain 11a preferentially dechlorinates TCE over 1,2-DCA when both are available. This may be a result of the ~ 5 times faster dechlorination rate of TCE than that of 1,2-DCA by strain 11a, although both TCE and 1,2-DCA are likely catalyzed by the same RDase gene, *vcrA*, in strain 11a (Lee, et al., 2013).

In summary, this study is the first report of metabolic reductive dechlorination of 1,1,2-TCA to chloroethanes and of identification of the enzyme responsible for this process. By co-culturing strain PR with *Dehalococcoides mccartyi* strain 11a, 1,1,2-TCA and TCE can be completely dechlorinated to a non-toxic end product, ethene. The characterized properties of strain PR enlarge our view of its versatile dechlorinating performance, making it a promising candidate for broad applications in chloroform and TCA as well as co-contaminants of each with TCE *in situ* bioremediation.

4 Chapter IV Genomic characterization of *Dehalococcoides mccartyi* strain 11a5, a plasmid-containing dechlorinating anaerobe

4.1 Introduction

Field-scale studies have revealed that *Dehalococcoides,* a group of strict organohalide respiring bacteria, is essential to effective bioremediation at many halogenated compounds contaminated sites (Major, et al., 2002, Lee, et al., 2008). *Dehalococcoides* possesses the ability to dehalogenate a diverse array of halogenated compounds, including chloroethenes, chloroethanes, chlorophenols, chlorobenzenes, dioxins, polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs) (Hug, et al., 2013). Notably, *Dehalococcoides* species has drawn attention since it is the only known genera to completely detoxify chlorinated solvents such as tetrachloroethene (PCE) and trichloroethene (TCE) beyond dichloroethenes (DCEs) and carcinogenic vinyl chloride (VC) to non-toxic ethene. The emergence of high throughput genomics technologies has provided a wealth of insight into physiological, biochemistry, evolutionary and ecological characteristics of this unique genus.

Following the release of the first two complete *Dehalococcoides mccartyi* genomes, strains 195 and CBDB1 in 2005, nine additional *Dehalococcoides* genomes have been published on the National Center for Biotechnology Information database (NCBI). Comparison of *Dehalococcoides* genomes reveals conserved core genomic regions and two high plasticity regions (HPRs) (McMurdie, et al., 2009). The core genome encodes proteins that catalyze essential metabolic functions and determines shared physiological features among *Dehalococcoides*, i.e. the requirement of

acetate as a carbon source, hydrogen as an electron donor, and specific halogenated compounds as electron acceptors. The HPRs are hypothesized to provide strain-specific genetic resources for adaptation to different environmental niches, including mobile genetic elements (MGE), insertion sequences and deletions as well as reductive dehalogenase homologous (*rdhA*s) (McMurdie, et al., 2009).

Although core genetic features of *Dehalococcoides* have been gained through the comparative analysis of available genomes, some aspects, e.g., how the *rdhA*s flow among organohalide respiring bacteria, still remain mysterious. Additionally, the genomic characteristics can provide the great guidance to reveal the features of strains. Therefore, it is essential to explore the genomic characteristics of novel strains. *Dehalococcoides mccartyi* strain 11a5 was isolated from a TCE fed microcosm, capable of dechlorinating TCE and DCE isomers to VC and ethene (Lee, et al., 2013). A microarray genomic analysis targeting *Dehalococcoides* revealed phylogenetic relationships of strain 11a5 with other *Dehalococcoides* strains and core metabolic characteristics (Lee, et al., 2013). However, as microarray analysis is based on available genomes of other *Dehalococcoides* strains, the data is insufficient to obtain a full perspective of the unique genomic features of detected strains. In this study, the strain-specific traits of *Dehalococcoides mccartyi* strain 11a5 were explored based on the complete genome sequence. The existence of a circular extrachromosomal genetic region and a putative prophage in the genome is discovered and verified in strain 11a5. In addition to the previously reported TCE dechlorination capability (Lee, et al., 2013), strain 11a5 is able to dechlorinate PCE by novel PCE reductive dehalogenase (RDase).

4.1 Materials and Methods

4.1.1 Chemicals, bacterial growth conditions and sample analytical procedures

All chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) or Merck (Darmstadt, Germany) at the highest purity available (>98%). Strain 11a5 was grown in an anaerobic bicarbonate-buffered defined medium spiked with acetate (10 mM) as the carbon source, as previously described (Lee, et al., 2013). For all dechlorination kinetic studies, enzyme assays and proteomic analyses, strain 11a5 was grown in 160-mL serum bottles containing 100 mL medium amended with hydrogen as an electron donor (0.33 atm) and chloroethene (PCE or TCE), as indicated, as an electron acceptor (~ 0.6 mM). All kinetics studies were performed in biological triplicate and with abiotic controls (without bacterial inocula and with autoclaved cultures). Cultures were incubated in the dark at 30 ºC without shaking.

Chloroethene concentrations were determined by headspace analysis using an Agilent gas chromatograph (GC7890) equipped with a flame ionization detector and a GS-GasPro column (30 m by 0.32 mm; J&W Scientific) as previously described (Cheng, et al., 2010). Headspace samples were injected manually using a gas-tight glass syringe (Model Gastight ® #1725, Hamilton Co., Reno, Nevada).

4.1.2 Molecular analyses

Genomic DNA was extracted using the QIAGEN DNeasy Blood and Tissue Kit (QIAGEN® GmbH, Hilden, Germany) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was

conducted on an ABI 7500 Fast System using the SYBR green reporter (Lo-ROX Kit, SensiFASTTM SYBR, BIOLINE) (Lee and He, 2010). The thermocycling was programmed at an initial step of 3 min at 95 ºC, followed by 40 cycles of 15s at 94 ºC and 30s at 55-60 ºC depending on the annealing temperature of primers, and then 30s at 72 ºC. Plasmids carrying targeted genes to serve as qPCR standards constructed as follows: amplified targeted genes being ligased into pGEM-T vector and then transformed into Escherichia coli TOP10 competent cells. The plasmids were obtained using the QIAprep Spin Minprep kit (QIAGEN GmbH) (Chow, et al., 2010). The calibration curves were established using serial dilutions of known plasmid DNA concentrations. *tceA*-specific primers (1270F & 1336R) were utilized to monitor cell growth (Holmes, et al., 2006). Specific primers for transcriptional analysis of *rdhA*s based on the genome sequence of strain 11a5 were designed using Primer Premier 5.0 (Table 4.1).

Table 4.1 Primers used in 11a5 genome closure and transcriptional anlyses

Category	Primer ID	Sequences (5'-3')
Genome scaffold gap closure	1F	GCAGGCATAACCGAAAGTC
	1B	TCCAGACAGACCCTTCACTT
	2F	CTTCCTCCATAGTATTTCGTTTAG
	2B	TAAAGACCTGGTTATTGAAGACG
	3F	CAATCCACCTGCCGTAAGC
	3B	GCAATACGGCTTGATACAA
	4F	GCTAAAGGCGATTACAGAGT
	4B	TACCATCAATACGCAAGCC
qPCR primers to target reductive dehalogenases	11a5_0188	ACAGGTGGCATATTACCCTTGA
		CCAAACCAGCGGCAAATC
	11a5_0227	TCTTTGGTGCTTCCGAGGTG
		TCTGAACGGCTACACTGACGA
	11a5_0232	AACGGTTCGTATGACGGCACT
		CCCTTTACAAAACGCTGGAGAT
	11a5_0255	CACATCTTCCCCAAGTGCTATC
		CACCAAACTTTAGTGCGACCAG
	11a5_0263	TTCAGGTATCGGCGAACTAGG
		GGTAAAGGCAGGTCGGTAAAA
	11a5_1006	TCCCAGATAGCCCAGCAGA
		CGCCCGATTTCACCCATAC

11a5_1306	TCACATCTTCGGCAATCGGTA
	AGGGGTTTCAAGGTCAGTGGA
11a5_1308	CGGTCTGCGGTAATCTTCT
	CCTTCATAACCCTGCTCCA
11a5_1352	ATCCAGATTATGACCCTGGTGAA
	GCGGCATATATTAGGGCATCTT
11a5_1355	TATCAATCCGTTCGTGGTG
	CGAGTGGCAGGTCAGTTAG
11a5_1395	TTGCTGGGCGGGCTGGAACTTTTTG
	CAAAAAGTTCCAGCCCGCCCAGCAA
11a5_1397	TAGACTGGAATAAGGCAAGCA
	CCATCCCTAAGGTCAACCC
11a5_1402	CCGTAGCCTCTGCCACATT
	GCGTCAAACCGCTCAATCT
11a5_1404	ATGCACGGTTTTCATTCAACAGTAA
	TTACTGTTGAATGAAAACCGTGCAT
11a5_1419	TTTTATGAAGGGGCTTGGACT
	TGATTCTTGCTGAGGGTGCTG
11a5_1424	AATACAACGGCAAAGGCGTAA
	AAGCACCTGAACCTGAAAGACC
11a5_1428	GTTCAAAGACATAGATGTACCCTAC
	ATAAGAATACCAGACCGCAAA
11a5_1437	CTTATGTCATCCTCTTCGGTAA
	CTGCATCAAGTCCCAATCA
11a5_1439	CTTTGTTGGAGCAGGAGTT
	CTTGAGGTATAGTAGTGGCATC
11a5_1448	CTTCCGTTTCTTAGGCTGTC
	GCCGTTTCATAGGCTTCAT
11a5_1453	GGGTCAGGTTATCGGTTCTC
	GGCATCTATCGGATTGGTT
11a5_1456	TCAGGGTCAGCCGAGTGAG
	TGCCAAGCGGTGAATAGGT
11a5_1460	ATGGGAGCTATCAGCAAGGA
	CGGACAATGGGAACAAACT
11a5_1467	AGGTTTGAAGGTGCGGCTAC
	GGAGAAAGGCTGTTGTTGC
11a5_1473	CCTTGGCGGCTGTGATGTA
	TGGCAGGAATGACCAGTTTG
11a5_1477	AAATACCTGGGCGAAGAAGA
	TCGGCAGCATACTCATAACG
11a5_1497	GCAGCTTACAGTGCCAACG
	GCAGGTCCCATGAAAGTGAT
11a5_1503	TATGAGACCGCAACCAAGATG
	CGGGAATATGAATACCAAACG
11a5_1506	TGAGCCGCTACCGTCTCAATCAGGA
	TATCCATTTCGCCCAGACC
11a5_1515	GTTATGTGGGTTCCGAGGGT
	TGGGATGGGTAGGTTCAAGAG
11a5_p001	TACCTTTCTGACGGAGCAG
	CCAAGCCCAGACATAACAC

Identification of plasmid	pDhc6-3554F	GACGGGATAGTGTTCATAGATTG
	pDhc6-411R	CAGGTTGGGTAGTGTCATTCG
	pDhc6-4404F	ATTGGGATGATAATACGAGG
	pDhc6-3981R	CGTGCGTTAGCCAGTTTA

Reverse transcription PCR (RT-PCR) followed by qPCR (RT-qPCR) was performed to monitor expression levels of *rdhA*s during the PCE-dechlorinating process. Cultures for transcriptional analysis were inoculated in 5% (v/v), from which cultures had been starved for 3 days in order to minimize the residual level of RNA. Cell samples were collected at 12 hour intervals for DNA (1 mL of culture) and RNA (1.5 mL of culture) at 10,000g, 4 ºC for 10 min. Pellets of RNA were immediately resuspended in Trizol reagent and stored at -80 ºC for further analysis. 2 μL luciferase mRNA control (Promega) was added to each RNA sample to quantify the absolute amount of transcripts and RNA was later extracted using a protocol combining Trizol and QIAGEN RNeasy mini kit. QIAGEN RNase-free DNase kit was used to remove possible residual genomic DNA by on-column digestion. Reverse transcription was performed immediately after RNA elution using QIAGEN Sensiscript kit with random hexa primer (Promega) and RNase inhibitor (Promega) (Ding, et al., 2014).

Plasmid presence was confirmed by long range PCR using the GoTaq® Long PCR Master Mix kit (Promega). The PCR conditions were set according to manufacturer's instructions with an extension time of 12 min. Two pairs of primers, pDhc6-4404F & pDhc6-3981R (5518 bp) and pDhc6-3554F & 11a5-411R (2798 bp), were designed to amplify a potentially circular sequence of DNA (Table 4.1) and experimental design was shown in Figure

S1. Bacterial DNA was extracted from TCE fed cultures during different growth phases and from multiple subcultures. *rdhA* 11a5_p001 and 11a5_1352 (*tceA*) were selected to monitor the quantities of plasmid and chromosome, respectively.

4.1.3 Genomic DNA extraction, sequencing and analysis

Cultures (total 800 mL) grown on PCE were harvested (12,000 rpm, 4 ºC, 10 min) and genomic DNA was extracted for sequencing using the QIAGEN® Genomic DNA Buffer Set. Genomic DNA was used to construct a 500 bp short insert library and was sequenced by BGI Shenzhen (China) using an Illumina HiSeq2000 sequencing platform. Sequence coverages for the chromosome and the putative plasmid are 102.2 and 97.3 respectively, as estimated by the Mpileup script in SAMtools (Li, et al., 2009). The genome was assembled using the SOAPdenovo package with optimized parameters (Li, et al., 2010). Gaps between scaffolds were closed by combinatorial PCR (Figure 4.1) using primers specific to the beginning and end of each scaffold (Table 4.1). Sequencing errors were manually screened and corrected using Bamview and the SNP function in SAMtools (Li, et al., 2009, Carver, et al., 2013). The origin of replication was identified by comparison to the eleven published complete *Dehalococcoides mccartyi* genomes. Open reading frames, ORFs, were identified by GLIMMER and Prodigal (Delcher, et al., 1999). The ORF annotation started with comparison to eleven released *Dehalococcoides mccartyi* annotations and was further confirmed by searching the translated protein sequences in NCBI BLASTp. Ribosomal RNA (rRNA), transport RNA (tRNA) and transport-messenger RNA (tmRNA) were identified using RNAmmer v1.2 (Lagesen, et al., 2007). A

putative prophage was identified and annotated using PHAST (Zhou, et al., 2011). Physical map of chromosome and plasmid were drawn using software CIRCOS.

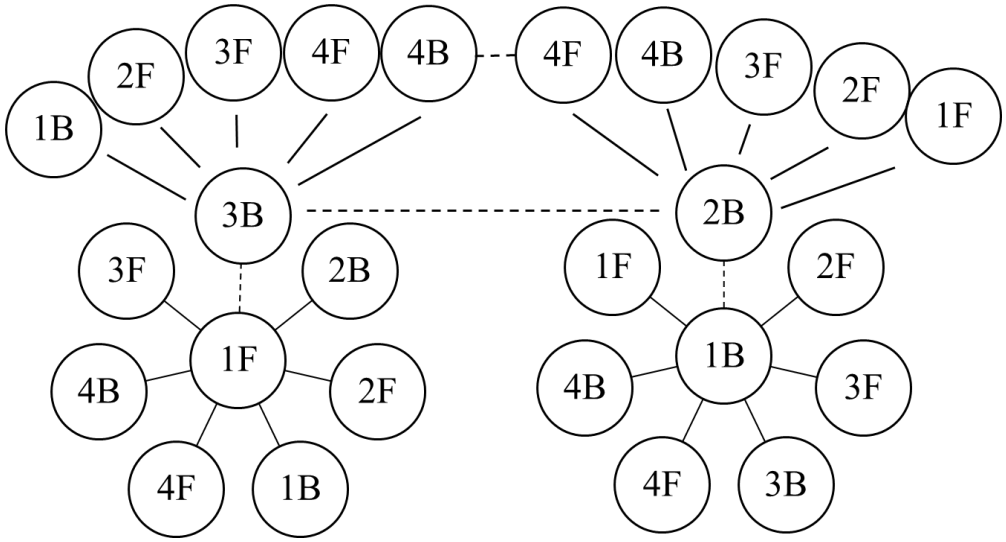


Figure 4.1 Experimental design of combinatorial PCR to close gaps between scaffolds. Note: each line represents a PCR reaction; each circle represents primers used in the PCR reaction (table 4.1), e.g., 1F and 1B are the primers at each end of scaffold 1 towards the ends of the scaffold; solid lines show negative results, while dash lines show positive results.

The sequences of functionally identified reductive dehalogenase in *Dehalococcoides* and other PCE RDases were obtained from the NCBI. Phylogenetic trees were constructed with Molecular Evolutionary Genetics Analysis 6 (MEGA6) using neighbor-joining methods and supported by1000 bootstraps.

4.1.4 Enzyme assays and proteomic analyses

Cells were harvested by centrifugation (12,000 rpm, 20 min, 4ºC) and re-suspended in degassed Tris-HCl buffer (100 mM, pH 7.0). Crude extracts were obtained by disrupting cells using a VCX 130 sonicator (130W; 20% duty cycle; 3 min). RDases *in vitro* activity assay were carried out in 4-mL vials containing 2 mL assay solution (2 mM methyl viologen; 1.5 mM

titanium (III) citrate; 100 mM Tris-HCl buffer (pH 7.0)) inside an anaerobic chamber as previously described (Adrian, et al., 2007). To differ PCE-RDases from TCE-RDases (TceA) in strain 11a5, 0.1 mM of each substrate (PCE, TCE, DCEs) was added to duplicate 4-mL vials containing the assay solution. The test was initiated by addition of cell extracts from PCE or TCE fed cultures, and the mixtures were incubated at 30 ºC for 24 h prior to headspace analysis.

Crude cell extracts from cultures during TCE, PCE-Stage A (without the production of VC) and PCE-Stage B (with the production of VC) dechlorination were sent for nano-LC-MS-MS analysis at Protein and Proteomics Centre, National University of Singapore. Output signals were searched in the Mascot database established by the genome of strain 11a5 for probable protein hits. The MS/MS based peptides and proteins were visualized using ProteinPilot software 4.5 (SCIEX). The identifications of peptides and proteins were validated if 95% and 99% probability were achieved by the Paragon Alogorithm, respectively (Shilov, 2007 #37)

4.1.5 Nucleotide sequence accession number

The sequence data of *Dehalococcoides mccartyi* strain 11a5 can be accessed using GenBank accession number CP011127 (chromosom, 11a5) and CP011128 (plasmid, pDhc6).

4.2 Results

4.2.1 Genome assembly revealing the presence of a putative circular plasmid

The SOAPdenovo assembly yielded only four scaffolds above 500 bp length – scaffolds 1 (1272 kbp), 2 (105 kbp), 3 (85 kbp), and 4 (5.3 kbp) based

on the Illumina reads of strain 11a5. During the gap closure stage, primers were designed at both upstream and downstream of each scaffold. PCR analyses showed that scaffolds 1, 2, and 3 connect to form a circular sequence, while scaffold 4 forms a separate circler sequence, i.e., the primers targeting the beginning and the end of scaffold 4 generate a PCR amplicon (~1,000 bp length). The sequence of gap in scaffold 4 (2,500 bp-3,192 bp) was of 100% identity with the partial sequence in chromosome (245,659-246,351), which was the only identical part between the two circular sequences. The repeats could possibly explain why scaffold 4 was not 'closed' during the first assembly phase, given that its small size and high coverage of about 100x using a 500bp library. This led us to speculate the possible presence of a circular extrachromosomal genetic element in strain 11a5. Two additional pairs of primers were designed within scaffold 4, pDhc6-4404F & pDhc6-3981R (5518 bp) and pDhc6-3554F & 11a5-411R (2798 bp), that cover the circular sequence (Figure 4.4). PCR with these two pairs of primers generated two amplicons of expected sizes (~5.5 kb and ~3 kb, respectively) (Figure 4.2), suggesting that the template DNA was circular. The sequences of the amplicons were sequenced and the sequence results matched the sequence of the circular DNA fragment identified during genome assembly and gap closure.

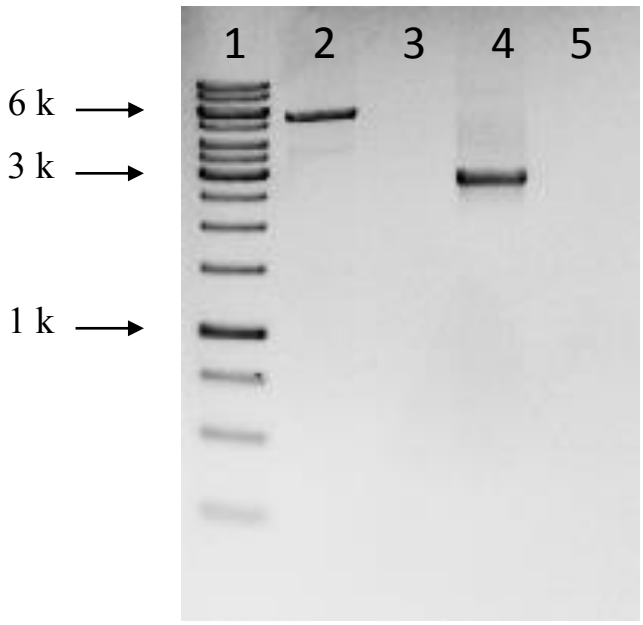


Figure 4.2 PCR analysis shows pDhc6 as a circular DNA and sizes of products are indicated as predicted. Note: lane 1, 1 kb ladder (Fermentas, catalogue number: SM0313); lane 2 and 3, pDhc6-4404F & pDhc6-3981R (5518 bp) and negative control; lane 4 and 5, pDhc6-3554F & pDhc6-411R (2798 bp) and negative control.

PCR alone cannot confirm the presence of a circular plasmid because tandem repeats of the putative plasmid sequence on the chromosome may also yield the same PCR results as an extrachromosomal element. However, the sequencing coverage of the repeats in such a case would be much higher than that of the rest of genome. Using the Mpileup script in the SAMtools package, we calculated the coverage of the putative plasmid and the rest of genome as 97.3 and 102.2, respectively, suggesting that the putative plasmid sequence does not exist in multiple copies in the genome. Additionally, none of the other scaffolds 1, 2 or 3 can be connected to scaffold 4 by PCR despite increase in the PCR extension time to 12 min, which should be sufficient to amplify up to a 12 kbp fragment. We can conclude from the PCR and genome sequencing that strain 11a5 carries a ~6 kb circular plasmid designated pDhc6 (plasmid-*Dehalococcoides*-6kb).

4.2.2 General features of the strain 11a5 genome

The *Dehalococcoides mccartyi* strain 11a5 genome consists of a circular chromosome of 1,461,973 base pairs (Figure 4.3) and an extrachromosomal circular plasmid of 5,940 base pairs (Figure 4.4). In total, 1,609 ORFs were identified (1,603 on the chromosome and 6 on the plasmid), of which 1,216 were annotated to known functions. The genome contains all three rRNA genes (one copy each for 5S, 16S, 23S rRNA genes), 1 tmRNA gene and 46 tRNA genes. Similar to other *Dehalococcoides* strains, the genome of strain 11a5 also possesses two high-plasticity regions (HPRs) of ~308.6 kbp and ~231.5kbp ((position: 58,510-250,309 (HPR1) and 1,184,581-1,416,101 (HPR2)), accounting for 28.8% of the total genome size.

The presence of multiple, nonidentical reductive dehalogenase homologous genes in the genome of *Dehalococcoides* explain the substrate versatility of the genus. Analysis of strain 11a5 revealed the presence of 31 *rdhA* genes and 31 *rdhB* genes; 30 of the *rdhA*s are paired with *rdhB*, leaving one *rdhA* (11a5-1388) and *rdhB* (11a5-1397) unpaired. Most of the 31 *rdhA*s are located in HPRs (5 *rdhA*s in HPR1 and 24 *rdhA*s in HPR2), while the other two *rdhA*s are located in conserved regions, one chromosomal and one extrachromosomal, respectively. Among all 31 *rdhA*s in the genome of strain 11a5 that share high homology (98%~100%) to *rdhA*s in GenBank (Table 4.2), including both functionally characterized and uncharacterized *rdhA* genes, only two are similar to characterized functional reductive dehalogenase genes: 11a5-1352, which has been identified as the TCE RDase gene in strain 11a5 and showing 97% identity with *tceA* (97% in amino acid) of strain 195,

and 11a5-1467 showing 86% identity with the *pceA* (91% in amino acid) of strain 195 (Lee, et al., 2013).

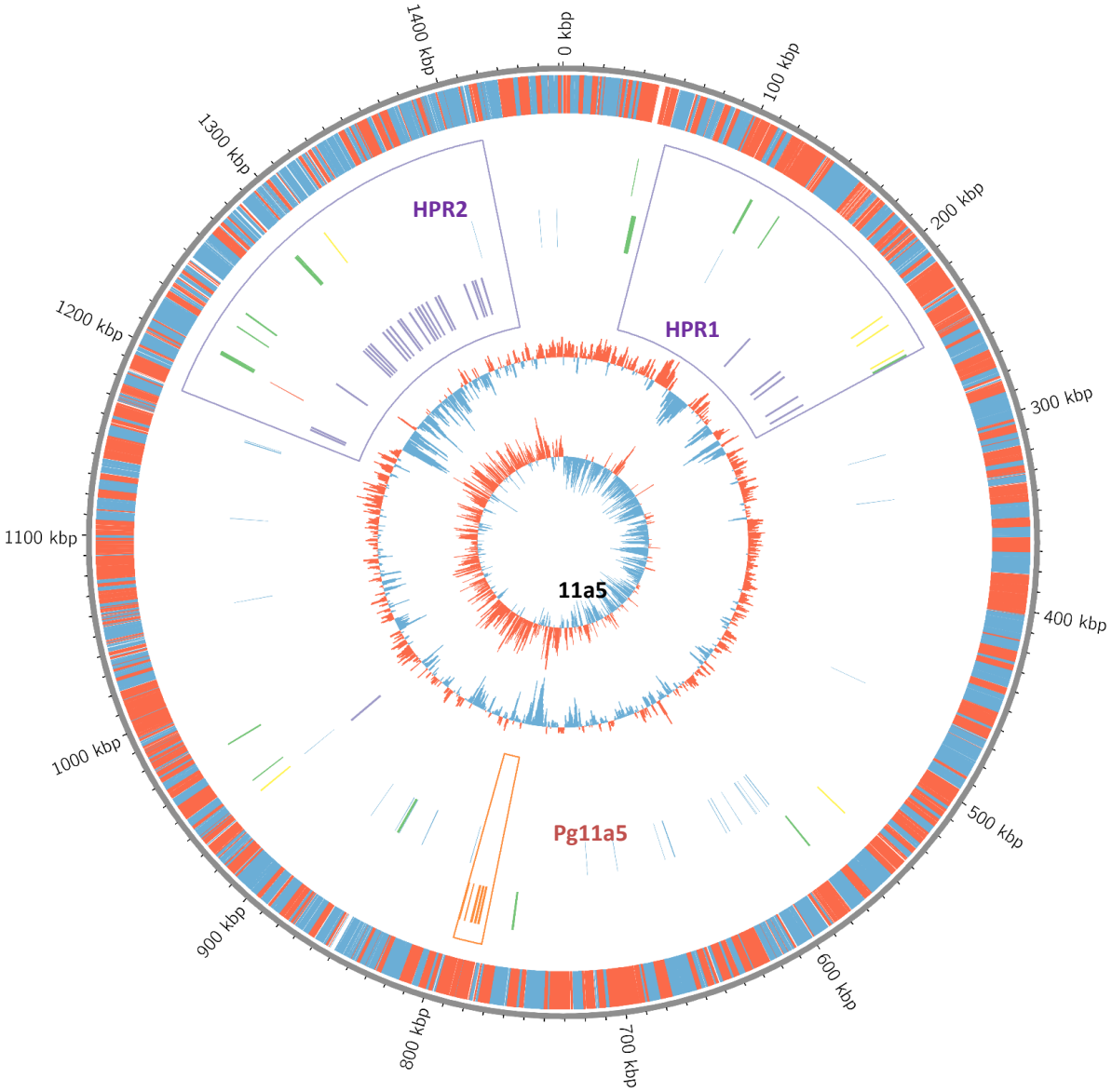


Figure 4.3 Genome map of chromosome DNA of strain 11a5. From outer to inner, circle 1 represents protein coding genes on plus strand (in red) and negative strand (in blue); circle 2 shows recombinase/resolvase genes (in green), cbiZ genes (in yellow), and phage-related genes (in orange); circle 3 shows the 5S/16S/23S rRNA genes (in green), the tmRNA gene (in red), and tRNA genes (in blue); circle 4 shows reductive dehalogenase genes (in purple); circle 5 shows GC contents (window size 2000 bp, step size 1000 bp), red: higher than genome average, blue: lower than genome average; circle 6 shows GC skew (C−G)/(C+G) (window size 2000 bp, step size 1000 bp), red: >0, blue: <0.

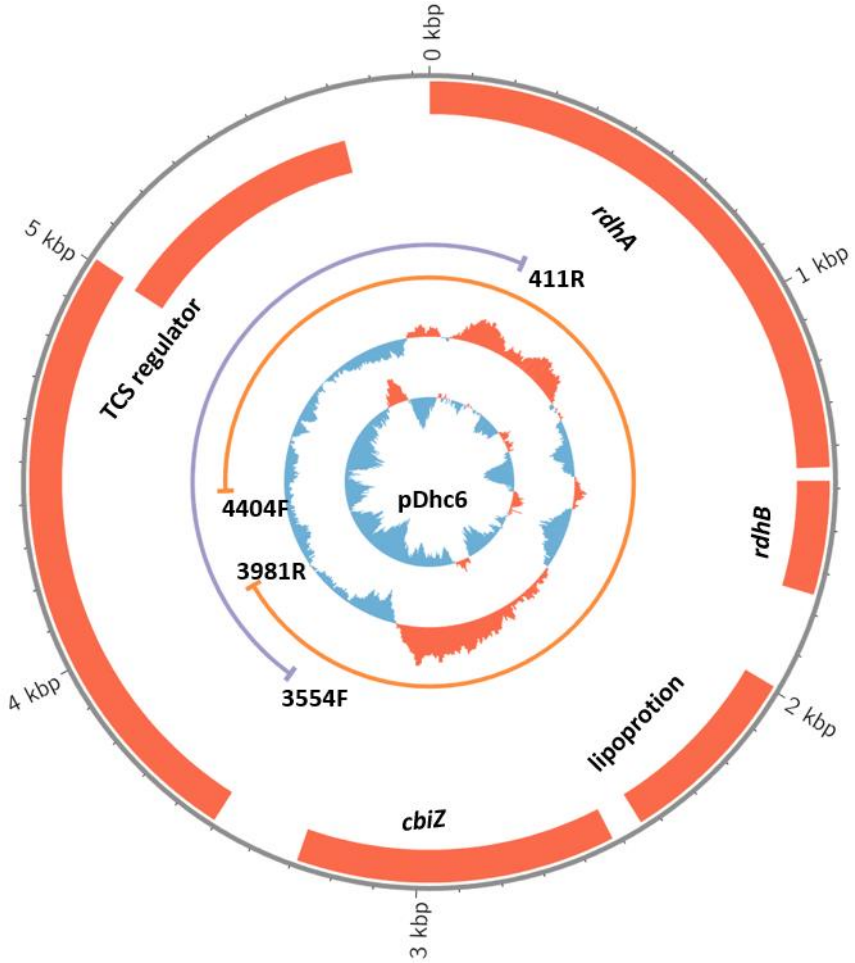


Figure 4.4 Genome map of plasmid pDhc6 of strain 11a5. From outer to inner, circle 1 and 2 represents protein coding genes (all on the plus strand), 11a5_p006 has an overlap of 14 bp with 11a5_p005, and is therefore placed on circle 2; circle 3 shows the amplified region by primers 4404F and 3981R; circle 4 shows the amplified region by primers 3554F and 411R; circle 5 shows GC contents (window size 250 bp, step size 10 bp), red: higher than plasmid average, blue: lower than plasmid average; circle 6 shows GC skew (C−G)/(C+G) (window size 250 bp, step size 10 bp), red: >0, blue: <0.

Table 4.2 Alignments of rdhAs in strain 11a5

Gene ID	Alignments description	Coverage (%)	Identity (%)
11a5_188	U*,CG5	100	100
11a5_227	U,CG5	100	100
11a5_232	U,DCMB5,CG5	100	99
11a5_255	U,GY50	100	100
11a5_263	U,GY50	100	99
11a5_1006	U,CBDB1	100	100
11a5_1306	U,GT	100	99
11a5_1308	U,DCMB5	100	100
11a5_1352	tceA,11a5	100	100
11a5_1355	U,BTF08	100	100

11a5_1395	U,DCMB5	100	100
11a5_1397	U,DCMB5	100	100
11a5_1402	U,DCMB5	100	99
11a5_1404	U,DCMB5	100	100
11a5_1419	U,BTF08	100	98
11a5_1424	U,CBDB1,CG5	100	99
11a5_1428	U,GT,CBDB1	100	99
11a5_1437	U,GT,CBDB1	100	100
11a5_1439	U,DCMB5,GT,CBDB1	100	100
11a5_1448	U,GT,CBDB1	100	99
11a5_1453	U,GT,CBDB1	100	100
11a5_1456	U,CG5	100	100
11a5_1460	U,GT,CBDB1,BTF08	100	99
11a5_1467	U,GT,CBDB1	100	100
11a5_1473	U,CBDB1	100	100
11a5_1477	U,GT,CBDB1	100	100
11a5_1497	U,CG5,FL2	100	100
11a5e_1503	U,CG5	100	100
11a5_1506	U,BTF08	100	100
11a5_1515	U,CG5	100	100

Note: U* indicates functionally unidentified.

The genome of *Dehalococcoides mccartyi* strain 11a5 also contains conserved features similar to other available *Dehalococcoides* genomes, e.g. three types of transcriptional regulators including CRP/FNR-type, multiple antibiotic resistance regulator (MarR)-type and two-component system regulator (TCS), six hydrogenase complexes and a complete gene cluster encoding corrinoid salvaging and remodeling systems.

4.2.3 Characterization of the pDhc6 plasmid

The extrachromosomal ~6 kb circular DNA was identified in strain 11a5 throughout all phases of growth and dechlorination. PCR confirmed the presence of the complete circular form of the plasmid during all growth phages (data not shown). qPCR confirmed that the plasmid was stably present in single copy during different growth phases (Figure 4.5) and over the course of at least 20 subcultures (Figure 4.6).

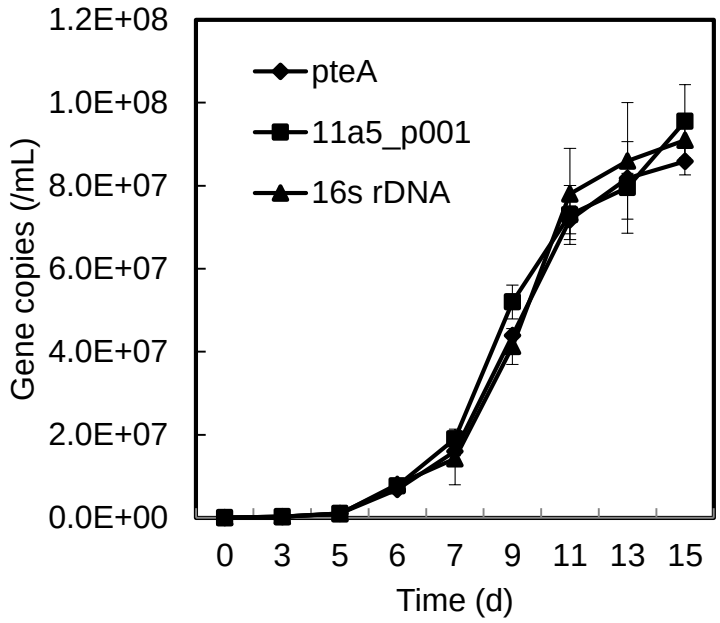


Figure 4.5 Quantification of *pteA*, 11a5_p001, and 16S rRNA gene in strain 11a5 during PCE dechlorination

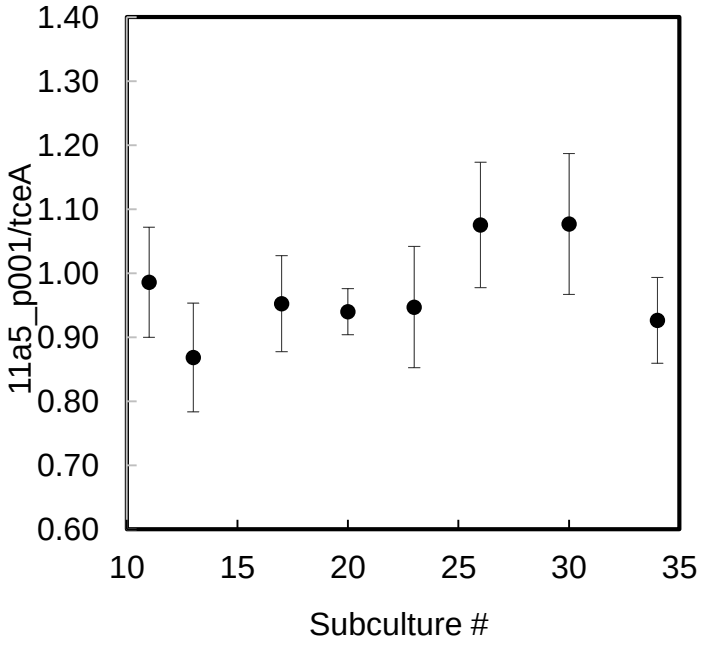


Figure 4.6 Ratio of *tceA* gene and 11a5_p001 gene numbers in subcultures

pDhc6 is 5,940 bp in length and has a GC content of 40.4%. No significant similarity to pDhc6 was found in the NCBI database, however high levels of similarity exists between pDhc6 and chromosomal sequences of other

Dehalococcoides strains. Region I of pDhc6 (from 1 bp to 3278 bp), which includes genes encoding one reductive dehalogenase (*rdhA*), one reductive dehalogenase anchoring protein (*rdhB*), one unidentified lipoprotein and one adenosylcobinamide amidohydrolase (*cbiZ*), shares 95% identity with a chromosomal region in strain CG4. Region II (from 2506 bp 5940 bp), which includes genes encoding the *cbiZ* and a TCS regulator composed of one sensor histidine kinase and one DNA-binding response regulator, shares 93% identity with a chromosomal region in strain CBDB1. No low-copy plasmid maintenance system could be found on the plasmid. Additionally, the characterized *Dehalococcoides* DnaA box "5'-TTATCGAAA-3'" is not present in pDhc6 (Gao and Zhang, 2008).

4.2.4 Presence of a putative prophage sequence in the genome of strain 11a5

A 16.3kbp putative prophage region (position: 777,442-793,840) was identified in the chromosome of strain 11a5 (designated pg11a5). The putative prophage comprises a total of 20 coding domain sequences (CDs), 14 of which are functionally annotated, while the other six are hypothetical proteins (

Table 4.3). pg11a5 has no significant phylogenetic similarity with any known prophage, but BLAST results assign it to the group of double stranded DNA (dsDNA) tailed phage which account for the majority of known annotated bacteriophage (Hatfull and Hendrix, 2011).

pg11a5 has a clear synteny among genes encoding the virion structure and assembly functions (

Table 4.3), including head genes, tail genes, head-tail connector genes and genes assisting the affiliation with and penetration of host cells. However, pg11a5 lacks conventional phage systems, containing neither a host lysis system nor any bacteriophage integration and excision systems. Such systems mediate bacteriophage transition from the lysogenic cycle to the lytic cycle (Young, 1992, Murphy, 2012). The absence of these two essential systems in the prophage sequence might have resulted from disruption or modification of the phage during co-evolution with host cells. Alternatively, as the number of characterized bacteriophage is significantly smaller than the pool of bacteriophage in existence, it may be that pg11a5 utilizes an uncharacterized mechanism for insertion and excision within the host sequence (Hatfull and Hendrix, 2011, Sarris, et al., 2014).

Table 4.3 Annotations of *Dehalococcoides mccartyi* strain 11a5 prophage pg11a5

#	ID	Annotation	Blast Hit
1	0840	Hypothetical protein HP2p14	Phage_Haemop_HP2_NC_003315;gi17981828
2	0841	Terminase small subunit	Phage_Bacill_phIS3501_NC_019502;gi422934327
3	0842	Terminase large subunit	Phage_Burkho_KS9_NC_013055;gi255033734
4	0843	Phage portal protein	Phage_Psychr_Psymv2_NC_023734;gi593779762
5	0844	Prohead protease	Phage_Vibrio_12B8_NC_021074
6	0845	Phage capsid protein	Phage_Escher_HK75_NC_016160; gi356870682
7	0846	Hypothetical protein	N/A
8	0847	Hypothetical protein	N/A
9	0848	Phage gp6-like head-tail connector protein	Phage_Rhizob_16_3_NC_011103;gi195546539
10	0849	phage head-tail adaptor	Phage_Psychr_Psymv2_NC_023734;gi593779767

1	085	DUF646	Phage_Psychr_Psymv2_NC_023734;gi593779770
1	0	domain-containing protein;	
1	085	Hypothetical	N/A
2	1	protein	
1	085	Phage tail	Phage_Entero_c_1_NC_019706;gi428781745
3	2	component	
1	085	Hypothetical	N/A
4	3	protein	
1	085	tail tape	Phage_Synech_S_CBS1_NC_016164;gi356870809
5	4	measure protein	
1	085	Putative	Phage_Geobac_GBSV1_NC_008376;gi115334640
6	5	phage tail protein	
1	085	gp46 family	Phage_Mycoba_Wildcat_NC_008206;gi109521483
7	6	protein	
1	085	Hypothetical	N/A
8	7	protein	
1	085	gp28 family	Phage_Mycoba_Kostya_NC_011056;gi194303081
9	8	protein	
2	085	Putative tail	Phage_Lister_LP_101_NC_024387;gi65860782
0	9	endopeptidase;	6

Table 4.4 Features of prophages in *Dehalococcoides mccartyi* strains

	195	BTF08-1	BTF08-2	11a5
Completeness/score[a]	Intact/110	Intact/150	Incomplete/30	Questionable/90
Length (kb)	31.5	35.4	45	16.3
GC content (%)	52.66	48.49	47.28	46.1
HPRs?	N	Y	Y	N
Total CDs	32	38	26	20
Phage hit protein NO.	18	22	12	15
Recombinases	3	5	1	0
Terminase	Y	Y	N	Y
Capsid protein	Y	Y	N	Y
Portal protein	Y	Y	N	Y
Head-tail connector	Y	Y	N	Y
Terminase protein	Y	Y	N	Y
Tail tape measure protein	Y	Y	N	Y
Holin protein	Y	Y	Y	N
Endolysin protein	Y	Y	N	N
Envelop glycoprotein	N	Y	N	N
Transposases	0	3	N	0

rdhAB	N	Y	N	N

[a]: If the region's total score is less than 70, it is marked as incomplete; if between 70 to 90, it is marked as questionable; if greater than 90, it is marked as intact.

PHAST analysis of published *Dehalococcoides* genomes found two intact *Dehalococcoides* prophages, one in strain 195 and another in strain BTF08 (Table 4.4). Like the prophage in strain 195, pg11a5 is located in the core genome, whereas the prophage in strain BTF08 lies in the HPR of the genome. The three prophage all have genes encoding essential phage structural proteins, yet, in accordance with the mosaicism of bacteriophage structural regions, there is low homology within the phage structural proteins in these *Dehalococcoides* prophage regions (Hatfull and Hendrix, 2011). Besides the shared common structural proteins, prophages in strain 195 and BTF08 contain host cell lysis proteins, CLP peptidase and a holin protein, as well as site-specific recombinases, responsible for extrachromosomal integration and excision, while pg11a5 has none of these. Interestingly, the BTF08 prophage possesses both *rdhAB* homologues and transposases, while the pg11a5 and 195 prophage regions do not.

4.2.5 Reductive dechlorination of tetrachloroethene (PCE) by strain 11a5

Previous studies (Lee, et al., 2013) identified dechlorination of TCE to VC and ethene by strain 11a5. In this study, we found that strain 11a5 also reductively dechlorinated PCE and coupled this process to growth (Figure 4.7).

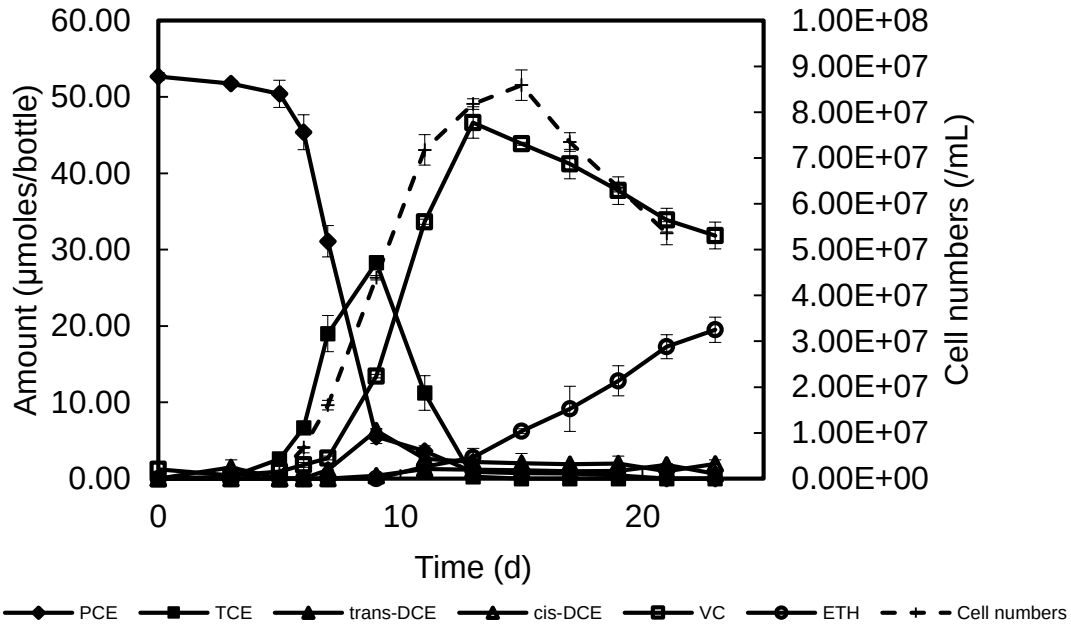


Figure 4.7 Dechlorination of PCE by *Dehalococcoides mccartyi* strain 11a5. Data points are averaged from triplicates

Unlike most other PCE dechlorinating *Dehalococcoides* (Damborsky, 1999, Cheng and He, 2009, Marco-Urrea, et al., 2011, Poritz, et al., 2013) strain 11a5 reductively dechlorinated PCE to VC and ethene at a ratio of 1.6: 1 with TCE as the intermediate, but negligible DCEs, within 23 days (Figure 4.7). The concentration of TCE, a metabolic intermediate, reached the highest point on day 9, but the maximum concentrations of TCE were variable in different kinetic analyses. Assays inoculated from cultures actively dechlorinating PCE to TCE led to higher amounts of TCE accumulation compared with inocula from cultures dechlorinating TCE to VC, suggesting a relationship between the maximum concentration of accumulated TCE and inoculum growth phase. Strain 11a5 dechlorinated PCE at a maximum rate of 88 µmoles·L^{-1}·day^{-1}, from day 4 to day 9, while the TCE dechlorination rate peaked at a maximum rate of 70 µmoles·L^{-1}·day^{-1}, from day 9 to day 13. Regression between cell number and the amount of PCE dechlorinated shows

a linear correlation ($R^2 = 0.98$) indicating the close coupling of cell growth and chlorine removal during PCE dechlorination (Figure 4.8). The growth yield was calculated as 8.7×10^7 cells /µmole chlorine removed based on the slope of the regression curve (Figure 4.8This indicates that PCE is a growth-supporting electron acceptor for strain 11a5. No further cell growth was observed during VC dechlorination and no VC dechlorination was observed when strain 11a5 was fed with VC as the sole electron acceptor, suggesting that dechlorination of VC to ethene is a co-metabolic process.

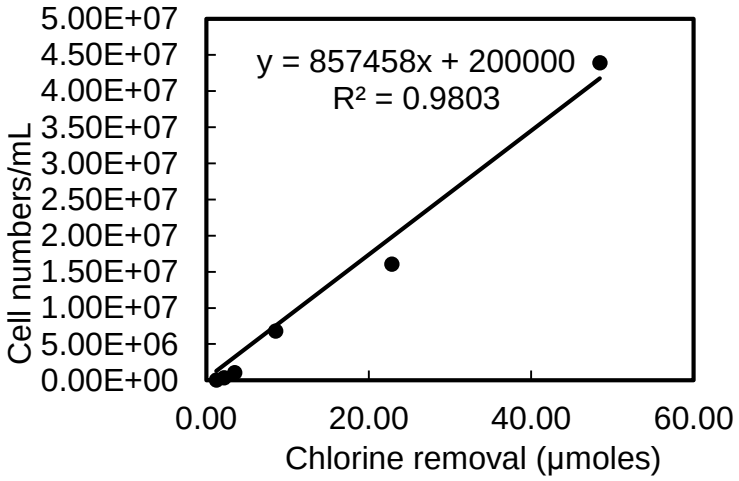


Figure 4.8 Linear correlation between cell numbers of strain 11a5 and chlorine removed from PCE

Crude cell extracts from cultures fed with PCE or TCE as an electron acceptor showed differing activities in *in vitro* assays. Crude cell extracts from cultures grown on TCE dechlorinated TCE and DCEs to VC, but did not dechlorinate PCE. Crude cell extracts harvested from PCE fed cultures in the stage without the production of VC (PCE-stage A) could dechlorinate PCE to TCE, but not TCE or DCEs to VC. Contrarily, crude cell extracts harvested from PCE fed cultures in the stage with the production of VC (PCE-stage B) dechlorinated not only PCE to TCE, but also TCE and DCEs to VC. The

observations from these enzyme assays seem to indicate that multiple RDases in strain 11a5 are involved in the PCE dechlorination, and that the dechlorination of PCE to TCE and of TCE to VC are mediated by different RDases. Previously, PceA and TceA were purified from *Dehalococcoides mccartyi* strain 195 and identified to catalyze PCE to TCE and TCE to cis-DCE / VC dechlorination, respectively (Magnuson, et al., 1998). The tceA gene has been identified to be responsible for dechlorination from TCE to VC in strain 11a5, however PCR with *Dehalococcoides*-pceA primers, which target the pceA in *Dehalococcoides*, yielded no amplification products, suggesting a novel PCE-to-TCE RDase exists in strain 11a5.

Among the 31 *rdhA*s in the strain 11a5 genome, gene 11a5_1355 and *tceA* were highly expressed when strain 11a5 was fed with PCE, while the transcription levels of other *rdhA*s were negligible (all less than 5 copies/cell) (Figure 4.9). The transcription level of *tceA* and 11a5_1355 increased to 37.5 ±2.4 and 14.7 ±1.2 transcripts/cell, respectively, prior to all PCE being dechlorinated to lower chlorinated ethenes (Figure 4.9). Contrarily, 11a5_1355 was transcriptionally expressed in negligible levels during TCE dechlorination (Figure 4.10). Consistent with transcriptional analyses results, the LC-MS/MS analyses of crude cell extracts showed transcribe of 11a5_1355 during PCE dechlorination but not during TCE dechlorination (Figure 4.11 & Table 4.5). Since it is known that TceA catalyzes reductive dechlorination of TCE to VC in strain 11a5, the appearance and increased transcription of 11a5_1355 in PCE dechlorination but not in the TCE dechlorination process indicates that RDase gene 11a5_1355 should play a role in PCE to TCE dechlorination process. Together with the crude cell

extracts assay and transcriptional analysis, we concluded that 11a5_1355, designated as *pteA,* is a novel PCE-RDase gene which is responsible for the dechlorination of PCE to TCE.

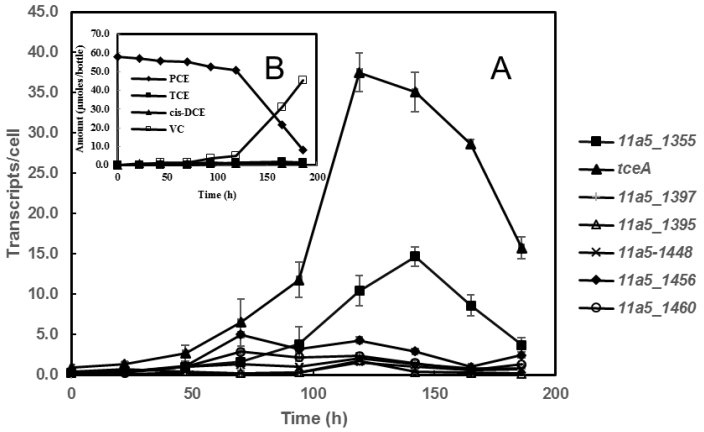


Figure 4.9 Transcriptional analysis of reductive dehalogenase genes in *Dehalococcoides mccartyi* strain 11a5 when fed with PCE (A) Gene expressional profiles of pteA and tceA in strain 11a5 when fed with PCE. (B) PCE dechlorination kinetics of strain 11a5. Data points are averaged from triplicates

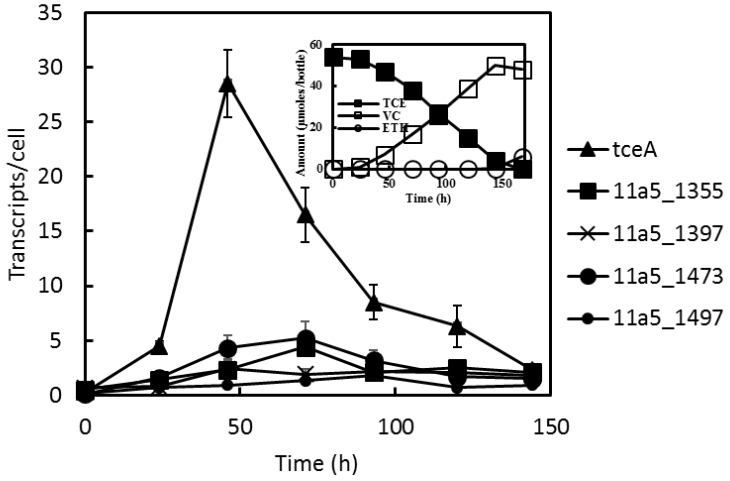


Figure 4.10 Transcriptional analysis of reductive dehalogenase genes in *Dehalococcoides mccartyi* strain 11a5 when fed with TCE (A) Gene expressional profiles of pteA and tceA in strain 11a5 when fed with TCE. (B)PCE dechlorination kinetics of strain 11a5. Data points are averaged from triplicates

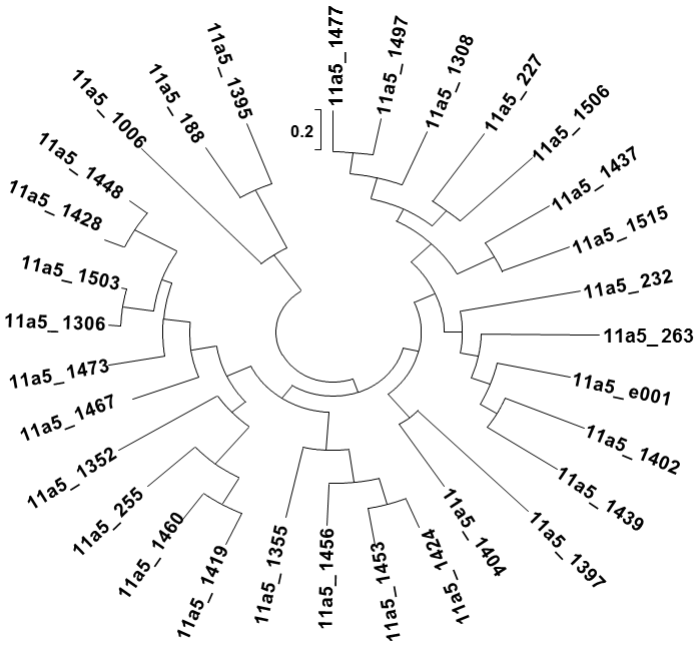


Figure 4.11 Phylogenetic analysis and transcribed *rdhA*s in strain 11a5. Note: Genes in green colour represents transcribe in PCE-stage A dechlorination and PCE-stage B dechlorination; Genes in purple colour represents transcribe in both TCE dechlorination and PCE-stage B dechlorination; Genes in red colour represents transcribe in PCE-stage B dechlorination only. Phylogenetic analysis of functionally characterized chloroethene-reductive dehalogenases by maximum likelihood method with 1000 bootstraps.

Table 4.5 Top 15 protein hits and all reductive dehalogenase hits detected by nano-LC-MS/MS

(a) *Dehalococcoides mccartyi* strain 11a5 was amended with TCE

Rank	Score	Name	Size (aa)	Coverage (%)	Amino acid hit (aa)
1	49.51	chaperonin GroEL (HSP60)	537	49.16	264
2	38.68	Molecular chaperone DnaK family	636	35.38	225
3	26.36	BNR/Asp-box repeat-containing protein	1024	17.58	180
4	17.74	acetyl-CoA decarbonylase / synthase complex subunit	733	15.42	113
5	16.32	S-adenosylmethionine synthetase	406	29.8	121
6	15.52	myo-inositol-1-phosphate synthase	370	31.62	117
7	15.48	Ni/Fe hydrogenase, large subunit	526	24.14	127
8	14.95	glutamine synthetase	443	23.48	104
9	13.43	glutamine synthetase	443	19.21	85
10	13.06	reductive dehalogenase 11a5_1352	498	23.09	115

11		nicotinate-nucleotide	286		
	12	pyrophosphorylase		26.57	76
12		DNA polymerase III subunit	378		
	11.98	beta		20.37	77
13		phosphoenolpyruvate	758		
	11.16	synthase/pyruvate phosphate		10.29	78
14		molybdopterin oxidoreductase,	993		
	10.44	formate dehydrogenase		7.322	73
15	10.38	[Fe] hydrogenase subunit	623	12.84	80

(b) *Dehalococcoides mccartyi* strain 11a5 was amended with PCE in stage A

Rank	Score	Name	Size (aa)	Coverage (%)	Amino acid hit (aa)
1	59.0	chaperonin GroEL (HSP60)	537	46.7	251
2	36.6	Molecular chaperone DnaK family	636	31.8	202
3	24.7	S-adenosylmethionine synthetase	406	37.9	154
4	24.2	BNR/Asp-box repeat-containing protein	1024	17.4	178
5	23.7	acetyl-CoA decarbonylase / synthase complex subunit	733	20.7	152
6	23.2	Ni/Fe hydrogenase, large subunit	526	32.5	171
7	16.8	glutamine synthetase	443	23.5	104
8	15.5	molybdopterin oxidoreductase, formate dehydrogenase	993	11.3	112
9	15.1	glutamine synthetase	443	23.4	104
10	15.0	translation elongation factor Tu	400	28.3	113
11	14.8	F-type H+-transporting ATPase subunit beta	464	21.6	100
12	14.0	adenylosuccinate synthase	432	17.1	74
13	13.9	glyceraldehyde-3-phosphate dehydrogenase, type I	335	30.5	102
14	13.5	acetyl-CoA decarbonylase / synthase complex subunit	448	16.5	74
15	13.2	isocitrate dehydrogenase (NAD+)	359	25.1	90
18	12.4	reductive dehalogenase 11a5_1355	498	20.7	103

The phylogenetic relationships between characterized chloroethene-RDases (PteA, PceA, TceA, MbrA, BvcA and VcrA) found in *Dehalococcoides*, as well as all functionally characterized PceA orthologues

are shown in Figure 4.12. It has previously been shown that RDases found in *Dehalococcoides* that catalyze different dechlorination processes cluster together, but are phylogenetically distant from RDases catalyzing similar processes in other genera (McMurdie, et al., 2009). Among characterized *Dehalococcoides* RDases, PteA is most closely related to PceA from strain 195, however these two proteins are distinct, sharing only 38% amino acid sequence, despite catalyzing the same dechlorination process.

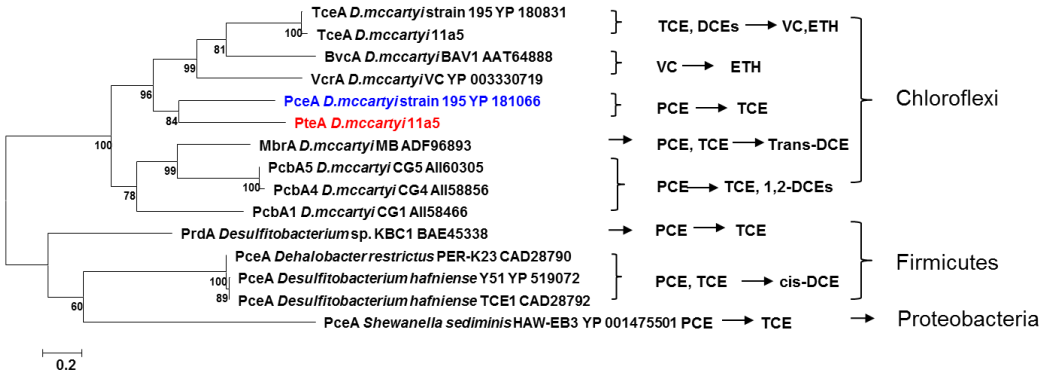


Figure 4.12 Phylogenetic analysis of functionally characterized chloroethene-reductive dehalogenases by maximum likelihood method with 1000 bootstraps.

4.3 Discussion

Dehalococcoides mccartyi strain 11a5 was first reported as capable of reductively dechlorinating TCE to VC and ethene in 2013 (Lee, et al., 2013). Here we expand on that initial report by identifying further dechlorination capability of this strain, from PCE to VC/ethene, and performing a genomic analysis, which revealed several interesting features in the genome of strain 11a5 - the presence of an extrachromosomal circular genetic element (pDhc6), a putative prophage (pg11a5) and a novel *Dehalococcoides* PCE RDase (PteA).

The genomes of *Dehalococcoides* are among the smallest of all bacterial genomes and, although being known to contain large regions of high

plasticity, they have not previously been found to harbor extrachromosomal genetic elements. This study reports the first instance of a stably maintained, circular, double stranded, single-copy plasmid of approximately 6 kb which encodes several functional genes – including all the elements of a classic reductive dehalogenase operon – present in *Dehalococcoides mccartyi* strain 11a5. Although it does not seem to contain any identifiable replication initiation protein coding sequences, we suspect that pDhc6 possibly is maintained by extrachromosomal replication machinery capable of recognizing an uncharacterized plasmid DnaA box which accounts for the consistent 1:1 ratio of chromosome and plasmid in strain 11a5. To maintain vertical transmission of low-copy plasmids from the mother cell to daughter cells, bacteria can use partitioning or post-segregational killing systems located on plasmids, e.g. pSZ77 in *Geobacter lovleyi* strain SZ (Wagner, et al., 2012b) (Sengupta and Austin, 2011). However, pDhc6 has no such genes involved in plasmid maintenance and stability. Unlike in partitioning systems and post-segregational killing systems, genes responsible for multimer resolution systems, which are site-specific recombinases converting plasmid multimers into monomers, can be located either on chromosomes or plasmids (Austin, et al., 1981, Summers, 1998). Strain 11a5 contains four resolvase family site-specific recombinases in chromosome, which could possibly contribute to maintenance and stability of pDhc6. The function of pDhc6 in growth and dechlorination of strain 11a5 remains unknown since transcriptional and proteomic analyses showed that the reductive dehalogenase operon in pDhc6 is not involved in dechlorination of PCE or TCE. Strong evidence for the horizontal transfer of reductive dehalogenase genes exists,

although no mechanism for such transfer has been conclusively shown. The presence of a plasmid carrying the full component of genes for a *rdh* operon provides a tantalizing clue that in the future may help to elucidate the mechanisms of *rdh* flow in organohalide respiring bacteria.

So far, prophage is found in 3 *Dehalococcoides* strains 195, BTF08, and 11a5. It is difficult to phylogenetically categorize pg11a5 present in strain 11a5 into a single bacteriophage class as the different coding sequences in the pg11a5 region have the highest homology with proteins from a variety of bacteriophage. No regions share significant similarity with the prophage located in the genome of *Dehalococcoides* strain 195, and only a small portion of pg11a5 (~3.5 kb/ 16.4 kb) with 90.1% identity with the genomic prophage region in strain BTF08. This lack of nucleotide sequence identity is consistent with the mosaicism associated with bacteriophage genomes, even among bacteriophage found in the genomes of closely related organisms (Hatfull, 2008). Interestingly, the prophage regions found in strains 11a5 and 195 contain neither transposes nor RDase genes, whereas the prophage region in strain BTF08 contains both transposes and genes encoding a putative RDase and anchoring protein. This suggests a role for bacteriophage and transposases in the movement of *rdhs* within and, possibly, among *Dehalococcoides* genomes.

Through transcriptional and proteomic analyses, a PCE RDase, PteA, was identified and functionally characterized in strain 11a5. PteA catalyzes the same PCE to TCE dechlorination as the PceA RDase in strain 195, yet shares only 38% similarity with PceA in *Dehalococcoides mccartyi* strain 195. This suggests that PteA represents a new type of PCE RDase in

Dehalococcoides. This finding enriches the existing database of functional genes that can be used to assess the potential for bacterial remediation of chloroethenes at contaminated sites. Interestingly, a chromosomal *rdhA* in strain 11a5, 11a5-1467, shares 91% amino acid similarity with PceA in strain 195, yet was not found to be involved in PCE dechlorination. A similar phenomenon was also shown in *Dehalococcoides mccartyi* strain CG5, in which RD-CG5-1 (32% amino acid similarity with PceA of strain 195) catalyzes PCE dechlorination while RD-CG5-20 (94% amino acid similarity with PceA of strain 195) does not (Wang, et al., 2014a). These results highlight how unreliable to predict RDase functions based solely on sequence similarity. This agrees with findings based on phylogenetic analysis of a curated set of RDases which concluded that sequence similarity and substrate specificity are generally not correlated (Hug, et al., 2013).

In conclusion, this study reports the complete genome sequence of a chloroethene-dechlorinating bacterium, *Dehalococcoides mccartyi* strain 11a5. Strain 11a5 is distinct from other *Dehalococcoides* strains as it contains a circular plasmid pDhc6 and a putative prophage pg11a5, which may provide an avenue to investigate horizontal transfer of reductive dehalogenase genes among organohalide respiring bacteria. The discovery of pDhc6, the first extrachromosomal genetic element found in *Dehalococcoides* may also open the door to possible genetic manipulation of this historically genetically intractable microbe. Finally, this study identifies previously uncharacterized PCE dechlorination by strain 11a5, implicates a novel PCE RDase, PteA in this process, and analyzes the phylogenetic and functional relationship among

characterized RDases, which provides new molecular tools and genomic insights to evaluate remediation strategy.

5 Chapter V Characterization of a Dehalobacter containing co-culture SN that dechlorinates chloroform and identification of chloroform reductive dehalogenase gene

5.1 Introduction

1,1,1-trichloroethane (1,1,1-TCA), and bromoform were once produced in large quantities for use as solvents and chloroform is still used industrially each year, primarily in the synthesis of refrigerants (HCFC-22) and Teflon. Due to this extensive industrial utilization and inappropriate disposal, large amounts of these compounds have been released into the environment and were subsequently intensively distributed into groundwater and anoxic zones of aquifers. The United States Environmental Protection Agency has identified chloroform, 1,1,1-TCA and bromoform in at least 783, 792 and 136 of 1322, respectively, National Priorities List sites in the United States (ATSDR, 2006a, 2011a, 2013). This intense environmental distribution has raised public concerns because these halogenated solvents can depress central nervous system and cause liver and kidney diseases or even be fatal (IARC, 2010). Besides health effects on humans, chloroform and 1,1,1-TCA also inhibits many vital microbial processes, especially methanogenesis and dechlorination of chloroethenes(Bagley, et al., 2000, Weathers and Parkin, 2000, Grostern and Edwards, 2006a).

Biodegradation of chloroform and 1,1,1-TCA has been observed either co-metabolically, in methanogenic and sulphate-reducing environments, or metabolically, in anaerobic conditions. However little is known about microbial transformation of bromoform. Sharing similar structure with the same functional group (CCl_3-), chloroform and 1,1,1-TCA are usually

degraded by the same mechanisms. Several strains belonging to the genera *Methylosinus, Methanosarcina, Desulfobacterium* and *Pseudomonas* have been reported to co-metabolically degrade chloroform or 1,1,1-TCA, resulting in the production of less chlorine substituted molecules, carbon dioxide, monoxide and organic acids (Oldenhuis, et al., 1991, Bagley and Gossett, 1995, McClay, et al., 1996). However, these co-metabolic processes usually have a low tolerance to inhibition by chloroform and 1,1,1-TCA, being effectively quenched by chloroform and 1,1,1-TCA at concentrations as low as 2.5 μM and 5.2 μM, respectively. This substrate inhibition restricts their application in dealing with highly contaminated sites (Duhamel, et al., 2002). Additionally, co-metabolic degradation requires the addition of other substrates for growth. For instance, strain OB3b can degrade chloroform and 1,1,1-TCA in concentrations as high as 750 μM, yet the cultures need to be pregrown with methane prior to exposure to these chlorinated solvents (Oldenhuis, et al., 1991). Most metabolic degradation can overcome the low tolerance to chloroform and 1,1,1-TCA as the initial concentrations can be a hundred times higher than is inhibitory to co-metabolic processes. The metabolic degradation of chloroform and 1,1,1-TCA can be achieved through stepwise reductive dechlorination, resulting in the production of DCM from chloroform and 1,1-DCA and/or CA from 1,1,1-TCA, respectively. Only a few bacterial cultures, belonging to *Dehalobacter* and *Desulfitobacterium* genera, have been reported to be involved in such stepwise reductive dechlorination (Sun, et al., 2002, Grostern, et al., 2010, Lee, et al., 2012a, Ding, et al., 2014). Therefore, it is of importance to enlarge the bacterial pools

which can dechlorinate chloroform and 1,1,1-TCA, especially debrominate bromoform.

In this study, we discovered a novel *Dehalobacter* sp., strain SNCF, that respires chloroform, 1,1,1-TCA and for the first time bromoform for growth in a co-culture, SN, with a non-dechlorinating *Desulfovibrio* sp. A novel reductive dehalogenase, ThmA, was identified in strain SNCF responsible for chloroform, 1,1,1-TCA and bromoform dehalogenation through transcriptional and proteomic analyses. A set of chloroform RDase gene primers cf159F/cf387R was also designed to monitor chloroform dechlorinating activity.

5.2 Materials and Methods

5.2.1 Establishment of microcosm

Anaerobic microcosms were obtained with mangrove sediment from north of Singapore in 2012. Microcosm studies were conducted in 60-ml serum bottles containing ~10g of sediment and 25ml autoclaved bicarbonate-buffered mineral salts medium, as previously described (Cheng, et al., 2010). The bottles were sealed with black butyl rubber septa (Geo-Microbial Technologies, Inc, Ochelata, OK, USA) and secured with aluminum crimp caps. Microcosms were spiked with lactate (10mM) and chloroform as electron acceptors (~24μmoles / bottle). Abiotic controls were established by autoclave sterilization of serum bottles prepared in the same way as microcosm cultures. All bottles were incubated in the dark at 30 ºC without shaking.

5.2.2 Chemicals, culture and growth conditions

All chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) or Merck (Darmstadt, Germany) at the highest purity available. Mixed and co-culture SN, unless otherwise specified, were grown in 60 mL serum bottles containing 30 mineral salts medium spiked with acetate as the sole carbon source and amended with hydrogen as an electron donor (0.33 atm) and halogenated compounds, as indicated, as an electron acceptor and incubated at 30 °C as previously described (He, et al., 2007). Cultures dechlorination kinetics studies was constructed in 100 mL mineral salts medium in 160-mL serum bottles with all other conditions the same.

In substrate range tests of co-culture SN, chloroethenes (~0.3 mM), chloroethanes (~0.3 mM), chlorophenols (0.05 mM), polybrominated diphenyl ethers (PBDEs) (100 nM), polychlorinated biphenyl (PCB) Aroclor 1260 (10 ppm) and trihalomethanes (0.3 mM) were added in acetate/H_2 amended medium (Table 5.1). All kinetics studies were performed in biological triplicates and with abiotic controls (without bacterial inocula or with autoclaved cultures). Cultures were incubated in the dark at 30 ºC without shaking.

5.2.3 Molecular analysis

Clone library A clone library of 16S rRNA genes of mixed culture SN was established using the p-GEM-T easy vector system (Promega) according to manufacturer's instructions. The universal primer sets 8F (5'-AGAGTT TGATCCTGGCTCAG-3') and 1392R (5'-ACGGGCGGTGTGTRC-3') were used to amplify 16S rRNA gene in the construction of clone library. Plasmids were extracted using plasmid miniprep kit. Restriction fragment length

polymorphism (RFLP) analysis was performed on the extracted plasmid DNA using the restriction endonucleases HhaI and MspI (NEB, Ipswich, MA, USA). The 16S rRNA gene inserts representing groups of distinct enzyme restriction patterns were subsequently sequenced by Integrated DNA Technology (IDT), Singapore. Sequences were aligned with the BioEdit assembly software package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and were analyzed with BLASTN (http://www.ncbi.nlm.nih.gov/).

PCR-denaturing gradient gel electrophoresis (DGGE) Genomic DNA was extracted using the QIAGEN DNeasy Blood and Tissue Kit (QIAGEN® GmbH, Hilden, Germany) according to the manufacturer's instructions. PCR of bacterial 16S rRNA genes for DGGE was carried out using the primer pair of 341GC and 518R with a touch-down thermal program as previously described (Duhamel, et al., 2004). PCR amplicons were separated by DGGE using a denaturing gradient ranging from 20% to 80% for 12h at 120 V and 60 ^{o}C.

Quantitative real-time PCR (qPCR) and reverse transcription PCR (RT-PCR) Quantitative real-time PCR (qPCR) was conducted on an ABI 7500 Fast System using the SYBR green reporter (Lo-ROX Kit, SensiFASTTM SYBR, BIOLINE) as previously described (Lee and He, 2010). Construction of plasmids carrying targeted genes to serve as qPCR standards was performed as previously described (Chow, et al., 2010). *Dehalobacter*-genus specific primers (Dhb441F &Dhb645R), *Desulfovibrio*-genus specific primers (Dsv691F &Dsv826R) and 16S rRNA gene primers (338F & 518R) were utilized to monitor cell growth (Fite, et al., 2004, Smits, et al., 2004).

Reverse transcription PCR (RT-PCR) followed by qPCR (RT-qPCR) was performed to monitor expression levels of *ThmA* during chloroform dechlorination using designed chloroform RDase gene specific primers (cf159F 5'-GGAACGGTCAAGGAACTGGA-3' and cf387R 5'-ATAATCAACGGCAAAGCTTG-3'). Cultures for transcriptional analysis were spiked with 200 μM chloroform after complete consumption of the initial chloroform amendment and a 3 day period of starvation in order to minimize residual RNA. Cell samples were collected at 12 hour intervals for DNA (1 mL of culture) and RNA (1.5 mL of culture). RNA extraction and reverse transcription were conducted as previously described (Ding, et al., 2014).

5.2.4 Proteomic analyses and enzyme assays

Native polyacrylamide gels (native-PAGE) composed of 5% stacking gel and 10% resolving gel were prepared as described previously (U.K.Laemmli, 1970), only differing that protein denaturation was prevented by running native-PAGE in an ice box and removing sodium dodecyl sulfate (SDS) from all buffers. Cells were harvested by centrifugation (12,000 rpm, 20 min, 4ºC) and re-suspended in degassed Tris-HCl buffer (100 mM, pH 7.0). Membrane proteins of co-culture SN were obtained and the procedure of native-PAGE analysis was conducted as previously described, except that the gel was stained using PlusOne Silver Staining kit (Adrian, et al., 2007). Briefly, the same protein samples were electrophoresed on two adjacent gel lanes. After electrophoresis, one lane was left unstained for further activity analyses, while the other lane was silver stained to mark the position of protein bands in the unstained lane. Bands in the unstained lane were excised for *in vitro* activity assays. *in vitro* activity assays were carried out in 4-mL vials

containing 2 mL assay solution (2 mM methyl viologen; 1.5 mM titanium (III) citrate; 100 mM Tris-HCl buffer (pH 7.0)) inside an anaerobic chamber as previously described (Adrian, et al., 2007) 0.1 mM of each substrate (chloroform, 1,1,1-TCA, bromoform) was added to duplicate 4-mL vials containing the assay solution. The test was initiated by addition of gel fragments cut from native-PAGE, and the mixtures were incubated at 30 ºC for 24 h prior to headspace analysis.

Prominent bands in the stained lanes corresponding to gel regions exhibiting chloroform, 1,1,1-TCA and bromoform dehalogenating activity recognized in the parallel non-stained bands were cut and sent for MALDI-TOF-MS analysis at Protein and Proteomics Centre, National University of Singapore. Output signals were searched in the Mascot database which was constructed based on National Center for Biotechnology Information Database for probable protein hits.

5.2.5 Sample analytical procedures

Chloroethenes and chloroethanes concentrations were determined by headspace analysis using an Agilent gas chromatograph (GC7890) equipped with a flame ionization detector and a GS-GasPro column (30 m by 0.32 mm; J&W Scientific) as previously described (Zhao, et al., 2015). Trihalomethanes, chlorophenols and PCBs were monitored by GC-MS using an Agilent GC6890-MSD5975 and an Rxi-5ms column (15 m by 0.25 mm; Restek) (Wang and He, 2013). Headspace samples were injected manually using a gas-tight glass syringe (Model Gastight ® #1725, Hamilton Co., Reno, Nevada).

5.3 Results

5.3.1 Subculture degradation characteristics and microbial community analysis

Microcosm SN was initially discovered to dechlorinate chloroform to dichloromethane after 45 days' incubation. Culture SN was then enriched by more than 10 consecutive transfers over a period of one year in a mineral salts medium amended with acetate (10 mM)/hydrogen (0.33 ppv) and spiked with chloroform (~0.4 mM). The enriched mixed culture SN was able to dechlorinate chloroform (~ 0.4 mM) to the final product dichloromethane within 11 days (Figure 5.1). Clone library of 16S rRNA genes showed 4 species predominant in mixed culture SN (Figure 5.2). 16S rRNA analysis of mixed culture SN showed a species belonging to the order *Clostridiales* to be the most dominant population, with *Dehalobacter* and *Desulfovibrio* accounting for 24% and 22%, respectively, and *Caulobacter* the minority population of 7%. *Clostridiales* exists ubiquitously in anaerobic environments, and most notably, in soils. Both the *Dehalobacter* and *Desulfovibrio* genera have been reported to be involved in dehalogenation.

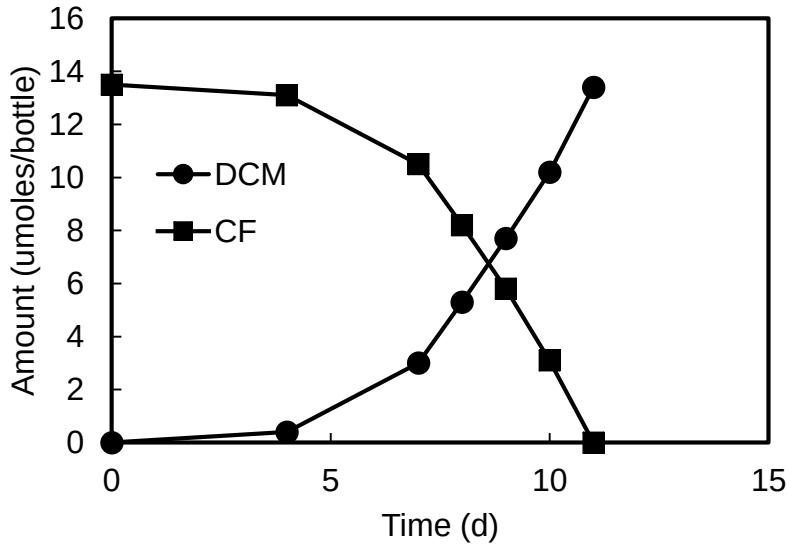


Figure 5.1 Dechlorination of chloroform by mixed culture SN. Note: CF, chloroform; DCM, dichloromethane.

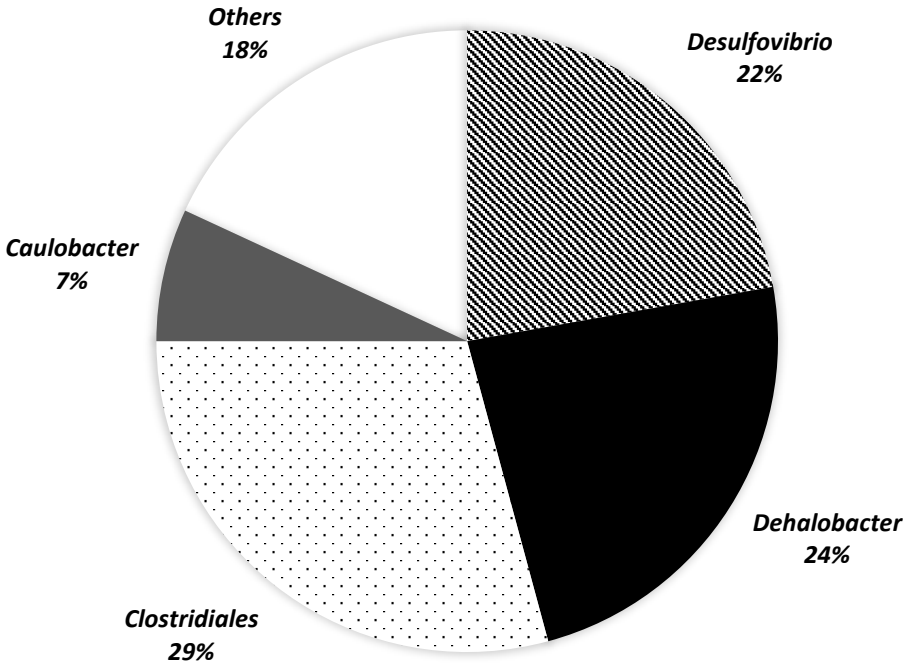


Figure 5.2 Microbial community analysis of mixed culture SN through clone library

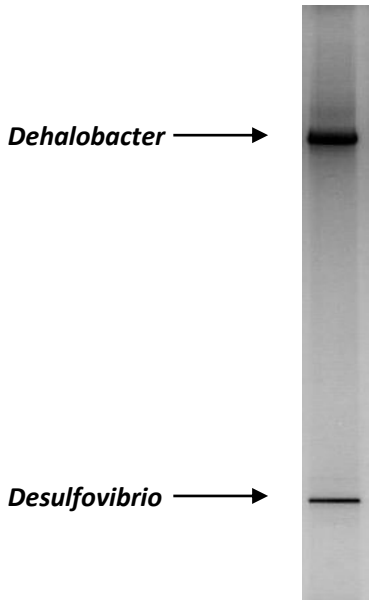


Figure 5.3 DGGE profile of 16S rRNA genes in co-culture SN

Five consecutive 10^{-1} to 10^{-7} serial-to-extinction dilution series were performed and three subcultures, including two chloroform dechlorinating co-cultures and one co-culture without chloroform dechlorinating capability, were obtained. Clone libraries constructed from these cultures showed that the co-cultures containing *Caulobacter/Dehalobacter* and *Desulfovibrio/Dehalobacter* retained chloroform dechlorinating capability, while the co-culture containing *Caulobacter/Desulfovibrio* but not *Dehalobacter* lost the ability to dechlorinate chloroform. Taken together, this suggests that the *Dehalobacter* is probably the chloroform dechlorinator in culture SN. The subculture which contains *Desulfovibrio* and *Dehalobacter* was selected for further study and designated co-culture SN (Figure 5.3).

5.3.2 Identification of chloroform dechlorinating anaerobe in co-culture SN

To confirm the involvement of *Dehalobacter* in chloroform dechlorination, the growth of *Dehalobacter* and *Desulfovibrio* were monitored

by q-PCR of DNA samples during dechlorination using genus-specific 16S rRNA gene primers. The DNA samples without chloroform amendment were compared as controls. 0.6 mM chloroform was dechlorinated by co-culture SN within 10 days with an average dechlorination rate of 84.4 μmoles·L^{-1}·day^{-1}, as measured from day 3 to day 10 after 2 days' lag phase (Figure 5.4A). During the chloroform dechlorination process, the cell numbers of *Dehalobacter* increased by almost 62-fold to a density of (3.5±0.4) ×10^7 16S rRNA gene copies/mL (Figure 5.4B). Linear regression between cell numbers and the amounts of chloroform dechlorinated showed a linear correlation indicating a close coupling of *Dehalobacter* cell growth and chlorine removal during chloroform dechlorination (Figure 5.5). The growth yield was calculated of (5.6±0.7) ×10^7 16S rRNA gene copies per μmol Cl^- released from chloroform. In the absence of chloroform, the cell numbers dropped below qPCR detection limit after 8 days' incubation, indicating the requirement of chloroform as electron acceptor for growth (Figure 5.4C). In the meantime, the growth of *Desulfovibrio* showed no correlation with chloroform dechlorination and clearly, was severely inhibited by an estimated one magnitude order in the presence of chloroform (Figure 5.4 B&C). As a result, *Dehalobacter* was identified as the chloroform dechlorinating population in co-culture SN and the *Dehalobacter* sp. strain was designated SNCF.

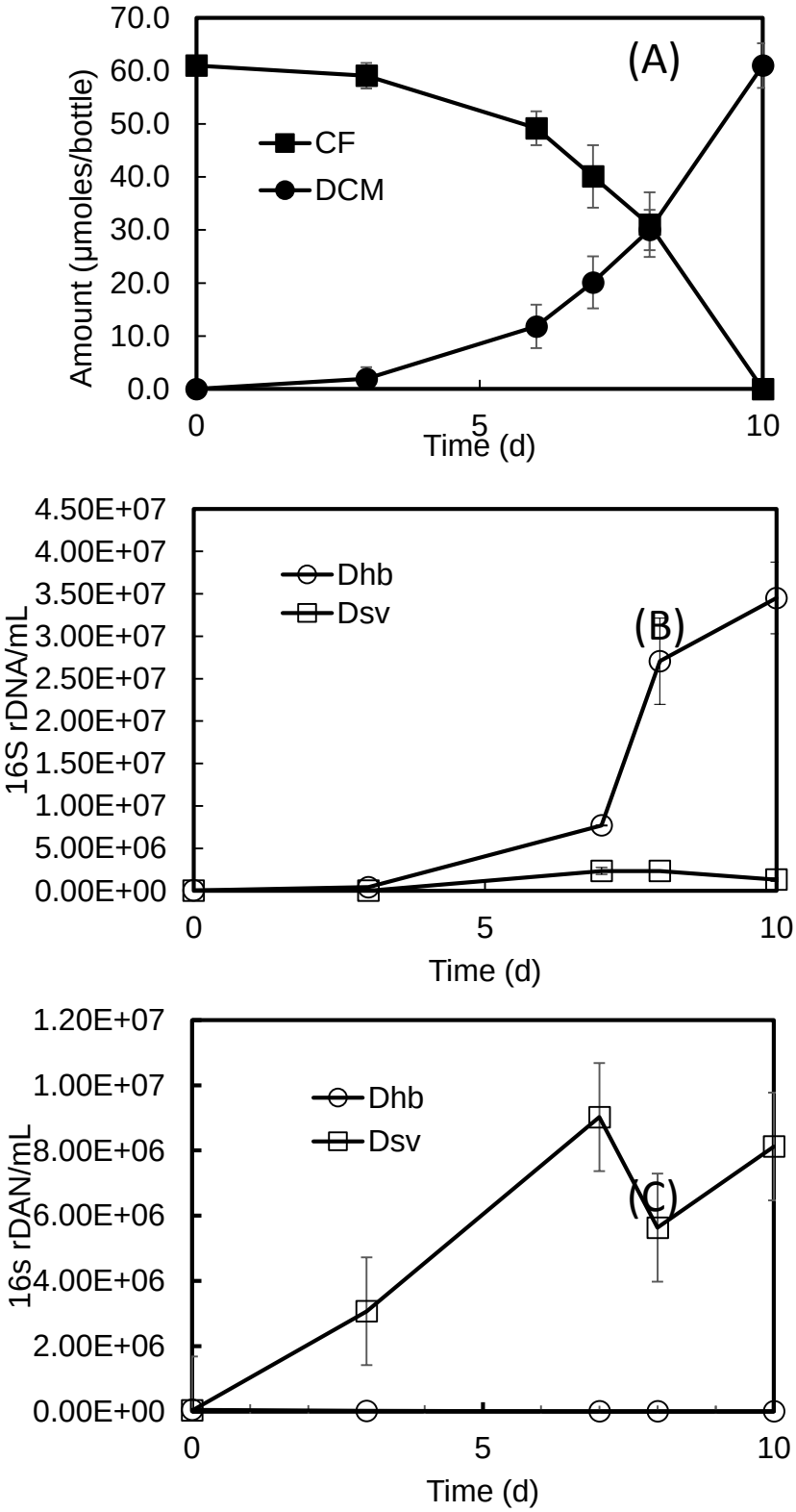


Figure 5.4 Identification of chloroform dechlorinating anaerobe in co-culture SN. (A) Dechlorination of chloroform by co-culture SN. (B) Cell growth of co-culture SN in the presence of chloroform. (C) Cell growth of co-culture SN in the absence of chloroform. Note: CF, chloroform; DMC, dichloromethane; Dhb, Dehalobacter; Dsv, Desulfovibrio.

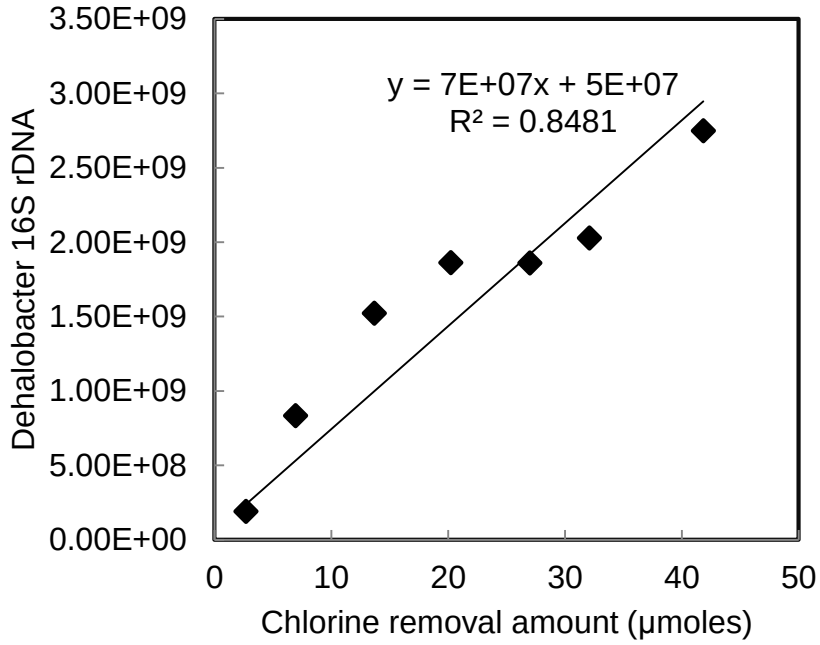


Figure 5.5 Linear correlation between cell number of strain SNCF and chlorine removed from chloroform.

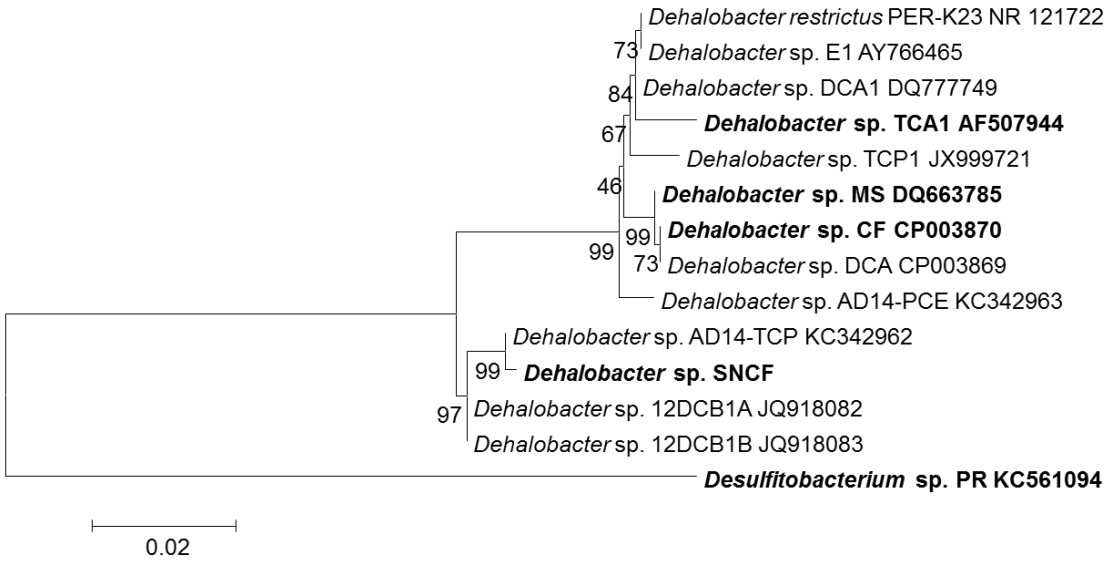


Figure 5.6 Molecular phylogenetic analysis of *Dehalobacter* strains by Maximum Likelihood method in 1000 bootstrap

Phylogenetic analysis of nearly full-length 16S rRNA gene was performed among *Dehalobacter* sp. strain SNCF and other identified *Dehalobacter* strains as well as identified trihalomethyl-group dechlorinating

anaerobes. The comparative analysis revealed a 99% similarity (1418/1422 bp) with its closet relative, a 2,4,6-trichlorophenol dechlorinating isolate *Dehalobacter* sp. AD14-TCP1, and a relatively distant phylogenetic relationship of 97% similarity with *Dehalobacter* CF, another chloroform dechlorinating culture identified in enriched culture Dhb-CF, (1385/1424 bp) (Figure 5.6).

5.3.3 Reductive dehalogenation of 1,1,1-TCA and bromoform by co-culture SN

The substrate utilization profile of co-culture SN was explored. Among all tested electron acceptors and none of the other tested substrates were dechlorinated, including chloroethenes as well as specific members of chloroethanes, chlorophenols, PBDEs, PCBs and THMs, co-culture SN showed a restricted substrate utilization profile that only chloroform, 1,1,1-TCA and bromoform were dehalogenated (Table 5.1).

Table 5.1 Substrate range of co-culture SN

Chloroethenes (3 months)	PCE	N
	TCE	N
	DCEs	N
	VC	N
Chloroethanes (3 months)	1,1,1-TCA	Y, to 1,1-DCA
	1,1,2-TCA	N
	1,1-DCA	N
	1,2-DCA	N
Chlorophenols (3 month)	PCP	N
	2,4,5-TCP	N
	2,4,6-TCP	N
PBDEs (3 month)	Penta-BDE	N
	Octa-BDE	N
	Nona-BDE	N

THMs (1 month)	$CHBr_3$	Y, to CH_2Br_2
	$CHClBr_2$	N
	$CHCl_2Br$	N
PCB (3 months)	PCB1260	N

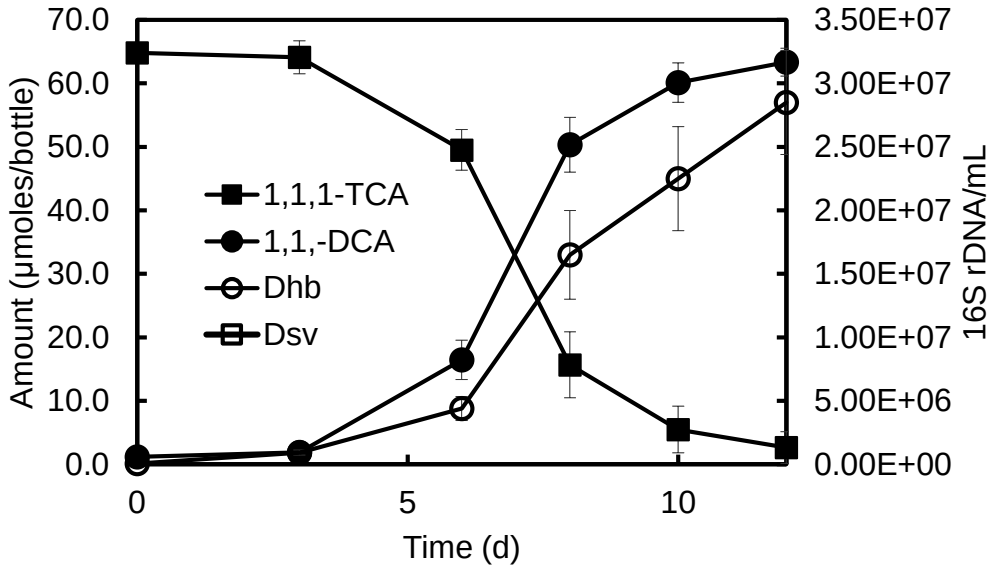


Figure 5.7 Dechlorination of 1,1,1-TCA by co-culture SN

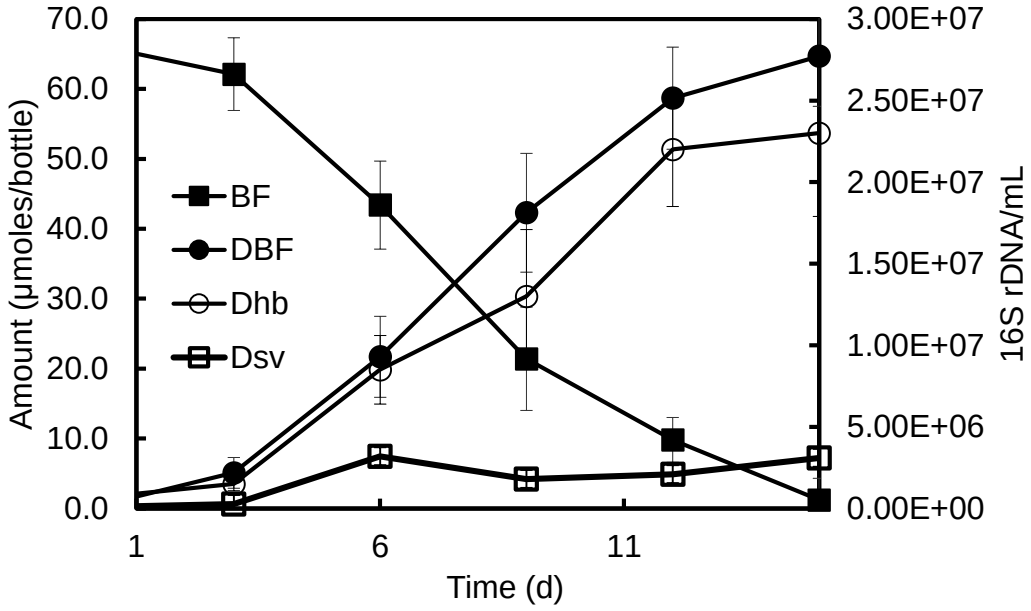


Figure 5.8 Debromination of bromoform by co-culture SN

1,1,1-TCA and bromoform were stepwise dehalogenated to 1,1-DCA within 12 days and dibromomethane within 15 days, respectively (Figure 5.7

& Figure 5.8). The average dehalogenation rates of 1,1,1-TCA and bromoform reached 83.7 μmoles·L^{-1}·day^{-1}, measured from day 3 to day 12 and 50.8 μmoles·L^{-1}·day^{-1}, measured from day 3 to day 15, respectively. With dechlorination, both 1,1,1-TCA and bromoform dehalogenation supported the growth of *Dehalobacter*. The growth yields were calculated of (4.4±0.6) ×10^7 16S rRNA gene copies per μmol Cl^- released from 1,1,1-TCA and (3.3±0.8) ×10^7 16S rRNA gene copies per μmol Br^- released from bromoform, similar to the growth yield of (5.6±0.7) ×10^7 16S rRNA gene per μmol Cl^- released from chloroform. Contrarily, no cell growth of *Dehalobacter* was observed during 1,1,1-TCA and bromoform dehalogenation (data not shown). Additionally, the growth of *Desulfovibrio* was not correlated with halogen removal from 1,1,1-TCA and bromoform (Figure 5.7 & Figure 5.8). Taken together, *Dehalobacter* sp. strain SNCF was also identified as 1,1,1-TCA and bromoform dehalorespiring bacteria.

5.3.4 Identification of a chloroform reductive dehalogenase gene in strain SNCF

(i) *Chloroform RDase gene-specific primer design and specificity*

Chloroform RDase gene specific primers were designed to identify putative chloroform reductive dehalogenase homologues in co-culture SN. Specific primers were developed following alignment of the selected sequences of identified chloroform RDase genes, *ctrA* from *Desulfitobacterium* sp. strain PR and *cfrA* from *Dehalobacter* sp. strain CF, and their homologous in high similarity but of distinguished function, *dcrA* (97% similarity either with *cfrA* or *ctrA*) mediating 1,1-DCA to CA from *Dehalobacter* sp. strain DCA and uncharacterized *rdhA* (76% similarity either with *cfrA* or *ctrA* from

Dehalobacter restrictus DSM5455 (Table 5.2). Five microcosms from different locations in Singapore were collected to further test the specificity of designed primers. Among the five cultures, samples #3 and #5 showed chloroform dechlorinating activity after two months' incubation while samples #1, #2, #4 did not. PCR with DNA extracted from five microcosms and strain PR using cf159F/cf387R primers yielded amplification products of expected size and sequences (Figure 5.9). The qPCR assay further confirmed the specificity of this set of primers and limited the detection range of 10 to 5E8 gene copies. Although small in scale, this initial proof of concept suggests that these primers are reasonably specific to indicate presence of chloroform RDase genes in a microbial community.

Table 5.2 Alignment of parts of *rdhAs* sequences for chloroform RDase gene specific primers design

	rdhA position						
primer sequence 5'	GGA	ACG	GTC	AAG	GAA	CTG	GA
ctrA	- - -	- - -	- - -	- - -	- - -	- - -	- -
cfrA	- - -	- - -	- - -	- - -	- - -	- - -	- -
thmA	- - -	- - -	- - -	- - -	- - -	- - -	- -
dcrA	- - -	- - -	- - -	- - -	- - -	- - -	- -
rdhA-DSM5455	- - -	GTA	A- -	- - -	AC-	-AC	- -

	rdhA position						
Primer sequence 5'	CAA	GCT	TTG	CCG	TTG	ATT	AT
ctrA	- - -	- - -	- - -	- - -	- - -	- - -	- -
cfrA	- - -	- - -	- - -	- - -	- - -	- - -	- -
thmA	- - -	- - -	- - -	- - -	- - -	- - -	- -
dcrA	- - -	- - -	GG-	- - -	- - -	- - -	- -
rdhA-DSM5455	- - -	- - -	- - -	-TC	- - -	- -T	GG

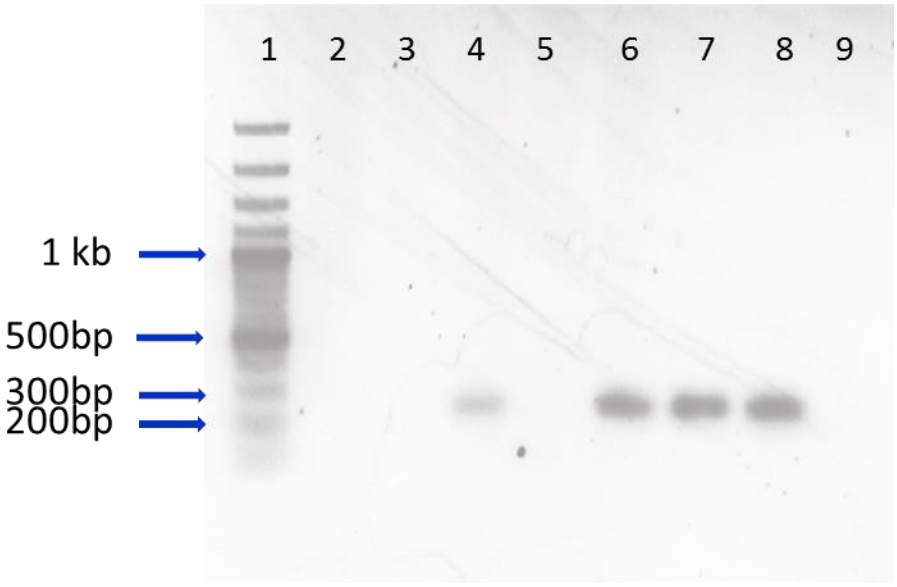


Figure 5.9 Identification of putative chloroform RDase gene in co-culture SN. Note: 1. 100bp DNA Ladder; 2-6, microcosms #1-#5; 7, co-culture ZH; 8, Desulfitobacterium sp. strain PR; 9, H2O.

Identification of putative chloroform RDase gene in strain SNCF and its expression upon exposure to chloroform Full putative chloroform RDase gene sequence of 1371 nucleotides in strain SNCF was obtained by PCR using cf159F/cf387R primers followed by genome walking. This *rdhA* gene is *ctrA*- and *cfrA*-like, sharing as high as 99% similarity (1362/1374 bp) with *ctrA* and 98% similarity (1341/1371) with *cfrA* (Figure 5.10).

```
CfrA-like       MDKEKSNNDKPATKINRRRFLKFGAGASSGIAIAAAATALGGKSLIDPKQVYAGTVKELD 60
CtrA            MDKEKSNNDKPATKINRRRFLKFGAGASSGIAIAAAATALGGKSLIDPKQVYAGTVKELD 60
CfrA            MDKEKSNNDKPATKINRRQFLKFGAGASSGIAIATAATALGGKSLIDPKQVYAGTVKELD 60
                ******************:***************:************************

CfrA-like       ELPFNIPADYKPFTNQRNIFGQALLGVPEPLALVERFDEVRWNGWQTDGSPGLTVLDGAA 120
CtrA            ELPFNIPADYKPFTNQRNIFGQALLGVPEPRALVERFDEVRWNGWLTDGSPGLTVLDGAA 120
CfrA            ELPFNIPADYKPFTNQRNIYGQAVLGVPEPLALVERFDEVRWNGWQTDGSPGLTVLDGAA 120
                ********************:***:****** ************** **************

CfrA-like       ARASFAVDYYFNGENSACRANKGFFEWHPKVPELNFRWGDPERNIHSPGVKSAEEGTMAV 180
CtrA            ARASFAVDYYFNGENSACRANKGFFEWHPKVPELNFRWGDPERNIHSPGVKSAEEGTMAV 180
CfrA            ARASFAVDYYFNGENSACRANKGFFEWHPKVAELNFKWGDPERNIHSPGVKSAEEGTMAV 180
                *******************************.****:************************

CfrA-like       KRMARFFGAAKAGIAPFDKRWVFTETAAFVKTPEGESLKFIPPDFGFEPKHVISMIIPQS 240
CtrA            KRMARFFGAAKAGIAPFDKRWVFTETAAFVKTPEGESLKFIPPDFGFEPKHVISMIIPQS 240
CfrA            KKIARFFGAAKAGIAPFDKRWVFTETYAFVKTPEGESLKFIPPDFGFEPKHVISMIIPQS 240
                *::*********************** *********************************

CfrA-like       LEGVKSAPSFLGSSEYGLSCAQYGYAPFGLSMFIKDLGYHAVPIGADSALAIPIAIQAGL 300
CtrA            LEGVKTSPSFLGSSEYGLGCAQYGYAPFGLSMFIKDLGYHAVPIGADSALAIPIAIQAGL 300
CfrA            PEGVKCDPSFLGSTEYGLSCAQIGYAAFGLSMFIKDLGYHAVPIGSDSALAIPIAIQAGL 300
                 ****  ******:****.*** ***.******************:**************

CfrA-like       GEYSRLGLMITPEFGPNVRLCEVFTDMPLNHDKPISFGVTEFCKTCKKCAEACAPQAISY 360
CtrA            GEYSRMGLMITPEFGPNVRLCEVFTDMPLNHDKPISFGVTEFCKTCKKCAEECAPQAISY 360
CfrA            GEYSRSGLMITPEFGSNVRLCEVFTDMPLNHDKPISFGVTEFCKTCKKCAEACAPQAISY 360
                ***** *********.*********************************** ********
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CfrA-like       EDPTIDGPRGQMQNSGIKRWYVDPVKCLEFWSRDNVRNCCGACIAACPFTKPEAWHHTLI 420
CtrA            EDPTIDGPRGQMQNSGIKRWYVDPVKCLEFWSRDNVRDCCGACIAACPFTKPEAWHHTLI 420
CfrA            EDPTIDGPRGQMQNSGIKRWYVDPVKCLEFMSRDNVRNCCGACIAACPFTKPEAWHHTLI 420
                ***************************** ******:********************

CfrA-like       RSLVGAPVITPFMKDMDDIFGYG-KPNDEKAIADWWK 456
CtrA            RSLVGAPVITPFMKDMDDIFGYGGKPNDKKAIADWWK 457
CfrA            RSLVGAPVITPFMKDMDDIFGYG-KLNDEKAIADWWK 456
                **********************: *************
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Figure 5.10 comparison of CfrA, CtrA and CfrA/CtrA-like RDase. The differences among the RDases are highlighted.

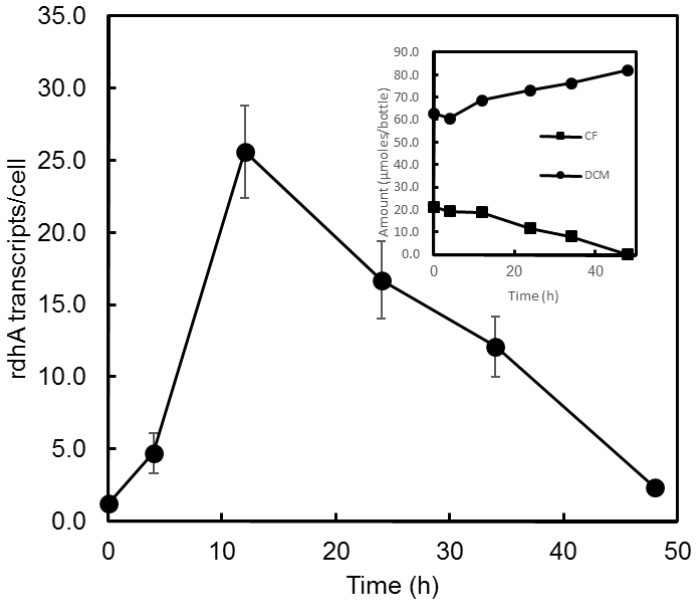

Figure 5.11 Transcription of putative chloroform RDase gene in co-culture SN when fed with chloroform. Note: CF, chloroform; DCM, dichloromethane.

In order to determine whether this *ctrA/cfrA*-like *rdhA* in strain SNCF is responsible for chloroform dechlorination, transcripts of this *rdhA* was monitored when co-culture SN was exposed to chloroform. Transcription of *ctrA/cfrA*-like *rdhA* reached the maximum level of 25.6±3.2 transcripts/cell at h 12 h, soon after chloroform dechlorination initiated that 12.9% of re-spiked chloroform was dechlorinated (Figure 5.11). Afterwards, the transcriptional level declined gradually to 2.3±0.3 transcripts/cell when re-spiked chloroform was completely consumed.

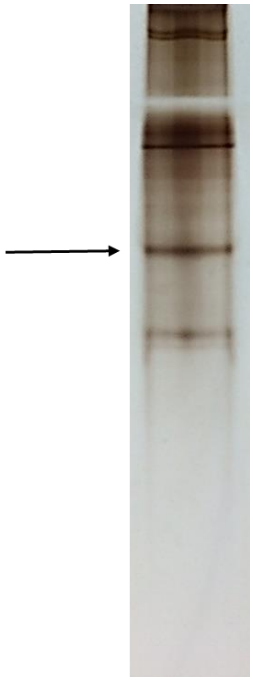

Figure 5.12 Native PAGE profile of membrane proteins extracted from co-culture SN fed with chloroform

(iii) Confirmation the role of CtrA/CfrA-like RDase in chloroform dechlorination The role of CtrA/CfrA-like RDase in chloroform dechlorination was confirmed using native-PAGE together with *in vitro* assay. Membrane proteins obtained from co-culture SN during the exponential phase of strain SNCF were separated on a native PAGE gel (Figure 5.12) and chloroform dechlorinating activity was checked from native gels directly. The reductive dechlorination products, dichloromethane, was detected from one single band while other gel fragments failed to show dechlorinating activity, indicating that the RDase in this band is responsible for the dechlorination of chloroform. Additionally, this single band was detected able to dehalogenate 1,1,1-TCA and bromoform to 1,1-DCA and dibromomethane, respectively. The silver stained counterpart of the gel fragment showing dehalogenating activity was analyzed by MALDI-TOF-MS. Results from MALDI-TOF-MS analysis identified CtrA/CfrA-like RDase as the only detected RDase in the

active gel fragment. Together, these analyses strongly suggest the *ctrA/cfrA*-like gene in strain SNCF is confirmed to be responsible for dehalogenating chloroform as well as 1,1,1-TCA and bromoform and designated *thm*A.

5.4 Discussion

Although mixed dehalogenating cultures may sometimes provide information about dehalogenation processes, it is essential to obtain enriched cultures with simpler microbial communities to gain insights into characteristics of specific bacteria. In this study, organohalide respiration of chloroform and 1,1,1-TCA by a *Dehalobacter* in a co-culture with defined bacterial composition broadens our knowledge in chloroform and 1,1,1-TCA dechlorinating anaerobes. Additionally, microbial removal of bromoform was reported for the first time, which enriches our understanding of the environmental fate of bromoform and assists further removal of bromoform. The role of *Dehalobacter* sp. strain SNCF in chloroform, 1,1,1-TCA and bromoform respiration was confirmed by qPCR indicating *Dehalobacter* growth in correlation with dehalogenation of halogenated substrates and lack of growth in the absence of specific halogenated organic compounds. The distinguishable 16S rRNA gene sequences differentiates strain SNCF from other identified *Dehalobacter*. Compared with another identified chloroform dechlorinating *Dehalobacter* sp. strain CF which shares the same chloroform and 1,1,1-TCA dechlorinating profile, strain SNCF exhibited a relatively far phylogenetic distance of 97% similarity within *Dehalobacter* genera.

Dehalobacter sp. strain SNCF can use chloroform, 1,1,1-TCA and bromoform, for respiration. Previous studies have demonstrated that the dehalogenating capabilities of some organohalide respiring bacteria can extend

from a single halogenated compound to structurally similar molecules with the same functional group substitutions. For example, *Desulfitobacterium* sp. strain PR can dechlorinate chloroform and 1,1,1-TCA, both of which have CCl_3- group. However, this study demonstrates for the first time an extension of substrate range to the compounds with the similar structures but different halogen substituents, e.g., chlorine and bromide. This information can help explore the extension of dehalogenating substrates range of organohalide respiring bacteria.

A set of chloroform RDase genes specific primers were designed and used to identify the putative chloroform RDase gene in strain SNCF. This pair of primers can be used as biomarker in evaluation, monitoring and modification in chloroform bioremediation. In previous studies, the identified chloroform RDases, CfrA and CtrA, can also respire 1,1,1-TCA to 1,1-DCA and CA respectively. Here, ThmA catalyzed chloroform, 1,1,1-TCA and bromoform to dichloromethane, 1,1-DCA and dibromomethane, respectively. This may suggest a common feature of chloroform RDases, that the catalytic site could possibly recognize a specific functional group (trihalomethyl) rather than a specific molecule. Although sharing a higher similarity with CtrA compared with CfrA, ThmA catalyzed chloroform and 1,1,1-TCA dechlorination via the same mechanisms as CfrA rather than CtrA. This phenomenon strongly demonstrates that although RDase-specific primers can be used to investigate the biodegradability to the targeted substances, it is still difficult and sometimes unreliable to predict the metabolic products. This finding broadens our understanding of RDase-specific primers serving as biomarkers.

In summary, this study presents a novel *Dehalobacter* sp. strain SNCF in co-culture with a non-dechlorinating *Desulfovibrio* sp. Strain SNCF exhibits a restricted dehalogenation substrate range of trihalomethyl-containing molecules including chloroform, 1,1,1-TCA and bromoform. The chloroform RDase gene from strain SNCF was identified and further characterized to be responsible for 1,1,1-TCA and bromoform dehalogenation. The physical and metabolic properties of strain SNCF make it a promising candidate for broad application in *in situ* bioremediation, and the newly designed chloroform RDase gene primers can be helpful in evaluation, monitoring and modification of chloroform bioremediation.

6 Chapter VI Conclusions and recommendations

This doctoral study aimed to gain insights to facilitate chlorinated solvents including 1,1,2-TCA, chloroform and PCE *in situ* bioremediation in contaminated sites. In order to achieve this goal, cultures including two isolates and one co-culture with novel capabilities were subjected to detailed investigation.

6.1 Conclusions

The key conclusions made in each section of this doctoral study are listed below:

(1) A novel 1,1,2-TCA dechlorination pathway, predominantly to 1,2-dichloroethane (1,2-DCA) and chloroethane, and to trace amounts of vinyl chloride and ethene, was identified in isolate *Desulfitobacterium* sp. strain PR. This pathway circumvents the production of carcinogenic compounds, vinyl chloride, through dihaloelimination pathway and provides an insight of a more environmentally friendly strategy to treat 1,1,2-TCA contaminated sites.

(2) TCE and 1,1,2-TCA are usually co-existing in contaminated sites. During the inhibitory study of strain PR, it was found that the presence of TCE could inhibit the dechlorination of 1,2-DCA to CA but not 1,1,2-TCA to 1,2-DCA, which makes the complete detoxification of these co-contaminants possible. Therefore, *Dehalococcoides mccartyi* strain 11a, which can dechlorinate both TCE and 1,2-DCA, was introduced to establish a co-culture with strain PR. Later, complete detoxification of co-contaminants, 1,1,2-TCA and TCE, was achieved.

(3) A novel reductive dehalogenase CtrA was identified as the 1,1,2-TCA reductive dehalogenase. CtrA is the first reported RDase to catalyse

dechlorination process from 1,1,2-TCA to 1,2-DCA. *ctrA* can serve as a biomarker to indicate 1,1,2-TCA *in situ* dechlorinating activity.

(4) CtrA was previously reported to be responsible for 1,1,1-TCA and chloroform dechlorination. Taken together with CtrA's dechlorination on 1,1,2-TCA, this finding crosses the border that single reductive dehalogenase catalyses one specific halogenated compound, suggesting that reductive dehalogenase can function based on structural analogs. Therefore, it broadens our knowledge that structural analogs should be considered during the exploration of potential substrate range in novel RDases.

(5) *Dehalococcoides mccartyi* strain 11a5 was found to possess the capability of dechlorinating PCE to VC and ethene. So far, this is the 3rd reported strain which can metabolically dechlorinate PCE beyond DCEs.

(6) A novel PCE *rdhA, pteA*, was identified in strain 11a5. PteA catalyses the reductive dechlorination of PCE to TCE and shares only 38% identity with its phylogenetically closest relative, PceA in *Dehalococcoides mccartyi* strain 195. This discovery enriches the existing database of functional genes that can be used to assess the potential for bacterial remediation of chloroethenes at contaminated sites, broadens our understanding of dehalogenation capabilities of other OHRBs which possesses *pteA* and serve as a biomarker to indicate PCE in situ dechlorinating activity.

(7) *Dehalococcoides* is a unique genus which possesses versatile dehalogenation capabilities in multiple halogenated compounds and specializes in complete dehalogenation among all OHRBs. The complete genome of *Dehalococcoides mccartyi* strain 11a5 was obtained in this study.

This extends the database of *Dehalococcoides* and broadens our horizon in physicological, biochemical, evolutionary and ecological traits of this genus.

(8) The fact, that PteA (38% amino acid similarity with pceA in strain 195) was identified as PCE RDase rather than 11a5_1467 (91% amino acid similarity with pceA in strain 195) in strain 11a5, highlights how unreliable to predict RDase functions based solely on sequence similarity. It concludes that sequence similarity and substrate specificity are generally not correlated.

(9) Plasmid was firstly reported existing in *Dehalococcoides*. pDhc6, this 5.94 kb extrachromosomal genetic element carries a classical *rdh* operon. The presence of a plasmid carrying the full component of genes for a *rdh* operon provides a tantalizing clue that in the future may help to elucidate the mechanisms of rdh flow in OHRB.

(10) A co-culture SN containing *Dehalobacter* and non-dechlorinating *Desulfovibrio* capable of rapidly dechlorinating chloroform, 1,1,1-TCA and bromoform to less toxic end product, dichloromethane, 1,1-DCA and dibromomethane has been cultivated, representing the first simple demonstration to characterize *Dehalobacter* in –CX3 dechlorination.

(11) *Dehalobacter* sp. Strain SNCF identified in co-culture SN is a novel strain compared with other *Dehalobacter* strains by sharing non-identical 16S rRNA gene sequences, with 99% similarity with its closest relative, strain AD14-TCP1. Strain SNCF is also distinguished with another chloroform dechlorinator, strain CF, by sharing relatively distinct relationship of 97% within *Dehalobacter* genera.

(12) CF RDase specific primers cf159F (5'-GGAACGGTCAAGGAACT-3') and cf370R (5'-ATAATCAACGGCAAAGC-3') was firstly designed to investigate chloroform dechlorinating activity.

6.2 Recommendations

The findings in this doctoral study provides useful information for the utilization of cultures in *in situ* bioremediation processes. Future studies will strive to obtain more in-depth knowledge about these cultures, including extension of dehalogenating substrate range, characterization of *RdhA*s, horizontal transfer of *rdhAs* in OHRBs, as well as various environmental factors that affect the dehalogenation processes. Specifically, the areas that require continued research work include:

(1) The potential of dehalogenating other halogenated contaminants of the cultures, including *Desulfitobacterium* strain PR, *Dehalococcoides mccartyi* strain 11a5, and co-culture SN, will be investigated. This is due to the versatile dehalogenation potential of numerous uncharacterized *rdhA*s in these cultures and the demand of remediation of multiple halogenated compounds contaminated sites. Additionally, co-cultures composing two or more strains will be established to deal with multiple halogenated compounds simultaneously.

(2) Efforts will be made to isolate *Dehalobacter* from co-culture SN. It is essential to isolate bacterial strains to get insights of their characteristics to better assist the application of cultures in the dehalogenation processes. Without knowing physiological properties of the specific bacteria in depth, planning, monitoring and optimizing bioremediation strategies would be

greatly hindered because of the lack of bacterial information of nutritional requirement and responses of specific environment.

(3) The discovery of plasmid pDhc6 containing a *rdh* operon provides the possibility to build up genetically modified microorganisms harboring multiple *rdh* operons to deal with complicated contaminated sites. This can be achieved by making the dehalogenating strains competent to take up vectors containing the rdhA genes in query.

(4) The final destination is to apply the cultures to the contaminated sites so that the halogenated compounds can be efficiently detoxified. Considering the differences between optimized laboratory experimental conditions and realistic environmental conditions, the dehalogenation performance by the cultures will be monitored under mimicked realistic conditions and enhancement strategies will be developed to overcome non-ideal growth conditions. After confirming the dehalogenation performance of these strains, pilot scale experiments at selected contaminated sites will be carried out to further evaluate the feasibility, time, and cost of the bioremediation techniques, prior to full scale applications in situ.

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Her publications during the doctoral studies are listed as follows:

Zhao, S., Ding, C., He, J. Characterization of a Dehalobacter containing co-culture SN that dechlorinates chloroform and identification of its reductive dehalogenase gene. In preparation.

Zhao, S., Ding, C., He, J. Genomic characterization of *Dehalococcoides mccartyi* strain 11a5, a plasmid-containing dechlorinating anaerobe. Submitted.

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