GENETIC IDENTIFICATION OF REDUCTIVE DEHALOGENASE GENES IN *DEHALOCOCCOIDES*

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This work is dedicated to the men in my life, “Howard and Yosi”.

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TABLE OF CONTENTS

		Page
DEDICATION		iii
ACKNOWLEDGMENTS		iv
LIST OF TABLES		xiv
LIST OF FIGURES		xvi
LIST OF SYMBOLS AND ABREVIATIONS		xviii
SUMMARY		xx
CHAPTER I	INTRODUCTION	1
CHAPTER II	BACKGROUND	8
	2.1. Chloroethenes in the environment	8
	2.1.1. Vinyl Chloride	8
	2.1.2. Dichloroethenes	10
	2.2. Remediation alternatives for chloroethenes	11
	2.2.1. Physicochemical methods	11
	2.2.2. Bioremediation	12
	2.3. Aerobic biodegradation of chlorinated solvents	13
	2.4. Anaerobic biodegradation of chlorinated solvents	14
	2.4.1. Reductive dechlorination	14
	2.4.2. Metabolic reductive dechlorination (dechlororespiration, chlororespiration, chloridogenesis)	15
	2.5. Chlororespiring microorganisms	15
	2.6. Detection of microorganisms in contaminated aquifers	21

2.6.1. Molecular techniques to detect chlororespiring populations 21

2.7. Reductive dehalogenases: key enzymes in chlororespiration 23

2.7.1. Characterized reductive dehalogenases 25

2.8. References 28

CHAPTER III DETECTION OF DECHLORINATING POPULATIONS IN CHLOROETHENE DECHLORINATING ENRICHMENT CULTURES AND IN SITE MATERIALS 35

3.1. Introduction 35

3.2. Materials and methods 38

3.2.1. Chemicals and enzymes 38

3.2.2. Sources of dechlorinating cultures and site material samples 38

3.2.3. Growth medium and culture conditions 41

3.2.4. Analytical methods 41

3.2.5. DNA extraction from soil and groundwater 41

3.2.6. DNA extraction from enrichment cultures 42

3.2.7. PCR-based tools to detect dechlorinating populations 43

3.2.8. Clone libraries 44

3.2.9. Sequence analysis of 16S rRNA genes 45

3.3. Results 47

3.3.1. Soil and groundwater from contaminated sites 47

3.3.2. 16S rRNA-targeted PCR analysis of TCE, *cis*-DCE and VC-dechlorinating enrichment cultures 48

3.3.3. Sequence analysis of cloned 16S rRNA genes and related isolates 52

3.3.4. Effect of ampicillin on dechlorinating cultures 53

3.4. Discussion 55

3.5. References 57

CHAPTER IV ENVIRONMENTAL DISTRIBUTION OF THE TRICHLOROETHENE REDUCTIVE DEHALOGENASE GENE (*tceA*) IN DEHALOCOCCOIDES SPECIES AND EVIDENCE FOR HORIZONTAL GENE TRANSFER 60

4.1. Introduction 60

4.2. Materials and methods 62

4.2.1. Chemicals gases and enzymes 62

4.2.2. Sources of DNA 62

4.2.3. Primer design 65

4.2.4. Bioinformatic tools 68

4.2.5. DNA extraction, PCR, cloning, and amplicon analysis 68

4.3. Results 71

4.3.1. *tceA* and *tceB* detection in ethene producing enrichment cultures 71

4.3.2. Identification of a genetic mobile element next to the *tceAB* genes 72

4.3.3. GC content in the *tceAB* genes and adjacent regions 73

4.4. Discussion 76

4.5. References 81

CHAPTER V PUTATIVE REDUCTIVE DEHALOGENASE GENES IN THE *DEHALOCOCCOIDES ETHENOGENES* STRAIN 195

GENOME 85

5.1. Introduction 85

5.2. Materials and methods 88

5.2.1. Screening for putative RDase genes 88

5.2.2. Recognition of motifs and comparison of translated RDases 89

5.2.3. Search for putative B genes 89

5.2.4. Degenerate primer design 90

5.2.5. RDase genes in *Anaeromyxobacter dehalogenans* genome 90

5.3. Results 92

5.3.1. Putative RDase genes in the *Dehalococcoides ethenogenes* genome 92

5.3.2. Recognition of conserved motifs 94

5.3.3. Presence of putative B genes 95

5.3.4. RDase genes in Anaeromyxobacter dehalogenans genome 96

5.4. Discussion 105

5.5. References 108

CHAPTER VI GENETIC IDENTIFICATION OF A PUTATIVE VINYL CHLORIDE REDUCTASE GENE IN *DEHALOCOCCOIDES* SP. STRAIN BAV1 110

6.1. Introduction 110

6.2. Methods 113

6.2.1. Chemicals 113

6.2.2. VC-dechlorinating cultures and sources of DNA 113

6.2.3. Primer design 114

6.2.4. PCR, cloning, and amplicon analysis 114

6.2.5. RNA isolation 116

6.2.6. Expression analysis of putative RDase genes 117

6.2.7. Chromosome walking and assembling the *bvcA* coding sequence 118

6.2.8. Nucleotide sequence accession numbers 117

6.3. Results 119

6.3.1. PCR amplification of putative RDase gene fragments 119

6.3.2. Expression analysis of putative RDase genes 119

6.3.3. Detection of *bvcA* in other VC-dechlorinating cultures 121

6.4. Discussion 128

6.5. References 131

CHAPTER VII MULTIPLE REDUCTIVE DEHALOGENASE GENES IN *DEHALOCOCCOIDES* SP. STRAIN FL2 AND EXPRESSION ANALYSIS OF REDUCTIVE DEHALOGENASE GENES IN STRAIN FL2 GROWN ON TRICHLOROETHENE (TCE) AND DICHLOROETHENE (DCE) AS ELECTRON ACCEPTORS 136

7.1. Introduction 136

7.2. Materials and methods 140

7.2.1. Dechlorinating cultures 140

7.2.2. Nucleic acids extraction 140

7.2.3. Amplification of RDase genes 141

7.2.4. Sequencing of RDase genes 142

7.2.5. Sequence analysis 143

7.2.6. Expression analysis of putative RDase genes 143

7.2.7. Nucleotide sequence accession numbers 144

7.3. Results 145

7.3.1. Amplification of RDase genes 145

7.3.2. Sequence analysis of RDase genes 145

7.3.3. Iron-sulfur cluster binding motifs 147

7.3.4. N-terminal region of RDase genes 148

7.3.5. B gene sequences 148

7.3.6. Expression analysis of RDase genes in FL2 grown on TCE as electron acceptor 149

7.3.7. Expression analysis of RDase genes in FL2 grown on *cis*-DCE as electron acceptor 150

7.4. Discussion 156

7.5. References 160

CHAPTER VIII OPTIMIZATION OF RNA EXTRACTION PROCEDURES FROM A DECHLORINATING CONSORTIUM 165

8.1. Introduction 165

8.2. Materials and methods 167

8.2.1. Chemicals 167

8.2.2. Biodechlor-dechlorinating inoculum and sources of DNA 167

8.2.3. RNA isolation 167

8.2.4. Expression analysis of putative RDase genes 170

8.2.5. Real Time (RTm) PCR 170

8.3. Results 173

8.3.1. RNA extraction methods 173

8.3.2. Expression analysis of putative RDase genes 174

8.4. Discussion 179

8.5. References 181

CHAPTER IX CONCLUSIONS AND RECOMMENDATIONS 183

APPENDIX A NOMENCLATURE OF RDASE GENES IN *DEHALOCOCCOIDES ETHENOGENES* STRAIN 195 GENOME 190

APPENDIX B RDASE GENES IN *DEHALOCOCCOIDE*S SP. STRAIN BAV1 191

APPENDIX C RDASE GENES IN *DEHALOCOCCOIDES* SP. STRAIN FL2 197

APPENDIX D METHODS 207

Buffers 207

Electrophoresis of DNA in Agarose gels 208

Extraction of genomic DNA from dechlorinating cultures 211

Extraction of DNA from soil samples 213

Qiagen RNeasy + SDS and homogenization (QHS) 215

Qiagen RNeasy + homogenization (QH) 217

Qiagen RNeasy +SDS (QS) 219

Micro to midi (M) 221

Micro to midi no SDS (MH) 223

DNase treatment 225

Reverse transcription 226

LIST OF TABLES

		Page
Table 2.1.	Growth-linked reductive dechlorination of chloroethenes by pure bacterial cultures	20
Table 2.2.	Molecular characteristics of purified reductive dehalogenases	25
Table 3.1.	Enrichment cultures used for this study	40
Table 3.2.	Contaminated site materials analyzed	40
Table 3.3.	Primers, annealing temperatures, and expected amplicon sizes	44
Table 3.4.	Detection of dechlorinating populations in site material samples	47
Table 3.5.	Detection of specific dechlorinating populations in enrichment cultures	51
Table 4.1.	*tceA* detection in chloroethene degrading cultures and chloroethene contaminated site materials	64
Table 4.2.	Specific primers used in this study and their position relative to *tceA* and *tceB*	66
Table 4.3.	Percent identity among *tceA* nucleotide sequences from several *Dehalococcoides*-containing cultures	74
Table 5.1.	Common motifs in characterized RDases	86
Table 5.2.	Iron sulfur clusters and twin arginin motifs in putative RDases	93
Table 5.3.	Degenerate primers designed using translated putative RDase genes identified in the *Dehalococcoides ethenogenes* genome	102
Table 5.4.	Characteristics of putative reductive dehalogenase genes found in the *Anaeromyxobacter dehalogenans genome*	103
Table 5.5.	Identity matrix of translated RDase proteins in *D. ethenogenes* genome and previously characterized RDases	107
Table 6.1.	Degenerate primers used in this study	123

Table 6.2.	Specific primers used in this study	123
Table 7.1.	RDase genes detected in cDNA clone libraries established with RNA from FL2 cultures grown with TCE or *cis*-DCE as electron acceptors	155
Table 8.1.	RNA extraction methods (kits) and lysis enhancement methods used	169
Table 8.2.	RTm PCR primers and Taqman probes used to quantify bacterial 16s rRNA genes, *Dehalococcoides* 16S rRNA genes, *tceA* and *bvcA* gene transcripts	172

LIST OF FIGURES

		Page
Figure 2.1.	Reductive dechlorination of PCE and TCE	10
Figure 3.1.	Detection of *Dehalococcoides* spp. in VC dechlorinating enrichment cultures with *Dehalococcoides* 16S rRNA gene-targeted primers (FL2F and FL2R)	49
Figure 3.2.	Detection of *Dehalococcoides* spp. in *cis*-DCE-dechlorinating enrichment cultures with *Dehalococcoides* 16S rRNA gene-targeted PCR primers (FL2F and FL2R)	50
Figure 4.1.	Organization of the *tceAB* and adjacent gene regions	67
Figure 4.2.	Dhc-TE1 Alignment	75
Figure 5.1.	Alignment of translated putative RDases identified in the *Dehalococcoides ethenogenes* genome	98
Figure 5.2.	Hydrophobicity plots of TceB and translated putative B proteins	100
Figure 5.3.	Alignment of translated putative B proteins in *Dehalococcoides ethenogenes*	101
Figure 5.4.	Partial alignment of translated putative RDases identified in the *Anaeromyxobacter dehalogenans* genome	104
Figure 6.1.	Arrangement of the *bvcA* gene and the corresponding B gene, *bvcB*	124
Figure 6.2.	PCR amplification of the putative VC RDase gene with specific primers bvcAR and bvcAF and templates generated from VC-grown BAV1 cultures	125
Figure 6.3.	Specificity of primers (bvcAR/bvcAF) targeting the putative VC RDase gene *bvcA*	126
Figure 6.4.	Detection of *bvcA* in VC-dechlorinating mixed cultures using primers bvcAR/bvcAF	127
Figure 7.1.	Schematic diagram of gene fragments amplified using RRF2 and B1R degenerate primers	152

Figure 7.2. Phylogenetic analysis of RDase genes from *Dehalococcoides* sp. strain FL2 (*rdhA1-11* FL2), *Dehalococcoides* sp. strain CBDB1 (*rdhA1-14* CBDB1), *Dehalococcoides* sp. strain BAV1 (*rdhA1-7* BAV) and *Dehalococcoides ethenogenes* strain 195 (*rdhA1-17* DE) 153

Figure 7.3. Alignment of the C-terminal region of deduced amino acid sequences of RDH genes from *Dehalococcoides* strains 154

Figure 8.1. RNA concentrations obtained with different extraction methods 176

Figure 8.2. *tceA* expression in Biodechlor culture grown with PCE as an electron acceptor 177

Figure 8.3. Comparison of RNA the quality of various RNA extraction methods using RTm-PCR quantification of 16s rRNA, Dhc 16srRNA and *tceA* transcripts in Bio-DECHLOR culture enriched in PCE 178

LIST OF SYMBOLS AND ABREVIATIONS

ATSDR	Agency for Toxic Substances and Disease Registry
BSA	Bovine Serum Albumin
bp	Nucleotide base pairs
BvcA	BAV1 VC Reductive Dehalogenase
CERCLA	Comprehensive Environmental Response, Compensation, and Liability Act
CprA	Chlorophenol reductive dehalogenase
cprA	Chlorophenol reductive dehalogenase encoding gene
CprB	Chlorophenol reductive dehalogenase associated B protein
cprB	Chlorophenol reductive dehalogenase associated B protein encoding gene
DCE	Dichloroethene
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EPA	Environmental Protection Agency
MCL	Maximum contaminant level
MCLG	Maximum contaminant level goal
NAPL	Non Aqueous Phase Liquid
NCBI	National Center for Biotechnology Information
OSHA	Occupational Safety and Health Administration
PCE	Tetrachloroethene

PceA	PCE reductive dehalogenase
pceA	PCE reductive dehalogenase encoding gene
PceB	PCE reductive dehalogenase associated B protein
pceB	PCE reductive dehalogenase associated B protein encoding gene
PCR	Polymerase Chain Reaction
pfam	Protein Families Database
RDase	Reductive Dehalogenase
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RDase	Reductive Dehalogenase
RDH	Reductive Dehalogenase Homolog
TCE	Trichloroethene
TceA	TCE reductive dehalogenase
tceA	TCE reductive dehalogenase encoding gene
TceB	TCE reductive dehalogenase associated B protein
tceB	TCE reductive dehalogenase associated B protein encoding gene
U	Enzymatic units of activity
VC	Vinyl chloride

SUMMARY

Halogenated compounds have been present in the environment for ages because they are naturally formed through biotic or abiotic processes. Geothermal abiotic processes, such as volcano eruptions and forest fires provide favorable conditions for formation of halogenated compounds. Biologically, halogenated compounds can be produced by seaweeds, corals, algae, jellyfish, sponges, fungi, bacteria, insects, and mammals. Some microorganisms, such as fungi and bacteria produce halogenated compounds while other microorganisms are able to break them down. Microorganisms are an essential part of the halogen geochemical cycle.

In the last century, chlorinated compounds have been widely used for industrial and agricultural applications, such as solvents, biocides, etc., resulting in a large entry of these chemicals into the environment. These large quantities of halogenated organics have created undesirable environmental conditions. One such class of organic halides are chloroethenes. Chloroethenes such as tetrachloroethene (PCE), trichloroethene (TCE), dichloroethene (DCE) and vinyl chloride (VC), are major contaminants in subsurface systems threatening water quality and human health. Under anaerobic conditions, PCE and TCE can be reductively dechlorinated to ethene. Recent findings indicate that members of the *Dehalococcoides* group are responsible for ethene formation at chloroethene-contaminated sites. *Dehalococcoides* species exhibit diverse dechlorination activities, but share highly similar 16S rRNA genes. Hence, additional gene targets that go beyond the 16S rRNA gene are needed to reliably detect and quantify

Dehalococcoides populations involved in high rate chloroethene detoxification at contaminated sites.

Dehalococcoides sp. strain BAV1 couples growth to reductive dechlorination of VC to ethene. To shed light on the genes involved in reductive dechlorination in strain BAV1, degenerate primers targeting reductive dehalogenase (RDase) genes of *Dehalococcoides* were designed using available sequence information. PCR amplification with these primers yielded seven putative RDase genes with genomic DNA from strain BAV1 as template. Transcription analysis identified one RDase gene possibly involved in VC dechlorination, which was named *bvcA*. The *bvcA* gene was not present in *Dehalococcoides* strains that failed to couple growth with reductive dechlorination of VC (i.e., *Dehalococcoides* isolates CBDB1, FL2 and 195). Primers specific for *bvcA* detected this gene in several, but not all, *Dehalococcoides*-containing, ethene-producing mixed cultures. Apparently, the *bvcA*-targeted primers do not capture the diversity of VC RDase genes. Nevertheless, a relevant target was identified, and *bvcA*-targeted primers are commercially applied to monitor *Dehalococcoides* sp. strain BAV1 and related organisms at contaminated sites undergoing bioremediation treatment.

Additional RDase genes were identified in *Dehalococcoides* sp. strain FL2, and expression analysis was performed when FL2 was grown with *cis*-DCE and TCE as electron acceptors. Multiple RDase genes were transcribed with each electron acceptor, suggesting that RDase expression in strain FL2 is provably not triggered by a specific electron acceptor.

This work identified novel process-specific target genes that are useful for site assessment and bioremediation monitoring at chloroethene-contaminated sites. In

particular, *bvcA* emerged as a relevant target for monitoring the critical detoxification step from VC, a human carcinogen, to ethene, an environmentally benign product. Additionally, the RDase genes retrieved in this work form a basis for further exploration of the specific functions and regulation mechanisms involved in reductive dechlorination processes.

CHAPTER I

INTRODUCTION

Halogenated compounds have been present in the environment for ages given that they are naturally formed through biotic or abiotic processes (Asplund and Grimvall, 1991; Keppler *et al.*, 2002; Gribble, 2003). High temperatures and pressure facilitate the formation of halogenated compounds, geothermal abiotic processes, such as volcano eruptions and forest fires, provide such conditions (Häggblom and Bossert, 2003). Biologically, halogenated compounds can be produced by seaweeds, corals, algae, jellyfish, sponges, fungi, bacteria, insects, and mammals (Asplund and Grimvall, 1991; Gribble, 1996; Gribble, 1998; van Pee and Unversucht, 2003). Some microorganisms, such as fungi and bacteria, produce halogenated compounds, where as other microorganisms are able to break down halogenated compounds. Biological agents play an essential role in cycling halogenated compounds in the environment (Häggblom and Bossert, 2003; van Pee and Unversucht, 2003).

In the last century, halogenated compounds, mainly the chlorinated ones have been widely used and misused for industrial and agricultural applications, such as solvents, and biocides, resulting in a large entry of these chemicals into the environment. These large quantities of chlorinated organics have altered their inherent concentrations in nature, creating undesirable environmental conditions.

Tetrachloroethene (PCE) and trichloroethene (TCE) have been widely used as solvents and degreasers. PCE, TCE, dichloroethenes (DCEs), and vinyl chloride (VC)

are some of the chlorinated organics most commonly found in aquifer sediments and groundwater (Lee *et al.,* 1998). PCE and TCE have been found in more than 771 and 852, of the 1,430 National Priorities List sites identified by the Environmental Protection Agency (EPA), respectively (ATSDR, 1997a; ATSDR, 1997b). According to EPA's Toxic Chemical Release Inventory from 1987 to 1993, PCE and TCE releases to land and water totaled over 1 million and 291,000 lbs, respectively.

Under anaerobic conditions, PCE can be reductively dechlorinated in a stepwise manner to ethene. At each step of the dechlorination, one chlorine is removed and replaced by one hydrogen atom. Intermediates in the pathway include TCE, DCE isomers, and VC (Fetzner, 1998; Lee *et al.*, 1998). PCE, TCE, and *cis*-DCE are toxic and suspected carcinogens. Furthermore, VC is a proven human carcinogen and because of its high toxicity is the first organic compound on the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) priority list (ATSDR, 2004). Hence, complete dechlorination is imperative to achieve detoxification.

Remediation technologies such as natural attenuation and engineered approaches exploit the biodegradation potential of plants and microorganisms. Microorganisms that have been inherently present in the environment and play a role in the natural dechlorination cycle can be sought for bioremediation methods. In order for bioremediation techniques to work appropriately, it is necessary to have the right microorganisms and monitoring tools, which will aid in assessing a successful approach.

In an effort to take advantage of naturally-occurring microorganisms that have a role in the halogen cycle, several microorganisms that are able to dechlorinate chlorinated ethenes in a respiratory manner i.e., chlororespiration, have been isolated. Some of these

microorganisms, such as *Desulfitobacterium dehalogenans* (Utkin *et al.,* 1994), *Dehalobacter restrictus* (Holliger *et al.,* 1998), and *Sulfusporillum multivorans,* formerly *Dehalosporilum multivorans* (Neumann *et al.,* 1994), only partially dechlorinate PCE or TCE to *cis*-DCE. *Dehalococcoides ethenogenes* strain 195 was the first identified microorganism able to dechlorinate PCE to VC in a respiratory manner (Maymó-Gatell *et al.,* 1997). However, the critical dechlorination step from VC to ethene was cometabolic and slow.

This dissertation is organized in the following way: A literature review is presented in Chapter II. This literature review was intended to provide general background information related to the research presented in this dissertation. A detailed discussion of background literature that is specific to the research presented was reserved for the introduction of each individual chapter.

The first objective of this project was to investigate the presence of *Dehalococcoides* organisms in VC-dechlorinating cultures through the use of 16S rRNA-based molecular techniques to assess if *Dehalococcoides* species had a role in VC dechlorination (Chapter III). The role of *Dehalococcoides species* in VC reductive dechlorination was confirmed. Simultaneously, Hendrickson *et al.* demonstrated through the use of 16S rRNA-based techniques the presence of *Dehalococcoides* species at many contaminated sites where complete dechlorination occurred. However, the 16S rRNA genes of the *Dehalococcoides* spp. available are almost identical. *Dehalococcoides* sp. strain CBDB1, a *Dehalococcoides* sp. that dechlorinated chlorobenzene (Adrian *et al.,* 2000) and dioxins and strain FL2, a *Dehalococcoides* sp. that dechlorinated

chloroethenes, are 100% identical. Given this high similarity, efforts focused on identifying process-specific reductive dehalogenase genes.

The only reductive dehalogenase gene identified in *Dehalococcoides* species at the time we started this study (2000) was the *tceA* gene. In chapter IV, the presence of the *tceA* gene was assessed in several site samples and in cultures that were using VC as a sole electron acceptor. The possibility of horizontal gene transfer was explored using the *tceA* sequences retrieved.

The methodology followed to gather genomic information of reductive dehalogenase genes in the genome of *Dehalococcoides ethenogenes* strain 195 is described in chapter V; the ultimate goal was to use the obtained sequences to design degenerate primers. Additional reductive dehalogenase genes were also retrieved from the genomes of *Desulfitobacterium hafniense* and *Anaeromyxobacter dehalogenans* for comparison purposes and their organization was also investigated.

The subsequent goal was to identify reductive dehalogenase genes, which would aid in distinguishing *Dehalococcoides* species that are able to respire VC and *Dehalococcoides* species that cannot use VC as an electron acceptor. During the course of this study, a *Dehalococcoides* species that is able to respire in VC as an electron acceptor, i.e. strain BAV1, was isolated in our laboratory (He *et al.,* 2003). Because of its importance in complete VC dechlorination as a metabolic process, the focus shifted into identifying reductive dehalogenase genes in BAV1. Seven putative reductive dehalogenase genes were identified. These genes provided additional targets for remediation monitoring and were the beginning of a collection of *Dehalococcoides* reductive dehalogenase genes. Special interest was placed on the *bvcA* gene, a reductive

dehalogenase gene that is unique to strain BAV1 and was transcribed at higher levels than the remaining reductive dehalogenase genes in strain BAV1 when strain BAV1 is grown in VC as a sole electron acceptor.

Metabolic genes are useful in assessing the bioremediation potential of a specific site and in distinguishing among microorganisms that have identical 16s rRNA gene sequences but different metabolic capabilities. In Chapter VII, identification of reductive dehalogenase genes in *Dehalococcoides* sp. FL2 and expression with FL2 cultures grown using TCE or cis-DCE as an electron acceptor is described. An optimization method for RNA extraction is described in Chapter VIII. The use of this RNA extraction method, together with cDNA clone libraries and reverse transcription in conjunction with quantitative real time PCR were used to evaluate transcription of RDase genes in Bio-Dechlor INOCULUM™, a culture that is commercially available for bioaugmentation. Conclusions of this research and future recommendations are presented in Chapter IX.

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CHAPTER II

BACKGROUND

2.1. Chloroethenes in the environment

PCE and TCE have been widely used mainly as solvents. Poor disposal practices have lead into their widespread in subsurface environments. Under anaerobic conditions PCE and TCE can be reductively dechlorinated in a stepwise manner to ethene (Figure 2.1). At each step of the dechlorination, one chlorine is removed and replaced by one hydrogen atom. Intermediates in the pathway include TCE, DCE isomers, and VC (Fetzner, 1998; Lee *et al.*, 1998). PCE, TCE, and *cis*-DCE are toxic and suspected carcinogens. Furthermore, VC is a proven human carcinogen and the first organic compound on the CERCLA priority list (ATSDR, 2004). Hence, complete dechlorination is imperative to achieve detoxification.

2.1.1. Vinyl Chloride

Vinyl chloride, also known as chloroethene, chloroethylene, ethylene monochloride, monochloroethene, monovinyl chloride (MVC), and trovidur is a colorless, flammable gas at standard temperature with a mild, sweet odor. Industrial importance relies on the fact that it is used to make polyvinyl chloride (PVC). PVC is used to make a variety of plastic products used in pipes, wire, and cable insulation coatings, building and construction, automotive industry, industrial and household equipment, and medical supplies. Production of vinyl chloride in 1993 was nearly 14

billion lbs, and, according to EPA's toxic release inventory, from 1987 to 1993 over 38,000 lbs were released into soil and water.

Besides being a result from the reductive dechlorination of TCE, and PCE, it has been proven that VC is also naturally formed (Keppler *et al.*, 2002). Exposure to VC inhalation affects the central nervous system, causing dizziness, headaches, and irritation of the eyes and upper respiratory system. Chronic exposure may result in liver and lung damage, as well as circulatory problems, and conclusive links have been made to cancer of the liver, brain, lung and digestive system (ATSDR, 1990; ATSDR, 1997).

Because of its high toxicity, the EPA has set a maximum contaminant level (MCL) of 2 μg/liter in drinking water for VC, not because VC is not toxic at that level anymore, but because it is the lowest level that can be realistically achieved during water treatment if VC is present in the water source. The maximum contaminant level goal (MCLG) for VC is zero. EPA also requires that spills or accidental releases into the environment of 1 pound or more must be reported.

The Occupational Safety and Health Administration (OSHA) has set the maximum allowable level of VC in workroom air during an 8-hour workday in a 40-hour workweek at 1 part VC per million parts of air (1 ppm) (ATSDR, 1997).

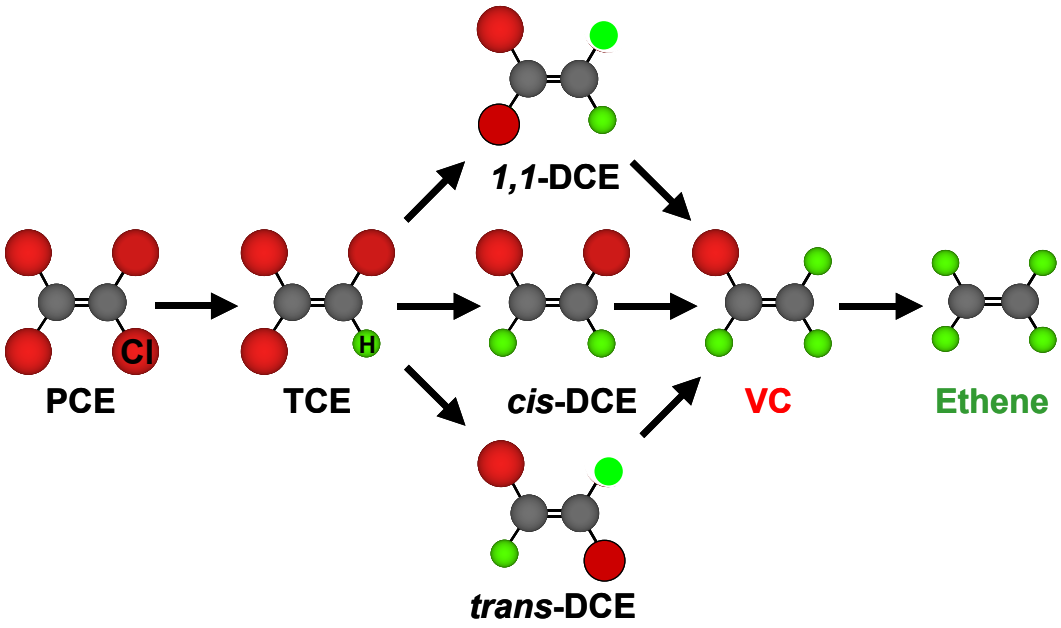

Figure 2.1. Reductive dechlorination of PCE and TCE

2.1.2. Dichloroethenes

DCE exists in three isomeric forms i.e. 1,1-DCE, *cis*- DCE and *trans* DCE. The isomers *cis*- DCE and *trans* DCE can cause central nervous system depression at levels higher than the MCL. Besides being the product of the reductive dechlorination of PCE and TCE in anaerobic environments, commercial uses of *cis* and *trans* DCE, which are usually present as a mixture include: solvent for waxes, resins, and acetylcellulose; extraction of rubber; refrigerants; manufacture of pharmaceuticals and artificial pearls and extraction of oils and fats from fish and meat. Also, they are used as a chemical intermediates in the synthesis of other chlorinated compounds. No data is available on recent production levels in the United States (EPA). *cis*-and *trans*-DCE are not listed

chemicals in the Toxics Release Inventory, data on releases during manufacture and handling is not available in public databases.

2.2. Remediation alternatives for chloroethenes

2.2.1. Physicochemical methods

PCE and TCE are DNAPLs (Dense Non Aqueous Phase Liquid). Their low solubility and high density makes them challenging to remediate. The most common method that has been used to treat subsurface water contaminated with DNAPLs is pump and treat (Abelson, 1990). Pump and treat remediation consists of pumping out the contaminated water from the aquifer and treating it ex situ. One of the problems of pump and treat technology is that the source of contamination and the chlorinated ethenes adsorbed to the soil is not treated. Pump and treat is a method that remediate only the plume of DNAPL in aqueous phase, however, in most cases pure face DNAPL remains at the site and serves as a continuous polluting source.

Additional physicochemical methods for remediation include: surfactant flushing, soil vapor extraction, and reduction by metallic iron. Also methods where microbial populations are involved in the remediation process such as natural attenuation, and bioremediation-engineered approaches i.e. biostimulation and bioaugmentation have been explored in the last decade.

2.2.2. Bioremediation

Bioremediation is the use of living organisms as the catalysts for detoxification reactions that result in pollutants clean up. Some of the different processes by which bioremediation can be implemented are: natural attenuation, biostimulation, and bioaugmentation.

2.2.2.1. Natural attenuation

Natural attenuation is defined by the EPA as, "Naturally occurring processes in soil and groundwater environments that act without human intervention to reduce the mass, toxicity, mobility, volume, or concentration of contaminants in those media. These in situ processes include biodegradation, dispersion, dilution, adsorption, volatilization, and chemical or biological stabilization or destruction of contaminants." Microbial reactions are an important component in natural attenuation (Smets and Pritchard, 2003). Hence, for natural attenuation to work successfully, microorganisms responsible for detoxification reactions must be present.

2.2.2.2. Biostimulation

When the microorganisms of interest are present at the site, but perhaps not at high numbers or not be metabolically active due to the aquifer's conditions, such as redox potential or lack of nutrients; biostimulation is an approach that can be followed. The environmental engineer's job is to stimulate the microorganisms present at the site of interest by the addition of nutrients, electron acceptors, electron donors, etc.

2.2.2.3. Bioaugmentation

When the microorganisms of interest are not present at the site and there is a will to treat the site using bioremediation, bioaugmentation is the approach to follow. In a bioaugmentation process, microorganisms that are able to perform the detoxification reactions of interest are grown in reactors ex-situ, and once high numbers of metabolically active cells are obtained, they are introduced into the site to be remediated. Detoxification, microbial growth, and microbial distribution in the aquifer need to be monitored to assess progress in the remediation process.

The benefits of bioremediation have awakened research focused on populations that are able to detoxify widespread compounds, such as chlorinated ethenes.

2.3. Aerobic biodegradation of chlorinated solvents

VC is mineralized to carbon dioxide by aerobic bacteria such as *Mycobacterium* sp. (Hartmans and Debont, 1992), *Rhodococcus* sp.(Malachowsky *et al.*, 1994), *Actinomycetales* sp. (Phelps *et al.*, 1991), *Nitrosomonas* (Vannelli *et al.*, 1990), *Pseudomonas* spp. (Verce and Freedman, 2000; Verce *et al.*, 2000). Methanotrophic microorganisms utilize monooxygenase to co-metabolically oxidize TCE through the formation of an epoxide (Vogel *et al.*, 1987). One of the disadvantages of the use of methanotrophic microorganisms for bioremediation is that the chloroethene is not metabolized (Verce *et al.*, 2000). Also, in order for the reaction to take place, methane is required as a co-substrate. If aerobic degradation of chlorinated solvents would be the choice for bioremediation, the co-substrates would have to be introduced in the

subsurface. Another disadvantage is the fact that chloroethenes are usually present in anaerobic zones, which means that if aerobic degradation were wanted, the introduction of oxygen into the aquifer would be required.

2.4. Anaerobic biodegradation of chlorinated solvents

2.4.1. Reductive dechlorination

Given their high oxidation state, PCE and TCE can easily be reduced through the exchange of a chlorine atom for hydrogen in a process called reductive dechlorination. Reductive dechlorination can occur by abiotic processes, cometabolic processes, or by metabolic conversion. Anaerobic microorganisms, such as methanogens (Fathepure *et al.*, 1987; Cabirol *et al.*, 1998), sulfate reducers and homoacetogens (Egli *et al.*, 1988; Terzenbach and Blaut, 1994), can reductively dechlorinate PCE and TCE in a co-metabolic process (Ensley, 1991; Bouwer, 1994). Methanogens and sulfate reducers possess metal ion-containing tetrapyrroles enzymes or transition metal coenzymes, such as vitamin B12, coenzyme F430. (Gantzer and Wackett, 1991) demonstrated that Vitamin B12, coenzyme F430, and hematin catalyze reductive dechlorination of chlorinathed ethenes. In this case, the dechlorination process occurs as a fortuitous reaction and there is no energy gain.

2.4.2. Metabolic reductive dechlorination (dechlororespiration, chlororespiration, chloridogenesis)

Chlororespiration also known as dechlororespiration and chloridogenesis, is a process in which microorganisms are able to use chlorinated compounds (such as chlorinated ethenes) as electron acceptors in their energy metabolism. The name chlororespiration comes from the fact that so cold chlororespiring microorganisms use chloroorganics as electron acceptor in their electron respiratory chain.

Numerous bacterial strains that are able to use a chlorinated compound as an electron acceptor have been isolated. The majority of the isolates use chlorophenolic compounds, tetrachloroethene, or both. Some other isolates use chlorobenzoate, trichloroacetate, and chlorobenzenes.

2.5. Chlororespiring microorganisms

Chlororespiring microorganisms belong to four different phylogenetic groups: Low G+C Gram-positives, δ-Proteobacteria, ε-proteobacteria, and Green non-sulfur bacteria or chloroflexi. Chlororespiring microorganisms use primarily fermentation products, such as H_2 and organic acids, as primary electron donors. Microorganisms able to dechlorinate chloroethenes in a metabolic fashion are summarized in table 2.1.

Desulfitobacterium sp. strain PCE1 is a Gram-positive microorganism that can dechlorinate PCE to TCE and small amounts of DCE. *Desulfitobacterium* sp. strain PCE1 can use a wide range of compounds, such as hydrogen, lactate, pyruvate, butyrate, formate, succinate, ethanol, and serine as electron donors; and PCE, 2-chlorophenol,

2,4,6-trichlorophenol, 3-chloro-4-hydroxy-phenylacetate, sulfite, thiosulfate, fumarate, cysteate, and isethionate as respiratory electron acceptors (Gerritse *et al.*, 1996).

Desulfitobacterium sp. strain Viet1 (Löffler *et al.*, 1997) reduces PCE to TCE using H_2, formate, lactate, pyruvate, or yeast extract as electron donors. Acetate can be the carbon source for strain Viet1 when H_2 or formate is supplied as electron donor. The PCE dechlorination activity of strain Viet1 was tested in the presence of 2-bromoethanesulfonate (BES), and it was not affected indicating that no methanogens are present in the culture and also that strain Viet1 is not inhibited with BES (Löffler *et al.*, 1997).

Desulfitobacterium sp. strain PCE-S is a Gram-positive organism that dechlorinates PCE to *cis*-DCE using formate and pyruvate as electron donors (Miller *et al.*, 1997). Besides PCE, strain PCE-S also utilizes TCE, sulfite, and fumarate as electron acceptors.

Desulfitobacterium frappieri strain TCE1 is a Gram-positive microorganism, which growths with H_2, formate, lactate, butyrate, crotonate, or ethanol as the electron donors, and PCE, TCE (to produce *cis*-DCE), sulfite, thiosulfate, nitrate, and fumarate as electron acceptors (Gerritse et al. 1999). Pyruvate and serine can also be used by *Desulfitobacterium frappieri* in a fermentation process.

Dehalobacter restrictus strain PERK23 is a strictly hydrogenotrophic organism, which transforms PCE or TCE to *cis*-DCE. Acetate is required as a carbon source for the transformation to occur, also the addition of thiamine, cyanocobalamin, arginine, histidine and threonine are necessary for growth (Holliger *et al.*, 1998a). *Dehalobacter*

restrictus strain PER-K23 was the first described bacterium, which can dechlorinate PCE and TCE to *cis*-DCE through a growth-linked process (Holliger *et al.*, 1993).

Dehalobacter restrictus strain TEA, reductively dechlorinates PCE and TCE to *cis*-DCE. Preferred electron donor used is H_2 and acetate or carbon dioxide can be used as carbon sources. *Dehalobacter restrictus* strain TEA is a Gram-positive, rod-shaped, motile bacterium, which was isolated from an anaerobic charcoal reactor originally inoculated with contaminated groundwater (Wild *et al.*, 1996). The 16S rRNA gene sequence of strain TEA is 99.7% similar to the sequence of *Dehalobacter restrictus* PERK23.

Clostridium bifermentans strain DPH-1 dechlorinates PCE and TCE to cis-DCE. Yeast extract, glucose, and H_2 can be used as electron donors. *Clostridium bifermentans* DPH-1 is a Gram-positive anaerobic bacterium (Chang *et al.*, 2000).

Desulfuromonas chloroethenica strain TT4B (Krumholz, 1997) and related populations, such as *Desulfuromonas michiganensis* (Sung *et al.*, 2003), require acetate as the electron donor for dechlorination of PCE or TCE to *cis*-DCE. *Desulfuromonas chloroethenica* TT4B is a Gram-negative, rod shaped bacteria, which can use PCE as electron acceptor, dechlorinating it to *cis*-DCE. Electron donors used by *Desulfuromonas chloroethenica* include acetate and pyruvate. *Desulfuromonas chloroethenica* strain TT4B is a strict anaerobe, when it is exposed to air, dechlorinating activity is lost.

Desulfuromonas michiganensis strain BB1 and strain BRS1 were isolated from pristine river sediment and choroethene-contamined aquifer material, respectively (Sung et al. 2003). The acetotrophic PCE dechlorinators, like the *Desulfuromonas michiganensis* strains and *Desulfuromonas chloroethenica* (Krumholz 1997), can couple

chloroethene reduction to the oxidation of acetate. Strains BB1 and BRS1 dechlorinate PCE and TCE to *cis*-DCE in the presence of acetate, lactate, pyruvate, succinate, malate, and fumarate as electron donors. Besides PCE and TCE, *Desulfuromonas michiganensis* strains BB1 and BRS1 can use other electron acceptors such as ferric iron, sulfur, fumarate, and malate.

Sulfurospirillum multivorans (formerly *Dehalospirillum multivorans*), a Gram-negative spirillum, dechlorinates PCE and TCE to *cis*-DCE using either hydrogen or formate as electron donors and acetate as a carbon source. *Sulfurospirillum multivorans* can also use other electron donors, such as lactate and pyruvate, ethanol or glycerol. Electron acceptors used by *Sulfurospirillum multivorans* are PCE, fumarate, arsenate, selenate, and nitrate (Neumann *et al.*, 1994; Scholz-Muramatsu *et al.*, 1995).

Dehalococcoides spp. are strict hydrogenotrophs that can use chlorinated compounds as electron acceptors. The first *Dehalococcoides* isolated in pure culture was *Dehalococcoides ethenogenes* strain 195 (Maymó-Gatell *et al.*, 1997). *Dehalococcoides ethenogenes* strain 195 was isolated from a sewage sludge reactor fed with PCE and methanol. Strain 195 was the first organism capable of complete dechlorination of PCE to ethene. Unfortunately, the last dechlorination step from VC to ethene was slow and followed first-order kinetics, proving that it was a cometabolic process (Maymó-Gatell *et al.*, 1997; Maymó-Gatell *et al.*, 2001).

Recent investigations have revealed expanded metabolic capabilities among *Dehalococcoides* populations. *Dehalococcoides* sp. strain CBDB1 can dechlorinate chlorinated benzenes (Adrian *et al.*, 2000) and polychlorinated dibenzodioxins (Bunge *et al.*, 2003). *Dehalococcoides* sp. strain FL2 grows using TCE, *cis*-DCE, and *trans*-DCE

as electron acceptors and dechlorinates them to ethene. However, similar to *Dehalococcoides ethenogenes* strain 195, strain FL2 is not able to grow with VC as electron acceptor and the VC dechlorination step is cometabolic and slow (He *et al.*, 2005). *Dehalococcoides* sp. strain BAV1, is able to use VC as an electron acceptor (He *et al.*, 2003). *Dehalococcoides* sp. strain BAV1, also uses DCE isomers as electron acceptors, dechlorinating them all the way to ethene.

Table 2.1. Growth-linked reductive dechlorination of chloroethenes by pure bacterial cultures (adapted and updated from (Löffler *et al.*, 2003)).

Lineage	Organism	Dechlorination end product	Electron donors	Reference
Low G+C	*Desulfitobacterium* sp. strain PCE1	TCE	H_2	(Gerritse *et al.*, 1996)
Gram-positive	*Desulfitobacterium* sp. strain Viet1	TCE	H_2	(Löffler *et al.*, 1997)
bacteria	*Desulfitobacterium* sp. strain PCE-S	*cis*-DCE	H_2	(Miller *et al.*, 1997)
	Desulfitobacterium frappieri sp. strain TCE1	*cis*-DCE	H_2	(Gerritse *et al.*, 1999)
	Dehalobacter restrictus strain PER-K23	*cis*-DCE	H_2	(Holliger *et al.*, 1998b)
	Dehalobacter restrictus strain TEA	*cis*-DCE	H_2	(Wild *et al.*, 1996)
	Clostridium bifermentans strain DPH1	*cis*-DCE	H_2	(Chang *et al.*, 2000)
Protobacteria,	*Desulfuromonas chloroethenica* strain TT4B	*cis*-DCE	acetate	(Krumholz, 1997)
δ-subdivision	*Desulfuromonas michiganensis* strain BB1	*cis*-DCE	acetate	(Sung *et al.*, 2003)
	Desulfuromonas michiganensis strain BRS1	*cis*-DCE	acetate	(Sung *et al.*, 2003)
Proteobacteria,	*Sulfurospirillum multivorans (Dehalosporillum multivorans)*	*cis*-DCE	H_2	(Scholz-Muramatsu *et al.*, 1995)
ε-subdivision	*Sulfurospirillum multivorans (Dehalosporillum multivorans* strain PCE-M2*)*	*cis*-DCE	H_2	(Smidt *et al.*, 2000)
Green non-sulfur bacteria	*Dehalococcoides ethenogenes* strain 195	Ethene (VC)[a]	H_2	(Maymó-Gatell *et al.*, 1997)
chloroflexi	*Dehalococcoides* sp. strain FL2	Ethene (VC) [a]	H_2	(He *et al.*, 2005)
	Dehalococcoides sp. strain BAV1	Ethene	H_2	(He *et al.*, 2003)

[a] VC is the end product of the metabolic reaction. Dechlorination of VC to ethene is cometabolic.

2.6. Detection of microorganisms in contaminated aquifers

Several methods to study and identify the microbes that are involved in a remediation process exist. Identification methods can be divided into cultivation methods and methods where no cultivation is required. Cultivation methods are useful to study the physiology and biochemistry of microorganisms but require more labor, are time consuming, and can be biased by the type of cultivation media and methods used. Only 1-15% of microorganisms present in the environment can be grown in the laboratory (Amann *et al.*, 1995), therefore cultivation methods usually underestimate the microbial potential in the environment. Approaches to analyze biodegradation potential have been shifting to the use of culture-independent techniques such as molecular techniques (Pieper *et al.*, 2004). Molecular techniques are more reliable since they target both cultivable and non-cultivable microorganisms, and information can be obtained quicker. The main focus of this study was to design molecular (DNA targeted) methods, which are quick, reliable, and unbiased by cultivation techniques.

2.6.1. Molecular techniques to detect chlororespiring populations

Bioremediation techniques, such as natural attenuation, biostimulation, and bioaugmentation, require monitoring of the populations of interest for successful implementation. The presence, growth, and distribution of microorganisms in the aquifer can be monitored through molecular techniques.

The 16S rRNA gene is a gene that has been used for microorganisms' detection and phylogenetic analysis. The 16S rRNA gene is a useful gene to characterize microbial populations and evolution because it is involved in the ribosomal machinery, making it a

gene that is essential for bacteria survival. Hence, it cannot undergo drastic changes, or the bacteria would not survive. A slow changing gene is a good indicator of phylogeny. Other advantages for using the 16S rRNA gene for analysis are: the 16S rRNA gene is present in all bacteria, it is not translated into protein, which makes the analysis easier, and there are extensive databases containing known 16S rRNA sequences available.

Because of the above-mentioned characteristics of the 16S rRNA gene, 16S rRNA-based methods can be used to examine the diversity of bacterial communities in the environment or in a controlled laboratory setting. Such techniques include: terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), Amplified ribosomal DNA restriction analysis (ARDRA), clone libraries (Chandler *et al.*, 1997a; Chandler *et al.*, 1997c), and primers designed to target a specific population (Löffler *et al.*, 2000).

Drawbacks of using 16S rRNA based detection methods include: biases introduced in the PCR reaction (Chandler *et al.*, 1997b), which is usually the first step for this type of analysis, and lack of correlation among metabolic functions, given that certain microorganisms have an identical 16S rRNA gene and diverse metabolic capabilities. 16S rRNA gene primers have been specifically designed to detect dechlorinating groups, such as *Dehalococcoides* spp. and *Desulfuromonas* spp (Löffler *et al.*, 2000).

Metabolic genes, i.e. genes that encode enzymes can be used as indicators of a biodegradation process, such as reductive dechlorination. The presence of metabolic genes can also be used to track a specific microbial population in the environment; however, in order to use metabolic genes for detection of microorganisms, the gene in

question has to be previously identified. The main goal of this research was to identify reductive dehalogenase genes in *Dehalococcoides* species.

2.7. Reductive dehalogenases: key enzymes in chlororespiration

Reductive dehalogenases (RDases) are the key catalysts in the respiratory chain of halorespiring microorganisms. Knowledge on reductive dehalogenating enzymes and their encoding genes has only started to accumulate in the past ten years. RDase enzymes with a wide range of substrate specificities have been purified and characterized from a number of halorespiring microorganisms. PCE–to-*cis*-DCE reductive dechlorinating enzyme systems and/or the encoding gene sequences have been characterized from *Dehalosporillum multivorans* (Neumann *et al.*, 1996; Neumann *et al.*, 1998), *Dehalobacter restrictus* (Schumacher et al., 1997; Maillard et al., 2003), *Desulfitobacterim* sp. strain PCE-S (Miller *et al.*, 1998), *Clostridium bifermentans* (Okeke *et al.*, 2001), and *Dehalococcoides ethenogenes* strain 195 (Magnuson *et al.*, 1998; Magnuson *et al.*, 2000). Common features in all characterized reductive dehalogenase enzymes include iron sulfur binding motifs and a corrinoid factor (summarized in table 2.2.), and a twin arginin signal peptide sequence that is characteristic for proteins that are excreted out of the periplasm (Berks *et al.*, 2000).

All characterized enzymes but one are associated with the cytoplasmic membrane, reinforcing their role in membrane-associated electron transport–coupled phosphorylation. The only one exception is the PCE/TCE RDase in *Dehalosporilum multivorans* (Neumann *et al.*, 1996), which was present in the cytoplasm. Two iron-sulfur (Fe_4S_4) binding motifs, which are characteristic of enzymes involved in respiration, are

also present. The molecular characteristics of isolated reductive dehalogenases, including the presence of cofactors, are summarized in table 2.2.

Energy yielding reactions, such as electron transport phosphorylation, require an association of the mediating proteins with the cytoplasmic membrane. All characterized enzymes but one are associated with the cytoplasmic membrane, reinforcing their role in membrane-associated electron transport–coupled phosphorylation. The only one exception is the PCE/TCE RDase in *Dehalosporilum multivorans* (Neumann *et al.*, 1996), which was present in the cytoplasm. Based on DNA sequence data, it has been proposed that a small hydrophobic protein anchors the reductive dehalogenase to the membrane. Studies with methyl viologen (a membrane impermeable electron donor) as an electron donor suggest that the RDase is located attached to the periplasmic membrane facing the inside of the cell however it cannot be ruled out that the RDase could also face the outside of the cell.

Table 2.2. Molecular characteristics of purified reductive dehalogenases. Adapted from Holliger *et al.*, (2003)

Enzyme	Organism	Cofactors	Accesion #	Reference
PCE RDase	*Dehalococcoides ethenogenes*	Corrinoid[a] Fe/S	N.A.	(Magnuson *et al.*, 1998)
PCE/TCE RDase	*Sulfurospirillum multivorans*	1 Corrinoid[a,b] 8Fe/8S [b]	AAC60789	(Neumann *et al.*, 1996)
	Dehalobacter restrictus	1 Corrinoid[a,b,c] 2 4Fe/4S [c]	CAD28790	(Maillard *et al.*, 2003)
	Desulfitobacterium sp. PCE-S	1 Corrinoid[a,b] 8Fe/8S [d]	AAO60101	(Miller *et al.*, 1998)
	Clostridium bifermentans	Corrinoid[a]	CAC37919	(Okeke *et al.*, 2001)
TCE RDase	*Dehalococcoides ethenogenes*	Corrinoid[a] Fe/S	AAW39060	(Magnuson *et al.*, 1998; Magnuson *et al.*, 2000)
3-CB RDase	*Desulfomonile tiedjei*	Heme[e]	N.A.	(Ni *et al.*, 1995)
O-CP RDase	*Desulfitobacterium dehalogenans*	1 Corrinoid[c,b] 1 4Fe/4S [c] 1 3Fe/3S [c]	AAD44542	(van de Pas *et al.*, 1999)
	Desulfitobacterium hafniense	1 Corrinoid[a] 12Fe/13S [d]	AAL87763	(Christiansen *et al.*, 1998)

[a] Indicated by photo-reversible inhibition of the reduced enzyme by iodo-alkanes.
[b] Quantified by analysis of cobalt content and by extraction of corrinoid from purified enzyme followed by spectroscopic analysis.
[c] Determined by EPR spectroscopy.
[d] Quantified by atomic absorption spectroscopy and by standard procedure for acid-labile sulfide.
[e] Indicated by optical spectroscopic analysis.

2.7.1. Characterized reductive dehalogenases

A TCE RDase (TceA) is responsible for reductive dechlorination of TCE to vinyl chloride in *Dehalococcoides ethenogenes* strain 195 (Magnuson *et al.*, 1998) . The gene encoding the TCE RDase, *tceA*, has been cloned and sequenced (Magnuson *et al.*, 2000). The *tceB* gene is located downstream, and both genes are co-transcribed. *tceB* encodes for a hydrophobic protein whose function has not been proven at the protein level, however, it has been hypothesized to have a role in anchoring *tceA* to the membrane. TceA is the first characterized RDase, which presents two [4Fe-4S] binding motifs. In the previously characterized RDases, in the second iron-sulfur-cluster binding-motif, a

Glycine (G) replaces the first expected cystein (C) residue present in other bacterial ferredoxins.

PceA is the purified PCE-reductive dehalogenase from *Sulfurospirillum multivorans.* This reductive dehalogenase contains a corrinoid cofactor and two iron-sulfur clusters (Neumann *et al.*, 1996). The corrinoid cofactor has been purified, and its crystal structure has been determined (Maillard *et al.*, 2003). This corrinoid cofactor is different from other known B12 cofactors in the fact that it lacks a methyl group of the cobamide moiety. The PceA protein and its corresponding encoding gene have been characterized (Neumann *et al.*, 1998). One of the common features shared with other reductive dehalogenase proteins is the presence of two iron-sulfur binding motifs, one Fe_4S_4 and one Fe_3S_3.

A PceA reductive dehalogenase has also been characterized in *Dehalobacter restrictus* (Maillard *et al.*, 2003). The enzyme was isolated in the membrane fraction and contained cobalamin, cobalt, iron, and acid labile sulfur. The purified enzyme catalyzed the dechlorination of PCE and TCE to *cis*-DCE. The N-terminal amino acid sequence aided identifying the coding gene for PceA, which has been cloned and sequenced.

(Maillard *et al.*, 2003) compared the characterized *pceA* and *pceB* genes. The *pceA* genes of *Desulfitobacterium hafniense* strains PCE-S and Y51, *Desulfitobacterium hafniense* strain TCE1 and *Dehalobacter restrictus* form a coherent group of reductive dehalogenase homologous with almost 100% sequence identity. Also, the *pceB* genes, and the intergenic regions of *Dehalobacter restrictus,* and the three *Desulfitobacteria* have identical sequences (Maillard *et al.*, 2003).

cob(II)alamin is involved in PCE dechlorination by PceA in *Dehalobacter restrictus* (Schumacher *et al.*, 1997), however PceA does not contain a corrinoid binding motif (DXHXXGSXLGG) found in vitamin B12-dependent mutases and methionine synthases (Marsh and Holloway, 1992).

The *cprA* and *cprB*, chlorophenol reductive dehalogenase genes in *Desulfitobacterium dehalogenans, Desulfitobacterium hafniense*, *Desulfitobacterium chlororespirans*, *Desulfitobacterium* sp. strain PCE1, and *Desulfitobacterium* sp. strain Viet1, are organized in the inverse order as *pceA* and *pceB*. *cprB* genes are located upstream from *cprA* genes and all *pceB* characterized so far are located downstream from *pceA*.

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CHAPTER III

DETECTION OF DECHLORINATING POPULATIONS IN CHLOROETHENE DECHLORINATING ENRICHMENT CULTURES AND IN SITE MATERIALS

3.1. Introduction

Widespread use, careless handling, lack of proper storage, and disposal practices have lead to an accumulation of chlorinated solvents such as tetrachloroethene (PCE) and trichloroethene (TCE) in groundwater. PCE, TCE, and their lesser-chlorinated degradation products have been recognized as a threat to public health and environmental quality in the U.S. and many other industrialized countries (Abelson, 1990; Fetzner, 1998). To date, thousands of contaminated sites exist where remedial action is required. Fortunately, efforts to treat these sites can take advantage of naturally-occurring anaerobic bacteria that have evolved strategies for respiring with chlorinated compounds serving as electron acceptors in an energy-yielding process (Mohn and Tiedje, 1992; Fantroussi *et al.*, 1998; Fetzner, 1998). This metabolic, energy-yielding process is known as chloridogenesis or (de)chlororespiration (Löffler *et al.*, 2003).

Incomplete bacterial dechlorination can result in the accumulation of compounds that are more toxic and pose a greater health hazard than the original pollutant. VC and *cis*-dichloroethene (*cis*-DCE) are frequent byproducts of microbial respiration of PCE or TCE by *Desulfuromonas* (Krumholz, 1997; Sung *et al.*, 2003) or *Dehalobacter* populations (Holliger *et al.*, 1998). VC is a proven human

carcinogen (Kielhorn *et al.*, 2000). In order to prevent accumulation of carcinogenic compounds in water reservoirs, it is imperative that bacterial populations and byproducts of their metabolism are correctly identified when bioremediation strategies are employed.

Many previously-described bacteria that degrade chlorinated ethenes, such as *Dehalobacter* spp. (Holliger *et al.*, 1998), *Desulfuromonas* spp. (Krumholz, 1997; Sung *et al.*, 2003), and *Sulfosporilum* spp. (Scholz-Muramatsu *et al.*, 1995), contribute to accumulation of *cis*-DCE in groundwater. Because *cis*-DCE is a common product of PCE dechlorination, it has become increasingly important to actively pursue organisms that have the ability to either completely dechlorinate PCE or TCE to ethene or that can utilize *cis*-DCE and VC as electron acceptors. The first PCE-to-ethene dechlorinating pure culture was identified as *Dehalococcoides ethenogenes* strain 195 (Maymó-Gatell *et al.*, 1997). *Dehalococcoides* strain FL2 can use TCE as an electron acceptor, reducing it to ethene (Löffler *et al.*, 2000). Both *Dehalococcoides* sp. strain 195 and *Dehalococcoides* sp. strain FL2 can dechlorinate VC to ethene. However, neither population can grow with VC as a sole electron acceptor, and the VC-to-ethene step is not respiratory, but cometabolic, and slow (Maymó-Gatell *et al.*, 2001). Recent investigations have revealed expanded metabolic capabilities among *Dehalococcoides* populations. *Dehalococcoides* populations that can dechlorinate VC to ethene have been identified (He *et al.*, 2003a; He *et al.*, 2003b). Also, groundwater sample-analysis from contaminated industrial sites throughout North America and Europe indicated the presence of *Dehalococcoides* populations at sites where ethene production was detected (Hendrickson *et al.*, 2002). The work by Henrickson *et al.* (2002) grouped the known 16S rRNA gene sequences of *Dehalococcoides* populations into three distinct clusters based on signature

variations in two regions of the small subunit rRNA gene. The Cornell group is embodied by the first *Dehalococcoides* isolate, strain 195. The second group, Victoria, is only represented by clone sequences. The third group, Pinellas, includes strains CBDB1 and FL2 (Hendrickson *et al.*, 2002).

Aquifer and sediment samples collected at pristine and contaminated sites from various geographic locations were used to establish anaerobic microcosms (Löffler *et al.*, 1997; Löffler *et al.*, 1999). Chloroethene dechlorination occurred in more than half of the microcosms, including ones initiated from material that had not been exposed to chlorinated solvents from anthropogenic sources. Sediment-free, non-methanogenic enrichment cultures were established from five river-sediment-derived microcosms that completely dechlorinated PCE to ethene (ETH). Subcultures were established and fed with PCE, *cis*-DCE or VC as an electron acceptor. All these enrichments were transferred more than 35 times in bicarbonate-buffered mineral salts medium with H_2 as the electron donor, a chlorinated ethene as the electron acceptor, and acetate or lactate as the carbon source.

The main objective of this study was to assess the presence of *Dehalococcoides* and *Desulfuromonas* in VC and *cis*-DCE enrichment cultures and in soils and subsurface water from chloroethene-contaminated sites. Clone libraries with VC-dechlorinating cultures were established to investigate the presence of diverse *Dehalococcoides* spp. and the overall microbial community present in the enrichment cultures.

3.2. Materials and methods

3.2.1. Chemicals and enzymes

Chlorinated ethenes, ampicillin, Proteinase K, lysozyme, achromopeptidase, and other chemicals were purchased from Aldrich (Milwaukee, WI) and Sigma Chemical Co. (St. Louis, MO). H_2 was obtained from Air Products (Atlanta, GA). Vinyl chloride (VC) was obtained from Fluka Chemical Corp. (Ronkonkoma, NY), and ethene was purchased from Scott Specialty Gases (Durham, NC). The molecular biology reagents were obtained from the following sources: restriction enzymes were purchased from Promega Biosciences, Inc. (San Luis Obispo, CA), tissue and DNA kit from QIAGEN (Valencia, CA); Taq DNA polymerase and PCR buffer from Applied Biosystems (Foster City, CA); bovine serum albumin (BSA) from Roche (Mannheim, Germany); the TOPO TA Cloning kit, chemically competent One Shot® *E. coli* cells from Invitrogen (Carlsbad, CA), and oligonucliotide primers for PCR and sequencing from Integrated DNA Technologies (Coralville, IA).

3.2.2. Source of dechlorinating cultures and site material samples

Three sediment-free cultures were established from PCE-to-ethene-dechlorinating microcosms established with river sediment materials, and one sediment-free culture was established from PCE-to-ethene-dechlorinating microcosm established with aquifer material from a chloroethene-contaminated site. Cultures used had been transferred over 35 times. The sources of the enrichment cultures are summarized in Table 3.1. Four sediment samples enriched with VC or *cis*-DCE were the focus of this study and were compared with a TCE-dechlorinating enrichment culture derived from the Pine River, Michigan (culture YK). The Bachman Road Site enrichment (culture BRS) was from a PCE contaminated site in Michigan. The Red

Cedar River, Okemos, MI, (culture RC) is a site that may have encountered fumigants in agricultural runoff. The Père Marquette River (culture PM) and the Ausable River (culture AuS) are presumed to be pristine rivers of Northern Michigan.

Soil and subsurface water samples were obtained from contaminated sites under aseptic conditions. Cores were kept at cold temperatures in coolers and shipped to the laboratory. Once in the laboratory, cores were opened in an anaerobic glove box and the site materials were deposited in sterile Mason jars. Subsurface water was kept in shipped containers until analysis was performed. The sources and type of site material samples used for this study are summarized in Table 3.2.

Table 3.1. Enrichment cultures used for this study. The material source used to establish microcosms and the chloroethenes used for enrichment are indicated. All samples used to establish microcosms used in this study were collected from the state of Michigan.

Culture	Enrichment	Source
FL2	TCE	Red Cedar River
YK	TCE	Pine River
Aus	*cis*-DCE, VC	Ausable River
BRS	PCE, *cis* DCE, VC	Bachman Road Site
PM	*cis* DCE, VC	Père Marquette River
RC	*cis*-DCE, VC	Red Cedar River

Table 3.2. Contaminated site materials analyzed.

Site	Material	Source	State
FMC	Soil, Water	FMC site	CA
SRS	Water	Savannah River Site	SC
SRS boxes	Water	Savannah River Site	SC
Alameda	Water	Alameda site	CA
GA P-66	Soil, Water	Plant 66	GA

3.2.3. Growth medium and culture conditions

All experiments were carried out in 160 mL serum bottles, containing 100 mL of growth medium. Medium and growth conditions were as described by (He, 2003). Cultures were transferred into medium containing ampicillin (25 mM).

3.2.4. Analytical methods

Chloroethenes were measured with a Hewlett Packard 6890 gas chromatograph equipped with a HP-624 column (60m length, 0.32 mm diameter, 1.8 μm film thickness) with a flame ionization detector (FID). Headspace samples of 100 μL were withdrawn with gas-tight 250 μL Hamilton glass syringes with Teflon-lined valves (model #1725) and manually injected into a split injector operated at a split ratio of 2:1. All syringes were flushed with H_2- and O_2-free N_2 gas to prevent contaminating the cultures with these gases. Analysis was performed as described previously (He *et al.*, 2002).

3.2.5. DNA extraction from soil and groundwater

When the sample to be analyzed was soil, a gram of soil was used with the UltraClean Soil DNA Kit from Mo Bio Laboratories, Inc. (Solana Beach, CA). If the sample analyzed was groundwater, approximately one liter of water was filtered through a 0.2 μm membrane filter. One filter per sample was placed in a 1.5 mL plastic tube and 1 mL of TE buffer (10 mM TRIS, 1 mM EDTA, pH 8) was added. The suspended cells were rigorously shaken for 5 minutes, the filter was removed using sterile tweezers, and the supernatant was centrifuged for ten minutes. A pellet was formed and used for DNA extraction. DNA was extracted using the UltraClean

Soil DNA Kit. A second filter from the same sample was inserted in the microbed tube (provided in the soil DNA kit) to increase biomass available for the analysis.

3.2.6. DNA extraction from enrichment cultures

Cells from cultures that had dechlorinated > 90% of the initial VC or cis-DCE were concentrated by aseptically filtering 40 mL of culture fluid with a vacuum filtration unit onto a 0.2-µm polycarbonate membrane. The biomass concentrated on the membrane was removed by placing the filter into a 1.5 mL micro centrifuge tube, adding 1 mL TE buffer (Tris 10mM, EDTA 1mM, pH 8.0), and shaking horizontally on a vortex at maximum speed for ten minutes. The filter was removed from the tube, and the cells were pelleted by centrifugation. DNA was extracted from sediment-free enrichment culture pellets grown with *cis*-DCE or VC as the terminal electron acceptor using the QIAGEN tissue kit, lysis steps were modified as follows: After suspending the pellet in 180 µL buffer ATL (supplied with QIAGEN tissue kit), 20 µL lysozyme (100mg/mL), 45 µL Proteinase K (25mg/mL), 10 µL Achromopeptidase (25 mg/mL, or 7500 U/mL) were added. The mixture was incubated at 55°C for 3h, mixing by inverting the tube 2-3 times an hour. Complete lysis was verified microscopically.

DNA was eluted in 200 µL of heated (70°C) 10 mM Tris buffer, pH 8.5. To obtain a more concentrated product from the samples, which were further enriched with ampicillin treatment, the final elution was performed in 100 µL of heated (70°C) 10 mM Tris buffer, pH 8.5 buffer was used instead of 200µL.

DNA was quantified using spectrophotometric analysis at 260 nm and the quality of DNA was verified with the 260/280 ratio and by gel electrophoresis on

1.5% agarose gels using ethidium bromide as a double stranded DNA stain. The DNA was stored at –20˚C.

3.2.7. PCR-based tools to detect dechlorinating populations

Community DNA extracted from the site materials and each of the TCE, *cis*-DCE and VC-dechlorinating enrichment cultures was screened with primers targeting previously identified *Dehalococcoides* (Löffler *et al.*, 2000; He *et al.*, 2003b), *Desulfuromonas* (Löffler *et al.*, 2000) and *Dehalobacter* populations (Schlötelburg, 2001). *Dehalococcoides* 16S rRNA genes were detected using the *Dehalococcoides*-targeted primer pairs *Dhc* 730F and *Dhc* 1350R and FL2F and FL2R. *Desulfuromonas* 16S rRNA genes were detected using the *Desulfuromonas*-targeted primer pair *DsfF* and *DsfR*. *Dehalobacter* 16S rRNA genes were detected using the *Dehalobacter*-targeted primer pair *DebF* and *Deb*. Primer sequences and expected amplicon sizes are listed in Table 3.3. Negative controls included PCR reactions with water replacing the template DNA. Positive controls consisted of plasmid DNA containing a *Dehalococcoides* sp. strain FL2 16S rRNA gene insert for *Dehalococcoides* targeted PCRs, plasmid DNA containing a *Dehalobacter restrictus* 16S rRNA gene insert for *Dehalobacter*-targeted PCRs, and plasmid DNA containing a *Desulfuromonas michiganensis* strain BB1 16S rRNA gene insert for *Desulfuromonas*-targeted PCRs. DNA molecular weight markers VI (0.15-2.1kbp) from Roche Applied Sciences and the 50-2,000 bp molecular marker from Bio-Rad were used to estimate amplicon size. PCR reactions were performed in a final volume of 25 μL. The final concentration of each chemical in a single reaction tube was: PCR buffer (1x), $MgCl_2$ 2.5 mM, BSA (0.13 mg/mL), dNTPs (0.25 mM each), Primers (1 mM each), and Taq DNA polymerase (0.5 μL). PCR conditions included

an initial denaturation of 92°C for 2 min, followed by 30 cycles of 94°C for 30 sec, annealing temperature for 45 sec, and 72°C for 2min. A final extension at 72°C was performed. In a 25 µL reaction volume, 50 ng of community DNA or 2 µL of 1:50 diluted 16S rRNA gene product (for the more sensitive nested-PCR) were used as template to determine presence or absence of particular dechlorinating populations.

Table 3.3. Primers, annealing temperatures, and expected amplicon sizes.

PCR primers	Primers sequences 5'→ 3'	Annealing temperature	Amplicon size (bp)
8F	AGAGTTTGATCCTGGCTCAG	55°C	1517
1525R	AAGGAGGTGATCCAGCCGCA		
Dhc 730 F	GCGGTTTTCTAGGTTGTC	58°C	620
Dhc 1350 R	CACCTTGCTGATATGCGG		
FL2R(Dhc 1164)	CGTTTCGCGGGGCAGTCT	58°C	434
FL2F (Dhc728)	AAGGCGGTTTTCTAGGTTGTCAC		
DsfF	AACCTTCGGGTCCTACTGTC	58°C	466
DsfR	GCCGAACTGACCCCTATGTT		
Deb 179F	TGTATTGTCCGAGAGGCA	50°C	828
Deb 1007R	ACTCCCATATCTCTACGG		

3.2.8. Clone libraries

The 16S rRNA gene clone libraries were established using genomic DNA from the VC-dechlorinating AuS, PM, and RC enrichment cultures. The TOPO TA cloning kit (Invitrogen, Carlsbad, CA) was used following manufacturer recommendations. Primers to obtain the nearly full-length 16S rRNA genes were 8F and 1525R (Table 3.3). PCR products were ligated into the TA cloning vector pCR2.1 and introduced into chemically competent *E. coli* cells. Seventy-two white colonies that presumably had an insert were picked for each library. Three VC-dechlorinating enrichments (cultures RC, PM and AUS) were thoroughly analyzed. Clones were first screened using direct PCR with primers targeting the TA cloning vector, flanking the inserted 16S rRNA gene fragment as described previously (Zhou *et al.*, 1997). The resulting amplicons were screened with a *Dehalococcoides*-

targeted primer pair to assess the presence and abundance of *Dehalococcoides* spp. in the dechlorinating cultures.

The 16S rRNA gene fragments amplified with the TA-vector targeted primers were restricted for Amplified Ribosomal DNA Restriction Analysis (ARDRA). Digests were performed individually with *MspI*, according to the manufacturer recommendations. The reactions were terminated by incubation at 65°C for 10 min. Digest fragments were resolved by electrophoresis for 3 hours on 2.5% low-melting agarose gels (Seaplaque; Cambrex, Rockland, MN). Electrophoresis and Gel staining in ethidium bromide solution were performed at 4°C.

The unique *Dehalococcoides*-like clones as determined by the presence of a *Dehalococcoides* specific PCR product (620 bp) as well as by ARDRA analysis were sequenced to determine the extent of diversity among the *Dehalococcoides* populations. Also, individual clones of a library produced from PCR-amplified 16S rRNA genes identified by ARDRA analysis were sequenced.

When *Dehalococcoides*-like populations were not identified in the clone libraries of the VC-dechlorinating enrichment cultures, a second clone library was established using a subsequent culture that shared dechlorination characteristics but had been transferred one additional time in medium containing ampicillin. Ampicillin treatment was necessary for enrichment to obtain *Dehalococcoides* 16S rRNA gene inserts in clone libraries Aus and PM.

3.2.9. Sequence analysis of 16S rRNA genes

Representative *E. coli* clones and *E. coli* cells that tested positive with the *Dehalococcoides*-targeted PCR primers in each clone library and at least two clones of each of the dominant *Msp*I patterns were sequenced to infer their phylogenetic

affiliation. Plasmid DNA from clones selected to be sequenced was extracted using the QIAprep Spin Miniprep Kit (QIAGEN Inc.,Valencia, CA) according to manufacturer recommendations. Sequencing reactions consisted of 30 pmol of primer added to 300 ng of plasmid DNA in a final 12 µl volume. The plasmids were submitted to the High Throughput Sequencing Facility at Michigan State University (www.bch.msu.edu) to obtain nearly complete 16S rRNA gene sequences using the universal primers 8F, 529R (5'-CGC GGC TGC TGG CAC), 533F (5'-CAG CAG CCG CGG TAA), 1114F (5'-GCA ACG AGC GCA ACC C), and 1392R (5'-ACG GGC GGT GTG T). After manual alignment and assembling a contiguous sequence for the 16S rRNA gene using the Megalign software of the Lasergene package (Madison, WI), the entire sequence was subjected to the chimera-check tool of the ribosomal database project (RDP) website and compared to known databases using the Phylip tool of the RDP site (www.rdp.cme.msu.edu). The search results were expanded using the NCBI database search (www.nlm.ncbi.gov).

3.3. Results

3.3.1. Soil and groundwater from contaminated sites

Dehalococcoides populations were detected in all chloroethene contaminated sample materials from sites where ethene production had been detected. *Desulfuromonas* were detected in five out of six sites, but in most of the cases *Desulfuromonas* was only present in a fraction of the samples (wells) analyzed. *Dehalobacter* was detected only at the Alameda and GA P66 sites (Table 3.4).

Table 3.4. Detection of dechlorinating populations in site material samples

Site	Sample (Soil or water)	Number of wells analyzed	*Dhc*[a]	*Dsf*[b]	*Deb*[c]
FMC	Soil	4	2/4	2/4	0
FMC	Water	4	4/4	1/4	0
SRS	Water	8	8/8	4/8	0
SRS Boxes	Water	5	3/5	3/5	0
Alameda	Water	4	4/4	4/4	1/4
GA P-66	Soil	1	1/1	0	1/1
GA P-66	Water	1	0	0	0

[a] *Dehalococcoides* 16S rRNA gene
[b] *Desulfuromonas* 16S rRNA gene
[c] *Dehalobacter* 16S rRNA gene

3.3.2. 16S rRNA-targeted PCR analysis of TCE, cis-DCE, and VC-dechlorinating enrichment cultures.

Dehalococcoides-like populations were identified in DNA from four cultures that were enriched with *cis*-DCE or VC as the terminal electron acceptor and with H_2 as the electron donor (Figures 3.1 and 3.2) and in DNA from the YK culture enriched with TCE as electron acceptor and with H_2 as electron donor. Table 3.5 summarizes the results generated when primers targeting specific dechlorinating organisms were used to characterize the enrichment cultures. Prior to ampicillin treatment, amplicons generated with the *Desulfuromonas*-targeted primer pair were not observed in the PCR reactions. However, using a nested PCR approach, an amplicon was generated with *Desulfuromonas*-targeted primer pair in all the *cis*-DCE and VC enrichment cultures used for this study, indicating that *Desulfuromonas* populations were present in the VC and *cis*-DCE enrichment cultures, although in low numbers. No amplification was observed with the *Desulfuromonas*-targeted primer pair when DNA from the TCE enriched culture YK was used as template. After ampicillin treatment, *Desulfuromonas* populations were no longer detected, indicating that ampicillin eliminated *Desulfuromonas* populations from the VC enrichment communities. *Dehalobacter* populations were only present in the TCE enriched YK culture.

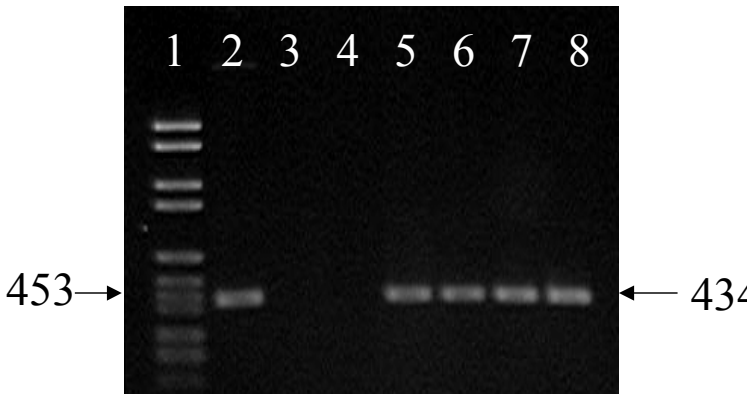

Figure 3.1. Detection of *Dehalococcoides* spp. in VC-dechlorinating enrichment cultures with *Dehalococcoides* 16S rRNA gene-targeted primers (FL2F and FL2R). Lane1: DNA Molecular Weight Marker VI, 0.15-2.1 kbp bp ladder (Roche); lane 2: *Dehalococcoides* sp. strain FL2; lane 3-4: negative controls *Desulfuromonas michiganensis* strain BB1 DNA and H_2O; lane 5: BRS culture enriched with VC; lane 6: Red Cedar culture enriched with VC; lane 7: Ausable culture enriched with VC; lane 8: Père Marquette culture enriched with VC.

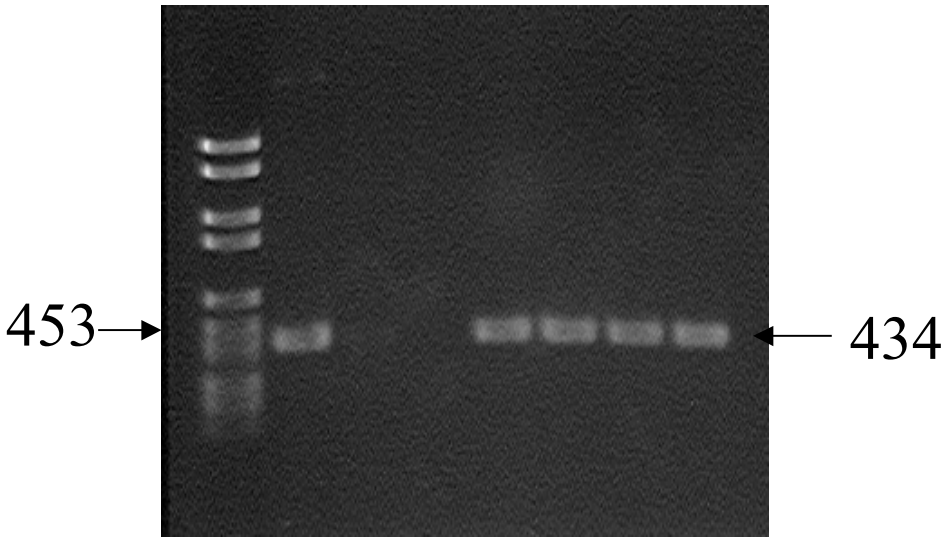

Figure 3.2. Detection of *Dehalococcoides* spp. in *cis*-DCE-dechlorinating enrichment cultures with *Dehalococcoides* 16S rRNA gene-targeted PCR primers (FL2F and FL2R). Lane1: DNA Molecular Weight Marker VI, 0.15-2.1 kbp bp ladder (Roche); lane 2: *Dehalococcoides* sp. strain FL2; lane 3-4: negative controls *Desulfuromonas michiganensis* strain BB1 DNA and H_2O; lane 5: BRS culture enriched with *cis*-DCE; lane 6: Red cedar culture enriched with *cis*-DCE; lane 7: Ausable culture enriched with *cis*-DCE; lane 8: Père Marquette culture enriched with *cis*-DCE.

Table 3.5. Detection of dechlorinating populations in enrichment cultures. Chloroethenes used as electron acceptors and the number of transfers in the presence of ampicillin is indicated. (+) Amplification was visible in an agarose gel. (–) Not detected under the conditions specified in the material and methods section.

Culture source	Electron acceptor	Ampicillin transfers	(Dhc)[a]	(Dsf)[b]	(Deh)[c]	N(Dsf)[b]	N(Deb)[c]
BRS	VC	1	+	-	-	+	-
BRS	cis-DCE	0	+	-	-	+	-
RC	VC	1	+	-	-	+	-
RC	cis-DCE	0	+	-	-	+	-
PM	VC	1	+	-	-	+	-
PM_2	VC	2	+	-	-	-	-
PM	cis-DCE	0	+	-	-	+	-
AuS	VC	1	+	-	-	+	-
AuS_2	VC	2	+	-	-	-	-
AuS	cis-DCE	0	+	-	-	+	-
YK	TCE	1	+	-	-	-	+

[a] Dhc=*Dehalococcoides* 16S rRNA gene, [b] Dsf=*Desulfuromonas* 16S rRNA gene,
[c] Deh=*Dehalobacter* 16S rRNA gene, N=nested PCR

3.3.3. Sequence analysis of cloned 16S rRNA genes and related isolates

When *Dehalococcoides*-targeted primers were used to screen the clone libraries, the expected amplicon size of about 430 bp was obtained with four clones of the YK-TCE clone library. Sequence analysis indicated that the *Dehalococcoides* 16S rRNA gene present in the YK culture shared higher similarity with *Dehalococcoides* strains CBDB1 and FL2 than with *Dehalococcoides ethenogenes* strain 195. *Dehalococcoides*-targeted primers identified one *Dehalococcoides* clone in the RC library and no positive clones in libraries established with the AuS, BRS, or PM enrichments. These four cultures had experienced one transfer in medium containing ampicillin. Clone libraries derived from enrichment cultures that experienced an additional transfer in ampicillin-amended medium revealed a higher proportion of *Dehalococcoides* positive clones in the libraries with 12, 5, and 24 *Dehalococcoides*-positive clones in the AuS, PM, and BRS libraries, respectively. At least one cloned 16S rRNA gene sequence that amplified with the *Dehalococcoides*-targeted primers from each enrichment community was fully sequenced. Additionally, all cloned 16S rRNA gene fragments were amplified using TA vector-targeted primers and scrutinized with restriction digest analysis (RFLP) using the enzyme *MspI*. At least two clones of each dominant pattern were fully sequenced. Two slightly different profiles were generated from a single cloned sequence because of the orientation of the 16S rRNA gene fragment into the cloning vector. Hence, the terminal (5' and 3') fragments generated in each of the restriction profiles had either an additional 27 bases or 55 bases added to them, corresponding to the size of the cloning vector at each end.

In addition to *Dehalococcoides* spp., which were consistently detected in all four VC-dechlorinating cultures, assorted members of the *gamma*-Proteobacteria

including *Pseudomonas, Serratia, Acinetobacterium, Lysobacter,* and members of the Firmicutes (Clostridiales) including *Clostridium*, *Acetobacterium*, *Acidaminobacter*, *Anaerovibrio*, *Sporomusa* and *Denitrobacterium* were detected in the enrichments that received one round of ampicillin treatment. A particular community was not comprised of all of these organisms. Despite differences in absolute composition, *Clostridium* spp. were detected in the clone libraries established with all of the cultures. Surprisingly, 16S rRNA gene sequences that were 99% similar to that of *Pseudomonas lundensis* were found in all of the libraries.

Prior to the ampicillin treatment, the RC clone library apparently contributed only one *Dehalococcoides* clone, RCVC38, and the chimera check tool of the RDP indicated that it was a chimeric sequence. While bases 300-1473 were most closely related to previously described dechlorinating populations i.e., *Dhc. ethenogenes* strain 195, *Dhc.* strain CBDB1, and *Dhc.* strain FL2, the first 300 bases of the 5' end included a short portion of an *Acetobacterium* sequence.

3.3.4. Effect of ampicillin on dechlorinating cultures

Cultures were first screened with *Dehalococcoides* and *Desulfuromonas* primers prior to ampicillin enrichment. *Dehalococcoides* spp. were present before and after further ampicillin enrichment and *Desulfuromonas* spp. were no longer detected after ampicillin treatment. Cultures that had undergone one round of ampicillin treatment were used for clone libraries analyses.

The AuS enrichment culture was studied through two consecutive rounds of ampicillin amendment, and some unexpected community shifts were observed. Transferring the highly enriched cultures to medium amended with ampicillin a second time achieved further enrichment of the VC-dechlorinating populations.

There was an increase in the number of clones with a *Dehalococcoides* 16S rRNA gene sequence from zero to twelve. *Clostridium* spp., *Pseudomonas* spp., *Acidaminobacterium* spp., and *Serratia* spp. clone sequences that were most prominent in the initial ampicillin-treated culture declined to undetectable levels following a second round of enrichment in the presence of the antibiotic.

Pseudomonas and *Serratia* were also present in a previously described TCE dechlorinating culture that had been exposed to oxygen briefly. However, the dechlorinating culture stopped dechlorinating at VC (Richardson *et al.*, 2002). The enrichment cultures in the current study were able to dechlorinate VC to ethene and were not (knowingly) exposed to oxygen. *Pseudomonas* might be present in the cultures because of trace amounts of oxygen introduced during sampling.

Most surprising was the appearance of a cloned 16S rRNA gene 99% similar to an iron oxidizing lithotroph strain ES1 and *Lysobacter* sp. G3 (GenBank Accession numbers AF012541 and AY074793). This clone sequence comprised 75% of the Au Sable (2x ampicillin) clone library. The majority of the remaining 25% of identified sequences were those of *Dehalococcoides* spp. In the consecutive ampicillin-treated cultures, two clone sequences 95% similar to that of clone GOUTA13 (GenBank Accession number AY050603.1) were identified, and their presence seemed unaffected by the antibiotic. Clone GOUTA 13 was identified in a reactor treating monochlorobenzene contaminated groundwater and possibly represents a dechlorinating population, which is resistant to ampicillin.

3.4. Discussion

Dehalococcoides-targeted primers identified *Dehalococcoides* populations in enrichment cultures that were fed *cis*-DCE and VC as terminal electron acceptors (Figure 3.1) and in site materials from sites contaminated with chloroethenes where ethene has been detected. Hence, it is likely that *Dehalococcoides* species are responsible for dechlorination reactions in the *cis*-DCE and VC- enriched cultures.

Neither *cis*-DCE nor VC dechlorination was inhibited in the presence of ampicillin. Bacteria resistant to *beta*-lactam antibiotics would be selectively enriched under these conditions, and it has been demonstrated that *Dehalococcoides* possess an unusual cell wall that renders them resistant to this class of antimicrobial agents (Maymo- Gatell 1997). An increased number of *Dehalococcoides* clones were identified when the cultures were treated with ampicillin, and VC dechlorination remained consistent in the enrichment cultures following an extended period of ampicillin treatment. These results indicate that the addition of ampicillin is a good selecting method to enrich for *Dehalococcoides* spp.

Clostridium spp. were detected in all the communities analyzed. *Clostridium* spp. are able to produce H_2, and *Dehalococcoides* spp. require H_2 as electron donor, hence a convenient symbiotic relationship was probably taking place. Another possible explanation of why *Clostridium* spp. were present in the clone libraries is that *Clostridium* spp. were actively involved in the dechlorination of VC to ethene. Some *Clostridium* spp. have been implicated in dechlorination, i.e. *Clostridium bifermentans* strain DPH1 (Chang *et al.*, 2000) dechlorinates PCE in a metabolic way using H_2 as electron donor, however, dechlorination halts at *cis*-DCE. Other *Clostridium* spp. which can dechlorinate tetrachloromethane (Egli *et al.*, 1988) and 1,1,1 trichloroethane (Egli *et al.*, 1988; Galli and McCarty, 1989) have been

identified. Besides *Clostridium* spp., *Pseudomonas* spp. and *Dehalococcoides* spp., the remainder of the populations was site specific, indicating that both enrichment procedures and source have an effect on community structure. The communities amended in VC and ampicillin were highly enriched. Dramatic shifts in the population profile were detected in response to ampicillin treatment.

The presence of *Dehalococcoides* populations in several chloroethene-contaminated sites and in VC and *cis*-DCE dechlorinating enrichment cultures obtained from natural sediments was confirmed. *Dehalococcoides* populations are implicated in the reduction of these chlorinated compounds to ethene. Ampicillin-treated cultures contained a higher amount of *Dehalococcoides* spp. and influenced the distribution of other organisms that were detected in the enrichment communities.

While specific PCR 16S rRNA-targeted primer pairs may be able to identify *Dehalococcoides* present in a culture or at a specific site, the analysis of 16S rRNA does not allow for a prediction of dechlorination activity. Additional target genes must be retrieved from *Dehalococcoides* populations to enhance monitoring and detection efforts.

3.5. References

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CHAPTER IV

ENVIRONMENTAL DISTRIBUTION OF THE TRICHLOROETHENE REDUCTIVE DEHALOGENASE GENE (*tceA*) IN *DEHALOCOCCOIDES SPECIES* AND EVIDENCE FOR HORIZONTAL GENE TRANSFER

4.1. Introduction

Tetrachloroethene (PCE) and trichloroethene (TCE) are solvents of choice for many degreasing operations. Irresponsible handling, storage, and disposal have led to widespread contamination of subsurface environments (Abelson, 1990; Fetzner, 1998). Under anaerobic conditions PCE and TCE are reductively dechlorinated to ethene. Laboratory (Duhamel *et al.*, 2002; Richardson *et al.*, 2002; Cupples *et al.*, 2003; He *et al.*, 2003b) and field (Hendrickson *et al.*, 2002; Major *et al.*, 2002; Lendvay *et al.*, 2003) studies demonstrated a link between the presence of *Dehalococcoides* spp. and complete dechlorination to nontoxic ethene.

Dehalococcoides ethenogenes strain 195 was the first isolate capable of complete dechlorination of PCE to ethene (Maymó-Gatell *et al.*, 1997). Magnuson *et al.* identified a TCE RDase responsible for reductive dechlorination of TCE to vinyl chloride. The gene encoding the TCE RDase, *tceA*, has been cloned and sequenced (Magnuson *et al.*, 2000); the *tceB* gene is located downstream of *tceA*, and both genes are co-transcribed. *tceB* encodes for a membrane-spanning protein that has been hypothesized to play a role in anchoring the RDase to the cytoplasmic membrane. A highly similar (99.4 % identity) *tceA* gene has been detected in *Dehalococcoides* sp. strain FL2, despite the fact that

several reductive dehalogenase homologous genes in strain FL2 share low identity with putative reductive dehalogenase genes in *Dehalococcoides ethenogenes* strain 195.

High similarity of *tceA* genes could be explained by a horizontal gene transfer event, according to Seshadri *et al.* (Seshadri *et al.*, 2005), the *tceAB* genes are located in a genomic integrated element, and recent computational findings indicate that the *tceAB* genes are located in DNA regions that seem to be of a foreign origin.

Dechlorination of a diverse range of chloroorganic has been reported for *Dehalococcoides* spp. For example, *Dehalococcoides* sp. CBDB1 dechlorinates chlorinated benzenes and chlorinated dioxins (Adrian *et al.*, 2000; Hölscher *et al.*, 2003; Jayachandran *et al.*, 2003) while *Dehalococcoides ethenogenes* strain 195, *Dehalococcoides* sp. FL2 and *Dehalococcoides* sp. BAV1 dechlorinate a range of chlorinated ethenes. The genetic systems encoding for pathways responsible for complete detoxification in *Dehalococcoides* spp., have gained interest because of *Dehalococcoides* spp. potential use in bioremediation systems (Rhee *et al.*, 2003; Hölscher *et al.*, 2004; Krajmalnik-Brown *et al.*, 2004; Regeard *et al.*, 2004). However, the question of why multiple RDase genes are present in *Dehalococcoides* spp. and how RDase genes have been disseminated among *Dehalococcoides* species still remains unanswered.

The objectives of this study were to investigate environmental distribution, and the possibility of horizontal gene transfer (HGT) of the trichloroethene reductive dehalogenase gene (*tceA*) in *Dehalococcoides* species. The *tceA and tceB* genes were targeted in several ethene producing cultures and environmental samples from diverse geographic locations. A specific PCR approach was used to detect *tceA*/*tceB* and

investigate their environmental distribution. Sequence information downstream of *tceAB* was explored to assess the possibility of gene transfer.

4.2. Materials and methods

4.2.1. Chemicals gases and enzymes

Chlorinated ethenes, ampicillin, Proteinase K, lysozyme, achromopeptidase and other chemicals were purchased from Aldrich (Milwaukee, WI) and Sigma Chemical Co. (St. Louis, MO). H_2 was obtained from Air Products (Atlanta, GA). Vinyl chloride (VC) was obtained from Fluka Chemical Corp. (Ronkonkoma, NY), and ethene was purchased from Scott Specialty Gases (Durham, NC). Molecular biology reagents were obtained from the following sources: Taq DNA polymerase and PCR buffer from Applied Biosystems (Foster City, CA); bovine serum albumen (BSA) from Roche (Mannheim, Germany); and oligonucliotide primers for PCR from Integrated DNA Technologies (Coralville, IA).

4.2.2. Sources of DNA

Genomic DNA was isolated from soil and water samples collected at chloroethene-contaminated sites such as FMC and the Savannah River Site, among others. Also DNA was obtained from *Dehalococcoides* sp. strain FL2, and several TCE-, *cis*-DCE-, and VC-dechlorinating enrichment cultures derived from river sediments (the Red Cedar, Au Sable and Père Marquette Rivers, all three in Michigan (Löffler *et al.*, 1999; Löffler *et al.*, 2000); the Sangamon River and the Salt Fork River, both in Illinois, provided by Robert Sanford) and chloroethene-contaminated aquifers (the FMC site in

California, the Bachman Road site in Michigan (Lendvay *et al.*, 2003), CH a contaminated site in Nebraska, LH a contaminated site in Houston Texas, and Owls an enrichment from a UASB reactor with no previous exposure to chlorinated solvents (Carr and Hughes, 1998). Genomic DNA was also available from *Dehalococcoides ethenogenes* strain 195 and *Dehalococcoides* isolates CBDB1, FL2, and BAV1. In addition, genomic DNA was available from two PCE-to-ethene-dechlorinating consortia successfully employed in bioaugmentation approaches in the field (i.e., KB-1(Major *et al.*, 2002), and Bio-Dechlor INOCULUM (www.regenesis.com)). The origins of the site samples and the chloroethene-dechlorinating consortia and pure cultures are summarized in Table 4.1.

Table 4.1. *tceA* detection in chloroethene degrading cultures and chloroethene contaminated site materials.

	Electron acceptor	Source	State	*tceA* [a]
Isolates				
FL2	TCE	Red Cedar River	MI	+
BAV1	VC	Bachman Road Site	MI	-
CBDB1				-
195		Wastewater treatment plant	NY	+
Consortia				
YK	TCE	Pine River	MI	+
Aus	VC	Ausable River	MI	-
BRS	PCE	Bachman Road Site	MI	+
BRS	*cis*-DCE	Bachman Road Site	MI	-
BRS	VC	Bachman Road Site	MI	-
PM	VC	Père Marquette River	MI	+
RC	VC	Red Cedar River	MI	+
RC	1,2 D*	Red Cedar River	MI	-
SFR	*cis*-DCE	Salt Fork River	IL	+
SNR	PCE	Sangamon River	IL	+
FMC	PCE	FMC site	CA	+
CH	PCE	Cornhuskers	NE	+
GSI	PCE	Houston	TX	+
Owls	PCE	Houston	TX	+
Minerva	VC	Minerva site	OH	-
Hydrite	PCE	Hydrite site	WI	-
Hydrite	VC	Hydrite site	WI	-
Bio-Dechlor	PCE	Mixture of various cultures		+
KB1	VC	Ontario TCE-contaminated site	Canada	-
KB1	TCE	Ontario TCE-contaminated site	Canada	-
Environmental samples				
FMC[b,c]	N.A.	FMC site	CA	-
SRS[c]	N.A.	Savannah River Site	SC	-
Alameda[c]	N.A.	Alameda site	CA	-
GP-66 b,[c]	N.A.	Plant 66	GA	-

[a] *tceA* was PCR amplified with *tceA* specific primers (Magnuson *et al.*, 2000). N.A. Not applicable. [b] soil sample. [c] water sample. * (Ritalahti and Löffler, 2004)

4.2.3. Primer design

Primers used in this study were designed based on *D. ethenogenes* genome sequence www.tigr.com**.** Primers were designed in order to obtain genetic information downstream of the *tceAB* genes. A 3.4 kb DNA fragment was targeted for amplification with the use of primers TRD-P and 4012R. An additional primer downstream from 4012R was designed and designated DTOR. Also, primers internal to the 3.4 kb amplicon were designed for sequencing purposes. Primer names and sequences used in this study are presented in Table 4., with primer designations presented in the first column, sequences of primers included in the second column, and target sequences indicated in the third column. Additionally, primer targeted positions with respect to *tceA* and *tceB* are illustrated in Figure 4.1. *tceA* and *tceB* genomic organization is presented, including open reading frames (ORFs) upstream and downstream. Sequencing primers are not included in Figure 4.1.

Table 4.2. Specific primers used in this study and their position relative to *tceA* and *tceB*.

Primer designation	**Primer sequence 5'→3'**	**Target sequence**	***Reference***
797F	ACGCCAAAGTGCGAAAAGC	*tceA*	Magnuson[1]
2490R	GAGAAAGGATGGAATAGATTA	*tceA*	Magnuson[1]
TRD-P	CAAACCTTCCCGTTGAGT	Upstream *tceA*	This study
1572R*	GGAAGAAGCCCCGAAGTAGC	*tceA*	This study
1905R*	GAGGGGATAGGCAATACAAGAGCA	*tceA*	This study
2345F*	TGCACAACTTGGTCAAGTCC	*tceA*	Magnuson [1]
2791F*	GCACATATAGCGGCAGGAAGGAAT	*tceB*	This study
3011R	TTTTCGGCACTTTTAGAGACACTT	Downstream *tceB*	This study
3277F*	TTTCAAGAATATGCGTGTCA	Downstream *tceB*	This study
3785R*	GGATGATAAAGCTAGCGGTA	Downstream *tceB*	This study
4012R	TGCCGGAATTGCTTATGC	Downstream *tceB* (Dhc-TE1)	This study
DTOR	AGTTACTTAACGCAAAAACC	Downstream *tceB* Upstream Dhc-TE1	This study

*Sequencing primers

[1](Magnuson *et al.*, 2000)

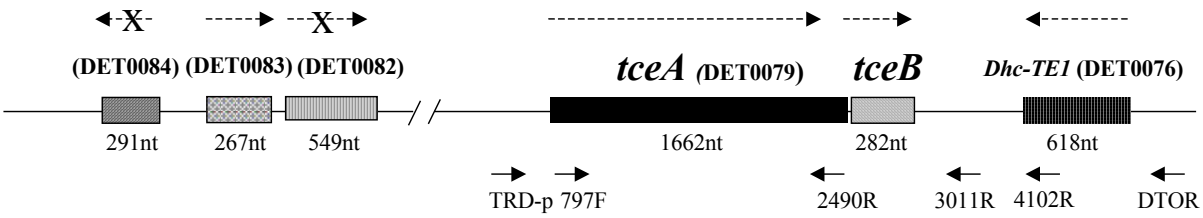

Figure 4.1. Organization of the *tceAB* and adjacent gene regions. Genes are shown as hatched boxes, assigned ORF number in the *Dehalococcoides* ethenogenes genome is indicated in parenthesis. Dashed arrows indicate the direction of transcription; an X through an arrow indicates incomplete open reading frames (ORFs). Arrows in the lower part of the diagram indicate primer positions and direction. Figure is not drawn to scale but proportions are maintained and size of each gene is indicated bellow the boxes. DET0084 is located 3582bp upstream of the *tceA* start codon*;* DET0083 is located 3154bp upstream of the *tceA* start codon; DET0082 is located 2756bp upstream of the *tceA* start. *Dhc-TE1* is located 1313bp downstream of the *tceB* stop codon.

4.2.4. Bioinformatic tools

All *tceA* and *tceB* genes obtained were aligned using Clustal W (Thompson *et al.*, 1997). Genomic information for *Dehalococcoides ethenogenes* strain 195 (www.tigr.com) was used to explore the sequence information found upstream and downstream of the *tceAB* genes. Frameplot (Ishikawa and Hotta, 1999), a gene finding program, was used to identify Open Reading Frames (ORFs) that most closely matched transposable elements (TEs) or integrases. To explore similarity with proteins that have known functions in transposition events, ORFs that most closely matched TEs found in non-redundant databases (NCBI and EMBL) were compared using BLASTX. The complete protein sequences of the best matches were retrieved, aligned, and compared using ClustalW. Conserved domains were identified with pFAM (Bateman *et al.*, 2004). The GC content of *tceA* and *tceB* genes was determined using the Geecee program (http://bioweb.pasteur.fr/seqanal/interfaces/geecee.html).

4.2.5. DNA extraction, PCR, cloning, and amplicon analysis

DNA was extracted from sediment-free, ethene-producing enrichment cultures as described (He *et al.*, 2003b). From environmental samples, DNA was extracted in the following way: water samples (50-100 mL) were filtered through 0.2 μm membrane filters (Fischer scientific) to collect microbial biomass. The filters were placed (using sterile tweezers) in 2 mL plastic tubes containing 1 mL of TE buffer (10 mM TRIS, 1mMEDTA, pH 8.0). Rigorous vortexing for 5 min yielded a cell suspension suitable for DNA extraction using the UltraClean Soil DNA Kit from Mo Bio Laboratories, Inc.

(Solana Beach, CA). DNA from soil samples was extracted using the UltraClean Soil DNA Kit following manufacturers recommendations using 1g of soil for each extraction.

The presence of DNA in each extract was verified by agarose gel electrophoresis. A final concentration of 1-2 ng/µL DNA was used for PCR amplification of *tceA* gene. DNA extracted from each of the cultures, water, and soil was initially tested with primers targeting *Dehalococcoides* 16S rRNA gene (Löffler *et al.*, 2000) and the *tceA* gene (Magnuson *et al.*, 2000). PCR conditions used were as described for *tceA* (Magnuson *et al.*, 2000) and for *Dehalococcoides* 16S rRNA gene (He *et al.*, 2003b). In cultures where *tceA* was amplified, primer pairs that would generate an amplicon containing *tceA* and *tceB*, i.e. primer pair 4012R and TRD-P, was used to assess *tceB* presence, similarity and sequence similarity downstream of *tceB*. When no amplification was achieved with primer pair 4012R and TRD-P, primer 3011R (5' TTTTCGGCACTTTTAGAGACACTT-3'), which was located closer to the *tceB* gene was used in conjunction with forward primer 797F previously used in the *tceA*-targeted PCR.

Amplicons generated with genomic DNA from ethene producing cultures using primers TRD-P and 4012R were purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA), ligated into vector pCR2.1 by TA cloning (TOPO TA cloning kit, Invitrogen, Carlsbad, CA), and cloned in chemically competent One Shot® *E. coli* cells provided with the cloning kit, following the manufacturer's recommendations. Recombinant *E. coli* clones were screened by verifying the correct insert size using direct PCR with primers targeting sequences of the pCR2.1 cloning vector flanking the inserted fragment (Zhou *et al.*, 1997). The resulting, correct-sized amplicons were digested

individually with the enzyme *Msp*I (Promega Biosciences, Madison, WI) as per manufacturer recommendations for Restriction Fragment Length Polymorphism (RFLP) analysis. Plasmid DNA from recombinant clones containing the different inserts was extracted using the Qiaprep spin miniprep kit (Qiagen), and sequenced using vector-specific primers (33) and internal sequencing primers (Table 4.2.) with an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Given that *Dehalococcoides* sp. strain FL2 has a *tceA* gene almost identical to the tceA gene in *Dehalococcoides ethenogenes* strain 195, strain FL2 was used as a positive control for all PCR reactions; when the target sequence was adjacent to *tceA*, in every case when no amplification was achieved with strain FL2, PCR reactions were repeated using *Dehalococcoides ethenogenes* strain 195 as a positive control.

4.3. Results

4.3.1. tceA and tceB detection in ethene producing enrichment cultures

The *tceA* gene was detected in two out of four *Dehalococcoides* isolates, 11 out of 19 mixed cultures, and was not detected in any of the environmental samples tested (Table 4.1.). The *tceA* amplicons were sequenced and compared with the *tceA gene* of *Dehalococcoides ethenogenes* strain 195 (AF228507) and *Dehalococcoides* sp. strain FL2 (AY165309), *tceA* amplicons were 95-99.5 percent identical to the *tceA* gene in *D. ethenogenes* strain 195 and 95.5 to 99.9 percent identical to the *tceA* gene in strain FL2 (Table 4.3.) *tceA* was not detected in soil or groundwater samples where *Dehalococcoides* spp. had been detected (Table 4.1.), but in one case, the FMC site, the *tceA* gene was detected after the soil sample was enriched in a microcosm in a laboratory microcosm. The *tceA* gene was present in two out of five VC-dechlorinating cultures (Table 4.1.). The eleven *tceA* gene sequences obtained from the ethene producing enrichment cultures were 95 to 100 percent identical to each other. A similarity matrix summarizes *tceA* genes identity in Table 4.3. The highest identity values were shared between the *tceA* genes from CH and Owls, which were identical to each other, as well as *tceA* genes from the Sangamon (SNR) and the Saltfork river (SFR) enrichment cultures also identical to each other. The lowest identity value shared is 95.3%, shared among the *tceA* in the FMC culture and the *tceA* in *D. ethenogenes* 195.

The primer pair 4012R and TRD-P generated an amplicon of the correct size (~3370 bp) (Figure 4.1.) using DNA from eight out of 11 cultures where the *tceA* gene was previously detected. Despite the fact that DNA from OWLS, CH, LH cultures produced amplicons that were 97% identical to *tceA* of *D. ethenogenes* strain 195 with primers

797F and 2490R (Magnuson *et al.*, 2000), DNA from OWLS, CH, LH cultures did not produce an amplicon with the primer pair TRD-P and 4012R. Additional primers were used in these three cultures to obtain complete *tceB* sequences and compare similarity. TRD-P was tested using DNA from OWLS, CH, LH, in conjunction with 2490R. Despite the fact that 2490R had produced an amplicon with 797F, no amplification was detected using the primer pair TRD-P and 2490R. The primer pair 797F and 3011R generated and amplicon of the expected size when DNA from OWLS, CH and LH cultures was used as template, successfully amplifying the *tceA* and *tceB* genes.

4.3.2. Identification of a genetic mobile element next to the tceAB genes

One TE, corresponding to *D. ethenogenes* gene number DET0076, was identified downstream of *tceAB*, designated Dhc-TE1. Figure 4.1. illustrates the position of Dhc-TE1 and its location relative to *tceA* and *tceB*. The downstream mobile element, Dhc-TE1, was 30% identical to a putative site specific recombinase in *Rhodococcus rhodochrous* at the protein level (18) (Figure 4.2.). The primer used to amplify the 3.4 kb fragment targeted a sequence internal to Dhc-TE1. To explore sequence similarities between Dhc-TE1 and *tceB*, primer DTOR (Figure 4.1.) was tested with DNA from all samples where the *tceAB* genes had been detected. Amplification with 3277F and DTOR primer pair produced an amplicon of the expected length of about 1640 bp when DNA from *Dehalococcoides ethenogenes* strain 195, and the FMC and YK consortia were used as template. A smaller amplicon of about 1300 bp in length was generated with DNA from the SNR consortium. Sequence analysis revealed that the 1640 bp FMC and YK amplicons were identical to sequence downstream of *tceB* in *D. ethenogenes* 195. The

1300 bp shorter amplicon produced with DNA from the SNR sample was identical to the FMC and YK sequences however, a 267 bp portion of the sequence in strain 195 was missing, and out of the 267 bp, 106 bp were part of Dhc-TE1. The DTOR primer did not produce an amplicon when DNA from *Dehalococcoides* sp. strain FL2, or community DNA from the PM, RC or SFR cultures were used as templates, suggesting that the sequence identity was interrupted by the transposable element Dhc-T1.

4.3.3. GC content in the tceAB genes and adjacent regions

The GC content in *Dehalococcoides ethenogenes* is 48.9% (Seshadri *et al.*, 2005). Several calculated GC% values were obtained when *tceA*, *tceB* and adjacent genes resembling TEs were analyzed. The calculated GC content in DET0084 was 43%, DET0083 was 50%, and DET0082 was 47%. In the DNA region between DET0082 and *tceA* DET0079 was 41%, in *tceA* and *tceB* genes was 47%, and Dhc-TE1 (DET0076) was 46%.

Table 4.3. Percent identity among *tceA* nucleotide sequences from several *Dehalococcoides*-containing cultures. Identity is reported as percentage on DNA level, identity was calculated on the protein level also and results for DNA and translated proteins are very similar. Calculated identity values are based on nucleotide positions 100 to 1,662, relative to *tceA* start. Matrix was generated using bioedit program using Blosum 62 similarity matrix.

	Owls	**GSI**	**CH**	**195**	**FMC**	**SFR**	**FL2**	**YK**	**PM**	**RC**	**SNR**	**BRS**
Owls	100											
GSI	99.9	100										
CH	100	99.9	100									
195	96.8	96.7	96.8	100								
FMC	97.9	97.8	97.9	95.3	100							
SFR	96.9	96.9	96.9	99.4	95.6	100						
FL2	96.9	96.8	96.9	99.4	95.5	99.9	100					
YK	96.3	96.3	96.3	98.1	96.3	98.2	98.2	100				
PM	96.9	96.8	96.9	99.4	95.5	99.9	99.8	98.2	100			
RC	96.8	96.7	96.8	99.3	95.5	99.8	99.8	98.1	99.8	100		
SNR	96.9	96.9	96.9	99.4	95.6	100	99.9	98.2	99.9	99.8	100	
BRS	97.2	97.2	97.2	97.2	97.7	97.2	97.2	97.9	97.2	97.1	97.2	100

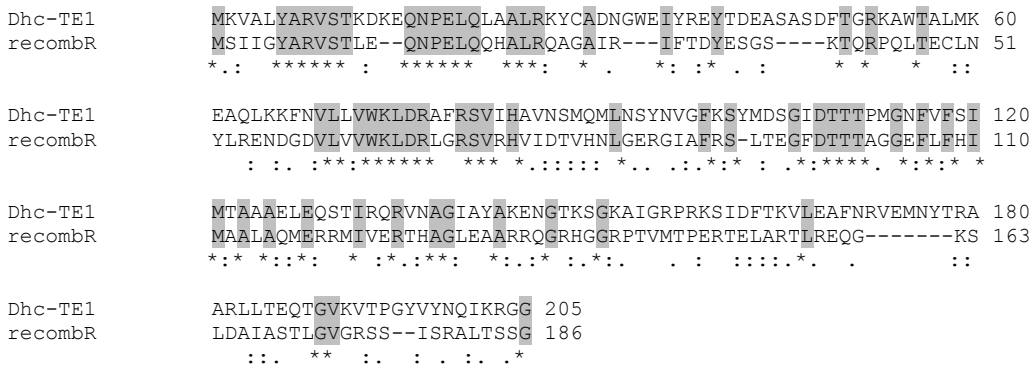

Figure 4.2. Dhc-TE1 Alignment. Alignment was generated using translated amino acid Dhc-TE1 sequence and the closest BLASTX match. The complete proteins were used in the alignment. Clustal W was used to generate alignments.
recombR: site specific recombinase [*Rhodococcus rhodochrous*] (Accession number AAC15837)

4.4. Discussion

The *tceB* genes sequenced in this study were 100% identical in all cultures. TceB has been hypothesized to be a transmembrane protein which function is to act as an anchoring protein by attaching the TceA to the periplasmic membrane (Magnuson *et al.*, 2000). The required conserved regions in an anchoring protein would be hydrophobic regions and a site for interaction with the protein to be attached. If TceB has only an anchoring function, why is it so conserved? One hypothesis is that TceB has a function other than just anchoring *tceA* to the periplasmic membrane. The second hypothesis is that *tceA* and *tceB* have been recently transferred and insufficient time has elapsed after the transfer to allow genetic variation.

Two of the cultures where *tceA* and *tceB* were detected i.e., PM and RC had been enriched using VC as electron acceptor. It was surprising to find the *tceA* gene in cultures where respiration occurred at the expense of VC, since TceA is not responsible for VC dechlorination and was not present in *Dehalococcoides* strain BAV1 (He *et al.*, 2003a), the only pure culture able to grow on VC as a sole electron acceptor. One explanation for this result is that most likely there are also other reductive dehalogenase genes present in the PM and RC VC-enriched cultures, which are responsible of the reduction of VC to ethene.

The *tceA* and *tceB* genes were not detected in soil or subsurface water samples in this study. Failing to amplify a gene might be due to a detection limit artifact or due to high specificity of the primers used. The *tceA* and *tceB* genes were not detected in soil samples from the FMC site. However, when FMC soil samples were enriched in the laboratory, the *tceA* and *tceB* genes were successfully amplified. Environmental samples

need to be enriched for feasible detection of *tceA* and *tceB* genes with PCR using the reported primers (Magnuson *et al.*, 2000); this observation strengthens the hypothesis that *tceA* was not detected in environmental samples due to a detection limit problem.

The *tceA* gene was not detected in culture H10 (Sung 2005, personal communication). H10 is a *Dehalococcoides* culture able to dechlorinate TCE *cis*-DCE and VC. Therefore, other TCE-reductive dehalogenase genes must be present in H10. Lack of detection of *tceA* for this case suggested that the primers used were very specific and did not target a variety of TCE reductive dehalogenase genes. The design of primers was based on *Dehalococcoides* ethenogenes sequence and, because of that, only TCE reductive dehalogenase genes that share a high similarity with *Dehalococcoides* ethenogenes *tceA* were amplified.

The *tceA* gene was present in a PCE enriched culture (Mike Aiello, Thesis), which was established with the same inoculum used to establish enrichment cultures with *cis*-DCE and VC as electron acceptors. The *tceA* gene was detected in the PCE-enriched reactor (Mike Aiello Thesis), however, the *tceA* gene was not detected in the *cis*-DCE and VC enriched cultures. The *tceA* gene was also absent in *Dehalococcoides* strain BAV1, which was isolated from the BRS VC-enrichment culture. The change in presence of *tceA* occurred from a mixed to a pure culture. On the other hand, the *tceA* gene was detected when the RC culture was enriched with VC and it was not detected when the culture was enriched in 1,2 dichloropropane (1,2D) (Ritalahti and Löffler, 2004). There are two possibilities for the change in detection of *tceA*. The first possibility is that enrichment with VC exerted environmental pressure on BAV1 and as a result BAV1 lost the *tceA* gene. This hypothesis is weakened by the fact that the *tceA*

gene was detected in two cultures enriched in VC (RC and PM), which indicates that enrichment in VC would not cause a loss in *tceA*. The second possibility is that multiple *Dehalococcoides* populations were present in the initial BRS inoculum, and the *Dehalococcoides* strain that contained the *tceA* gene was lost upon enrichment. The possibility of multiple *Dehalococcoides* populations is more likely. A similar case has been reported for culture KB-1, where one of the *Dehalococcoides* sp. present was lost upon enrichment in VC (Duhamel *et al.*, 2004).

TceA identity was conserved despite geographic locations and phylogenetic groups. *Dehalococcoides ethenogenes* strain 195 isolated in New York belongs to the *Dehalococcoides* Cornell group and *Dehalococcoides* sp. strain FL2 isolated in Michigan belongs to the *Dehalococcoides* Pinellas group. The *tceA* gene in these two microorganisms is 99% identical and the *tceB* gene is 100% identical. Also, *tceA* detection did not correlate with 16S rRNA gene sequences grouping. *Dehalococcoides* spp. in the cultures PM, RC, AuS, strain FL2 and strain CBDB1, all have an identical 16S rRNA gene sequence. However, *tceA* was detected in cultures PM, RC and in *Dehalococcoides* sp. strain FL2; and it was not detected in the Aus cultures and *Dehalococcoides* sp. strain CBDB1 (Hölscher *et al.*, 2004).

Conservation of identity downstream of *tceAB* in the genomic sequences of the cultures where *tceA* was present was verified by using primers targeting positions downstream the *tceAB* genes. Primer 4012R was used because the primer is located internal to Dhc-TE1 (Figure4.1.). Primer 4012R produced an amplicon with all cultures except Owls, CH and GSI. The sequence identity in these three cultures was most likely interrupted before Dhc-T1, and that is why no amplification was achieved. These results

demonstrate that, at least for these three cultures, *tceA* might be present in a different position in the chromosomal DNA. Furthermore, primer DTOR, which is located downstream of Dhc-T1, generated an amplicon only when DNA from *Dehalococcoides ethenogenes* 195, FMC, Sangamon and YK cultures was used as , suggesting that in *Dehalococcoides* sp. strain FL2, PM, RC, and Saltfork cultures, the *tceAB* genes are present in a different location in the chromosomal DNA. We suggest two possible explanations for this diverse chromosomal conformation. One hypothesis is that Dhc-T1 was involved in gene transfer from another microorganism to *Dehalococcoides* and *tceAB* got integrated into the chromosome in a different position. The second hypothesis is that Dhc-T1 has been involved in shuffling *tceAB* to different positions in the chromosome.

Dhc-TE1 has the average size of a typical TE (a site specific recombinase) and was also recognized by the protein families database pFAM (Bateman *et al.*, 2004) as having a typical transposase domain i.e., a resolvase N terminal domain. Dhc-TE1 was 40% identical to a putative site specific recombinase in *Rhodococcus rhodochrous* (Kulakov *et al.*, 1999). *Rhodococcus rhodochrous* degrades haloalkanes. The haloalkane dehalogenase in *Rhodococcus rhodochrous* is located on a plasmid (Kulakova *et al.*, 1997). The insertion sequence connected to the dehalogenase has been detected in other haloalkane-degrading *Rhodococcus* strains (Kulakov *et al.*, 1999) showing that it has the capability of transferring itself. Additionally, three putative TEs (DET0082, DET0083, DET0084) are present upstream of tceA. One of these i.e., DET0083 ISDet1 transposase, was annotated as a transposase and is most similar to insertion sequences from the IS3 family. DET0084 was annotated as a hypothetical protein. However, the closest matches

to DET0084 are transposable elements. DET0082 was annotated as a transposase with a true frame shift and the closest matches were integrases. The presence of TE gene remnants adjacent to *tceA* suggested earlier horizontal gene transfer events. Regions adjacent to genes that have been horizontally transferred usually contain parts of the sequences affecting their integration, such as remnants of transposable elements (Ochman *et al.*, 2000; Dutta and Pan, 2002).

A difference in GC content between chromosomal DNA and a specific gene is an indication of horizontal gene transfer. The difference in GC content between the *tceAB* genes, the TEs and the rest of the genome, is another line of evidence that *tceA* and *tceB* were horizontally transferred.

The acquisition of *tceA* and *tceB* genes through horizontal gene transfer was investigated and the following evidence was retrieved: (i) transposition elements were found surrounding the *tceA* and *tceB* genes; (ii) sequence identity between the analyzed *Dehalococcoides* cultures was interrupted before or after the Dhc-TE1; (iii) *tceB* sequences retrieved in various geographic locations were identical to each other; (iv) *Dehalococcoides* spp. from diverse *Dehalococcoides* groups (Hendrickson *et al.*, 2002) share high *tceA* and *tceB* identity; (v) the GC content in the TEs is different from the average GC content in the rest of the genome. The ability to acquire new genetic information provides microorganisms with environmental advantages, and could explain why *Dehalococcoides* are widely distributed in the environment despite their specific growth requirements, and why they have such a diverse dehalogenating capacity. Also, the genomic information presented in this study could be helpful for understanding anthropogenic influence in genetic changes.

4.5. References

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CHAPTER V

PUTATIVE REDUCTIVE DEHALOGENASE GENES IN THE *DEHALOCOCCOIDES ETHENOGENES* STRAIN 195 GENOME

5.1. Introduction

Chapter IV demonstrated that the presence of the *tceA* gene (Magnuson *et al.,* 2000) is not directly correlated with the microorganisms' capability of reductively dechlorinating VC in a metabolic fashion. Since the only *Dehalococcoides* reductive dehalogenase available at the time was *tceA*, common features in reductive dehalogenases from other chlororespiring microorganisms were analyzed and used to identify additional reductive dehalogenase genes in the *Dehalococcoides ethenogenes* genome. The information gathered was then used to design degenerate primers, with the objective of identifying reductive dehalogenase genes involved in metabolic reductive dechlorination of VC.

RDase proteins from chlororespiring microorganisms have been purified and the coding genes have been recognized (Neumann *et al.,* 1998; van de Pas *et al.,* 1999; Suyama *et al.,* 2002; Maillard *et al.,* 2003). Alignment of RDase proteins revealed certain conserved sequence information: a twin arginine (RR) motif RR**X**F**X**K near the N terminus of the protein and two iron-sulfur binding motifs Fe_4S_4 [**C**XX**C**XX**C**XXX**CP**] close to the C terminus. Characterized motifs in protein sequences available at the time this work was started are summarized in Table 5.1. The RR motif is a signal peptide and part of the Tat export system which is involved in translocating the protein through the membrane (Berks *et al.,* 2000). The iron sulfur binding motifs are characteristic of

ferredoxin proteins involved in electron transfer (Beinert, 2000). Some RDase proteins contain one Fe_3S_4 and one Fe_4S_4 (Maillard *et al.*, 2003). In addition, a gene coding for a small hydrophobic protein (B gene) has been associated with the RDase genes. The translated B protein contains two or three transmembrane regions (Neumann *et al.*, 1998; van de Pas *et al.*, 1999; Magnuson *et al.*, 2000). The function of the B protein has not been proven at the protein level. However, it has been speculated that it acts as a membrane anchor for the RDase proteins.

Table 5.1. Common motifs in characterized RDases.

Organism	**RDase protein Genbank Accession number**	**Twin arginine motif**	**Fe_4S_4 or Fe_3S_4 binding motifs**
Dehalococcoides ethenogenes	TceA	RRDFMK	CKTCGICAEHCP
	AAF73916		CINCTICEAVCP
Sulfurospirillum multivorans	PceA	RRDFGK	CETCKKCARECP
	AF022812		GGYCGVCVAVCP
Desulfitobacterium sp. strain Y51	PceAb	RRNFLK	CRLCKKCADACP
	BAC00916		GSPCSNCVAVCS
Desulfitobacterium dehalogenans	CprAd	RRSFLK	CRVCKKCADNCP
	AAD44542		GSSCGTCLKVCP
Desulfitobacterium hafniense	CprAh	RRSFLK	CRVCKKCADNCP
	AAL87763		GSSCGTCMKVCP

Conserved Reductive dehalogenase motifs are indicated in gray shading.

TceA, trichloro dehalogenase [*Dehalococcoides ethenogenes* 195] (Magnuson *et al.,* 2000)
PceA, tetrachloroethene dehalogenase [*Dehalospirillum multivorans*] (Neumann *et al.,* 1998)
PceAb, tetrachloroethene dehalogenase [*Desulfitobacterium* sp. Y51]. (Suyama *et al.,* 2002)
CprAd o-chlorophenol dehalogenase [*Desulfitobacterium dehalogenans*] (van de Pas *et al.,* 1999)
CprAh reductive dehalogenase [*Desulfitobacterium hafniense*].

Because of their potential use in bioremediation, three chlororespiring microorganisms, *Dehalococcoides ethenogenes* strain 195, *Desulfitobacterium hafniense* strain DCB-2, and *Anaeromyxobacter dehalogenans* strain 2CP have been completely sequenced. The genomic information of *Dehalococcoides ethenogenes* strain 195, *Anaeromyxobacter dehalogenans* strain 2CP and *Desulfitobacterium hafniense* strain DCB-2 are available in public databases. *Dehalococcoides ethenogenes* strain 195, a strict hydrogenotrophic chlororespiring microorganism was sequenced by the institute of genomic research (TIGR) (www.tigr.org); *Desulfitobacterium hafniense* strain DCB-2 and *Anaeromyxobacter dehalogenans* were sequenced by the joint genome institute (JGI) (http://www.jgi.doe.gov/). The main objectives of this work were to screen the *Dehalococcoides ethenogenes* strain 195 genome for the presence of putative reductive dehalogenase genes and to identify conserved regions in corresponding translated proteins for degenerate primer design. The *Desulfitobacterium hafniense* strain DCB-2 genome and the *Anaeromyxobacter dehalogenans* genomes were also screened for the presence of putative RDase genes. Features shared among the putative RDase genes and translated proteins in the three genomes were analyzed.

5.2. Materials and methods

5.2.1. Screening for putative RDase genes

Genes encoding for reductive dehalogenases in the genomes of *Dehalococcoides ethenogenes* strain 195 were identified through the use of *in silico* biology. Exhaustive BLAST searches were performed through the sequence database for microbial genomes (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/genom_table_cgi) and unfinished microbial genomes (http://www.tigr.org) using the TceA (Magnuson *et al.,* 2000), CprA (van de Pas *et al.,* 1999), and PceA (Neumann *et al.,* 1998; Suyama *et al.,* 2002) protein sequences as query. With the use of frameplot, a gene finding program (Ishikawa and Hotta, 1999), the coding gene and the presence of a complete protein were verified. When the protein was not complete, the obtained DNA sequence was extended until a complete translated protein was found. The complete protein was BLASTed against non-redundant databases (http://www.ncbi.nlm.nih.gov/BLAST/) to verify similarity to previously described RDases. Translated protein sequences that had significant similarity to RDase proteins were considered for further analysis. For comparison purposes, the same procedure was applied using the genome of *Desulfitobacterium hafniense* strain DCB-2 (http://genome.jgi-psf.org/draft_microbes/desha/desha.home.html) and for *Anaeromyxobacter dehalogenans* (http://genome.jgi-psf.org/draft_microbes/anade/anade.home.html) The procedure followed to identify putative RDase genes in *Anaeromyxobacter dehalogenans* is described in section 5.2.5. A different method was followed given that the *Anaeromyxobacter dehalogenans* genome had been annotated prior to this analysis.

5.2.2. Recognition of motifs and comparison of translated RDases

The retrieved RDase genes were translated into proteins and visually analyzed for conserved characteristic motifs. The program signalP (Nielsen *et al.,* 1997) was used to identify signal peptide motifs and cleavage sites. The protein families database Pfam (Bateman *et al.,* 2004) was used to identify previously characterized conserved domains.

Multiple alignments of dehalogenase genes and translated putative dehalogenase proteins were built using the clustalW or clustalX program (Thompson *et al.,* 1997), and an identity matrix was created using bioedit program. The matrix was built with BLOSUM 62.

5.2.3. Search for putative B genes

In order to evaluate the presence of putative B genes adjacent to RDase encoding genes, the available sequence information for each RDase gene was extended both upstream and downstream. Adjacent genes of approximately 300 bp size were translated into proteins. The translated putative B proteins were blasted against non-redundant databases. The "DAS" Transmembrane Prediction server (Cserzo *et al.,* 1997) was used for analysis of transmembrane regions, and hydrophobicity plots were created for each protein. Alignments of the B genes and translated proteins found in the genome of *Dehalococcoides ethenogenes* were performed using clustalX and ClustalW.

5.2.4. Degenerate primer design

Conserved regions were identified in putative RDase and B translated proteins found in the *Dehalococcoides ethenogenes* genome. Degenerate primer design was performed using protein and DNA alignments simultaneously. Primer design was completed without the use of primer design programs. Once the conserved amino acids were identified, translation codons were retrieved, and possibilities for DNA sequences and their corresponding degeneracy were assessed. In the cases where arginine (R) was one of the amino acids targeted for primer design, the task became more challenging given that six different combinations of nucleotide triplets encode for R. Nucleotide alignments of the putative RDase genes helped identify the nucleotide triplets that are most commonly used in *Dehalococcoides ethenogenes*.

5.2.5. RDase genes in Anaeromyxobacter dehalogenans genome

In order to find putative RDase genes in the *Anaeromyxobacter dehalogenans* genome, RDase proteins were blasted (blastp) against *Anaeromyxobacter dehalogenans* genome. RDase proteins blasted were the following:

PceA (AF022812) PCE reductive dehalogenase (*Sulfurospirillum multivorans*), PceAb (BAC00916) PCE reductive dehalogenase (*Desulfitobacterium* sp. Y51), PceAc (AJ439607) PCE reductive dehalogenase (*Dehalobacter restrictus*), PceAd (AJ439608) PCE reductive dehalogenase (*Desulfitobacterium frappieri*), PceC (CAC37919) PCE reductive dehalogenase (*Clostridium bifermentans*), CprAd (AAD44542) O-chlorophenol dehalogenase precursor (*Desulfitobacterium dehalogenans)*, CprAc (AAL84925) O-chlorophenol dehalogenase (*Desulfitobacterium chlororespirans)*, CprAv (AF259791) O-

chlorophenol reductivedehalogenase (*Desulfitobacterium* sp.Viet-1), TceA (AAF73916) TCE reductive dehalogenase *Dehalococcoides ethenogenes,* BvcA (AY563562) VC reductive dehalogenase *Dehalococcoides* sp. strain BAV1, and VcrA (AY322364) VC reductive dehalogenase *Dehalococcoides* sp. strain VS. The resulting hits were blasted against the genome to achieve an exhaustive blast search.

5.3. Results

5.3.1. Putative RDase genes in the Dehalococcoides ethenogenes genome

Seventeen gene sequences that encoded proteins significantly similar to known RDase proteins were retrieved. Two conserved regions were identified in all the translated RDase proteins. The typical twin arginine (RR) motif **RR**X**F**X**K** was present near the N-terminal end of all proteins with the exception of RDA3 and RDA4 (Figure 5.1.). In RDA3, a K (Lysine) was present instead of an R (arginine) in the position of the second arginine of the RR motif. In RDA4, the last K in the RR motif was not present, instead of the typical **RR**X**F**X**K** motif, **RK**D**F**L**K** and S**RR**D**F**L were the sequences present. At least one characteristic iron sulfur-binding motif **C**XX**C**XX**C**XXX**CP** was present in all the putative RDases. In some of the translated RDase proteins, two iron sulfur cluster-binding motifs were present, and in others a very similar motif was present in the position of the second iron sulfur-binding motif. The differences present in the second iron sulfur binding motifs are summarized in Table 5.2. and presented in figure 5.1. In some of the translated proteins, the second motif had a valine (V) or threonine (T) instead of the characteristic proline (P), following the last cysteine (C) in the motif, i.e. CP. The positions of the cysteins throughout the motif were conserved in most cases. However, the C that is most distant from the P was not always present in the same position.

Table 5.2. Iron sulfur clusters and twin arginine motifs in putative RDases.

RDase	**Size** aa	**RR motif** **RRXFXK**	**1st Iron sulfur binding motif** **CXXCXXCXXXCP**	**2nd Iron sulfur binding motifs** **CXXCXXCXXXCP**
TceA	554	T**RR**D**F**M**K**	**C**KT**C**GI**C**AEH**CP***	**C**IN**C**TI**C**EAV**CP**
RDA13	514	S**RR**D**F**M**K**	**C**HT**C**RK**C**AEA**CP***	C(X)$_9$**C**AR**C**MGT**C**X*
RDA1	469	S**RR**D**F**M**K**	**C**ET**C**GV**C**GTQ**CP***	**C**MGN**C**CS**C**MGA**CP**
RDA2	505	S**RR**D**F**M**K**	**C**ET**C**GI**C**AET**CP**	**C**AFTP**C**ASA**C**KSN**CP**
RDA10	500	S**RR**D**F**M**K**	**C**HS**C**QK**C**ADH**CP**	**C**(X)$_{10}$**C**RI**C**WGE**C**X*
RDA5	507	S**RR**D**F**M**K**	**C**ET**C**GI**C**ADA**CP**	**C**PH**C**PT**C**QGT**CP**
RDA4	505	S**RR**D**F**LN	**C**KT**C**KI**C**AEA**CP**	**C**PH**C**PV**C**QGT**CP**
RDA14	510	S**RR**D**F**M**K**	**C**HT**C**RK**C**AEA**CP**	**C**(X)$_{10}$**C**AR**C**MGT**C**V*
RDA12	510	T**RR**D**F**M**K**	**C**HT**C**HK**C**ADE**CP**	**C**(X)$_{10}$**C**GV**C**MAT**C**T*
RDA11	492	S**RR**D**F**M**K**	**C**HS**C**RK**C**ADT**CP**	**C**(X)$_{10}$**C**QI**C**TGV**C**V*
RDA6	494	S**RR**D**F**M**K**	**C**QT**C**GI**C**ADS**CP**	**C**PH**C**PV**C**QGS**CP**
RDA8	482	S**RR**D**F**M**K**	**C**IT**C**KK**C**ADL**CP**	C(X)$_{10}$**C**GV**C**QAV**C**V*
RDA7	515	S**RR**D**F**M**K**	**C**RD**C**GL**C**AKA**CP**	**C**(X)$_{11}$**C**AA**C**VAS**C**V*
RDA9	490	S**RR**D**F**M**K**	**C**TTA**C**KK**C**AEF**CP**	**C**LTDTF**C**GI**C**MGE**C**V*
RDA3	495	T**R**K**D**FL**K**	**C**ET**C**GI**C**AEN**CP**	**C**PH**C**PI**C**QGT**CP**
RDA15	486	S**RR**D**F**M**K**	**C**ST**C**GV**C**ANA**CP**	**C**YR**C**AAA**C**V*
RDA17	532	N**RR**D**F**L**K**	**C**SR**C**KL**C**AQV**CP**	**C**(X)$_{12}$**C**RA**C**ISV**CP***
RDA16	455	N**RR**D**F**V**K**	**C**KV**C**KK**C**ADN**CP**	**C**(X)$_8$**C**SK**C**QSV**CP***

Characteristic motifs are indicated with bold letters. Positions where the characteristic motif was not the expected are shaded. * Indicates that Pfam did not recognize the motif as a Pfam:fer4 conserved domain. The nomenclature used for *Dehalococcoides* putative RDases was adopted from Villemur *et al*. (2002)

5.3.2. Recognition of conserved motifs

The RRXFXK motif was recognized as a signal peptide using the program signalP. A signal peptide was also identified in a similar position to the rest of the translated RDases in translated proteins RDA3 and RDA4, both of which had a different **RR**X**F**X**K** motif. All analyzed RDase proteins, except TceA, have the predicted cleavage site between the amino acid in position 30 and the amino acid in position 50.

The first iron sulfur-binding motif **C**XX**C**XX**C**XXX**CP** was recognized by Pfam as a Pfam:fer4 domain in all translated proteins. When the second motif was slightly different, it was not recognized as a Pfam:fer4 (table 5.2). However, not being recognized by Pfam does not mean that it is not an iron-sulfur binding motif since the **G**XX**C**XX**C**XXX**CP** domain was previously recognized as a truncated iron sulfur binding motif for 3fe-4S (van de Pas *et al.,* 1999), and is not recognized by Pfam as a conserved domain.

Degenerate primers targeting conserved regions were designed through the use of multiple alignments of the retrieved RDase genes and translated proteins. Amino acids used for degenerate primer design are indicated in figures 5.1.and 5.2. In addition to the conserved regions in the putative RDase genes, a conserved region in the associated B gene was also targeted. Degenerate primers designed in this study are summarized in table 5.3.

5.3.3. Presence of putative B genes

A gene encoding for a small protein of about 90-100 amino acids long (putative B gene), was present next to all the putative RDase genes in the *Dehalococcoides ethenogenes* and *Desulfitobacterium hafniense* genomes. A gene with similar characteristics was not detected next to the putative RDases in the genome of *Anaeromyxobacter dehalogenans*.

The gene encoding for the putative B protein in *Desulfitobacterium hafniense* was found upstream in all the cases with the exception of Gene 1880 in contig 1065 where the putative B gene, i.e. gene 1881 is located downstream of the catabolic gene. On the other hand, in *Dehalococcoides ethenogenes,* the gene encoding for the putative B protein was always downstream of the RDase gene. The translated putative B proteins in *Dehalococcoides ethenogenes* had no significant similarity with any protein in the database, with the exception of the protein following RDA6, which is highly similar to the tetrachloroethene reductive dehalogenase membrane-bound subunit in *Dehalospirillum multivorans* (genbank accession number AAC60789). The translated putative B proteins in *Desulfitobacterium hafniense* were similar to previously sequenced B proteins in all the cases. A hydrophobicity analysis of these putative B proteins using DAS suggested that all analyzed B proteins except the B protein associated to RDA15 contained three transmembrane regions, and the hydrophobicity plots presented a similar profile to the hydrophobicity plot for TceB in all the cases (Figure 5.2.). The B protein encoded by the gene downstream of RDA15 had two transmembrane regions and was similar to PceB in *Sulfurosporillum multivorans*.

An alignment of translated B proteins found in the *Dehalococcoides ethenogenes* genome, excluding RDA15, is shown in figure 5.3. RDA15 was initially included in the alignment; however, since it is so different to the remaining proteins, a better alignment was obtained when this protein was excluded. A conserved motif containing two tryptophan (W) amino acids was identified and further used for degenerate primer design. Also, transmembrane predictions were performed; the dual tryptophan motif was always present in the hypothetical external part of the periplasmic membrane. The position of the tryptophan-containing motif is represented in figure 5.2.

5.3.4. RDase genes in Anaeromyxobacter dehalogenans genome

All blasted RDase proteins except PCEc (CAC37919) yielded two genes as a hit, gene 3604 (Ana_3604) and gene 3602 (Ana_3602). A third hit, i.e., gene 1639 (Ana_1639) was also obtained when the following RDase proteins were blasted: PceAc (AJ439607), PceAd (AJ439608), and CprAV (AF259791). No hits resulted from blasting PCEc (CAC37919).

Ana_3604 and Ana_3602 are located in contig 94, Ana_1639 is located in contig 76. No RR motif was present in these three genes. However, Pfam identified a signal peptide in the beginning of translated proteins Ana_3604 and Ana_3602 (Table 5.4). To verify that the RR motif was really absent and that the predicted signal peptide was actually in the beginning of the translated protein, the annotation of the genes was verified using frameplot, a gene-finding program. The annotation proved to be correct, and the absence of the RR motif was verified. Transmembrane regions were identified

by Pfam in Ana_3604 and Ana_3602 (table 5.4). The presence of a B gene was also investigated, and no adjacent genes that resemble a B gene were present.

Since no associated B gene was found next to any of the three encoding genes, the presence of transmembrane regions and absence of a signal peptide could be expected. Two typical iron sulfur-binding motifs were present in Ana_3602, and they were both recognized by Pfam as Pfam:fer4 conserved domains (Figure 5.4.). The first iron sulfur-binding motif was also present in the typical form in Ana_1639 and in Ana_3604 and was also recognized by Pfam as Pfam:fer4 Pfam did not recognize the second putative iron sulfur-binding motif in Ana_1639 and in Ana_3604.

No Cobalamin binding site motifs i.e., DXHXXG. . .SXL. . .GG, which are typical vitamin B12-dependent mutases and methionine synthases (Marsh and Holloway, 1992) were present in any of the three translated proteins.

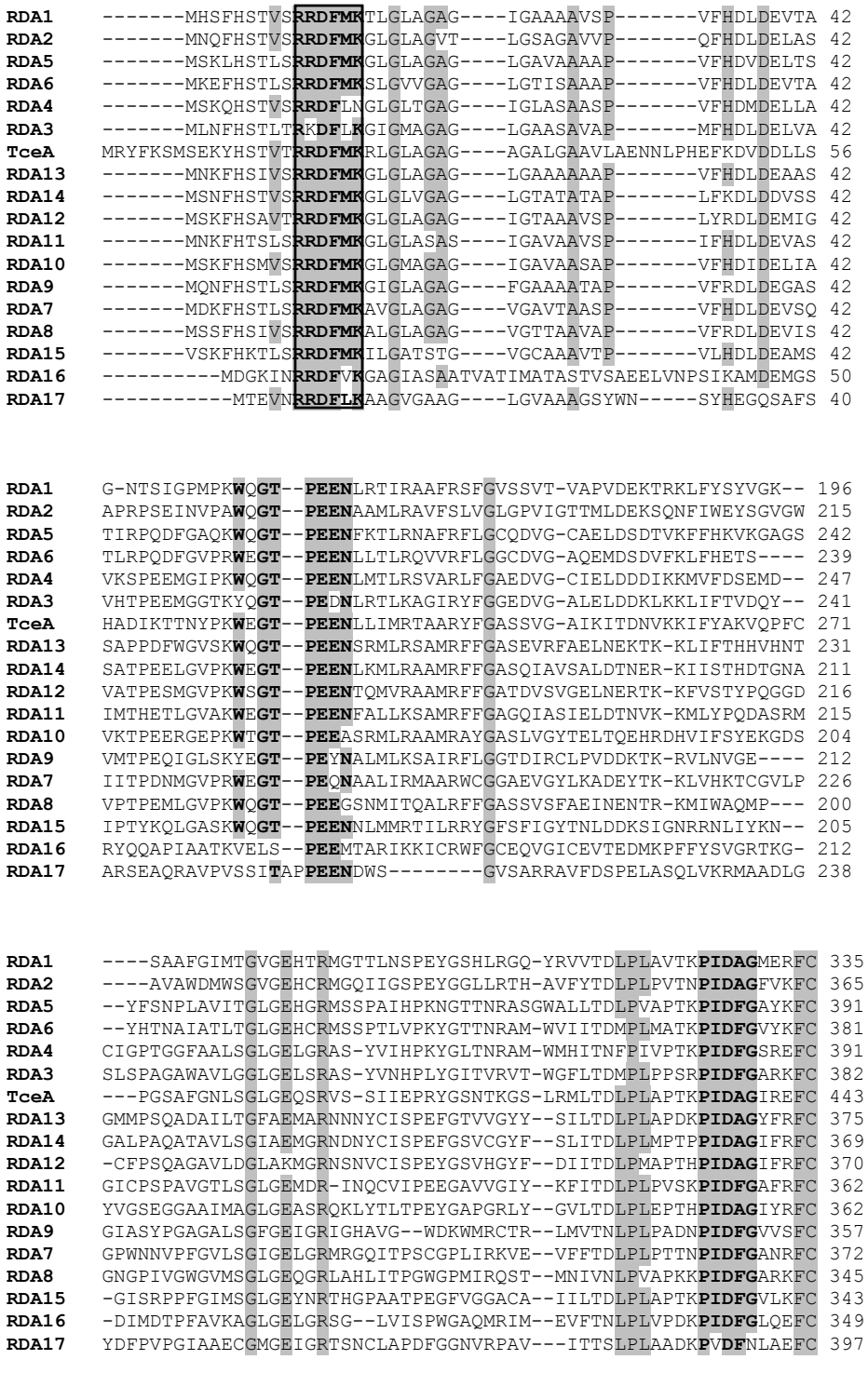


Figure 5.1. Alignment of translated putative RDases identified in the *Dehalococcoides ethenogenes* genome. Gray boxes indicate conserved regions. Amino acids used for degenerate primer design are indicated in bold. Iron sulfur binding motifs and the characteristic RR motif are indicated in boxes.

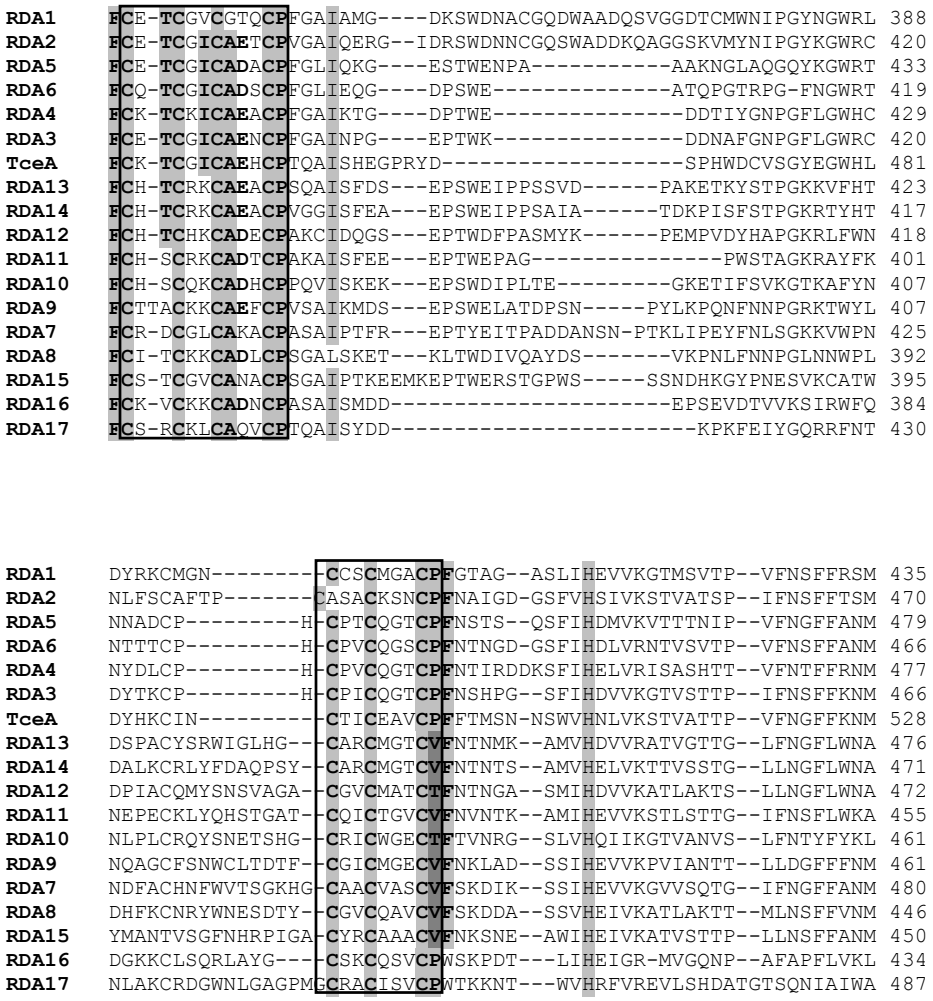
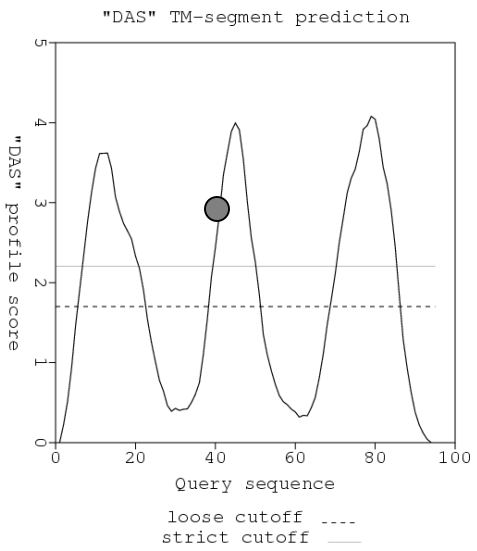
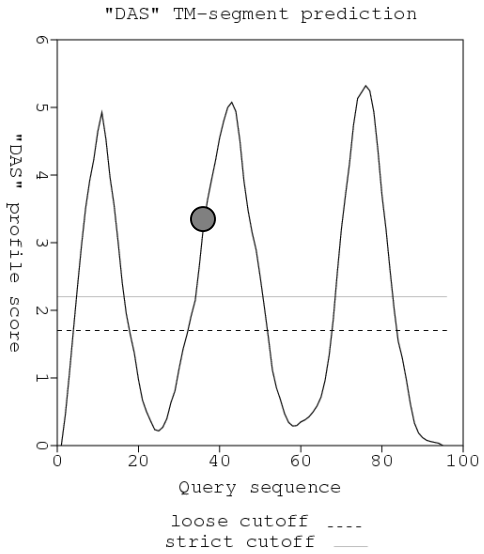


Figure 5.1. Alignment of translated putative RDases identified in the *Dehalococcoides ethenogenes* genome, continuation.

A)



B)



C)

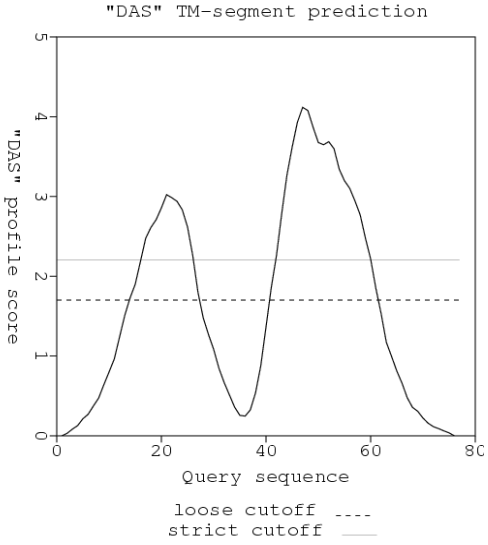


Figure 5.2. Hydrophobicity plots of TceB and translated putative B proteins. Hydrophobicity plots and transmembrane regions were estimated using "DAS." Three transmembrane regions were estimated for all putative B proteins in the *D. ethenogenes* genome, with the exception of the B protein associated to RDA15. A) TceB hydrophobicity plot. B) Hydrophobicity plot of translated protein following RDA16 (RDA16B) is a representative sample of all putative B proteins found in the *D. ethenogenes* genome. C) Hydrophobicity plot of translated protein following RDA15B. Only two transmembrane regions were present. Gray circles represent the position of a conserved motif containing two W (tryptoptophan aminoacids).

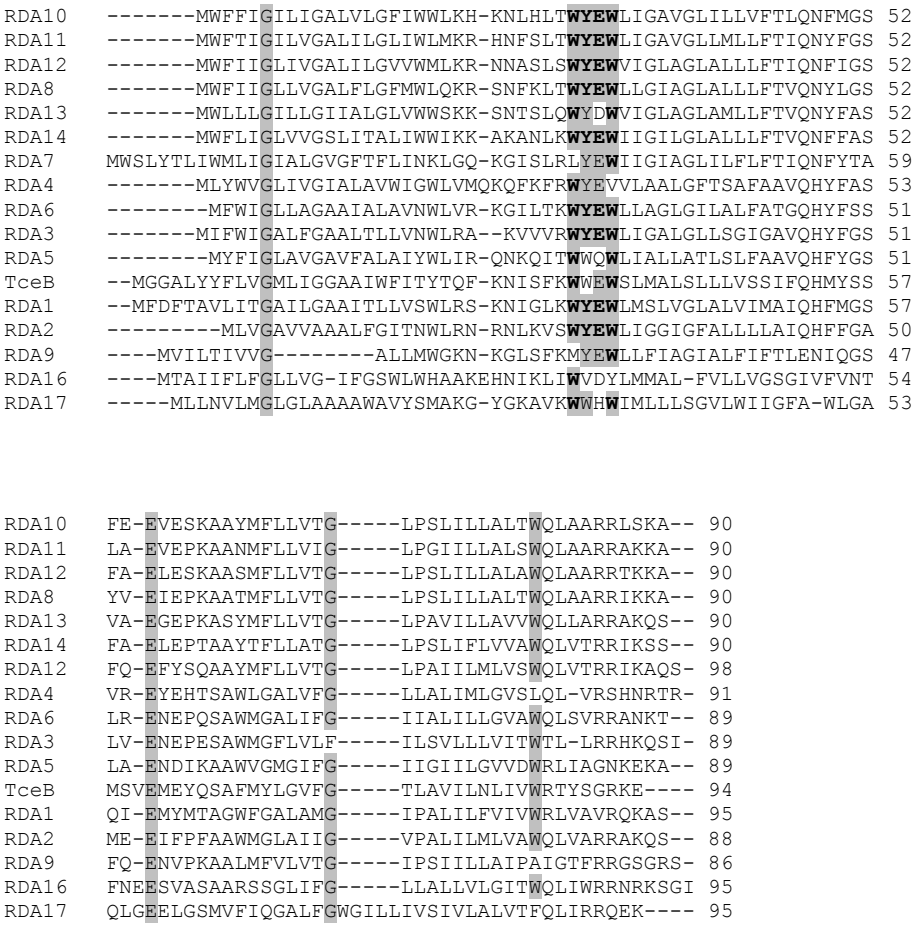


Figure 5.3. Alignment of translated putative B proteins in *Dehalococcoides ethenogenes*. A conserved motif containing two tryptophan (W) amino acids was identified and is indicated with shaded gray boxes. Bold letters indicate amino acids used for degenerate primer design

Table 5.3. Degenerate primers designed using translated putative RDase genes identified in the *Dehalococcoides ethenogenes* genome.

Primer	Sequence	Location in the translated protein
RRF1	5'-AGR MGD GAY TTY ATG AAR RSV-3'	RRXF
RRF2	5'-SHM GBM GWG ATT TYA TGA ARR S-3'	RRXF
GTPE_1_F	5'-MVTGGSAGGGWACWCCDG-3'	WXGTPEEN
GTPE_2_F	5'-GGSARGGBACDCCHGA-3'	WXGTPEEN
GTPE_3_F	5'-TGGDMBGGTACKCCYGA-3'	WXGTPEEN
PID_1_R	5'-RRDHVCCSRMATCTATAGGY-3'	PIDxG
PID_2_R	5'-CCGGCATCTATVGGNTKRG-3'	PIDxG
PID_3_R	5'-DRYMCCRRMRMRTCTATVGG-3'	PIDxG
PID_1_F	5'-CYMANCCBATAGATGCCGG-3'	PIDxG
CP_1a_R	5'-GRCANBBNKYNSCGCA-3'	First Iron-sulfur
CP_1b_R	5'-YCVKSACAVDTNBSGCTG-3'	First Iron-sulfur
CP_1c_R	5'-RCARNBNYYVKVKSRCAK-3'	First Iron-sulfur
CP_2_R	5'-GRCASVYRSCCWBRCA-3'	Second Iron-sulfur
B1R	5'-CHADHAGCCAYTCRTACCA-3'	B protein WYEW
B2R	5'-CCDATDAYCCAKTCRTACCA-3'	B protein WXXW
B3R	5'- RDB RRC CAT TVY CAC CA -3'	B protein WXXW

This chapter adapted the nomenclature proposed by Villemur *et al*. (2002) for easy comparative purposes, despite the fact that this was not the initial nomenclature assigned to these genes in this work. The different names that have been assigned to the reductive dehalogenase genes in the *Dehalococcoides ethenogenes* genome are indicated in appendix A. Appendix A includes the names originally assigned in this work, the nomenclature assigned by (Villemur *et al.,* 2002), and the recently published nomenclature corresponding to the annotated genome.

Table 5.4. Characteristics of putative reductive dehalogenase genes found in the *Anaeromyxobacter dehalogenans genome*

	size	1st Iron sulfur	2nd Iron sulfur	Pfam
Ana_1639	343	CGTCEACLPACP	VFGCDDCQTVCP	
Ana_3602	640	CANCDRCARACP	GNMCGACLAVCP	
Ana_3604	491	CRVCNKCADACP	NPKCMACTGACP	

Indicates transmembrane segments

 Indicates segments of low compositional complexity

 Indicates signal peptides

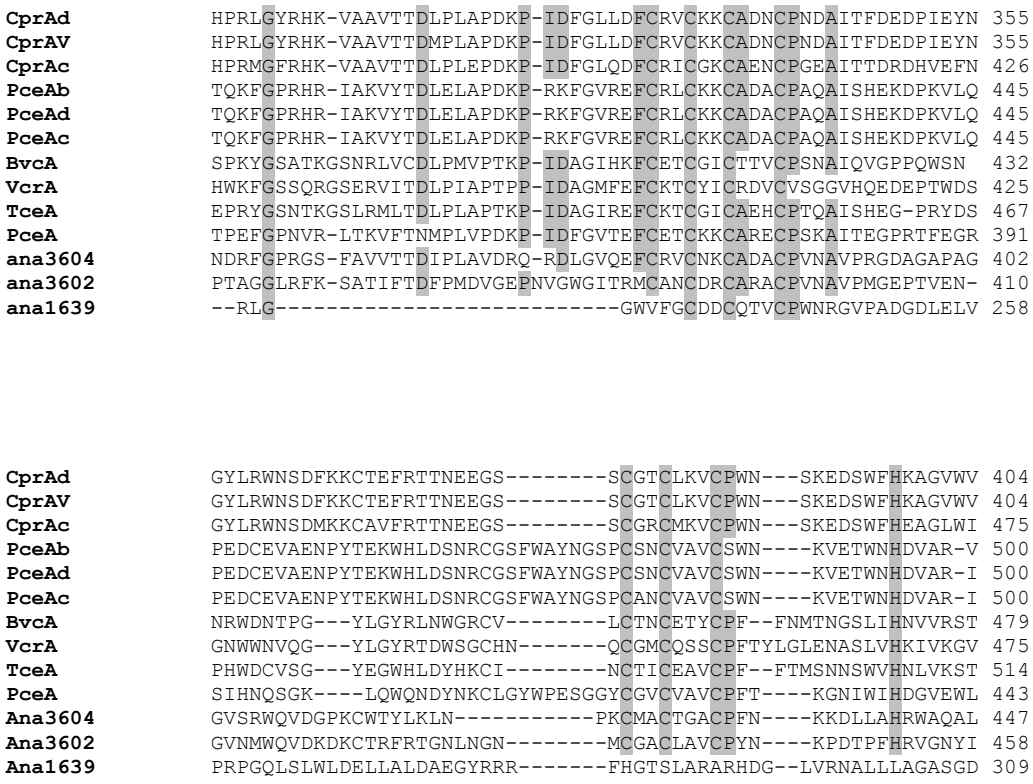


Figure 5.4. Partial alignment of translated putative RDases identified in the *Anaeromyxobacter dehalogenans* genome. Gray boxes indicate conserved regions. TceA, TCE RDase [*Dehalococcoides ethenogenes* 195] (Magnuson *et al.,* 2000). PceA, PCE dehalogenase [*Sulfusporillum multivorans*] (Neumann *et al.,* 1998). $PceA_b$ PCE dehalogenase [*Desulfitobacterium* sp. Y51]. (Suyama *et al.,* 2002). $CprA_d$ o-chlorophenol dehalogenase [*Desulfitobacterium dehalogenans*] (Smidt *et al.,* 2000). $CPRA_c$ o-chlorophenol dehalogenase [*Desulfitobacterium chlororespirans*]. $CprA_h$ reductivedehalogenase [*Desulfitobacterium hafniense*]. BvcA VC RDase [*Dehalococcoides sp.* strain BAV1]

5.4. Discussion

Seventeen putative dehalogenases were found in the genome of *Dehalococcoides ethenogenes* in addition to *tceA*. All have at least one iron sulfur cluster identical to the typical consensus motif **C**XX**C**XX**C**XXX**CP**, and some of them have two identical **C**XX**C**XX**C**XXX**CP** motifs. The difference in the second motif is usually a valine (V) or threonine (T) in the typical position of a proline (P). The fact that the second putative iron sulfur cluster motif contains a valine or threonine in the position of a proline in a few cases raises a question on the functionality of this second iron sulfur cluster. In the most recently described PCE reductive dehalogenase (PceA, BAC00916) (Suyama *et al.,* 2002), a serine (S) is present instead of a P in the same position where the T and V have been substituted.

The twin arginine motif **RR**X**F**X**K** was present in the form of RRDFMK in *tceA* and in 13 out of the 17 putative reductive dehalogenases found in *Dehalococcoides ethenogenes* strain 195. This motif is not so conserved in the previously identified dehalogenases from other reductive dechlorinating organisms suggesting that RRDFMK is a *Dehalococcoides*-characteristic signature.

Two conserved tryptophans were present in all translated putative B proteins. It has been hypothesized that the B protein anchors the dehalogenase protein to the membrane. Tryptophan is one of the amino acids, which most frequently is involved in protein-protein interactions, and when transmembrane predictions were performed, the dual tryptophan motif was always present in the hypothetical external part of the periplasmic membrane, suggesting that this is a potential interaction site for the B protein and the RDase.

RDase genes found in *Dehalococcoides ethenogenes* strain 195 are diverse; however, most of them share a higher identity to *tceA* than to other previously identified RDase proteins. Identity of the 17 RDases and TceA ranged from 28 to 54 percent (Table 5.5). Only the region containing the iron sulfur binding motifs was used for this comparison. For this case, RDA15 is 44 percent identical to TceA, despite the fact that its corresponding translated B protein is the least similar to the remainding putative B proteins found in the *Dehalococcoides ethenogenes* genome, given that it only presented two predicted transmembrane regions. RDA9 shares higher identity with PceA and even higher with CprAs than with TceA; Also, RDA17 and RDA16 share higher identity with CprA than with TceA.

The fact that a few putative dehalogenases in *Dehalococcoides ethenogenes* 195 share great similarity with CprA is surprising given the distant phylogenetic relation between *Dehalococcoides ethenogenes* 195 and *Desulfitobacterium* species. The identification of other dehalogenase coding genes in *Dehalococcoides* species is of significant importance for better primer design in order to evaluate the potential of natural attenuation and engineered bioremediation approaches.

Three putative reductive dehalogenase genes were found in the genome of *Anaeromyxobacter dehalogenans,* which differ to the RDase genes in *Dehalococcoides ethenogenes,* and neither RR signal peptide nor an associated B gene was present.

Table 5.5. Identity matrix of translated RDase proteins in *D. ethenogenes* genome and previously characterized RDases.

	RDA13	RDA1	RDA2	RDA10	RDA5	RDA4	RDA14	RDA12	RDA11	RDA6	RDA8	RDA7	RDA9	RDA3	RDA15	RDA17	RDA16	TceA	PceA	PceAb	CprAd	CprAc	CprAh
RDA13	1																						
RDA1	0.34	1																					
RDA2	0.25	0.63	1																				
RDA10	0.53	0.35	0.24	1																			
RDA5	0.42	0.48	0.47	0.30	1																		
RDA4	0.34	0.56	0.48	0.24	0.60	1																	
RDA14	0.82	0.34	0.28	0.48	0.36	0.27	1																
RDA12	0.59	0.36	0.30	0.48	0.42	0.23	0.66	1															
RDA11	0.63	0.40	0.33	0.50	0.35	0.31	0.68	0.60	1														
RDA6	0.42	0.72	0.66	0.34	0.71	0.79	0.45	0.38	0.48	1													
RDA8	0.38	0.34	0.25	0.3	0.32	0.42	0.42	0.39	0.29	0.37	1												
RDA7	0.42	0.3	0.28	0.26	0.26	0.27	0.35	0.32	0.35	0.31	0.41	1											
RDA9	0.48	0.19	0.28	0.34	0.25	0.29	0.48	0.4	0.34	0.31	0.52	0.41	1										
RDA3	0.39	0.68	0.64	0.34	0.66	0.86	0.38	0.35	0.47	0.9	0.51	0.42	0.38	1									
RDA15	0.38	0.34	0.34	0.25	0.34	0.37	0.40	0.36	0.39	0.37	0.39	0.39	0.34	0.36	1								
RDA17	0.23	0.12	0.14	0.12	0.18	0.18	0.16	0.1	0.22	0.16	0.22	0.16	0.18	0.13	0.22	1							
RDA16	0.33	0.28	0.17	0.27	0.25	0.28	0.29	0.26	0.32	0.26	0.33	0.25	0.35	0.31	0.25	0.41	1						
TceA	0.44	0.50	0.50	0.33	0.43	0.49	0.35	0.34	0.36	0.48	0.53	0.34	0.25	0.54	0.44	0.31	0.28	1					
PceA	0.22	0.22	0.17	0.20	0.20	0.22	0.22	0.28	0.28	0.20	0.37	0.25	0.26	0.24	0.28	0.30	0.28	0.35	1				
PceA$_b$	0.26	0.13	0.17	0.17	0.19	0.16	0.17	0.20	0.20	0.12	0.25	0.19	0.15	0.15	0.24	0.26	0.29	0.28	0.34	1			
CprA$_d$	0.31	0.22	0.22	0.25	0.25	0.26	0.26	0.29	0.29	0.24	0.29	0.24	0.26	0.32	0.29	0.33	0.36	0.32	0.41	0.35	1		
CprA$_c$	0.27	0.21	0.25	0.24	0.27	0.27	0.28	0.27	0.24	0.26	0.27	0.30	0.28	0.35	0.35	0.31	0.35	0.34	0.42	0.4	0.99	1	
CprA$_h$	0.32	0.20	0.21	0.25	0.25	0.25	0.28	0.30	0.29	0.23	0.30	0.21	0.30	0.33	0.29	0.35	0.38	0.31	0.42	0.37	1	0.96	1

To avoid biases produced by the size of the translated reductive dehalogenases, a fraction containing both iron sulfur-binding motifs was used to create the alignment; the size of the fraction used was about 150 amino acids. TceA, TCE dehalogenase [*Dehalococcoides ethenogenes* 195] (Magnuson *et al.,* 2000). PceA, PCE dehalogenase [*Sulfusporillum multivorans*] (Neumann *et al.,* 1998). PceA$_b$ PCE dehalogenase [*Desulfitobacterium* sp. Y51]. (Suyama *et al.,* 2002). CprA$_d$ o-chlorophenol dehalogenase [*Desulfitobacterium dehalogenans*] (Smidt *et al.,* 2000). CPRA$_c$ o-chlorophenol dehalogenase [*Desulfitobacterium chlororespirans*]. CprA$_h$ reductivedehalogenase [*Desulfitobacterium hafniense*]. Comparative identity shared among reductive dehalogenase proteins in the *Dehalococcoides genome* is shaded in light gray. Reductive dehalogenase genes that share the higher identity values with CprA and PceA proteins than with TceA are shaded in dark gray.

5.5. References

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CHAPTER VI

GENETIC IDENTIFICATION OF A PUTATIVE VINYL CHLORIDE REDUCTASE GENE IN *DEHALOCOCCOIDES* SPECIES STRAIN BAV1

6.1. Introduction

Vinyl chloride (VC) is a toxic and carcinogenic priority pollutant that threatens drinking water quality in most industrialized countries (Kielhorn *et al.*, 2000). A major source of environmental VC is through naturally occurring transformation reactions acting on chlorinated solvents such as tetrachloroethene (PCE) and trichloroethene (TCE), which are abundant groundwater pollutants (Vogel *et al.*, 1987; Mohn and Tiedje, 1992; Roberts *et al.*, 1996; van Pee and Unversucht, 2003). Additional environmental VC originates from landfills (Coulston and Kolbye Jr., 1994; Allen *et al.*, 1997), PVC production facilities (Kielhorn *et al.*, 2000) and abiotic formation in soils (Keppler *et al.*, 2002). Due to the extent of the problem, innovative and affordable technologies are needed to restore contaminated sites and guarantee drinking water safety. Bioremediation approaches that rely on the activity of bacterial populations that use chlorinated compounds as growth-supporting electron acceptors (i.e., chlororespiration) have been implemented successfully in the field (Ellis *et al.*, 2000; Major *et al.*, 2002; Lendvay *et al.*, 2003). Recent efforts identified the key populations responsible for reductive dechlorination and detoxification of VC to ethene as members of the *Dehalococcoides* (Duhamel *et al.*, 2002; Cupples *et al.*, 2003; He *et al.*, 2003b), a deeply branching group on the bacterial tree most closely affiliated with the Chloroflexi

(Maymó-Gatell *et al.*, 1997; Adrian *et al.*, 2000). 16S rRNA gene-based PCR approaches were designed to detect (Löffler *et al.*, 2000; Fennell *et al.*, 2001; Hendrickson *et al.*, 2002)and quantify (Cupples *et al.*, 2003; He *et al.*, 2003a; He *et al.*, 2003b) members of this group. Such approaches are critical for assessing VC-contaminated sites, monitoring bioremediation efforts, and establishing cause-effect relationships (Major *et al.*, 2002; Lendvay *et al.*, 2003). Unfortunately, *Dehalococcoides* strains with different dechlorination activities share similar or identical 16S rRNA gene sequences (He *et al.*, 2003a). *Dehalococcoides* sp. strain CBDB1 dechlorinates trichlorobenzenes, pentachlorobenzene and some polychlorinated dibenzodioxin congeners but failed to dechlorinate PCE and TCE (Adrian *et al.*, 2000; Bunge *et al.*, 2003). *Dehalococcoides ethenogenes* 195 (Maymó-Gatell *et al.*, 1997; Maymó-Gatell *et al.*, 2001) and *Dehalococcoides* sp. strain FL2 grow with polychlorinated ethenes as electron acceptors but cannot grow with VC, and only *Dehalococcoides* sp. strain BAV1 respires all DCE isomers and VC (He *et al.*, 2003a). Despite the metabolic differences, these populations share a 16S rRNA gene sequence with more than 99.9% similarity (based on the analysis of 1,296 aligned positions (He *et al.*, 2003b)). The identification of functional genes involved in the dechlorination process of interest could overcome these limitations and complement 16S rRNA gene-based approaches.

A few RDase sequences involved in partial reductive dechlorination of PCE and chlorinated aromatic compounds have been obtained (Neumann *et al.*, 1998; van de Pas *et al.*, 1999; Magnuson *et al.*, 2000; Smidt *et al.*, 2000; Okeke *et al.*, 2001; Suyama *et al.*, 2002; Maillard *et al.*, 2003) but functional genes involved in VC reduction have not been found. Alignment of known RDase amino acid sequences revealed low sequence identity

(27 to 32%); nevertheless, conserved stretches were identified. For example, a twin arginine (RR) motif near the amino-terminus and two iron-sulfur cluster binding motifs have been described (Neumann *et al.*, 1998; Magnuson *et al.*, 2000; Smidt *et al.*, 2000; Suyama *et al.*, 2002; Maillard *et al.*, 2003). Associated with the RDase genes are the B genes, which encode putative membrane anchoring proteins that are hypothesized to play a role in the reductive dechlorination process (Neumann *et al.*, 1998; van de Pas *et al.*, 1999; Magnuson *et al.*, 2000; Suyama *et al.*, 2002; Maillard *et al.*, 2003). In *Dehalococcoides* (Magnuson *et al.*, 2000), *Sulfurospirillum* (formerly *Dehalospirillum*) (Neumann *et al.*, 1998), *Dehalobacter* (Maillard *et al.*, 2003) and *Desulfitobacterium* (Suyama *et al.*, 2002), the B gene is located downstream of the PCE/TCE RDase genes. In *cprA* operons (ortho chlorophenol RDases) of *Desulfitobacterium* species an opposite arrangement was observed (van de Pas *et al.*, 1999).

The goal of this study was to use the available sequence information to design a procedure to PCR-amplify putative RDase genes of *Dehalococcoides* spp., and identify functional genes implicated in VC reductive dechlorination.

6.2. Methods

6.2.1. Chemicals

Chemicals were purchased from Aldrich (Milwaukee, WI) or Sigma Chemical Co. (St Louis, MO), except for VC, which was obtained from Fluka Chemical Corp. (Ronkonkoma, NY). Restriction enzymes were purchased from Promega Biosciences, Inc. (San Luis Obispo, CA), and enzymes used for cell lysis were from Sigma Chemical Co. PCR reagents were purchased from Applied Biosystems (Foster City, CA), and BSA was purchased from Roche (Mannheim, Germany).

6.2.2. VC-dechlorinating cultures and sources of DNA

Genomic DNA was obtained from pure cultures of *Dehalococcoides* sp. strain BAV1, and several VC-dechlorinating enrichment cultures derived from river sediments (the Red Cedar, Au Sable and Père Marquette Rivers, all three in Michigan (Löffler *et al.*, 1999; Löffler *et al.*, 2000)) and chloroethene-contaminated aquifers (the Minerva site in Ohio, the Hydrite Chemical site in Wisconsin, and the Bachman Road site in Michigan (Lendvay *et al.*, 2003)). VC-dechlorinating cultures were grown in 160-mL serum bottles containing 100 mL reduced basal salts medium amended with acetate (2 mM) as a carbon source, hydrogen (0.2 mmoles) as electron donor, and VC (0.12 mmoles) as electron acceptor as described (He *et al.*, 2003a; He *et al.*, 2003b). Genomic DNA was also available from isolates *Dehalococcoides* sp.strain CBDB1, *Dehalococcoides* sp. strain FL2, *Dehalococcoides ethenogenes* strain 195, and PCE-to-ethene-dechlorinating mixed cultures successfully employed in bioaugmentation approaches in the field (i.e., KB-1 (Major *et al.*, 2002), and Bio-Dechlor INOCULUM (www.regenesis.com), a

culture based on the Bachman Road site inoculum (Lendvay *et al.*, 2003)), and the VC-to-ethene-dechlorinating Victoria culture containing strain VS (Cupples *et al.*, 2003).

6.2.3. Primer design

Multiple alignments of full-length RDase genes and translated protein sequences including TceA (AAN85590, AAN85588, AAF73916A) and putative identified from the genome of *Dehalococcoides ethenogenes* strain 195 were constructed using clustalW and clustalX (Thompson *et al.*, 1997). Conserved amino acid sequences were identified and used to design degenerate PCR primers. The following conserved regions were targeted for designing forward and reverse primers, respectively: a stretch near the amino-terminus of the RDases (i.e., RRXFXK) and a region in the B gene (i.e., WYEW). The expected size of amplicons generated with these primers ranged from 1,500-1,700 bp. The degenerate primer set used in this study and its target sequences are listed in Table 1. Specific primer sets (Table 2) targeting each of the putative RDases identified in the clone libraries (see below) were designed using Primerquest (http://biotools.idtdna.com/Primerquest/).

6.2.4. PCR, cloning, and amplicon analysis

DNA from VC-dechlorinating pure and mixed cultures was extracted using the Qiagen mini kit (Qiagen, Valencia, CA) as described previously (He *et al.*, 2003b), and used as template for amplification with degenerate primers RRF2 and B1R (Table 1). PCR reactions were performed in total volumes of 30 μL and final concentrations were as follows: GeneAmp® PCR buffer (1x), $MgCl_2$ (3.0 mM), BSA (0.13 mg/mL), dNTPs

(0.25 mM each), primers (0.5 μM each), Taq DNA polymerase (2 units), and DNA (1-2 ng/μL). PCR conditions included an initial denaturation step at 94°C for 2 min 10 sec, followed by 30 cycles (94°C for 30 sec, 48°C for 45 sec, and 72°C for 2 min 10 sec), and final extension at 72°C for 6 min. The same conditions were used for amplification with the specific primers listed in Table 2 except that the primer concentrations were 0.1 μM, the $MgCl_2$ concentration was 2.0 mM, and the annealing temperature was 51°C. Amplicons generated from strain BAV1 genomic DNA with primers RRF2 and B1R were purified using the QIAquick PCR purification kit (Qiagen), ligated into vector pCR2.1 by TA cloning (TOPO or TA cloning kit, Invitrogen, Carlsbad, CA), and cloned in competent *E. coli* cells provided with the cloning kit following manufacturer recommendations. Recombinant *E. coli* clones were screened by verifying the correct insert size using direct PCR with primers targeting the pCR2.1 cloning vector flanking the inserted fragment (Zhou *et al.*, 1997). The resulting correct-sized amplicons were digested individually with the enzymes *Msp*I and *Hha*I (Promega Biosciences), as per manufacturer recommendations for Restriction Fragment Length Polymorphism (RFLP) analysis. Plasmid DNA from recombinant clones containing the different inserts was extracted using the Qiaprep spin miniprep kit (Qiagen), and partially sequenced with vector specific primers (Zhou *et al.*, 1997) using an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA). A second clone library was established using the same procedure with genomic DNA from the Bachman enrichment culture, from which strain BAV1 was isolated. Correct-sized inserts were analyzed with BLASTX to verify similarity and the presence of consensus sequences indicative of RDase genes. Further, DNA sequences were translated using the TRANSLATE program

(http://us.expasy.org/tools/dna.html) into amino acid sequences to examine for known RDase motifs. Partial protein sequences were aligned using the programs clustalW and clustalX. The designation of putative RDase genes identified in this study was adapted from Villemur et al (Villemur *et al.*, 2002).

6.2.5. RNA isolation

Biomass was collected by centrifugation and cell pellets were immediately frozen at –70ºC. All solutions used for RNA extraction were prepared with diethyl pyrocarbonate (DEPC)-treated water to inhibit RNase activity. Total RNA was extracted using the RNeasy extraction kit (Qiagen) according to the manufacturer's recommendations with the following modifications to enhance cell lysis and RNA yields. The cell pellet was suspended in 100 µL lysozyme digestion buffer (30 mM Tris-HCl, 1 mM EDTA, pH 8.0, 15 mg/mL lysozyme), 20 µL proteinase K (25 mg/mL) and 10 µL achromopeptidase (7,500 U/µL). The suspension was mixed and incubated at room temperature for 10 min, before 50 µL 0.1% Triton X-100 was added, and the mixture was shaken vigorously for 10 sec. Lysis buffer RLT (350 µL, provided with the RNeasy extraction kit) was added, and the lysate was transferred into a MicroRNA Bead Tube (Mo Bio Laboratories, Carlsbad, CA) and shaken horizontally on a Vortex mixer at maximum speed for 10 min. DNA was removed by two consecutive on-column treatments with RNase-free DNase (Qiagen) as described by the manufacturer. RNA concentrations were determined spectrophotometrically at 260 nm using an HP 8453 photodiodearray UV/Vis spectrophotometer.

6.2.6. Expression analysis of putative RDase genes

Reverse transcription PCR (RT-PCR) was performed with the two-step RT-PCR sensiscript kit (Qiagen). First, reverse transcription reactions were performed with 1 mM random hexamer primers (Promega) and 5-50 ng of extracted RNA in a total volume of 20 μL for 3 hrs at 37°C according to the manufacturer's recommendations. Then, PCR was performed with degenerate primers RRF2 and B1R (Table 6.1) or with specific primers (Table 6.2) using the PCR conditions specified above. RT-PCR amplification products were examined by gel electrophoresis on 1.5% agarose gels, and amplicons generated with primers RRF2 and B1R were cloned using the TOPO TA cloning kit. Recombinant *E. coli* clones were identified as described above, and the inserts were characterized by restriction analysis and sequenced. For nested PCR, the initial amplification was performed with primers RRF2 and B1R, and (1 μL) of the amplified product was used as template in a second round of PCR with the specific primers listed in Table 6.2.

6.2.7. Chromosome walking and assembling the bvcA coding sequence

To extend the putative reductive dehalogenase gene fragment *rdhA6*$_{[BAV1]}$, the TOPO Walker kit from Invitrogen (Carlsbad, CA) was used with primers 5Bfcomp (5'ACCACCTGTACTTGAGGCA-3'), and 5BGR (5'ACCCGACAAAGAACTGGTTTCG-3'), both illustrated in Figure 6.1. Purified genomic DNA of strain BAV1 was digested with *Pst* I and *Sac* I for 2 hrs at 37^{o}C. The digested DNA was dephosphorylated using calf alkaline phosphatase and precipitated with phenol:chloroform (1:1 pH 6.7) following the TOPO Walker manual. Primer

extension with primer 5Bfcomp at an annealing temperature of 55°C created a 3' overhang required for TOPO linking. TOPO linking was performed as to manufacturer's recommendations, and the TOPO-linked DNA was then subjected to amplification with primer 5BGr at an annealing temperature of 57°C. Amplification was verified on 1% agarose gels. The 305 bp product was purified using the Qiaquick Gel Extraction Kit (Qiagen) and cloned into *E. coli* using the cloning Kit (Invitrogen). Primers M13F and M13R were used to PCR amplify the cloned fragment according to the protocol for 'alternative method of analysis' provided with the TOPO XL PCR Cloning kit. The purified PCR product containing the 305 bp insert was sequenced using primers M13F and M13R. This sequence was aligned with the previously obtained *rdhA6*$_{[BAV1]}$ gene fragment sequence, and the coding region was determined using Frameplot (http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl) (Ishikawa and Hotta, 1999).

6.2.8. Nucleotide sequence accession numbers

The coding sequences of the putative RDase gene and putative B gene fragments were deposited in GenBank under accession numbers AY553222-AY553228. GenBank accession number AY563562 was assigned to the complete sequence of the putative VC reductive dehalogenase *bvcA*. Nucleotide and translated protein sequences are presented in Appendix B.

6.3. Results

6.3.1. PCR amplification of putative RDase gene fragments

The degenerate primer pair RRF2 and B1R produced fragments of the expected size and a total of seven clones were recovered in the clone library generated with DNA from the VC-dechlorinating Bachman mixed culture. Restriction analysis identified five clone types with distinct inserts, designated *rdhA1-5* $_{[BAV1]}$. In a second clone library constructed with strain BAV1 pure culture DNA, 54 clones were recovered, and two additional putative RDase sequences were identified, i.e., *rdhA6*$_{[BAV1]}$ and *rdhA7*$_{[BAV1]}$. No clones harboring *rdhA3*$_{[BAV1]}$, *rdhA4*$_{[BAV1]}$, or *rdhA5*$_{[BAV1]}$ were identified in the BAV1 clone library but subsequent PCR analysis using primer pairs specifically targeting each of the *rdhA1-7* $_{[BAV1]}$ sequences, demonstrated the presence of all putative RDase fragments in isolate BAV1 and in the Bachman mixed culture from which BAV1 was isolated.

6.3.2. Expression analysis of putative RDase genes

PCR amplification with degenerate primers RRF2 and B1R using cDNA obtained from VC-grown BAV1 cells as template yielded a PCR fragment of the expected size (approximately 1,700 bp). In contrast, no amplification occurred without the RT-PCR step, confirming that all DNA was successfully removed from the RNA preparation, and that the observed 1,700 bp amplicon was generated from mRNA. Amplification of cDNA occurred with degenerate primers RRF2 and B1R targeting the reductase internal RRXFXK motif and the WYEW sequence in the putative B gene, respectively, indicating that both genes are co-transcribed. A clone library generated with the PCR-amplified

cDNA contained a single insert, and RFLP and sequence analyses of six clones confirmed that the cloned fragments were identical to *rdhA6*$_{[BAV1]}$.

Transcription of the putative VC RDase found in the cDNA clone library was explored in more detail using the specific primer pair 5Bf and 5Br (Table 6.2). PCR reactions using cDNA generated from VC-grown BAV1 cultures as template yielded amplicons of the correct size (Figure 6.2), and sequence analysis confirmed their identity. No amplicons were obtained when total RNA extracts were used as template, confirming that no residual genomic DNA was present (Figure 6.2). An additional control shown in Figure 2 involved cDNA obtained from a *cis*-DCE-grown culture of *Dehalococcoides* sp. strain FL2. No amplicons were obtained with primer pair 5Bf and 5Br, which was expected since strain FL2 cannot grow with VC as electron acceptor.

Seven putative RDase gene fragments were identified in strain BAV1, however *rdhA6*$_{[BAV1]}$ was the only putative RDase gene fragment present in a cDNA clone library established with total RNA obtained from VC-grown BAV1 cultures. PCR reactions performed with the specific primers listed in Table 6.2 and cDNA as template confirmed these findings, and amplification only occurred with the 5Bf/5Br primer pair targeting the *rdhA6*$_{[BAV1]}$ sequence. To test if the six other RDase genes were expressed at lower levels, the PCR product generated from cDNA with primer pair RRF2/B1R was used for a subsequent nested PCR with the specific primer pairs listed in Table 6.2. These analyses suggested that genes contributing to fragments *rdhA1*$_{[BAV1]}$, *rdhA3*$_{[BAV1]}$, *rdhA4*$_{[BAV1]}$, *rdhA5*$_{[BAV1]}$, and *rdhA7*$_{[BAV1]}$ were also expressed, but at significantly lower levels than *rdhA6*$_{[BAV1]}$. The only putative RDase gene not transcribed at detectable levels in VC-grown BAV1 cells correlated with fragment *rdhA2*$_{[BAV1]}$.

Since the fragments generated with primer pair RRF2 and B1R lacked approximately 30 bp on the 3' end of the putative RDase genes, the *rdhA6*$_{[BAV1]}$ gene fragment was extended and the missing upstream portion of the putative RDase gene was obtained. The complete gene implicated in VC reductive dechlorination in *Dehalococcoides* sp. strain BAV1 was designated *bvcA*, and deposited at GenBank (accession number AY563562). The translated BvcA protein sequence contained the twin arginine motif (RRXFXK) in the form RRDFMK. The chromosomal organization of the *bvcA* region is shown in Figure 6.1. The deduced coding sequence of *bvcA* is 1,550 nucleotides long, which translates into a 516 amino acid protein. A second incomplete open reading frame for the putative B gene *bvcB* was found 51 nucleotides downstream of the *bvcA* stop codon TAA.

6.3.3. Detection of bvcA in other VC-dechlorinating cultures

PCR amplification was performed with *bvcA*-targeted primers 5Bf and 5Br (Table 6.2) using genomic DNA from other *Dehalococcoides* isolates and *Dehalococcoides*-containing mixed cultures as templates. As shown in Figure 6.3., the correct sized amplicon was generated with isolate BAV1 genomic DNA, but not with genomic DNA from *Dehalococcoides ethenogenes* strain 195, strain FL2, or strain CBDB1, none of which have been reported to grow with VC. *bvcA* was detected in four of eight *Dehalococcoides*-containing cultures capable of complete reductive dechlorination and ethene production. As shown in Figure 6.4, *bvcA* was also present in cultures KB-1 and the Bio-Dechlor INOCULUM, two commercially available ethene-producing enrichment cultures that have been successfully used in bioaugmentation approaches. In addition, *bvcA* was identified in two ethene-producing enrichment cultures derived from

chloroethene-contaminated aquifer materials (i.e., the Minerva site and the Hydrite site). *bvcA*, however, was not detected in the Victoria culture containing *Dehalococcoides* sp. strain VS nor in three VC-dechlorinating enrichment cultures derived from Michigan river sediments (Figure 6.4).

Table 6.1. Degenerate primers used in this study.

Degenerate Primers	Primer sequence[a] 5'→3'	Amino acids targeted
RRF2	SHMGBMGWGATTTYATGAARR	RRXFXK
B1R	CHADHAGCCAYTCRTACCA	WYEW

[a] Abbreviations of degenerate nucleotides: R=A/G; K=G/T; M=A/C; S=C/G; W=A/T; Y=C/T; B=C/G/T; D=A/G/T; V=A/C/G; H=A/C/T.

Table 6.2. Specific primers used in this study.

Specific Primers [a]	Primer sequence 5'→3'	Gene targeted	Position relative to *bvcA* start
bavrdA1F	GTACCGATGATGATTCACG	$rdhA1_{[BAV1]}$	504
bavrdA1R	AGCCATACATGTCCCGCAA	$rdhA1_{[BAV1]}$	1377
bavrdA2F	TGCAAGCAGGTTCCCAT	$rdhA2_{[BAV1]}$	467
bavrdA2R	GGCTTGATGTTAAACCC	$rdhA2_{[BAV1]}$	1305
bavrdA3F	GATTATGCTTTGTTTGGG	$rdhA3_{[BAV1]}$	406
bavrdA3R	TTAGAACAACCACCAGGC	$rdhA3_{[BAV1]}$	1367
bavrdA4F	ATGCCATGTATTCGGTC	$rdhA4_{[BAV1]}$	412
bavrdA4R	TCAACCCTCCAGCCTTTA	$rdhA4_{[BAV1]}$	1305
bavrdA5F	GTTAATGTTGCCAAGGCT	$rdhA5_{[BAV1]}$	527
bavrdA5R	CATGGTCTTTTCCATATTGGC	$rdhA5_{[BAV1]}$	1491
bvcAF	TGCCTCAAGTACAGGTGGT	$rdhA6_{[BAV1]}$ *bvcA*	450
bvcAR	ATTGTGGAGGACCTACCT	$rdhA6_{[BAV1]}$ *bvcA*	1288
bavrdA7F	AAACTGCTCAGGGTTG	$rdhA7_{[BAV1]}$	463
bavrdA7R	TTGCCCGGAACACTGTA	$rdhA7_{[BAV1]}$	1339
5Bfcomp	ACCACCTGTACTTGAGGCA	$rdhA6_{[BAV1]}$ *bvcA*	468
5BGR	ACCCGACAAAGAACTGGTTTCG	$rdhA6_{[BAV1]}$ *bvcA*	138

[a] PCR amplification used an annealing temperature of 51° except for bvcAR and bvcAF (52°C), 5Bfcomp (55°C), and 5BGR (57°C).

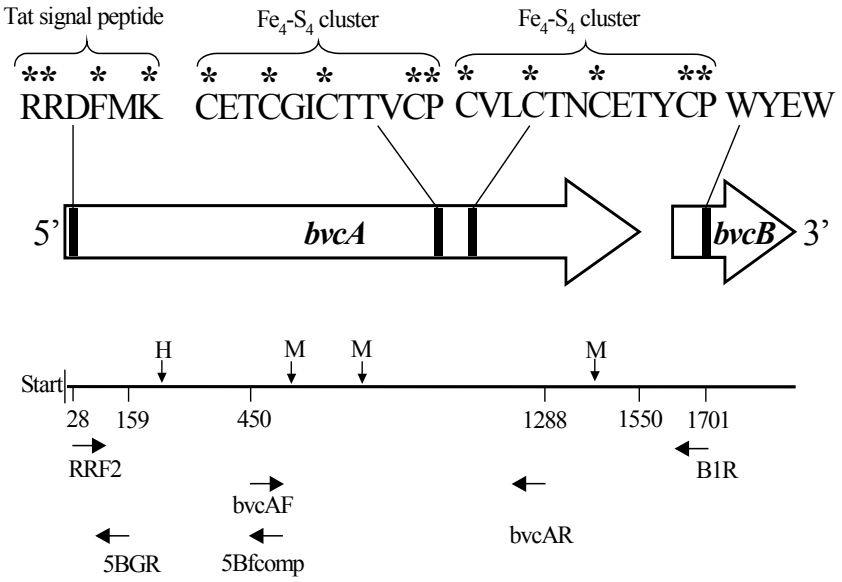

Figure 6.1. Arrangement of the *bvcA* gene and the corresponding B gene, *bvcB*. Also shown are conserved dehalogenase features including the Tat signal peptide RRDFMK, and two Fe_4-S_4 clusters near the C-terminal end. Amino acids conserved in RDases are labeled with an asterisk. Primer binding sites used for amplification of putative RDase genes (primers RRDF2 and B1R), detection of *bvcA* (primers bvcAr and bvcAF), chromosome walking for capturing the entire coding region of *bvcA* (primers 5bfcomp and 5BG) are indicated. The arrows indicate *Hha*I (H) and *Msp*I (M) restriction sites.

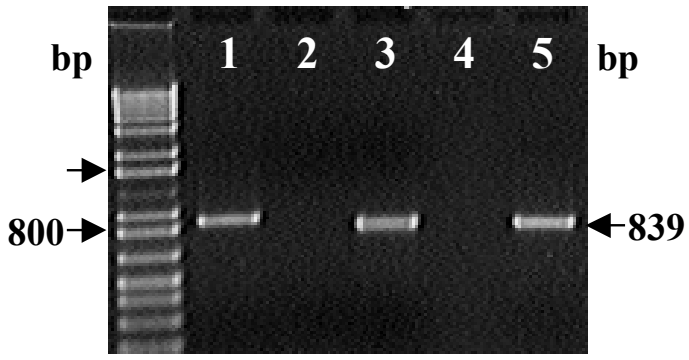

Figure 6.2. PCR amplification of the putative VC RDase gene with specific primers bvcAR and bvcAF and templates generated from VC-grown BAV1 cultures. DNA size marker 1Kb plus (Invitrogen) (left lane), BAV1 genomic DNA (lane 1), BAV1 total RNA (lane 2), BAV1 cDNA (lane 3), H_2O (lane 4), plasmid DNA containing *rdhA6*$_{[BAV1]}$ gene fragment (lane 5).

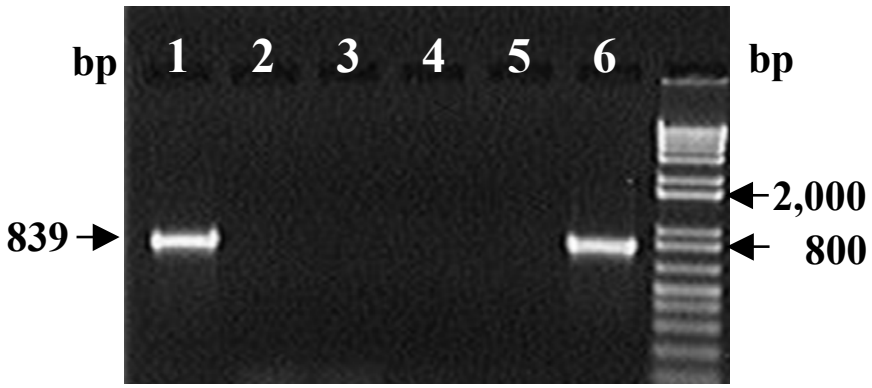

Figure 6.3. Specificity of primers (bvcAR/bvcAF) targeting the putative VC RDase gene *bvcA*. DNA size marker 1Kb plus (Invitrogen) (right lane), plasmid DNA containing *rdhA6*$_{[BAV1]}$ (lane 1), H_2O (lane 2), genomic DNA from strain FL2 (lane 3), *Dehalococcoides ethenogenes* (lane 4), strain CBDB1 (lane 5), and strain BAV1 (lane 6).

Figure 6.4. Detection of *bvcA* in VC-dechlorinating mixed cultures using primers bvcAR/bvcAF. DNA size marker 1Kb plus (Invitrogen) (left lane), H_2O (lane 1), plasmid DNA containing *rdhA6*$_{[BAV1]}$ (lane 2); genomic DNA from the Bachman enrichment culture (lane 3), the Au Sable culture (lane 4), the Père Marquette culture (lane 5), the Red Cedar culture (lane 6), the Hydrite culture (lane 7), the Minerva culture (lane 8), Bio-Dechlor INOCULUM (lane 9), KB-1 (lane 10), and the Victoria culture (lane 11).

6.4. Discussion

A putative VC reductive dehalogenase gene was identified in the VC-respiring isolate BAV1 (He *et al.*, 2003a) using a genomics approach. The degenerate forward primer was designed to target the RRXFXK motif, which is part of the TAT protein export pathway (Berks *et al.*, 2000); highly conserved, but not unique, to RDases. For this reason, Regeard et al (Regeard *et al.*, 2004). designed degenerate primer pairs targeting conserved RDase internal regions that yielded relatively short amplicons of a maximum size of about 900 bp. Since our goal was to specifically amplify putative *Dehalococcoides* RDase genes and obtain their complete sequences, we took advantage of the conserved twin arginin motif, and identified a conserved motif internal to the *Dehalococcoides* B genes for the design of a specific reverse primer. This degenerate primer set yielded fragments comprising nearly the entire RDase gene, and combined with a chromosome walking approach, the first complete sequence of a putative VC RDase gene from strain BAV1 was obtained. The biochemistry of *Dehalococcoides* RDases is of great interest but detailed investigations are hampered by the difficulties in obtaining sufficient biomass. This procedure designed to obtain the complete sequence of a putative VC RDase gene from strain BAV1 is applicable to capture other *Dehalococcoides* RDase genes, and will assist future efforts aimed at heterologous expression to allow in depth investigations of these interesting enzyme systems.

Of the seven putative RDase gene fragments identified in isolate BAV1, only *bvcA* transcribed at high levels when strain BAV1 was grown with VC as electron acceptor. Interestingly, five additional putative RDases transcribed in VC-grown BAV1 cells, but at significantly lower levels compared to *bvcA*. Although the significance of

these putative RDases in VC reductive dechlorination cannot be excluded, our findings point towards the involvement of *bvcA* in VC reductive dechlorination. Regulation of gene expression generally in *Dehalococcoides*, is poorly understood, and it is unclear if RDase genes are constitutively expressed or specifically induced by the respective chloroorganic substrate. Several lines of evidence suggest that *bvcA* is the gene encoding a VC RDase in strain BAV1: (i) *bvcA* was the only RDase expressed at high levels in VC-grown BAV1 cells, (ii) the primers targeting *bvcA* distinguished strain BAV1 from other *Dehalococcoides* populations that failed to grow with VC as electron acceptor, (iii) the translated amino acid sequence BvcA contained all of the characteristic RDase motives (e.g., the twin arginine and the two Fe_4S_4 iron-sulfur cluster binding motifs). A comparison of the translated amino acid sequence BvcA with all the putative RDases found on the *Dehalococcoides ethenogenes* genome (Villemur *et al.*, 2002) and with the putative RDases detected in strains FL2 and CBDB1 (Hölscher *et al.*, 2004) showed that sequence identity did not exceed 39% at the protein level. These findings indicate that *bvcA* is a unique gene not present in any other *Dehalococcoides* isolates. BvcA shared the highest similarity of 39% with TceA, the RDase responsible for TCE dechlorination in *Dehalococcoides ethenogenes* strain 195 (Magnuson *et al.*, 2000). BvcA also shared 16% identity with PceA, the PCE RDase of *Sulfurospirillum multivorans* (Neumann *et al.*, 1998) and 13% identity with CprA, the ortho chlorophenol RDase of *Desulfitobacterium dehalogenans* (van de Pas *et al.*, 1999).

Current efforts to assess and monitor chloroethene-contaminated sites rely on nucleic acid-based approaches targeting *Dehalococcoides* 16S rRNA genes (Fennell *et al.*, 2001; Hendrickson *et al.*, 2002; Major *et al.*, 2002; Lendvay *et al.*, 2003). A major

limitation of the 16S rRNA gene approach lies in the similarity of 16S rRNA genes from *Dehalococcoides* populations exhibiting different dechlorination activities (He *et al.*, 2003a). *bvcA* is a promising functional gene target to distinguish the VC-respiring strain BAV1. Nucleic acid-based tools targeting *bvcA* will be helpful to complement 16S rRNA gene-based approaches to monitor sites undergoing enhanced treatment where BAV1-type populations are already present (Lendvay *et al.*, 2003) or inocula containing BAV1 (e.g., Bio-Dechlor INOCULUM) are used for bioaugmentation. Since *bvcA* is currently the only available putative VC RDase gene, we can only speculate on the diversity of genes with the same function in the environment. Our experiments demonstrated the presence of *bvcA* in two independently enriched cultures from chloroethene-contaminated aquifer materials, however *bvcA* was absent in three VC-dechlorinating enrichment cultures derived from Michigan river sediments. Hydrogen consumption threshold measurements provided circumstantial evidence for the presence of VC-respiring *Dehalococcoides* populations (Löffler *et al.*, 1999), and *Dehalococcocides* 16S rRNA gene sequences were detected in all ethene-producing cultures (Ritalahti *et al.*, 2001). Similarly, the ethene-producing Victoria culture containing *Dehalococcoides* sp. strain VS did not contain *bvcA*. Growth of strain VS with VC as electron acceptor has been conclusively demonstrated (Cupples *et al.*, 2003) suggesting an RDase gene different from *bvcA* is present in this culture. These findings suggest that a diversity of VC RDase genes exists, and additional sequences must be obtained before designing a comprehensive suite of nucleic acid-based tools to target all VC RDase genes becomes feasible.

6.5. References

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CHAPTER VII

MULTIPLE REDUCTIVE DEHALOGENASE GENES IN *DEHALOCOCCOIDES* SP. STRAIN FL2 AND EXPRESSION ANALYSIS OF REDUCTIVE DEHALOGENASE GENES IN STRAIN FL2 GROWN ON TRICHLOROETHENE (TCE) AND DICHLOROETHENE (DCE) AS ELECTRON ACCEPTORS

7.1. Introduction

Bacterial reductive dechlorination reactions play important roles in the detoxification of chlorinated aromatic and aliphatic compounds in contaminated environments (Hendrickson *et al.*, 2002; Lendvay *et al.*, 2003). Several anaerobic bacteria use chlorinated compounds in their energy metabolism, coupling reductive dehalogenation to electron transport phosphorylation (Löffler *et al.*, 2003). The *Dehalococcoides* group forms a bacterial cluster that is phylogenetically distant from other dechlorinating bacteria (Maymó-Gatell *et al.*, 1997; Adrian *et al.*, 2000) and uses a spectrum of chlorinated compounds as growth-supporting electron acceptors, including chlorinated ethenes (Maymó-Gatell *et al.*, 1997; He *et al.*, 2003a; He *et al.*, 2005), chlorinated benzenes (Adrian *et al.*, 2000; Jayachandran *et al.*, 2003) and polychlorinated dibenzodioxins (PCDDs) (Bunge *et al.*, 2003). Four members of the *Dehalococcoides* group have been isolated. Three strains, *Dehalococcoides ethenogenes* strain 195, *Dehalococcoides* sp. strain FL2, and *Dehalococcoides* sp. strain BAV1, have been cultivated with chlorinated ethenes as growth-supporting substrates (Maymó-Gatell *et al.*, 1997; He *et al.*, 2003a; Löffler *et al.*, 2003; He *et al.*, 2005), whereas *Dehalococcoides* sp. strain CBDB1 was isolated with chlorinated benzenes as terminal electron acceptors

(Adrian *et al.*, 2000). Several strain-specific physiological differences exist. For example, tetrachloroethene (PCE) is used as metabolic electron acceptor by strain 195 but not by strains FL2, BAV1, and CBDB1(Adrian *et al.*, 2000; He *et al.*, 2003a; Löffler *et al.*, 2003). Both, strain 195 and strain FL2, use trichloroethene (TCE) for growth (Maymó-Gatell *et al.*, 1997; He *et al.*, 2005), while this compound is only cometabolically dechlorinated by strain BAV1 (He *et al.*, 2003a). Strain BAV1, however, respires vinyl chloride (VC) to ethene (He *et al.*, 2003a), whereas strain FL2 and strain 195 are unable to use VC as metabolic electron acceptor (Maymó-Gatell *et al.*, 1997; He *et al.*, 2005). Reductive dechlorination of chlorinated benzenes and PCDDs that had previously been shown for strain CBDB1(Adrian *et al.*, 2000; Bunge *et al.*, 2003; Jayachandran *et al.*, 2003) was recently demonstrated also for strain 195 (Fennell *et al.*, 2004). However, different patterns of chlorobenzene dechlorination and differences in utilization of chlorobenzene and PCDD congeners have been observed for strain CBDB1 (Holliger *et al.*, 1998; Adrian *et al.*, 2000; Bunge *et al.*, 2003; Jayachandran *et al.*, 2003) and strain 195 (Fennell *et al.*, 2004).

Reductive dehalogenases catalyzing the reductive dechlorination of chlorinated ethenes, phenols, or benzoates have been isolated from several species that use chlorinated compounds as growth-supporting electron acceptors (Ni *et al.*, 1995; Neumann *et al.*, 1996; Schumacher *et al.*, 1997; Christiansen *et al.*, 1998; Miller *et al.*, 1998; van de Pas *et al.*, 1999; Magnuson *et al.*, 2000). However, due to poor biomass yields, the isolation of catalytically active dehalogenases from *Dehalococcoides* is problematic (Holliger *et al.*, 1998; Magnuson *et al.*, 1998; Magnuson *et al.*, 2000). Only the TCE dehalogenase and the PCE dehalogenase of *D. ethenogenes* strain 195 have been

enriched from mixed cultures containing strain 195 in a semi-preparative manner that allowed the initial characterization of these interesting enzyme systems (Magnuson *et al.*, 1998). Nevertheless, purification of dehalogenases from *Dehalococcoides* will remain a major obstacle, and an integrated genetic/physiological approach seems most promising to shed light on biochemistry and genetics of reductive dechlorination. Dehalogenase encoding genes from different species have been identified following (partial) purification of the dechlorinating enzyme systems and peptide sequencing. Examples include the PCE dehalogenase from *Sulfurospirillum multivorans* (Neumann *et al.*, 1998); formerly *Dehalospirillum multivorans* (Luijten *et al.*, 2003), the PCE dehalogenase from *Desulfitobacterium* sp. strain Y51 (Suyama *et al.*, 2002), the *ortho*-chlorophenol dehalogenase from *Desulfitobacterium dehalogenans* (van de Pas *et al.*, 1999), and the TCE dehalogenase from strain 195 (Magnuson *et al.*, 2000). Sequence comparison of identified reductive dehalogenase genes revealed the presence of several conserved motifs to which specific functions have been attributed (reviewed in (Smidt *et al.*, 2000)). The open reading frame encoding the catalytic subunit of the dehalogenase, designated orfA, is linked to a second open reading frame, orfB. OrfB encodes a small hydrophobic B-protein, possibly acting as a membrane anchor for the dehalogenase (Neumann *et al.*, 1998). The N-termini of characterized reductive dehalogenases contain a twin-arginine signal sequence, comprising the consensus motif RRXFXK followed by a stretch of hydrophobic residues. Such signal sequences are involved in transporting cofactor-containing proteins across the cytoplasmic membrane and are proteolytically removed during protein maturation (Berks *et al.*, 2000). Two iron-sulfur cluster binding motifs characteristic of bacterial ferredoxins (Bruschi and Guerlesquin, 1988; Beinert, 2000) are

located near the C-terminal end of the dehalogenases. Further, dehalogenases contain highly conserved tryptophane and histidine residues that might be involved in catalysis (Smidt *et al.*, 2000) and other conserved sequence blocks with a yet unknown function (von Wintzingerode *et al.*, 2001; Regeard *et al.*, 2004) have been recognized. With the exception of the 3-chlorobenzoate dehalogenase of *Desulfomonile tiedjei* (Ni *et al.*, 1995), all biochemically characterized reductive dehalogenases apparently contain a corrinoid cofactor (Schumacher *et al.*, 1997; Christiansen *et al.*, 1998; Miller *et al.*, 1998; Neumann *et al.*, 2002; Maillard *et al.*, 2003). In chapter V, it was demonstrated that besides the TCE dehalogenase encoding *tceA* gene, 17 different RDase genes were present in strain 195, and all shared the dehalogenase-characteristic features described above (Villemur *et al.*, 2002; Smidt and de Vos, 2004). Hence, it was hypothesized that the genomes of *Dehalococcoides* populations contain multiple RDase genes, corresponding to the individual range of chlorinated electron acceptors used by individual strains. The goals of this study were i) to PCR-amplify and identify genes in the genome of strain FL2 that show high sequence similarity to known reductive dehalogenase genes and ii) to assess the expression of the RDase genes found in the genome of strain FL2 when strain FL2 is grown with two different electron acceptors. RDase genes found in the genomes of strain BAV1 and strain CBDB1 were included in the analysis.

7.2. Materials and Methods

7.2.1. Dechlorinating cultures

Dehalococcoides sp. strain FL2 was cultivated under anaerobic conditions with TCE or *cis*-DCE as electron acceptors. TCE and *cis*-DCE dechlorinating cultures were grown in 160-mL serum bottles containing 100 mL reduced basal salts medium amended with acetate (2 mM) as a carbon source, hydrogen (0.2 mmoles) as electron donor, and TCE or cis-DCE as an electron acceptor as described (Löffler *et al.*, 1999; He *et al.*, 2003b). The chloroorganic compounds were supplied via a hexadecane phase.

7.2.2. Nucleic acids extraction

Genomic DNA of strain FL2 was extracted from 50 ml of culture fluid as described in chapter III. For RNA isolation, biomass was collected by centrifugation, and cell pellets were immediately frozen at –70ºC. All solutions used for RNA extraction were prepared with diethyl pyrocarbonate (DEPC)-treated water to avoid RNase presence. Total RNA was extracted using the RNeasy extraction kit (Qiagen) according to the manufacturer's recommendations with the following modifications to enhance cell lysis and RNA yields: the cell pellet was suspended in 100 µL lysozyme digestion buffer, 20 µL proteinase K (25 mg/mL) and 10 µL achromopeptidase (7,500 U/µL). The suspension was mixed and incubated at room temperature for 10 min, before 50 µL 0.1% Triton X-100 was added, and the mixture was shaken vigorously for 10 sec. Lysis buffer RLT (350 µL, provided with the RNeasy extraction kit) was added, and the lysate was transferred into a MicroRNA Bead Tube (Mo Bio Laboratories, Carlsbad, CA) and shaken horizontally on a Vortex mixer at maximum speed for 10 min. DNA was removed by two consecutive on-column treatments with RNase-free DNase (Qiagen) as

described by the manufacturer. RNA concentrations were determined spectrophotometrically at 260 nm using an HP 8453 photodiodearray UV/Vis spectrophotometer.

7.2.3. Amplification of RDase genes

The degenerate primers (forward primer RRF2, reverse primer B1R) were derived from alignments of RDase genes of *D. ethenogenes* strain 195 (Krajmalnik-Brown *et al.*, 2004). PCR mixtures (30 µl) contained 0.05-3 ng of template DNA (i.e., genomic DNA of strain FL2), 0.5 µM of each primer, 2.5 mM $MgCl_2$, 0.25 mM each deoxynucleotide, 0.13 mg/ml BSA, and 0.4 U Taq DNA polymerase (Applied Biosystems, Foster City, CA) in 1x concentrated GeneAmp® PCR Buffer (Applied Biosystems). PCR was carried out with a GeneAmp® PCR System 9700 (Applied Biosystems) using the following parameters: 130 sec at 94°C; 30 cycles of 30 sec at 94°C, 45 sec at 48°C, and 130 sec at 72°C; and a final extension of 6 min at 72°C. The amplicons from five reactions were combined and purified with the Qiagen PCR purification kit following the manufacturer's recommendations. Purified PCR products were cloned in TOP10 *E. coli* cells using the TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Clones were screened for an insert of the expected size by colony PCR as follows: A small amount of cell material was transferred with a sterile toothpick to a plastic tube containing 50 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and incubated at 95°C for 10 min. A volume of 3 µl of the suspension was subsequently used as template for PCR with primers targeting the polylinker of the cloning vector (Zhou *et al.*, 1997). PCR mixtures were prepared as described above, and amplification was carried out using

the following parameters: 130 sec at 92°C; 30 cycles of 30 sec at 94°C, 1 min at 68°C, and 2 min at 72°C; and a final extension of 6 min at 72°C. A total of 99 clones containing inserts of about 1.7 kb were selected for further experiments. The 1.7 kb PCR products were digested with the restriction enzymes *MspI* or *HhaI* at 35°C for 4 h. Digestion mixtures (20 µl) contained 10 µl of the PCR product, 0.25 U of the restriction endonuclease (Promega Biosciences, Inc., San Luis Obispo, CA), and 0.1 mg/ml acetyl-BSA (bovine serum albumin) (Promega) in restriction buffer (Promega), and were incubated at 35°C for three hours. Deactivating restriction enzymes for five minutes, at 68°C, stopped the digestion reaction. The resulting digested fragments were separated by electrophoresis for 2 h on 3% low-melting agarose gels. Plasmids containing inserts with different restriction patterns were extracted from the respective *E. coli* clones with the Qiaprep Spin Miniprep kit (Qiagen) following the manufacturer's recommendations.

7.2.4. Sequencing of RDase genes

Sequence analysis was performed with an ABI 3100 genetic analyzer (Applied Biosystems) using the ABI PRISM BigDye Terminator v.3.1 Cycle Sequencing kit. Single-stranded sequencing of the terminal ends of the 1.7 kb fragment was performed using the M13 reverse primer 5'-CAGGAAACAGCTATGAC-3' and the vector-targeted 3'end primer (Zhou *et al.*, 1997). The resulting sequence information was used to design two additional internal primers for each fragment to obtain the complete sequence of the insert.

7.2.5. Sequence analysis

FL2 retrieved sequences of PCR-amplified fragments were compared to other published sequences using the NCBI blastx search tool. Deduced amino acid sequences were obtained with the program TRANSLATE http://us.expasy.org/tools/dna.html). Translated RDase sequences of *Dehalococcoides* sp. strain BAV1 presented in Chapter VI (GenBank accession numbers AY553222-AY553228) and from *Dehalococcoides* sp. strain CBDB1 (GenBank under the accession numbers AY374229-AY374244) were included for comparative analysis. Reductive dehalogenase gene sequences from other organisms were obtained from GenBank (http://www.ncbi.nlm.nih.gov). Amino acid sequences were aligned with ClustalW using the European Bioinformatics Institute (EBI) website (http://www.ebi.ac.uk/clustalw/). Phylogenetic trees (neighbor-joining, maximum parsimony, default settings) were generated from nearly complete RDase genes (sequences extending from immediately downstream of the twin-arginine signal sequence to the 3'end) using MEGA version 2.1 (Kumar *et al.*, 2001).

7.2.6. Expression analysis of putative RDase genes

Reverse transcription-PCR (RT-PCR) was performed with the two-step RT-PCR sensiscript kit (Qiagen). First, reverse transcription reactions were performed with 1 mM random hexamer primers (Promega) and 5-50 ng of extracted RNA in a total volume of 20 μL for 3 hrs at 37°C according to manufacturer's recommendations. Then, PCR was performed with degenerate primers RRF2 and B1R (Krajmalnik-Brown *et al.*, 2004). RT-PCR amplification products were examined by gel electrophoresis on 1.5% agarose gels, and amplicons generated with primers RRF2 and B1R were cloned using the TOPO

TA cloning kit. Recombinant *E. coli* clones were screened by verifying the correct size insert using PCR with primers targeting the pCR2.1 cloning vector flanking the inserted fragment (Zhou *et al.*, 1997). The resulting correct-sized amplicons were digested individually with the enzymes *Msp*I and *Hha*I (Promega Biosciences) as explained previously in this chapter for Restriction Fragment Length Polymorphism (RFLP) analysis. Plasmid DNA from recombinant clones containing the different inserts was extracted using the Qiaprep spin miniprep kit (Qiagen) and partially sequenced with vector specific primers (Zhou *et al.*, 1997) using an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Correct-sized inserts were analyzed with BLASTX to verify similarity and the presence of consensus sequences indicative of RDase genes. Further, DNA sequences were translated using the TRANSLATE program (http://us.expasy.org/tools/dna.html) into amino acid sequences to examine for known RDase motifs. Partial protein sequences were aligned using the programs clustalW and clustalX (Thompson *et al.*, 1997).

7.2.7. Nucleotide sequence accession numbers.

The coding sequences of RDase genes and putative B-gene fragments in strain FL2 were deposited in GenBank under the accession numbers AY374245-AY374255 and AY165309. Nucleotide and translated protein sequences are presented in Appendix C

7.3. Results

7.3.1. Amplification of RDase genes

Amplicons of the expected size of 1.7 kb were obtained with the primer pair RRF2/B1R using genomic DNA from strain FL2 as template. A total of 48 clones, (designated rdh_{FL2}), were obtained. Restriction fragment length analysis distinguished 14 distinct patterns among the rdh_{FL2} clones. All sequences contained a nearly complete open reading frame (length of 1350–1495 bp) missing only the first 45-60 nucleotides at the 5'end of the RDase genes, and about 90 nucleotides of the open reading frame of the B gene (Figure 7.1.). The one exception, orfA of $rdh10_{FL2}$ (*rdhA10*$_{FL2}$) lacked additional 250-260 nucleotides of the 5'end, probably due to mispriming of the forward primer RR2F used for PCR.

7.3.2. Sequence analysis of RDase genes

Inserts of representative rdh_{FL2} clones were sequenced. *rdhA*$_{FL2}$ and *rdhA*$_{CBDB1}$ genes showed highest similarity to a group of genes comprising the *tceA* gene of *D. ethenogenes* strain 195, the *tceA* gene of strain FL2, and *tceA* genes amplified previously from three chloroethene dechlorinating enrichment cultures (NCBI accession numbers AAN85590, AAN85592, and AAN85594). Blastx search yielded best hits (*E*-value range, 10^{-31} to 10^{-61}) for full-length alignments of *rdhA1-14*$_{CBDB1}$ and *rdhA1-11*$_{FL2}$ with the characterized *tceA* gene of strain 195. As shown by pairwise alignments of deduced amino acid sequences, *rdhA1-11*$_{FL2}$ and *rdhA1-14*$_{CBDB1}$ were 27-33% identical to the *tceA* gene of strain 195. Some *rdhA*$_{CBDB1}$ and *rdhA*$_{FL2}$ genes also shared high similarity (blastx *E*-values < 10^{-35}) with sequence *rdh63A* (NCBI accession number AAO15649), an

RDASE gene derived from a 2-bromophenol degrading microbial consortium (Rhee *et al.*, 2003). Hits with small E-values (*E*-value range, 10^{-10} – 10^{-30}) were also obtained for alignments of $rdhA_{CBDB1}$ and $rdhA_{FL2}$ genes with other known reductive dehalogenase genes, i.e., the PCE dehalogenase encoding genes (*pceAs*) from *S. multivorans*, *Dehalobacter restrictus* and *Desulfitobacterium* spp., and the chlorophenol dehalogenase encoding genes (*cprA*s) from *Desulfitobacterium* spp.

Deduced amino acid sequences of nearly complete orfA genes from rdh_{CBDB1} and rdh_{FL2} sequences (404-489 residues) were used to generate a tree in which the 17 RDASE genes ($rdhA1\text{-}17_{DE}$) found in *Dehalococcoides ethenogenes* 195 (Villemur *et al.*, 2002), the seven RDASE genes ($rdhA1\text{-}7_{BAV1}$) found in strain BAV1 (Krajmalnik-Brown *et al.*, 2004), and reductive dehalogenase genes from other dechlorinating bacteria were included (Figure 7.2.). All *Dehalococcoides* RDASE genes, except $rdhA16_{DE}$ and $rdhA17_{DE,}$ grouped together (Figure 7.2.). Furthermore, seven $rdhA_{CBDB1}$ genes ($rdhA8\text{-}14_{CBDB1}$), five $rdhA_{FL2}$ genes ($rdhA7\text{-}11_{FL2}$), and three $rdhA_{BAV1}$ genes ($rdhA4\text{-}6_{BAV1}$) formed a cluster with six $rdhA_{DE}$ genes ($rdhA1\text{-}6_{DE}$) and the *tceA* gene subcluster (Cluster I, Figure 7.2.). A second cluster (Cluster II) was composed of six $rdhA_{CBDB1}$ genes ($rdhA1\text{-}6_{CBDB1}$), six $rdhA_{FL2}$ genes ($rdhA1\text{-}6_{FL2}$), two $rdhA_{BAV1}$ genes ($rdhA1_{BAV1}$, $rdhA7_{BAV1}$), and five $rdhA_{DE}$ genes ($rdhA10\text{-}14_{DE}$). $rdhA7_{CBDB1}$ could not be unambiguously assigned to either of the two clusters. $rdhA16_{DE}$ and $rdhA17_{DE}$ genes shared only limited similarity with $rdhA_{CBDB1}$ and $rdhA_{FL2}$ genes but grouped with separate clusters comprising *cprA* genes and *pceA* genes from *Desulfitobacterium* spp. and *Dehalobacter restrictus*, respectively.

Several *rdhA*$_{CBDB1}$ genes shared high sequence similarity with *rdhA*$_{FL2}$ genes (Figure 7.3.). Six *rdhA*$_{CBDB1}$ genes with 98.5 - 99.8% identity to *rdhA*$_{FL2}$ genes and one *rdhA*$_{CBDB1}$ gene with 100% identity to an *rdhA*$_{FL2}$ gene based on the deduced amino acid sequences were identified. In addition, the restriction patterns of three FL2-clone library inserts matched three different restriction patterns of CBDB1-clone library inserts, indicating further pairs of highly similar sequences. Four *rdhA*$_{DE}$ genes were identified that shared 86.5 - 94.4% identity with *rdhA*$_{CBDB1}$ and *rdhA*$_{FL2}$ genes, thus forming four subclusters (Fig. 2). Also, one *rdhA*$_{BAV1}$ gene (*rdhA5*$_{BAV1}$; Cluster II) shared 90.8 - 93.1% sequence identity with the genes of one subcluster. A total of 10 subclusters, each comprising 2-4 RDase genes from different strains, were identified (Figure 7.2.).

7.3.3. Iron-sulfur cluster binding motifs

Like other dehalogenases, all *rdhA*$_{CBDB1}$- and *rdhA*$_{FL2}$-encoded amino acid sequences contained two iron-sulfur cluster binding (ISB) motifs in the C-terminal region (Fig. 3). The first ISB motif of all sequences corresponded to the conserved consensus sequence CXXCXXCXXXCP found in bacterial ferredoxins (Bruschi and Guerlesquin, 1988). Variations of this pattern, however, were observed in the second ISB motif. In 14 out of 21 RDase genes in Cluster I (Figure 7.2.), the second ISB motif shared the consensus CXXCXXCXXCP. In the other seven RDase genes of Cluster I, the first two cysteine residues of the second ISB motif were separated by three, four, or six residues instead of two (Figure 7.3.). In all five RDase genes of Cluster I with four or six residues between the first two cysteines of the second ISB (i.e., *rdhA12-14*$_{CBDB1}$, *rdhA10*$_{FL2}$, *rdhA2*$_{DE}$), a fifth cysteine residue was present upstream of the second ISB motif. This

motif $CX_4CX_nCX_{2\text{-}3}CXXXCP$ was also found in *rdhA3*$_{DE}$ and *rdhA4*$_{DE}$. In all *Dehalococcoides* RDase genes outside of Cluster I, the second ISB motif was characterized by a long stretch of residues (8-21 amino acids) separating the first two cysteine residues (i.e., *rdhA1-7*$_{CBDB1}$; *rdhA1-6*$_{FL2}$; *rdhA7-17*$_{DE}$; *rdhA1-3*$_{BAV1}$, *rdhA7*$_{BAV1}$; Fig. 3). All RDase genes that contained a second iron sulfur binding motif with closely linked cysteine residues (Cluster I) formed a coherent group of related genes whereas the other *Dehalococcoides* RDase genes that contained a second iron sulfur binding motif with a distant cysteine did not form a phylogenetically consistent group (Figure 7.2.).

7.3.4. N-terminal region of RDase genes

Deduced amino acid sequences of all gene fragments amplified with the primer pair RR2F/B1R contained stretches of hydrophobic residues at their N-terminal ends that were similar to those in known reductive dehalogenases. The forward primer RR2F targets the consensus motif RRXFXK of twin-arginine signal sequences, indicating that all amplified genes contained this sequence though the exact nucleotide composition is not known due to the degenerate nature of this primer and the low stringent conditions of the PCR reactions.

7.3.5. B gene sequences

The obtained N-terminal B gene fragments (*rdhB*$_{CBDB1}$, *rdhB*$_{FL2}$) encoded mainly hydrophobic amino acid residues, similar to the respective regions of B genes linked to known RDase genes, e.g., *tceB* of *D. ethenogenes* strain 195 or *pceB* of *S. multivorans.* The obtained *rdhB*$_{FL2}$ fragments were small (~90 bp) and therefore did not allow

calculation of a phylogenetic tree with high bootstrap values. Six *rdhB*$_{CBDB1}$ gene fragments were 100% identical to six *rdhB*$_{FL2}$ fragments, analogous to the corresponding highly similar pairs of *rdhA*$_{CBDB1}$ and *rdhA*$_{FL2}$ genes.

7.3.6. Expression analysis of RDase genes in FL2 grown on TCE as electron acceptor

cDNA generated with RNA extracted from FL2 cells grown with TCE was PCR amplified with *tceA* primers (Magnuson *et al.*, 2000), and an amplicon of the expected size was generated. Since *tceA* was a gene expected to be transcribed, this just confirmed that the cDNA generated was good quality cDNA and suitable for future analysis. PCR amplification with degenerate primers RRF2 and B1R using cDNA obtained from TCE and *cis*-DCE grown FL2 cells as template yielded a PCR fragment of the expected size (approximately 1,600 bp). In contrast, no amplification occurred when RNA that had not undergone the RT-PCR step was used as template, confirming that all DNA was successfully removed from the RNA preparation and that the observed 1,600 bp amplicon was generated from mRNA. Amplification of cDNA occurred with degenerate primers RRF2 and B1R targeting the reductase internal RRXFXK motif and the WYEW sequence in the putative B gene, respectively, indicating that both genes are co-transcribed. A clone library generated with the PCR-amplified cDNA contained eight clones with the correct size insert. Five different clones were identified, with the use of RFLP analysis. Four clones were sequenced (one was lost), and three putative reductive dehalogenases were identified. The three putative reductive dehalogenase genes sequenced were identical to *rdhA6*$_{[FL2]}$, *rdhA5*$_{[FL2]}$, and *rdhA10*$_{[FL2]}$, indicating that

rdhA6$_{[FL2]}$, *rdhA5*$_{[FL2]}$, and *rdhA10*$_{[FL2]}$ were transcribed when FL2 was grown with TCE as an electron acceptor.

Nested PCR using cDNA as template, RRF2 and B1R primers for the first round amplification, followed by *rdhA5*$_{[BAV1]}$ targeted primers, i.e., bavrdA5F (5'-GTTAATGTTGCCAAGGCT-3') and bavrdA5R (5'CATGGTCTTTTCCATATTGGC-3') revealed the presence of another FL2 putative reductive dehalogenase designated *rdhA12*$_{[FL2]}$ *rdhA12*$_{[FL2]}$ shares high identity to *rdhA5*$_{[BAV1]}$, *rdhA10*$_{[CBDB1]}$, and *rdhA6*$_{[DE]}$.

7.3.7. Expression analysis of RDase genes in FL2 grown on cis-DCE as electron acceptor

cDNA generated with RNA extracted from FL2 cells grown in *cis*-DCE was PCR amplified with *tceA* primers (Magnuson *et al.*, 2000), and an amplicon of the correct size was generated, as expected, indicating that the *tceA* gene was transcribed when FL2 was grown with *cis*-DCE as an electron acceptor.

PCR amplification with degenerate primers RRF2 and B1R using cDNA obtained from *cis*-DCE grown FL2 cells as template yielded a PCR fragment of the expected size (approximately 1,700 bp). In contrast, no amplification occurred without the RT-PCR step, confirming that all DNA was successfully removed from the RNA preparation and that the observed 1,700 bp amplicon was generated from mRNA. Amplification of cDNA occurred with degenerate primers RRF2 and B1R targeting the reductase internal RRXFXK motif and the WYEW sequence in the putative B gene, respectively. A clone library generated with the PCR-amplified cDNA contained 19 clones with the correct size

insert. Through RFLP, five different clones were identified and sequenced. Three putative reductive dehalogenase genes were identified (two clones were a repetition) and were identical to *rdhA6*$_{[FL2]}$ *rdhA12*$_{[FL2]}$ and *rdhA1* $_{[FL2]}$ (Hölscher *et al.*, 2004), indicating that *rdhA6*$_{[FL2]}$ *rdhA12*$_{[FL2]}$ and *rdhA1*$_{[FL2]}$ were transcribed when FL2 was grown with *cis*-DCE as an electron acceptor.

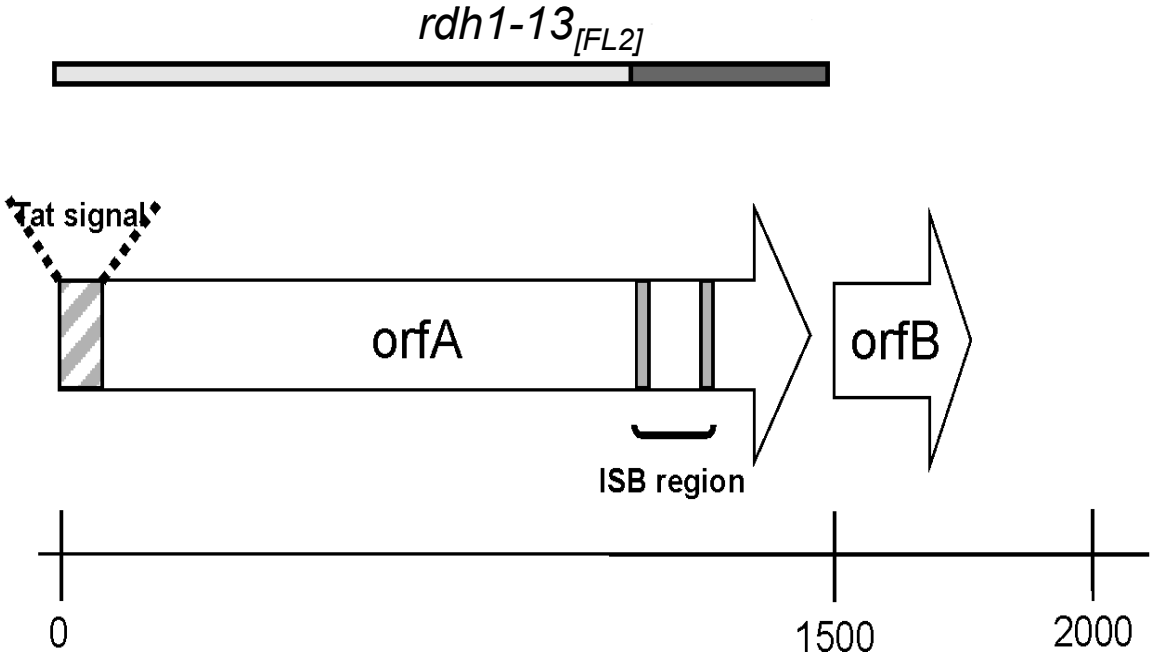

Figure 7.1. Schematic diagram of gene fragments amplified using RRF2 and B1R degenerate primers. OrfA represents the open reading frame encoding the catalytically active reductive dehalogenase; orfB is the open reading frame encoding a small hydrophobic B-protein. Tat signal, indicates the position of the twin-arginine translocation signal sequence, including the RRXFXK; ISB region, iron-sulfur cluster binding region. The scale bar indicates nucleotide position (1= start codon of orfA).

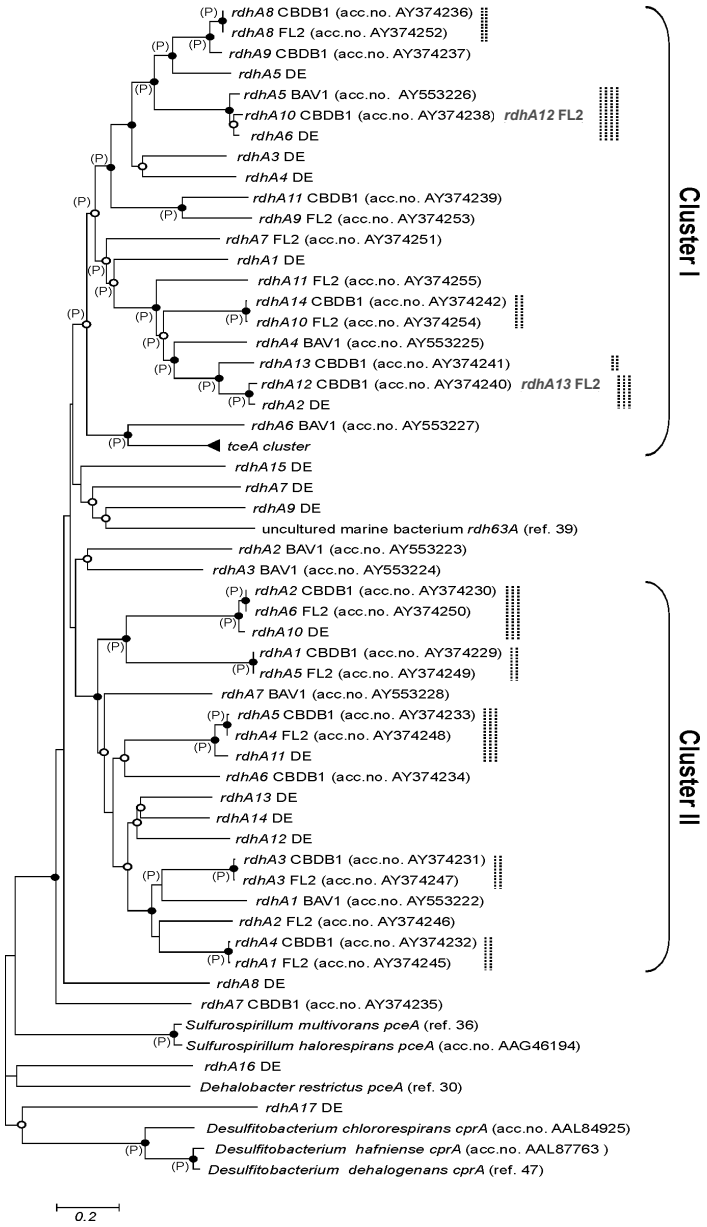

Figure 7.2. Phylogenetic analysis of RDase genes from *Dehalococcoides* sp. strain FL2 (*rdhA1-11* FL2), *Dehalococcoides* sp. strain CBDB1 (*rdhA1-14* CBDB1), *Dehalococcoides* sp. strain BAV1 (*rdhA1-7* BAV) and *Dehalococcoides ethenogenes* strain 195 (*rdhA1-17* DE). The neighbour-joining tree shown was generated from amino acid sequences of nearly complete orfAs. Branching points supported by 85-100% of 1000 bootstrap sampling events are indicated by solid circles. Open circles indicate 50-84% support by bootstrap sampling. Branching points supported by the maximum parsimony treeing method are marked with (P). clones from strain FL2 (*rdhA12*FL2 and *rdhA13*FL2) are indicated together with the almost identical $rdhA_{CBDB1}$ genes. Dotted double, triple, and quadruple lines indicate subclusters of highly similar genes from two, three, or four different *Dehalococcoides* strains, respectively. The *tceA* cluster comprises the *tceA* genes of *Dehalococcoides ethenogenes* strain 195 (AAF73916), strain FL2 (AY165309), and three chloroethene dechlorinating enrichment cultures (AAN85590, AAN85592, AAN85594). The scale bar represents 20% sequence divergence. Figure modified from Hölscher *et al*, 2004.

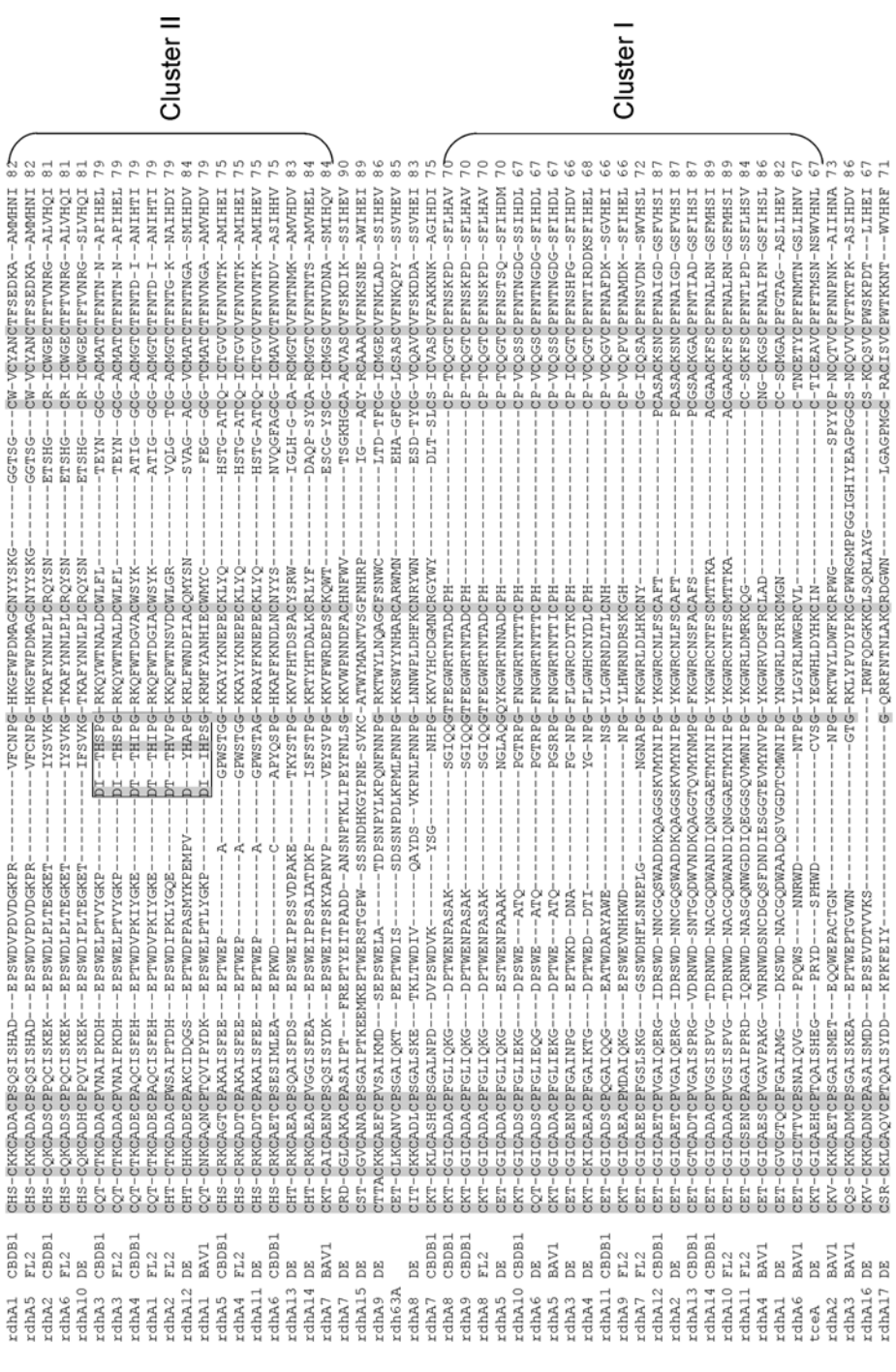

Figure 7.3. Alignment of the C-terminal region of deduced amino acid sequences of RDase genes from *Dehalococcoides* strains (*tceA* DE refers to the *tceA* gene of *Dehalococcoides ethenogenes* strain 195). Also included is sequence *rdh63A*, an RDase gene obtained from a bacterial consortium. ClustalW alignments were manually corrected to align putative cofactor binding sites. Conserved residues of the two iron-sulfur cluster binding motifs and cobalamin-binding consensus sequences are highlighted in grey. Boxes mark cobalamin-binding motifs in *rdhA3-4*$_{CBDB1}$, *rdhA1-3*$_{FL2}$, *rdhA12*$_{DE}$*, and rdhA1*$_{BAV1}$. In *rdhA12*$_{DE}$ and *rdhA1*$_{BAV1}$. Figure from Hölscher *et al.,* 2004.

Table 7.1. RDase genes detected in cDNA clone libraries established with RNA from FL2 cultures grown with TCE or *cis*-DCE as electron acceptors

RDase	**FL2 *cis*-DCE**	**FL2 TCE**
TceA	+	+
$RdhA1_{[FL2]}$	+	
$RdhA2_{[FL2]}$		
$RdhA3_{[FL2]}$		
$RdhA4_{[FL2]}$		
$RdhA5_{[FL2]}$		+
$RdhA6_{[FL2]}$	+	+
$RdhA7_{[FL2]}$		
$RdhA8_{[FL2]}$		
$RdhA9_{[FL2]}$		
$RdhA10_{[FL2]}$		+
$RdhA11_{[FL2]}$		
$RdhA12_{[FL2]}$	+	+
$RdhA13_{[FL2]}$		

7.4. Discussion

Thirteen novel RDase genes were amplified from *Dehalococcoides* sp. strain FL2 Deduced amino acid sequences of nine RDase genes from strain FL2 were 98.5-100% identical to nine different RDase genes from strain CBDB1, suggesting that both strains have an overlapping substrate range. All RDase genes identified in strains FL2 and CBDB1 were related to the RDase genes present in the genomes of *Dehalococcoides ethenogenes* strain 195 and *Dehalococcoides* sp. strain BAV1, however, sequence identity did not exceed 94.4% and 93.1%, respectively

Although the substrate specificities of enzymes encoded by the amplified genes cannot be inferred, there is strong evidence that all RDase genes identified in the four *Dehalococcoides* strains represent true homologs (genes with a common ancestry) of the biochemically characterized TCE dehalogenase gene of strain 195. i) All amplified RDase genes share 27-33% sequence identity with the *tceA* gene at the amino acid level over the whole length of the sequence (449-489 residues for all $rdhA_{CBDB1}$ and $rdhA_{FL2}$ genes except $rdhA10_{FL2}$ that is 404 residues in length), which allows the inference of a homologous relationship as described by Brenner *et al.*, (1998) and Rost, (1999). ii) All RDase genes share two functional domains that determine the structure and/or localization of the protein, i.e., the N-terminal twin-arginine signal sequence and the iron sulphur binding region near the C-terminus comprising two separate iron sulphur binding motifs. iii) All RDase genes are organized in operons with a downstream B gene encoding a putative membrane anchor. iv) The high number of RDase genes (17 RDase genes in strain 195, at least 14 RDase genes in both strains FL2 and CBDB1, and at least seven RDase genes in strain BAV1) makes convergent evolution unlikely.

Sequence comparison of RDase gene fragments from all four *Dehalococcoides* isolates demonstrated that highly similar genes are shared among strains and that unique RDase genes exist that distinguish different *Dehalococcoides* strains. The sub clusters of highly similar genes can be interpreted as orthologs, i.e., homologs derived by a speciation event (Storm and Sonnhammer, 2002). The 16S rRNA genes of strain CBDB1 (accession number AF230641) and strain FL2 (accession number AF357918) are 100% identical and share 98.5% sequence identity with the 16S rRNA gene of strain 195 (accession number AF004928). The topology of each orthologous group of RDase genes from strain CBDB1, strain FL2, and strain 195 is consistent with 16S rRNA gene-based phylogeny supporting that strains FL2 and CBDB1 are more closely related to each other than to strain 195. The 16S rRNA gene of strain BAV1 (accession number AY165308) exhibits only one base difference (99.9% identity) from the 16S rRNA genes from strains CBDB1 and FL2. However, no stronger relationship was identified between $rdhA_{\mathrm{BAV1}}$ genes and RDase genes from strains CBDB1 and FL2 than between $rdhA_{\mathrm{BAV1}}$ genes and $rdhA_{\mathrm{DE}}$ genes.

The presence of multiple non-identical RDase genes in *Dehalococcoides* strains is consistent with the observation that the different strains use different chlorinated electron acceptors. Several RDase genes are present in individual strains that do not have an ortholog among the known genes of the other strains. Many of the RDase genes found in strains CBDB1, FL2, and BAV1 do not have orthologs in the completely sequenced genome of strain 195, suggesting that these strains possess dechlorination activities not present in strain 195. Strains CBDB1 and 195, but not strain FL2, dechlorinate chlorinated benzenes, suggesting that chlorobenzene reductive dehalogenases are only

present in strains CBDB1 and 195. However, specific culture conditions might be necessary to induce chlorobenzene dechlorination in strain FL2.

All RDase genes contain the conserved functional domains characterized in reductive dehalogenases (Holliger *et al.*, 1998; Smidt *et al.*, 2000). The presence of a twin-arginine signal peptide sequence in RDase translated proteins is consistent with the hypothesis that RDase genes are involved in respiratory reductive dehalogenation, because such motifs are predominantly found in membrane-associated proteins involved in respiratory electron transport (Berks *et al.*, 2000; Smidt *et al.*, 2000). Further, deduced amino acid sequences of all RDase genes contain two ISB motifs and could, therefore, form two spatially linked iron-sulfur clusters. Iron-sulfur clusters have been detected in most reductive dehalogenases and probably mediate electron transfer to the active site containing the corrinoid (Neumann *et al.*, 1996; van de Pas *et al.*, 1999; Beinert, 2000). Indications for the involvement of a corrinoid cofactor in catalysis have been found for most reductive dehalogenases from bacteria that couple reductive dechlorination to growth (Holliger *et al.*, 1998; Smidt *et al.*, 2000).

Though not identifying the function of the *Dehalococcoides* RDase genes, this study provides essential sequence data that is useful for the identification of *Dehalococcoides* reductive dehalogenase genes from peptide fragments and for the design of primers for transcription analysis of specific RDase genes.

Substrate-dependent differential induction of dehalogenases has been demonstrated in *Desulfitobacterium* spp. (reviewed in Smidt and de Vos (2004)). In strain CBDB1, different 1,2,3-TCB, PeCB and HCB dechlorination rates were obtained with cells pre-grown on different chlorobenzene congeners as electron acceptors, suggesting

that dehalogenases are induced by their respective substrates (i.e., electron acceptors) (Jayachandran *et al.*, 2003). In contrast, PCE dechlorination was constitutive in cultures of strain 195 grown on TCE, 1,1-DCE or dichloroethane (Maymó-Gatell *et al.*, 1999). In this study, additionally to *tceA,* four and three RDase genes were transcribed when FL2 was grown on TCE and *cis*-DCE respectively. The fact that one additional RDase was transcribed when FL2 was grown on TCE was expected if it is assumed that transcription of RDase genes is triggered by the substrate to be dechlorinated, since one more dechlorination step is needed for the complete dechlorination of TCE. However, one of the RDase genes transcribed when FL2 was grown in cis-DCE, i.e., *RdhA1* $_{[FL2]}$ was not detected in FL2 cells grown on TCE. A few explanations are possible. One of them is that transcription is not triggered by the specific substrate to be dechlorinated, which would mean that RDase transcription is not tightly regulated. Another explanation is that RDase genes undergo posttranscriptional changes, which are essential to determine their activity. A third explanation could be that the activity of the transcribed RDase is dependent on the interaction with the corrinoid factors, and the last possibility is that some of these RDase proteins are constitutively transcribed.

7.4. References

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CHAPTER VIII

OPTIMIZATION OF RNA EXTRACTION PROCEDURES FROM A DECHLORINATING CONSORTIUM

8.1. Introduction

Many molecular biology techniques such as reverse transcription-polymerase chain reaction (RT-PCR), microarray analysis, and Northern blotting rely on good quality RNA and require a method that will allow for quick isolation of intact RNA with no DNA contamination. The methods of RNA isolation from eukaryotic cells have been well established because their RNA molecules are more stable than prokaryotic RNA molecules and eukaryotic cells are easy to lyse. However, the methods for isolating intact RNA from bacteria, especially from Gram-positive bacteria, are challenging because of their complex cell walls which are harder to lyse through physical methods or detergent solutions usually used for mammalian or plant cells. *Dehalococcoides* species have a peculiar cell wall, which has not been characterized as Gram-positive, however, in order to efficiently lyse *Dehalococcoides* spp. cells for DNA extractions severe lysis conditions were needed (He, 2003), suggesting that *Dehalococcoides* cells are harder to lyse than Gram-positive bacteria . Lysing procedures in RNA extraction methods which have been used for Gram positive bacteria include: enzymatic lysis (Bashyam and Tyagi, 1994), sonication (Rajagopalan *et al.*, 1995), and bead-beating. Usually guanidine isothiocyanate, phenol, and SDS are used as part of the extraction method to inhibit RNases. Methods for obtaining good quality RNA from environmental samples have also been explored (Alm and Stahl, 2000; Hurt *et al.*, 2001)

Dehalococcoides spp. have gained interest in bioremediation due to their widespread dechlorinating capabilities. The study of their enzymatic systems poses a challenge, given the difficulties presented when growing them in the laboratory. Most anaerobic microorganisms usually provide low biomass yields making it challenging to obtain a critical amount of biomass by which to study their protein systems. In order to study expression of enzymes, which are difficult to isolate, mRNA transcribed can be targeted. RNA extraction methods were used in previous chapter in this dissertation and proved to be challenging when *Dehalococcoides spp.* was the population to be studied.

The objective of this chapter was to identify an RNA extraction protocol that could be used to obtain *Dehalococcoides* mRNA. RNA isolation methods and several lysing conditions were tested and compared using Bio-dechlor INOCULUM (BDI). The impact of the RNA isolation method on metabolically active *Dehalococcoides* spp. and the quality of mRNA was determined by a two-step reverse transcription RT-PCR reaction. The first step included the formation of cDNA using random hexamer primers. The second step included PCR and Real-Time PCR (RTm PCR) targeting *Dehalococcoides* spp. 16S rRNA and reductive dehalogenase genes. The quality of mRNA was assessed by RT-PCR and RTm-PCR targeting reductive dehalogenase genes such as the TCE reductive dehalogenase *tceA* and the vinyl chloride (VC) reductive dehalogenase *bvcA*. Additionally, a clone library was established using cDNA and degenerate primers RRF2 and B1R to investigate transcription of RDase genes other than *tceA* and *bvcA*.

8.2. Methods

8.2.1. Chemicals

Chemicals were purchased from Aldrich (Milwaukee, WI) or Sigma Chemical Co. (St Louis, MO), except for VC, which was obtained from Fluka Chemical Corp. (Ronkonkoma, NY). Restriction enzymes were purchased from Promega Biosciences, Inc. (San Luis Obispo, CA), and enzymes used for cell lysis were from Sigma Chemical Co. PCR reagents were purchased from Applied Biosystems (Foster City, CA), and BSA was purchased from Roche (Mannheim, Germany).

8.2.2. Sources of DNA

Genomic DNA was obtained from pure cultures of *Dehalococcoides* sp. strains BAV1, and FL2. Bio-Dechlor INOCULUM (www.regenesis.com), a culture based on the Bachman Road site inoculum (Lendvay *et al.*, 2003), a PCE-to-ethene-dechlorinating mixed culture that has been successfully employed in bioaugmentation approaches was used for this study.

8.2.3. RNA isolation

Biomass was collected by centrifugation and cell pellets were immediately frozen at –80ºC, 5mL of cultures were used fro each treatment. All solutions used for RNA extraction were prepared with diethyl pyrocarbonate (DEPC)-treated water. The chlorophorm phenol based reagent trizol was compared with two column-based kits, the Rneasy extraction kit (Qiagen) and micro to midi RNA isolation kit (Invitrogen), in conjunction with the extraction methods; several lysis procedures were used and

compared. The Max reagent (Invitrogen) was tested in conjunction wit trizol to assess a possible increase in RNA yield.

Total RNA was extracted using the RNeasy extraction kit (Qiagen) and micro to midi RNA isolation kit according to the manufacturer's recommendations, with the following modifications in several combinations to enhance cell lysis and RNA yields. The cell pellet was suspended in 100 µL lysozyme digestion buffer (30 mM Tris-HCl, 1 mM EDTA, pH 8.0, 15 mg/mL lysozyme). When SDS was part of the protocol, it was added at this point. The suspension was mixed and incubated at room temperature for 10 or 5 min (depending on the lysis method). Lysis buffer RLT (350 µL, provided with the RNeasy extraction kit) was added. When homogenization was part of the protocol, the lysate was homogenized by passing the sample through a syringe with a 25G needle. After homogenization the sample was transferred into a MicroRNA Bead Tube (Mo Bio Laboratories, Carlsbad, CA) and shaken horizontally on a Vortex mixer at maximum speed for 10 min. Not every step on the lysis procedure was performed at each extraction and results were compared to asses the effect of the lysis steps on RNA yield. The different lysis procedures used for each extraction are summarized in Table 8.1. DNA was removed after each extraction with RNase-free DNase (Invitrogen) as described by the manufacturer. RNA concentrations were determined spectrophotometrically at 260 nm using an HP 8453 photodiodearray UV/Vis spectrophotometer. A detail protocol including the best extraction method is included in Appendix D.

Table 8.1. RNA extraction methods (kits) and lysis enhancement methods used.

Sample name	Extraction method (kit)	Lysis enhancement
TM	Trizol max (I)	Max reagent phenol
TMM	Trizol max and micro to midi (I)	Max reagent phenol
M	Micro to midi (I)	Lysozyme 5 minutes Homogenization, SDS
MH	Micro to midi (I)	Lysozyme 5 minutes Homogenization
QSH	RNeasy (Q)	Lysozyme 5 minutes Homogenization, SDS
MMB	Micro to midi (I)	Lysozyme 5 minutes, beads 10 minutes Homogenization.
QH	RNeasy (Q)	Lysozyme 10 minutes Homogenization
QS	RNeasy (Q)	Lysozyme 10 min, SDS
QR	RNeasy (Q)	Lysozyme 10 minutes
MKA	Micro to midi (I)	Lysozyme and achromopeptidase 5 minutes, protenaise K 5 minutes Homogenization, SDS

(I) Invitrogen, (Q) Qiagen

8.2.4. Expression analysis of putative RDase genes

Reverse transcription PCR (RT-PCR) was performed with the two-step RT-PCR omniscript kit (Qiagen). First, reverse transcription reactions were performed with 1 mM random hexamer primers (Promega) and 360 ng of extracted RNA in a total volume of 20 µL for 3 hrs at 37°C according to the manufacturer's recommendations. Then, PCR was performed with specific primers targeting reductive dehalogenases *tceA* (Magnuson *et al.*, 2000) and *bvcA* (Krajmalnik-Brown *et al.*, 2004). PCR conditions were as described by Magnuson and Krajmalnik-Brown respectively. RT-PCR amplification products were examined by gel electrophoresis on 1.5% agarose gels. Additionally, cDNA clone libraries were established using primers RRF2 and B1R as described in chapters six and seven.

8.2.5. Real-Time (RTm) PCR

Oligonucleotides targeting 16S rRNA genes, 16S rRNA genes sequences of *Dehalococcoides* species and two reductive dehalogenase genes, i.e., *tceA* and *bvcA* were used to asses the capability of extracting *Dehalococcoides* spp. rRNA and mRNA for each of the methods. Primers and probes used for this task are summarized in Table 8.2.

TaqMan Probes were labeled with 6-carboxyfluorescein (FAM) as a reporter fluorochrome on the 5'end, and N,N,N',N'-tetramethyl-6-carboxy-rhodamine (TAMRA) as quencher on the 3' end. Each MicroAmp optical tube had 30-µL reaction volume containing 1 x TaqMan Universal PCR Master Mix (including DNA polymerase, deoxynucleoside triphosphates, and $MgCl_2$) (Applied Biosystems), forward primer, reverse primer, TaqMan probe (300 nM each), and DNA template from each 10-fold

diluted sample. The PCR conditions were as follows: 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. PCR was carried out in a spectrofluorimetric thermal cycler (ABI Prism 7700 Sequence Detection System, Applied Biosystems). A calibration curve (log DNA concentration versus an arbitrarily set cycle threshold value, C_T) was obtained using serial dilutions of DNA of known concentration. The C_T values obtained for each sample were compared with the standard curve to determine the copy numbers. Experiments were performed in triplicate along with appropriate controls (e.g., no template DNA and RNA as template). Copy number estimates used the assumptions of an average molecular weight of 660 for a base pair in dsDNA, one 16S rRNA *tceA* and *bvcA* gene operon per *Dehalococcoides* genome, and a genome size of 1.5 Mbp (www.tigr.org). The equation used to estimate the copy numbers/ml of culture was

$$gene\ copies/\mu L = (DNA\ (\text{ng}/\mu L) \times 6.023 \times 10^{23})/(1.5 \times 10^{6} \times 660) \times 10^{6}$$

Table 8.2. RTm PCR primers and Taqman probes used to quantify bacterial 16S rRNA genes, *Dehalococcoides* 16S rRNA genes, *tceA* and *bvcA* genes present in cDNA generated with random hexamer primers.

Primer	**Sequence 5'-3'**	**Target gene**	**Ref**
		Dehalococcoides spp.	
RTmDhcF	CTGGAGCTAATCCCCAAAGCT	16S rRNA	1
		Dehalococcoides spp.	
RTmDhcR	CAACTTCATGCAGGCGGG	16S rRNA	1
		Dehalococcoides spp.	
RTmDhc (P)	FAM-TCCTCAGTTCGGATTGCAGGCTGAA-TAMRA	16S rRNA	1
VCR925F	AAAAGCACTTGGCTATCAAGGAC	*bvcA* gene	2
VCR1017R	CCAAAAGCACCACCAGGTC	*bvcA* gene	2
VCR977(P)	FAM-TGGTGGCGACGTGGCTATGTGG-TAMRA	*bvcA* gene	2
TCEA1270F	ATCCAGATTATGACCCTGGTGAA	*tceA* gene	2
TCEA1336R	GCGGCATATATTAGGGCATCTT	*tceA* gene	2
RtmTceA(P)	FAM-TGGGCTATGGCGACCGCAGG-TAMRA	*tceA* gene	2
		All Bacteria	
Bac1055F	ATGGYTGTCGTCAGCT	16S rRNA	3
		All Bacteria	
Bac1392R	ACGGGCGGTGTGTAC	16S rRNA	4
Bac1115(P)	FAM- CAACGAGCGCAACCC-TAMRA	All Bacteria	4,5

(P) Probe

1. (He *et al.*, 2003), 2. (Ritalahti *et al.* 2005), 3. (Ferris *et al.*, 2003), 4. (Lane *et al.*, 1985), 5. (Harms *et al.*, 2003)

8.3. Results

8.3.1. RNA extraction methods

Three samples were extracted under the same conditions in parallel and after the extraction was completed, the three samples were mixed and DNase treated, the results are presented in Figure 8.1. The concentrations presented in Figure 8.1. are similar to an average of three samples.

The best extraction method judged only by total RNA concentration, was QSH, i.e. the RNeasy extraction method with the following lysis modifications: SDS was added after lysosyme and the sample was homogenized through a syringe with a 25G needle. The next best method was M, which used the micro to midi columns with the following lysis modifications: SDS was added after lysosyme and the sample was homogenized through a syringe with a 25G needle. The only difference between M and QSH is the columns used. For QSH, RNeasy (Qiagen) columns were used while for M micro to midi (Invitrogen) columns were used.

Concentrations of RNA obtained were organized in descending order before plotting the graph in Figure 8.1. Best yielding extraction methods are on the left of the graph and worst yielding extraction methods are located on the right of the graph. It is not a surprise that the methods where homogenization was used to improve lysis yielded more RNA (figure 8.1.), indicating that homogenization had a positive effect in the overall extraction yield. Homogenization using a syringe and thin needles is a good alternative to improve lysis procedures, and can be done quickly. Time is a critical factor when extracting RNA given that the longer it takes to complete the extraction, the higher the chances that RNA will get degraded during the process. Another advantage of the

proposed homogenization method is that it does not require special equipment, just syringes and needles.

8.3.2. Expression analysis of putative RDase genes

Transcription of *bvcA* and *tceA* was explored in more detail using specific primers. PCR reactions using cDNA generated from a PCE-grown culture as template and *tceA* targeted primers yielded amplicons of the correct size for most of the extraction methods. (Figure 8.2.), and no amplicons were obtained when total RNA extracts were used as template (data not shown), confirming that no residual genomic DNA was present. Surprisingly, no amplification was observed, when *bvcA* targeted primers were used.

Levels of expression of *tceA* were used as an indicator of the adequacy of the method in efficiently extracting *Dehalococcoides* mRNA. Results from RTm PCR indicated that the three methods for which the highest concentrations of RNA where obtained were also the methods where more transcripts/μL in the RTm reaction were generated. The three best methods based on amount of total RNA obtained and amount of *tceA* transcripts obtained were QSH, M and QH.

Method QH provided much higher yields than QS, indicating that homogenization is a better lysis method for RNA extraction than the addition of detergents like SDS, however, the highest yields were obtained with the combination of SDS and Homogenization.

A cDNA clone library was established with cDNA generated with RNA isolated with the QSH method. Seventy-six white colonies were picked and only 20 had the correct size insert. RFLP identified nine clones, which seemed to be different to each other. Partial sequence analysis of the nine clones indicated the presence of three putative reductive dehalogenase genes in the cDNA clone libraries. One was identical to *vcrA*, and the other two were identical to putative RDH genes present in FL2 i.e. $RdhA1_{[FL2]}$ and $RdhA13_{[FL2]}$.

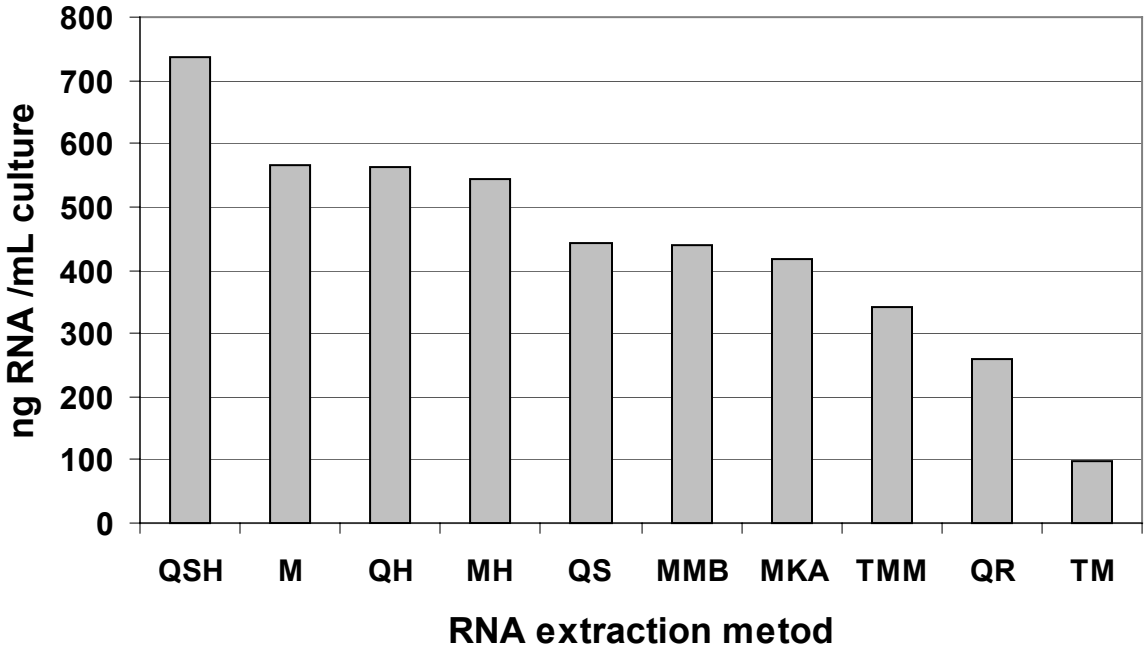

Figure 8.1. RNA concentrations (ng RNA/mL culture) obtained with different extraction methods.

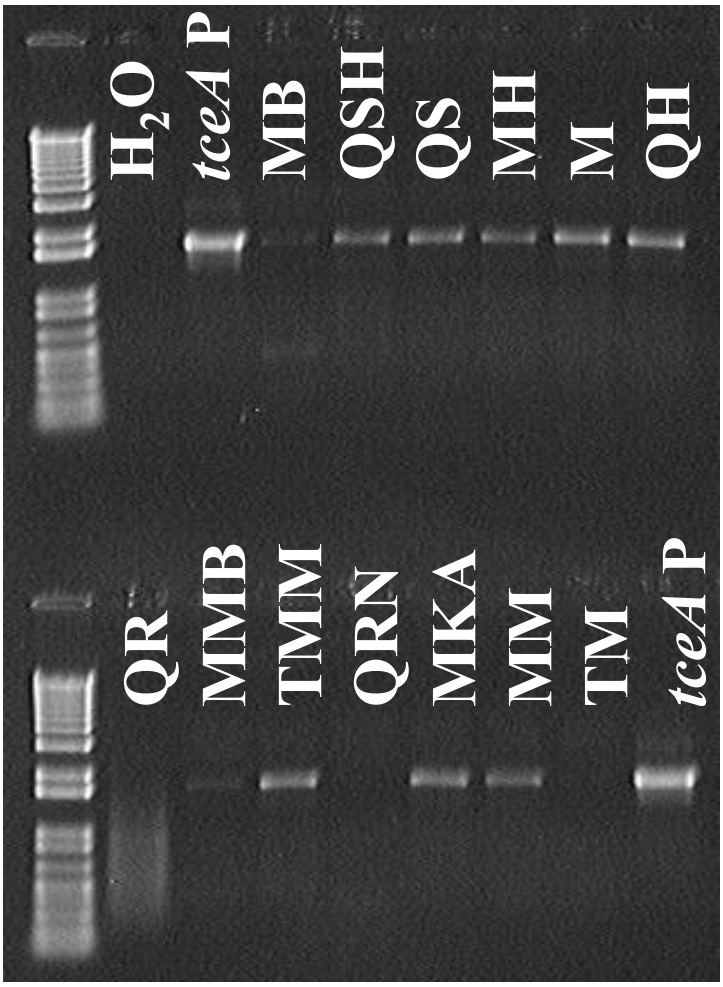

Figure 8.2. *tceA* expression in BDI grown with PCE as an electron acceptor. Amplification was performed using *tceA*-targeted primers and cDNA produced following the different extraction methods. *tceA* P was used as positive control, *tceA* P is a plasmid containing the *tceA* gene from strain FL2.

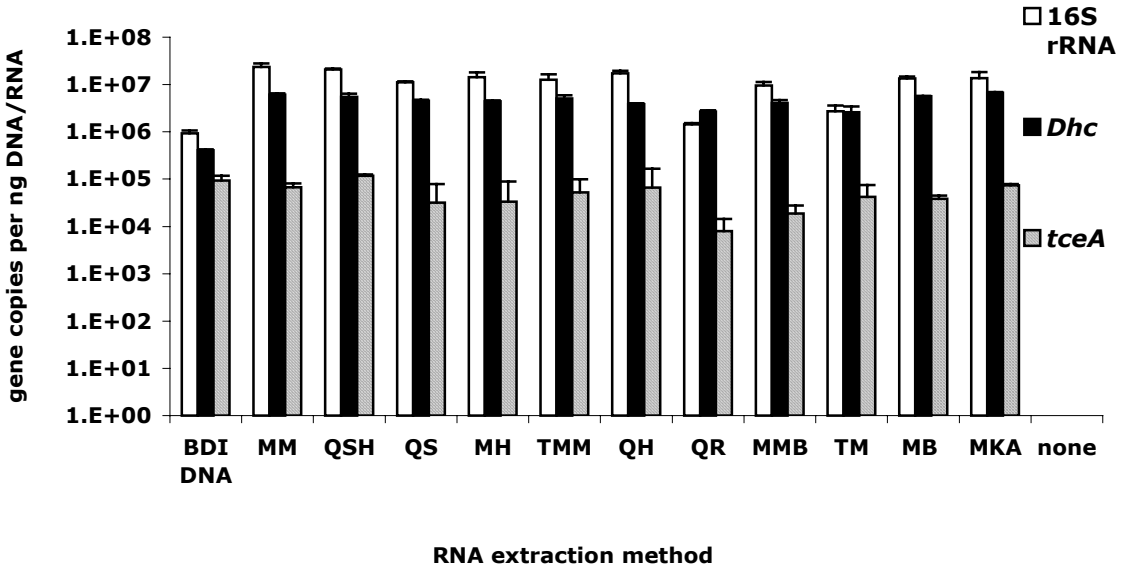

Figure 8.3. Comparison of the quality of various RNA extraction methods using RTm PCR quantification of 16S rRNA, Dhc 16SrRNA and *tceA* in cDNA from BDI consortium enriched in PCE. BDI DNA was used as a positive control. Water (none) used as a negative control.

8.4. Discussion

A simple and reproducible method for RNA extraction was designed. Column based methods yielded higher RNA concentrations than the chloroform-phenol method tested. Based solely on RNA concentration, higher yields were obtained with RNeasy kit adding lysozyme, SDS, and homogenization to the original protocol or with the Micro to Midi kit, under the same conditions. RTm PCR results for *tceA* transcripts support these findings. These results suggest that the type of column used might not be so important for a good RNA yield, a good and quick lysis procedure and creating an RNase free environment are factors which seem to be more important than the type of column being used.

The lysis procedure i.e., lysozyme, homogenization and the addition of SDS are crucial steps for achieving higher RNA yields. The addition of other lysis enzymes such as proteinase K and achromopeptidase to column-based protocols did not improve RNA yields. The findings of this study suggest that it is lysing using proteinase K and achromopeptidase has a negative effect in the final RNA concentration, because longer digestion times are necessary in which the RNA is exposed to the degrading activity of RNases.

16S rRNA gene and *tceA* gene transcripts were successfully quantified with a RT-RTm PCR approach. RT-RTm PCR results indicated that the abundance of *Dehalococcoides* 16S rRNA as well as the recovery of mRNA were influenced by the isolation method. An easy, quick and reproducible method for RNA extraction of *Dehalococcoides* species is provided. Cheaper columns are available and could be tested with the same lysis procedure recommended, to make the extraction more economic.

In addition to *tceA* at least three additional RDase genes were transcribed as shown in the cDNA clone libraries, two of the transcribed putative RDases i.e. $RdhA1_{[FL2]}$ and $RdhA13_{[FL2]}$, most likely came from FL2, given that the sequences obtained were nearly identical, and the third RDase detected was *vcrA*, which most likely was present in the culture given that BDI contains strain GT, and *vcrA* is present in strain GT. Strain GT cannot use PCE as an electron acceptor; however, it is most likely active in the culture because when PCE is dechlorinated by other bacteria, dechlorination products with lower degrees of chlorination such as TCE, DCE and VC are produced, and these chlorinated compounds can serve strain GT as electron acceptor.

8.5. References

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CHAPTER IX

CONCLUSIONS AND RECOMMENDATIONS

Reductive dehalogenase genes were investigated in site samples, enrichment and pure dechlorinating cultures. Degenerate primers targeting putative reductive dehalogenase genes were designed. With their use seven and thirteen putative reductive dehalogenases were identified in *Dehalococcoides* sp. strain BAV1 and *Dehalococcoides* sp. strain FL2 respectively; a putative VC reductive dehalogenase gene, *bvcA* was identified in strain BAV1. Specific primers targeting *bvcA* were designed. *bvcA* targeted primers are being used for monitoring efforts in the field, and will be a helpful tool to distinguish among *Dehalococcoides* spp. given that *bvcA* can be used to track growth and distribution of BAV1 like microorganisms in contaminated aquifers. Being able to monitor the *bvcA* gene will enhance monitoring techniques and will help establish cause effect relationships. Additionally, reductive dehalogenase genes were also identified in two parallel studies in strain CBDB1 and in the KB1 inoculum through the use of the primers developed in this study. The characterization of the putative VC RDase gene, *bvcA*, along with the additional putative RDase genes identified will improve the understanding about RDase systems in *Dehalococcoides* spp. Application of the findings of this study within the field of Environmental Engineering provides a powerful monitoring tool for chloroethene contaminated sites where an engineered bioremediation approach is or will be applied using *Dehalococcoides* spp. as the main player in the complete reductive dechlorination. Applications in other fields include a collection of

reductive dehalogenase genes, which will be helpful for studying genomics and evolution of RDase genes providing a better understanding of evolution of chlororespiring populations. Studies involving expression and regulation of RDase genes will also be aided by the collection of RDase gene sequences started through this research.

Based on the results of this research, the following specific conclusions can be drawn:

The presence of *Dehalococcoides* populations in chloroethene-contaminated sites and in VC and *cis*-DCE dechlorinating enrichment cultures obtained from natural sediments was confirmed through the use of *Dehalococcoides*-targeted primers and through 16S rRNA clone libraries. Evidence is presented that implicates *Dehalococcoides* populations in the reduction of these compounds to ethene. Cloned 16S rRNA gene sequences of *Dehalococcoides* populations in the cultures used for this study were identical to each other, all belonging to the Pinellas group.

Ampicillin treated cultures contained a higher amount of *Dehalococcoides* spp., and influenced the presence of other organisms that were detected in enrichment communities. This demonstrated that ampicillin is an effective selecting agent to enrich for *Dehalococcoides* spp.

The presence of the *tceA* gene was assessed in site material samples and enrichment cultures. No correlation was found between *tceA* presence and VC metabolic dechlorination. High similarity in the *tceA* gene sequences obtained from diverse locations suggested that the *tceA* gene was horizontally transferred. Also, it was

demonstrated that enrichment procedures influence the presence of *tceA* containing *Dehalococcoides*. The BRS culture initially contained the *tceA* gene, however, upon enrichment in VC the *Dehalococcoides* population containing the *tceA* gene was lost, and as a result, the pure culture BAV1 is not able to dechlorinate TCE in a respiratory manner.

Seventeen putative RDase genes were identified in the genome of *Dehalococcoides ethenogenes*. All of them have at least one iron sulfur cluster identical to the typical consensus motif **C**XX**C**XX**C**XXX**CP**; some of them have two identical **C**XX**C**XX**C**XXX**CP** motifs. A gene encoding for a hydrophobic protein designated the B gene, was present downstream of all RDases in the *Dehalococcoides ethenogenes* genome.

The twin arginin motif RRXFXK was present in sixteen of the seventeen identified RDases. The motif was in the form of RRDFMK in *tceA* and in thirteen out of the seventeen putative reductive dehalogenases found in *Dehalococcoides ethenogenes* strain 195, this motif is not so conserved in the previously identified dehalogenases from non-Dehalococcoides spp. indicating that RRDFMK is a possible *Dehalococcoides* signature motif.

Three putative RDase genes were identified in the genome of *Anaeromyxobacter dehalogenans*. The putative RDase dehalogenase genes present in the *Anaeromyxobacter dehalogenans* shared with other RDases the presence of at least

one characteristic iron-sulfur binding motif, however, they had features dissimilar to other RDases such as the absence of the characteristic RR signal peptide and the absence of a B gene associated to the RDases.

A putative VC reductive dehalogenase gene was identified in the VC-respiring isolate BAV1 using a genomics approach. The conserved twin arginin motif was used for forward primer design and a conserved motif internal to the translated B proteins present in *Dehalococcoides* was identified and used for the design of a degenerate reverse primer. Using these degenerate primer pair yielded fragments comprising nearly the entire RDase genes, and, combined with a chromosome walking approach, the first complete sequence of a putative VC RDase gene from strain BAV1 was obtained.

The procedure designed to obtain the complete sequence of a putative VC RDase gene from strain BAV1 is applicable in capturing other *Dehalococcoides* RDase genes and will assist future efforts aimed at heterologous expression to allow in depth investigations of these interesting enzyme systems.

Of the seven putative RDase gene fragments identified in isolate BAV1, only *bvcA* transcribed at high levels when strain BAV1 was grown with VC as electron acceptor. Interestingly, five additional putative RDases transcribed in VC-grown BAV1 cells, but at significantly lower levels compared to *bvcA*. Although the significance of these putative RDases in VC reductive dechlorination cannot be

excluded, the findings point towards the involvement of *bvcA* in VC reductive dechlorination.

The presence of *bvcA* in two independently enriched cultures from chloroethene-contaminated aquifer materials was demonstrated, however, *bvcA* was absent in three VC-dechlorinating enrichment cultures derived from Michigan river sediments. At the time this research took place, another VC RDase gene i.e., *vcrA*, was identified in *Dehalococcoides* strain VS; *vcrA* was not present in BAV1, and *bvcA* was not present in strain VS. These findings suggest that a diversity of VC RDase genes exists, and additional sequences must be obtained before a comprehensive suite of nucleic acid-based tools to target all VC RDase genes can be designed.

Most efforts to assess and monitor chloroethene-contaminated sites have relied mainly on nucleic acid-based approaches targeting *Dehalococcoides* 16S rRNA genes. A major limitation of the 16S rRNA gene approach lies in the similarity of 16S rRNA genes from *Dehalococcoides* populations exhibiting different dechlorination activities. The *bvcA* gene is a promising functional gene target to distinguish the VC-respiring strain BAV1. Nucleic acid-based tools targeting *bvcA* will complement 16S rRNA gene-based approaches to monitor sites undergoing enhanced treatment where BAV1-type populations are already present or inoculum containing BAV1 (e.g., Bio-Dechlor INOCULUM) are used for bioaugmentation.

Degenerate primers that successfully amplify a diversity of putative reductive dehalogenases in *Dehalococcoides* sp. strain BAV1 aided in the identification of fourteen putative reductive dehalogenases in CBDB1, thirteen putative Rdases in FL2, and seven putative RDases in BAV1. Also, through the use of these degenerate primers and collaboration with another laboratory, a collection of RDase genes has been started. This RDase gene collection will be helpful to improve monitoring efforts, and on the genetic and evolution level, the RDase collection will provide important genomic information about *Dehalococcoides* microorganisms, which will be helpful for expression and regulation studies.

Transcription of putative RDases was studied in strain FL2 through the establishment of cDNA clone libraries, which contained inserts generated with the degenerate primers RRF2 and B1R. Three and four RDH genes were transcribed in FL2 cultures grown on *cis*-DCE and TCE as electron acceptors respectively, in addition to *tceA*. Regulation of gene expression in *Dehalococcoides* is poorly understood and it is unclear if RDase genes are constitutively expressed or specifically induced by the respective chloroorganic electron acceptor. These results suggest that a specific chloroorganic electron acceptor does not induce expression of a specific RDase gene. Hence, expression of RDase genes needs to be further investigated.

Several combinations of lysis procedures with RNA extraction protocols were investigated in this study. A simple and reproducible method for RNA extraction

was developed. This method will be a helpful tool to explore expression and regulation of RDase genes.

Targeting RDase genes offers a higher resolution than 16S rRNA gene-based approaches that cannot distinguish between *Dehalococcoides* with differing dechlorinating activities. Detection of a specific complement of RDase genes provides more reliable strain identification. Detection of transcription of certain RDase genes will be able to provide quantitative information about the metabolic processes occurring *in situ*. Monitoring RDase genes at chloroethene-contaminated sites will help establish cause-effect relationships.

RTm PCR primers and probes together with the RDase genes presented in this study will aid future research on RDase gene transcription and regulation. A quantitative analysis must be performed for better understanding of the regulation processes involved in transcription of RDase genes.

The degenerate primers presented in this study are being used as a tool for the identification of other RDase genes in *Dehalococcoides* species. There is still a diversity of RDase genes, which has not yet been explored. The search for additional RDase genes must continue.

APPENDIX A

NOMENCLATURE OF RDASE GENES IN *DEHALOCOCCOIDES ETHENOGENES* STRAIN 195 GENOME

Krajmalnik nomenclature	(Villemur *et al.* 2002)	*D. ethenogenes* annotation
RDX_297,000	RDA4	DET0306
RDX_301,000	RDA13	DET0302
RDX_18,000	RDA1	DET1528
RDX_26,000	RDA2	DET1519
RDX_5,000	RDA10	DET1545
RDX_23,000	RDA5	DET1522
RDX_321,000	RDA 14	DET0876
RDX_73,000	RDA 12	DET0173
RDX_11,000	RDA11	DET1538
RDX_13,000	RDA6	DET1535
RDX_102,000	RDA8	DET1559
RDX_293,000	RDA7	DET0311
RDX_15,000	RDA9	DET0235
RDX_287,000	RDA3	DET0318
RDX_83,000	RDA15	
RDX_79,000	RDA17	DET1171
RDX_68,000	RDA16	DET0180

APPENDIX B

RDASE GENES IN *DEHALOCOCCOIDE*S SP. STRAIN BAV1

>bvcA (AY563562)

ATGCATAATTTCCATTGTACGATAAGTAGGCGAGATTTTATGAAGGGATTGGGGTTAGCGGGAGCAGGGAT
AGGTGCCGCGACTTCAGTTATGCCGAATTTTCACGACTTGGATGAAGTAATTTCTGCTGCTAGTGCCGAAA
CCAGTTCTTTGTCGGGTAAATCTCTTAATAATTTTCCTTGGTATGTGAAAGAAAGGGATTTTGAAAATCCT
ACCATTGATATAGATTGGTCTATACTTGCGCGTAATGACGGTTACAATCATCAGGGAGCCTATTGGGGACC
TGTACCTGAAAATGGAGATGATAAAAGGTATCCTGATCCCGCGGACCAGTGTCTTACTCTACCAGAAAAGA
GAGATCTTTATTTAGCGTGGGCAAAACAGCAATTTCCTGACTGGGAACCAGGAATTAATGGCCATGGGCCA
ACAAGGGACGAAGCTTTATGGTTTGCCTCAAGTACAGGTGGTATCGGTAGGTATAGAATTCCTGGTACCCA
GCAAATGATGTCCACAATGCGTCTTGACGGGTCTACTGGTGGTTGGGGTTATTTCAATCAACCACCGGCAG
CAGTCTGGGGAGGGAAATACCCAAGGTGGGAAGGAACTCCTGAAGAGAATACGTTGATGATGCGAACTGTT
TGTCAATTTTTTGGTTACTCCAGTATAGGTGTAATGCCAATCACCAGCAATACAAAGAAGCTTTTTTTTGA
AAAGCAAATACCTTTCCAATTTATGGCTGGAGATCCCGGTGTATTTGGGGGAACGGGAAATGTGCAGTTTG
ATGTCCCGCTGCCAAAGACACCTGTTCCAATAGTCTGGGAGGAAGTCGATAAAGGGTATTATAATGACCAG
AAAATTGTAATACCCAATAAGGCTAACTGGGTATTAACAATGACAATGCCTTTACCAGAAGATCGTTTTAA
ACGTTCTCTAGGGTGGTCACTTGACGCTTCAAGTATGATTGCCTATCCTCAGATGGCTTTTAATGGAGGCC
GAGTTCAGACTTTTTTAAAAGCACTTGGCTATCAAGGACTTGGTGGCGACGTGGCTATGTGGGGACCTGGT
GGTGCTTTTGGAGTTATGAGTGGTCTTTCCGAACAAGGTCGTGCTGCTAATGAAATCAGCCCCAAATACGG
TTCGGCAACTAAGGGCTCTAATCGATTAGTTTGTGATTTGCCCATGGTTCCGACCAAGCCAATTGATGCTG
GCATACACAAATTCTGTGAAACGTGTGGCATTTGTACAACAGTTTGTCCCTCAAATGCTATCCAGGTAGGT
CCTCCACAATGGAGTAATAATCGGTGGGATAATACCCCTGGTTATCTTGGTTATCGACTTAACTGGGGTAG
ATGTGTTCTTTGTACAAACTGTGAGACCTATTGCCCATTTTTTAACATGACTAATGGTTCTTTGATTCATA
ACGTAGTCAGATCCACAGTTGCAGCTACACCGGTTTTTAATTCATTTTTCCGCCAAATGGAACATACATTT
GGATATGGTATGAAAGATGATTTAAACGATTGGTGGAATCAATCACACAAGCCTTGGTAA

>rdhA1[BAV1] (AY553222)

GGGAGCAGGTATTGGTACCGCAGCTGCAACTGCAACTGCCCCAATGTTTCACGACCTTGATGAGGTGATCG
CTTCACCCTCAGCAGCAAATGAAAGACCATGGTGGGTAAAGGATAGAGAATTGTACCAGCCCACGCTTGAG
GTAGATTGGGATATTATGACTCCGCCGGATGGCAGAGTTAGCGGGCAGCAGACTGAAACCCAAATTCACTA
CCTTGGAAGCGAAGAGGTAAAAAGGCGTTTATCATCGAATATAATGTCTCCCAACGTTGAAGCCGCTATCA
ATAATACACCGGGGAAAACTTTGCGTGACCAAGCCTTGGGACTCAGCTCAATTGTACCGATGATGATTCAC
GGTATATCTTTCATGGGCCCGGGTCTTATTCCTACCCCTGCAACAACCGGCGCCCCTAAATGGGAGGGTAC
ACCTGAAGAAAACAGCCGGATGGTACGCAGTGTTCTTACTTTTCTGGGTGCCGGTATGGTTGGTTTTGGTG
AAATTTCCAGCCAGGAGAGAGAAAAAATATTCTACACTTATCATAAACAAGTCCCCAACAAGAGGCAGGTA
TTTGAGGATGTAGATGTTGGCTACGAAGGTACCGATAAATACGTTTTCCCTGACAGGAAGCTTTATAAGAT
ATCTATGTCCCTGCCTATGTCCCGGGAAATGTATCGAACTTCCGACAGATCTTCATTACAATTTGCAGCCA
ATGTATCCCGTTACCGTCACTTCAGTATGCTTCAGCCGGCTTTCCAAGAATTTATCAGAGGTATCGGGTAT
CATTGTTATGGCTATCCTGTACCACAGGCTGGCCCTATGCCTGCAGCAGTTAGTGCTATTCTTACCGGTCT
GGCGGAATCAAGCCGGAATAGCGGGTATTGTATCTCTCCGGACTACGGACCGGTTTCAGGTTTCTTTACAT
TTGTAACTGACTTGCCAGTTGAACCCACTACACCTATAGATGCTGGTATCTGGCGCTTTTGTCAGACTTGC
AATAAGTGTGCCCAAAACTGTCCGACCCAAGTAATCCCTTACGATAAAGAACCGAGTTGGGAACTCCCTAC
ATTATATGGTAAACCGGATATTATCCATCCTTCCGGCAAGCGGATGTTCTACGCAAACCATATAGAGTGTT
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>*rdhA2*$_{[BAV1]}$ (AY553223)

GGGTGCTGCAACAGCTTCAGCACCAGTGTTTCATGATTTGGATGAAATGATNACATCTGTACCTAAATCTA
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CTCACCAGCGTTCGGACGAAGAGCGACTTAACTGGTGGTACAGGGATCTTAATACATGGCAATATGATGAT
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>*RdhA3*$_{[BAV1]}$ (AY553224)

GGAGCAGGCCTAGGAGCAGCTGCGTCCACTACTCCGGTGTTTCATGACATGGATGAACTCATTGCTTCATC
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>*RdhA4*$_{[BAV1]}$ (AY553225)

TTTTATGAAGGGCTTGGGGTTAGCTGGTGCGGGACTTGGTGCCGTGTCGGCTGTTACGCCTGTCTTTAGAG
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TTACTGGGTTAA

>*rdhA5*$_{[BAV1]}$ (AY553226)

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>*rdhA6*$_{[BAV1]}$ (AY553227)

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TTCCGCCAAATGGAACATACATTTGGATATGGTATGAAAGATGATTTAAACGATTGGTGGAATCAATCACA
CAAGCCTTGGTAA

> *rdhA7*$_{[BAV1]}$ (AY553228)

ATGAAGGCACTCGGTCTTGTAGGGGCTGGTGCGGGTGCGGCAGCAGCTGTTGCTCCGGTGTTCAGAGACCT
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GCCGGTATGTGGCGGTTCTGTAAGACTTGTGCTATTTGCGCTGAAAACTGTCCTTCGCAGTCTATTTCATA
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CTGAGTCTGAGTGGAACAATTGGTGGGACATGAATCTGCCGGCCTATGCTTTTGATACTACTGTTGGTGTT
ACTGATGGTGGTTACAAAGCCAAAGGCCTGCTGCAGCAATAA

Deduced amino acid sequence of RDase genes are identified in strain BAV1 are presented below.

>BvcA
MHNFHCTISRRDFMKGLGLAGAGIGAATSVMPNFHDLDEVISAASAETSSLSGKSLNNFPWYVKERDFENP
TIDIDWSILARNDGYNHQGAYWGPVPENGDDKRYPDPADQCLTLPEKRDLYLAWAKQQFPDWEPGINGHGP
TRDEALWFASSTGGIGRYRIPGTQQMMSTMRLDGSTGGWGYFNQPPAAVWGGKYPRWEGTPEENTLMMRTV
CQFFGYSSIGVMPITSNTKKLFFEKQIPFQFMAGDPGVFGGTGNVQFDVPLPKTPVPIVWEEVDKGYYNDQ
KIVIPNKANWVLTMTMPLPEDRFKRSLGWSLDASSMIAYPQMAFNGGRVQTFLKALGYQGLGGDVAMWGPG
GAFGVMSGLSEQGRAANEISPKYGSATKGSNRLVCDLPMVPTKPIDAGIHKFCETCGICTTVCPSNAIQVG
PPQWSNNRWDNTPGYLGYRLNWGRCVLCTNCETYCPFFNMTNGSLIHNVVRSTVAATPVFNSFFRQMEHTF
GYGMKDDLNDWWNQSHKPW

>RdhA1[BAV1]
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PEENSRMVRSVLTFLGAGMVGFGEISSQEREKIFYTYHKQVPNKRQVFEDVDVGYEGTDKYVFPDRKLYKI
SMSLPMSREMYRTSDRSSLQFAANVSRYRHFSMLQPAFQEFIRGIGYHCYGYPVPQAGPMPAAVSAILTGL
AESSRNSGYCISPDYGPVSGFFTFVTDLPVEPTTPIDAGIWRFCQTCNKCAQNCPTQVIPYDKEPSWELPT
LYGKPDIIHPSGKRMFYANHIECWMYCFEGGCGTCMATCTFNVNGAAMVHDVVKATLATTSMFNEFLWKAD
KTFGYGVKSGEEKEDWWDLSLPSMGWDTTSFSKHGY

>RdhA2[BAV1]
GAATASAPVFHDLDEMXTSVPKSTTQHAWWVKERDYEDITTPVDWTVWSRREALKNPMPPGFAGNYVPKEQ
ARLQSFRNEIKRGITEKIPGATLRDWALSEAGRSNTTSSSWMGLDVKPPWLWGEASALPVEPWPEGAPKWE
STPEDNLRTVQAAGHYFGTPQVGAMEINEHMIRMFDKDGFEHNYSASYEKPMMRFRSEWFEDIPVGFQDAN
QVKHIPKSCKWAVTYIAAKENALQMTYGMRTGDPQDPWYKRIFPLGYTTGEAYSKADYVKVQFMKFIKMLG
YQTYYMGLAGGTSSNSPAGIFSGLAEEARPALACSPYYGNAVRHIGIIVTDMPLSPTKPIDAGIVNFCKVC
KKCAETCPSGAISMETEQQWEPACTGNNPGRKTWYLDWFKCRPWGSPYYCPNCQTVCPFNNPNKAIIHNAV
XXTAATTPIFNSFFSSLDKSFGYAHQRSDEERLNWYRDLNTWQYDDVFGMGTKDPKSWL

>RdhA3[BAV1]
GAGLGAAASTTPVFHDMDELIASSGFSGSESYSRYPWWVKEVDKPTAEIDWNLMKPYDMRNSDKWATPELL
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PEENLKMCAAAIHLLGGRDISVVEVDDNVKKVLYSHSAMLMGGKPSRAIVWEDVDNAYETPEKMVIPNKCK
WALVYSCPQSQLSRYRSVIMGKFGVFGAYSDIAVMDQRLQKFLRILGYQGVLDGFGGGNSISSNSGFGVLA
GSGEIGRHDYVNSPSFGALMRMSQFILTDLPLAPTKPIDAGMWKFCQSCKKCADMCPSGAISKEAEPTWEP
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FNSFFTTLDKSFHYGGAFVTPLGEVNVSPDEWWNRDLKTYPFKGRVMGDGWA

RdhA4[BAV1]
LGLAGAGLGAVSAVTPVFRDLDELTSSVTAHPKRAWYVKEREFGDIGIEIDWNILKRRDTRGYSYWNPMIW
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IGMPNWAGTPEENFQMIRAAFSLIGLGPSIGITELDDKSRRFVREYNNCGQHIIFDDNITETYRTANPPTI
HIPSSHRYVIATHNMGADEILRRAPSTIGACTESISYARVAYAKSFVEQFIRGLGYNVVYGHSLQAAPAMD
FWSGVGEHARMGQVCVTPENGAMMRTHAIFFTDLPLSPTKPIDAGITKFCETCGICAESCPVGAVPAKGVN
RNWDSNCDGQSFDNDIESGGTEVMYNVPGYKGWRVDGFRCLADCNGCKGSCPFNAIPNGSFIHSLVKATTS
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>RdhA5[BAV1]
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AQERYAMGIPGGSSGTWASPEQAQVLFDYMKKEFPGWEPGYAGLGDNRTTALFMATKFMRMGMWPGEINMG
GNRVNVAKAISAAGGTAAFTSFLGLRSSETLRPQDFGVPRWEGTPEENLLTLRQVVRFLGGCDVGAQEMDS
DVFKLFHEKSGKKQLVIENVDEAAETPTKLVIPAKAKYILQWTARQPYESTRRQAGEYEDAAVYWSYQRFP
FVGAIIQEFIHALGYTAVSTHLSGYHSSAVATLTGMGEHCRMSSPILVPKYGVTNRAMWVIMTDMPLMSTK
PIDFGVYDFCKTCGICADACPFGLIEKGDPTWEATQPGSRPGFNGWRTNTTICPHCPVCQSSCPFNTNGDG
SFIHDLVRNTVSTTPIFNSFFANMEKTMGYGRKDPRDWWNIDDYTYGINTSY

>RdhA6[BAV1]
GAGIGAATSVMPNFHDLDEVISAASAETSSLSGKSLNNFPWYVKERDFENPTIDIDWSILARNDGYNHQGA
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PGTQQMMSTMRLDGSTGGWGYFNQPPAAVWGGKYPRWEGTPEENTLMMRTVCQFFGYSSIGVMPITSNTKK
LFFEKQIPFQFMAGDPGVFGGTGNVQFDVPLPKTPVPIVWEEVDKGYYNDQKIVIPNKANWVLTMTMPLPE
DRFKRSLGWSLDASSMIAYPQMAFNGGRVQTFLKALGYQGLGGDVAMWGPGGAFGVMSGLSEQGRAANEIS
PKYGSATKGSNRLVCDLPMVPTKPIDAGIHKFCETCGICTTVCPSNAIQVGPPQWSNNRWDNTPGYLGYRL
NWGRCVLCTNCETYCPFFNMTNGSLIHNVVRSTVAATPVFNSFFRQMEHTFGYGMKDDLNDWWNQSHKPW

>RdhA7[BAV1]
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KYVGVDKVKEAAASAAAIKKEALETGKPGMDLRATALGSTSGLYNAPQPYFSYTKTAQGWGGGKSFTGQST
IKGPDVLGVPKWQGDPDANLRMLRAALRFYGAAQIGVVPYDTNVKNKLTCVREGGMASMSDKYIEKWPIPA
VDARPFVFEDVEKGYETAEKLVIPDKKELFVVSVIQPMSREMWRQGSGNLRVATNGHRYSLASVWQTKIQG
FLTTLGYQGLGYPTRAYGSMPTIPGFIFSGLGELGRSNNVCLSPEYGSTHGSFHFLTDLPLTPTKPIDAGM
WRFCKTCAICAENCPSQSISYDKEPSWEITPSKYAPNVPVEYSVPGKKVFWRDEPSCKQWTESCGYSCGIC
MGSCVFNVDNASMIHQVVKGTIATTSLFNGFMKQADKFFGYGLTPESEWNNWWDMNLPAYAFDTTVGVTDG
GYKAKGLLQQ

APPENDIX C

RDASE GENES IN *DEHALOCOCCOIDES* SP. STRAIN FL2

>*rdhA10*$_{FL2}$ (AY374254)

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>*rdhA11*$_{FL2}$ (AY374255)

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>*rdhA13*[FL2]
CCTTGGTCTTACAGGGGTAACCTTGGGTTCTGCCAGTGCCTTAAGTCCTCAATTTCGTGATCTGGATGAAC
TTGCGAACTCAGCTAAGGTAGTTAACAAACGTGGTTGGTGGGTCAAGGAAAGAGACTACGGCAATCCCACC
ATAGAAATAGACTGGAACTTGATGAAACGCCGTGATTTACGCGGTTTTTCAAACTGGGATTATGCAAGTTT
GATGATGGCCTTTCCCGGAGGCCCGCCGGCGTTTAAAGCAAACACTCCGAAACAGGCAGCCGCTGTAACCG
CAAAAGCAAAGGAAATCTGGCCTGACTATGCAGGTCCGACTATCCGGGATAAGGCTTTATCAAGTTCTTTT
TGGGCATCCGCCTACGGTCATTCCGGATATTGCCGCAGTCAAAATCAGCACGGTATGCCAACGGAAATACC
TGCACCCCGTCCCAGTGCAATAAACACACCTGCATGGGAGGGAACACCTGAAGAAAATGCAGCCATGCTCA
GGGCTGTTTTCAGTCTTGTCGGGTTGGGGCCGGTAATGGGCACTACTATGCTGGACGAAAAATCCCAAAAC
TTTATCTGGGAGTATTCCGGTGTAAGCTGGACAGGTGATGAATCCGTTCCAGGCAATAAGCATATTGTTTT
GGATTCAGGTATAACCGAATCCTATGTGGATGCGACCAGTTTCCACATCCCCACTTCGCAAAAGTACGTCA
TCGCAACTCACAATATTTCATGTGACGGGTTTCTGCGCCGCTCTATGGCGGGCGCAGGTTTTTCCAGCACC
GAAGAAATGTCTTATGTCCGGGTTGCCTATGCAAAGAGTATTGTGGAGCAGTTTATCCGCGGGTTAGGGTA
CAACGTAACCTACGGGCATGATCTGCAATCAGCTGTGGCTTGGGATATGTGGAGCGGTGTTGGTGAACATT
GCCGTATGGGTCAGGTTATCGGTTCTCCTGAGTATGGCGGTTTGCTGCGCACCCACGCTGTTTTTTATACT
GATTTGCCGTTGCCGGTAACCAATCCGATAGATGCCGGGTTCGTCAAATTCTGCGAAACCTGCGGTATCTG
TGCTGAAACCTGCCCTGTAGGTGCAATACAGGAACGCGGTATTGATCGTAGCTGGGATAATAATTGCGGCC
AGAGCTGGGCTGATGACAAACAGGCCGGCGGCAGCAAGGTAATGTATAACATTCCCGGTTATAAAGGCTGG
CGTTGCAACCTGTTCAGCTGTGCATTTACGCCTTGTGCCAGCGCCTGTAAGAGTAACTGCCCGTTCAACGC
CATAGGCGACGGAAGCTTTGTACACAGCATAGTAAAATCAACTGTTGCCACCAGCCCGATATTTAATAGCT
TCTTTACCAGCATGGAGGGTGTTCTGCACTATGGCAAGCAGGATAAAGATCCCGCATCATGGTGGAATAGC
CCTGATGAATGGTTTATCTATGGCACTCATCCTAATCTGTTGAGACAGTAAGCTACTTAAAAAAAGGAGAA
AATTGATATGGAGTATTTCATACCCCTTATGCTGGTCGGGGCTGTTGTTGCAGCGGCTCTTTTCGGTATTA
CCAACTGGCTGCGTAATCGGAATCTAAAGGTAAGC

Deduced amino acid sequences of RDase genes are in strain FL2 are presented below

>RdhA1$_{[FL2]}$
VGLAGAGLGASAAISPVFHDVGEFMSSPTAEWKRPWYVKNRELEDPTVELDWSLMYRSDGIWTGQNNPTQD
FSLGAEEGAKRRAAAAAYSANAVKTNQSGMTLRDRALSSGNYMYPITFMGPASSTTPESLGVPKWQGTPEE
NSKMIRAAMIHFGAAQVGMAEITDRVKTKLVREYDKDFTHKKYMFEDVPKGYEGTDKLVFPDKVPLYDFAF
THPLNKEMFRSSPSSDIGSAGNSLRYSQFSIIQPRIQMFMQVLGYTCYGYTRPFNGAIPTIATATLTGLGE
GARNNGAFISPEFGPCVGLFSLVTDLPLEPTPPIDAGMWRFCQTCTKCADECPAQCIPLSMNQLGTCPRFT
ARKIPPIYRDVNSSGRTVLPAGRIKPLSVVAVPVWVPVPSILILPIFIRLSGQPCQLLLYLTASCGRQINS
SAMVYTKTKRPGGICHNQHLALTLRMLQSAKITKTQGRIILCG

> RdhA2$_{[FL2]}$
LGLTGAGLGTAAAATPVFQDLDDVTASPSAEWKRPWWVKNREIDDPTTEIDWDMMYRSDGRMVGQVRSVQI
KYLGEEEVNRRNAVGAKFTADGLKNDTPGLKVRDQALAAGVMSMLPMAMGMIPSISFMGPATATPEARGVA
KYRGTPAENSRMLRSALVFYGAAQVGYGEVTQRYKDKLFRTFDKGNAATAYQGAWPPPLTQCKQYFFEDVP
VGYDTAEKMVFPANVPLYEFTFIVPMSKEMFRCSPSSALQNAANLSRYTAMAQIQPKIQAFIKSLGYQCYG
YTLPMNGAVPTIASAVLTGLGEGARNIGAFNNPEFGSITGLFHLITDLPLEPTPPIDAGMWRFCHTCTKCA
DACPWSAIPTDHEPSWDIPKLYGQEDTTHVPGKKQFWTNSVDCWLGRVQLGTCGACMGTCTFNTGKNAIHD
YVKATLSTTPVFNSFLWQADKAFGYGLRAGEDLENWWDMPQPIGGFDSTCGIQGGSY

> RdhA3$_{[FL2]}$
LGILGTGLGTAAATAPVFHDLDDIISSPKAEWKRPWWVKYREADNPTTEIDWSLMNRWDARQTAQAPGIQA
KYLGADEIKKRYANVLTNKVKAITHDTPGQTLRDYALSSGAGYFMNLPYVTTFMGPQKVATPQSLSVPVWQ
GTPEENSRMLRSAVIFYGGGQVGFGVIDQKIKDKLVFTNHKGAANSIGFVENFPPPPALGKSYLFEDVEQG
YEGATTFVLPSNKQLYEFCFTVPMSKDMFRTANESQIMYSANLSRYRLFGNIQNCIQEFIRSLGYTCYGYA
SPFSGMMPAIASAQLTGITEGNRNNGFCTSPEYGPILGVFSLVTDMPLEPTNPIDAGICGSARPVPSVQMP
AL

> RdhA4$_{[FL2]}$
LGLAGAGIGAVAAAAPVFHDLDEVASADSSVNKRPWWVREVDKPTIEIDWSKTSSLPFPQEGCHLPPLLAE
FVGWDRVNSAMAQGVAALNAGAKKTGSKEAISLLDTSMQEAAWPHFIAHAGWREPVYPALEGAAPIPELVG
QTYTHESFGVPRWESTPEENFALLKSAMRFFGAGQIASIELDANVKSMFYPVDASRMFFNGPPMAYGFEDC
DNGYVTDTHFIIPNKARWVVTYTTPMPKEMYRTAPSGVCYAANMSRYRLNQETMACVQKFLLGLGYQGLQS
APWPNGICPSPAVATLSGLGEMDRINQCVIPEEGAVVGIYKFITDLPLPVSKPIDFGAFRFCHSCRKCADT
CPAKAISFEEEPTWEPAGPWSTGGKKAYYKNEPECKLYQHSTGATCQICTGVCVFNVNTKAMIHEIVKSTL
STTGIFNSFLWKADVAFGYGHHDAAEWWDLDLPRYGFDTTMGVRDGGYGK

> RdhA5$_{[FL2]}$
IGMAGAGVGAVAATTPVFHDLDELMSSSSVTPAKRPWWVKERELFNPTSEIDWDLMQRFDRKNEAHSRRIA
TMYRSVETIDAAAVTQKKIDADRIAKQTPGFDTKYQALKAEYSGSTESPAWAYPGIVDEADWAKTPEELGM
PKWSGTPEENSRLLYAALRYYGAMFIGYAEVEDKWRNKLFVKTTTDAVRNWTWTPQNPDPPESDELRYVYE
NVDQPYSELRKGSTGRSAGKHVIPSKPLWLITIATGACMEATKTLDSTISKSNSSTADNGHEALKVRTFNF
VRALGGWRAFGDGGHQTSESNFSAAMILTGLAENSRQGNYCLTPETGPNHIPFTMLTDFPLVPTKPIDAGL
FRFCHSCKKCADACPSQSISHADEPSWDVPDVDGKPRVFCNPGHKGFWPDMAGCNYYSKGGGTSGCWVCYA
NCTFSEDKAAMMHNIIRGTVSTTSLFNGFFSSMSNTFGYGPYESPEVWWICPYRPMALIVLLVPPRAVTAN
KREVIRYVVFHRPDCWRWNYGFGLVAENQRRQT

> RdhA6$_{[FL2]}$
LGMAGAGIGAVAASAPVFHDIDELIASDTAVQPRPWWVKERPIDDPTIEVDFSMMERHDGRDQGQSARVRA
IYYGADRVLGAAALSAAELAERTASNYPGYTYRSRALAGSFKRISQGTSPGWAETKDPAPVKTPEERGEPK
WTGTPEEASRMLRAAMRAYGASLVGYTELTQEHRDHVIFSYEKGDSNNEKYIGTTIPVTAARPIVFENVPK
AYETTEKLVIPNVPLWEIAMSTQGSNELWRSAGTLLGGMANGNTFYNCANLHASTYNFLRYLGYQLIGTIG
NDARYVGSEGGAAIMAGLGEASRQKLYTLTPEYGAPGRLYGVLTDLPLEPTHPIDAGIYRFCHSCQKCADS
CPPQCISKEKEPSWDLPLTEGKETIYSVKGTKAFYNNLPLCRQYSNETSHGCRICWGECTFTVNRGALVHQ
IIKGTIANIPLFNTYFYKLGDAFGYGTDPEKAEAWWDLSLPTLGQDSTIVAADAGYGK

> RdhA7$_{[FL2]}$
LGLAGVGIGGSAAGAPVFHDLDELIASEGSSYSSSPSAIHNAPWWVKERDYENTTTEIDWSQIIRLDQGKD
WQKLYPDRPQYGSFRILLNLPQDPDMIPDPAPILEGYIKNKYPEGKGTQTRDHALQAADSATFGHISFLGV
NMFGDLRTRTPEQFGVPKHQGTPEENLKMCRAFLRLVGAHDVGNVPLTENTRKLIYKSGITDTAVVKEYQF
TDDEKLSENDTAWKIPNKLNNMLTYSMLNCTELCLGNTLSIPKYLASDGVGNGAIGTLVAYQRSFIANFQL
QNFLHSLGYQSVRGASYNICPSTPFGTLAGIGEHARMATVIVSPMYGATMRVCDRCLTDLPLAPTKPIDAG
INKFCETCGICAEECPFGSLSKGGSSWDHFLSNEPLGNGNAPGFKGWRLDLHKCNYCGICQSACPFNSVDN
SWVHSLIKSTVGTTSIFNGFFANMEKNFNYGFHNPENWWDMEQPVFGIKKEWLGGD

> RdhA8$_{[FL2]}$
LGVVGAGLGTLSAAAPVFHDLDEVTSSAIGINKNPWWVKERDFKNPTVPMDWPKITRHAGTFKTLPRPTVA
DFTKAGVVGGTSTDLETPEMALTLYDAMAKEFPGWTPGYAGMGDTRTTALCNASKFMMMGAWPGNMEMGGK
RINVQAAIMAAGGSPTFTPWLGPQLDTTTRPQDFGAPVWQGTPEENLKTCRSAIRFFGGSDVAALELDDDI
LKFFHSKIGGKDLVIEDVEEAYETTTKMVIPRKCKWVLMWSARQSLEGTRRQAGITENYAVWYSYSRLPKV
GVQIQEFIRGLGYQALNPGMKGYLTSPLAAFSGMGEHGRMSSPTITPKYGVTNRAMWR

> RdhA9$_{[FL2]}$
LGLIGAGVGAATAVSPVFRDLDEMASAPSARINMPWWVKQVDEPTTPIDWNVLPTLGTACDDNNGVVPNIR
TRAEYQKVMLDYTMKQYPDWDKGPTLTGVPGPSPDLNYPDYVGDIKDNALCVGATLCNAGLFPTEVIQATG
GKYTTIQDRPQGWRCVKPVEQRGGTKWQGTPEEALKVVRAAARFYGFDDVTAIPVDDKFLKVMWGQKRMLR
MATPTKFEFGDVDDFVCTPEIQPMSKIVIPKRVKWFLQFSSRQLGEVTKHGVGTCQNAGQLYTYVNWIRTV
KTIQEFLWGPGYISLDNINGRFAPTGATGIMAGAGELARWGAVMTPKYGIMVRVMHGVSTDLPLEQSSPIN
FGGREFCKTCGICAEACPMDAIQKGEPSWEVNHKWDNPGYLHWRNDRSKCGHCPVCQPVCPFNAMDKSFIH
ELVKGTVSTTPIFNSFFTGMDKNFNYGRKPPAEWWESEQPVGGFDSSV

> RdhA10$_{[FL2]}$
MESAAQKNSDRLKELWPDYKASTRDLALANALGSVGTFGTYALNTTQGGWKVDPAPTPEELGIPKWEGTPE
ENLMMIRAAFSVMGLGPMVGVSELNEKTKNFVYEYTGDSWTWLPQGQPSEHIIFDDNISEFYRTQNPNTLH
IPSSHKYVISTHNLPLDELTRRTYSPLGTPAEAISYSRVAIAKNFVEEFIRGLGYHVVYGHALQPALVWDF
LSGVAEHSRMGQNAVSPEYGGMMRAHATFYTDLPLAFTNPTDAGLTKFCETCGICADACPVGSISPVGTDR
NWDNACGQDWANDIQNGGAETMYNIPGYKGWRCNTFSCMTTKAACGAACKFSCPFNVSATAALCTV

> RdhA11$_{[FL2]}$
LGLGTMGLGAAAVSAPVFHDLDELISSPSATLKXPWYVKNREYGDIGIELDWDLLKPRLGALVPGFSPTWN
NAAKEAAIPSFNSAAFDKAVDAXTKELWPDYRPSTRDYAFANACSSAGIGSPFTDGALEGNLFNGSALTRI
VKAPTPEELGVPRWEGTPEENQRMVRAALTFIGMGPAVGCAELNTKTKNFFYQNHYPEYVWDDNITETYYS
ETEKTQYLPSSHRYVVVTNNMGTTELGRRSPSVLTGSRLVNVGAHVETISYSRTQMAKVFFEQFIRGIGYH
CFYSHTLQPAAAWAQLSGVGEHARMGSMFVSPIYGCNLRTHMIVYTDLPQAYTPPTDGGVTKFCETCGICS
ENCPAGAIPPRDIQRNWDNASGQNWGDDIQEGGSQVMWNIPGYKGWRLDMRKCQGCCSCKFSCPFNTLPDS
SFLHSVVKATSSTTPIFNSFFREMEGLLHYGKQDKDPESWWNDPNSWFVYGANSKLML

> RdhA12$_{[FL2]}$
LGLAGAGIGAVSAAAPVFHDVDELTASSGGVQKLPWWVKEREFKDPSVPIDWQNLPKMEGTFPYQARPTLS
AQERYAMGIPGGSSGTWASPEQAQVLFDYMKKEFPGWEPGYAGLGDNRTTALFMATKFMRMGMWPGEINMG
GNRVNVMQAILKAGGTATFPSFMGLRSSETLRPQDFGVPRWEGTPEENLLTLRQVVRFLGGCDVGAQEMDS
DVFKLFHEQSGGKQLVIENVDEAAETPTKLVIPAKAKYILQWTARQPYESTRRQAGEYEDAAVYYSYQRFP
FVGAIIQEFIHALGYTAVSTHMMGYHTNAIATLTGMGEHCRMSSPTLVPKYGTTNRAMWVMMTDMPLMSTK
PIDFGVYDFCKTCGICADSCPFGLIEKGDPSWEATQPGTRPGFNGWRTNTTTCPHCPVCQGSCPFNTNGDG
SFIHDLVRNTVSVTPVFNSFFANMEKTMGYGRKDSRDWWNIDDYTYGINTSY

> RdhA13$_{[FL2]}$
LGLTGVTLGSASALSPQFRDLDELANSAKVVNKRGWWVKERDYGNPTIEIDWNLMKRRDLRGFSNWDYASL
MMAFPGGPPAFKANTPKQAAAVTAKAKEIWPDYAGPTIRDKALSSSFWASAYGHSGYCRSQNQHGMPTEIP
APRPSAINTPAWEGTPEENAAMLRAVFSLVGLGPVMGTTMLDEKSQNFIWEYSGVSWTGDESVPGNKHIVL
DSGITESYVDATSFHIPTSQKYVIATHNISCDGFLRRSMAGAGFSSTEEMSYVRVAYAKSIVEQFIRGLGY
NVTYGHDLQSAVAWDMWSGVGEHCRMGQVIGSPEYGGLLRTHAVFYTDLPLPVTNPIDAGFVKFCETCGIC
AETCPVGAIQERGIDRSWDNNCGQSWADDKQAGGSKVMYNIPGYKGWRCNLFSCAFTPCASACKSNCPFNA
IGDGSFVHSIVKSTVATSPIFNSFFTSMEGVLHYGKQDKDPASWWNSPDEWFIYGTHPNLLRQ

APPENDIX D

METHODS

Buffers

50x TAE Buffer (Tris-Acetate-EDTA).

242 g Tris base

57.1 mL Acetic acid

100 mL 0.5M EDTA (146 g/L)

ddH2O to 1 liter

Dissolve Tris base, acetic acid, and EDTA in 800 ml of ddH2O, adjust to desired pH with concentrated and adjust to 1 L final volume with distilled water. Autoclave to sterilize.

TE Buffer.

10 mM Tris-Cl, pH 7.5-8.0 1 mM EDTA pH 8.0.

Make from 1 M stock of Tris-Cl (pH 7.5) and 500 mM stock of EDTA (pH 8.0).

1 M stock of Tris-Cl (pH 7.5)

Tris base 121.1 g

ddH2O to a final volume of 1 L

Dissolve Tris base in 800 ml of ddH2O, adjust to desired pH with concentrated HCl and adjust to 1 L final volume with distilled water. Autoclave to sterilize.

500 mM stock of EDTA (pH 8.0).

Na_2EDTA 186.1 g

ddH2O to a final volume of 1 L

Dissolve Na_2EDTA in approx. 400 ml ddH2O, adjust pH to 8.0 with 10 N NaOH, and adjust to 1 L final volume with distilled water. Autoclave to sterilize.

Electrophoresis of DNA in agarose gels

a) Agarose concentrations:

Use 0.8% agarose (w/v) for high molecular weight DNA fragments, and 1 - 1.5% for smaller DNA fragments, use Ultra pure agarose (Invitrogen Catalog # 15510-027). The liquid to prepare gels should always be buffer. Never make a gel with water.

For low melting agarose gels, which are used for RFLP, use a gel concentration of 2-3% (w/v). Metaphor agarose (Biowhittaker Molecular Applications Catalog # 50180) melts at 75°C and solidifies at about 35°C. The rigidity of low melting temperature agarose is low (gels brake easily); these gels need to be handled with extreme care. Making the gel the night before and refrigerating it, helps increase rigidity; also running the gel in cold conditions i.e. in the cold room or covering the electrophoresis unit with ice helps increase rigidity also.

b) Gel preparation:

Measure agarose and dilute in 1X TAE buffer to appropriate concentration i.e. when preparing a 2% agarose gel, weigh 2g agarose and dilute in 100 mL buffer. Boil in a 250 mL corning ware bottle flask (usually done in the microwave), make sure agarose is completely dissolved. Cool to 60-65°C before pouring onto a clean, leveled, glass

plate or plastic tray surrounded by masking tape. Insert the comb parallel to the plate's edge, with the bottom of the teeth about 2mm above the plate. Multiple combs can be used on a gel to accommodate additional samples needing only short separation distances. Use a 2mm comb is used for larger (greater than 30 µL) volumes of DNA. The thinner the comb used, the higher the resolution. Pour the molten agarose solution at room temperature or in the cold room. Use a Pasteur pipette to push any air bubbles that form to the side. The gel will solidify in the cold room in about 20-25 minutes, or about 45 minutes at room temperature. The gel is opaque when solid. Remove the comb carefully or you may rip out the bottom of the wells. Transfer the gel to the electrophoresis chamber. If the gel won't be run for 1-2 hours, submerse it in running buffer. If the gel will be used the next day store in the refrigerator in a zip lock bag containing buffer.

c) Buffers & electrophoresis conditions:

For PCR product electrophoresis separations use 1X TAE at 90 volts for 45minutes.

For RFLP run the gel in cold at 100 volts for approximately three hours

*d) Sample preparation***:**

Load 3-5 µL of DNA or PCR product per lane for analytical gels. Load your molecular weight markers of choice. Gently overlay with buffer, or alternatively, load samples through the buffer. Make sure you do not puncture the bottom of the wells.

e) Staining the gel:

Stain gels in a 1 µg/mL ethidium bromide solution after the gel has finished running in the electrophoresis unit. Staining is performed in a separate container to avoid

ethidium bromide contamination in the electrophoresis unit. Stain for 20-40 minutes and de-stain in a container with water for 5-30 minutes. For RFLP stain and de-stain cold (keep the staining and de-staining containers in the refrigerator an hour before using them and while staining and de-staining). Wear non-latex gloves when working with ethidium bromide, it is carcinogenic and mutagenic. Dispose of the ethidium bromide solution in a well-labeled container and the gels in the disposal unit specific for ethidium bromide waste. .

Extraction of genomic DNA from dechlorinating cultures

QIAamp Tissue Kit Protocol for genomic DNA isolation from Qiagen (Catalog # 29304)

1. Set up two water baths at 70°C and 95°C. A 37°C water bath or incubator is also needed.
2. Prepare lytic enzyme solutions:

   Proteinase K: stock solution of 20 mg/mL in DI water (best to prepare in aliquots and store at -20°C).

   Enzyme Lysis Buffer: 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1.2% Triton X-100. Add the following enzymes to this stock on the day that you are going to extract DNA. Do not prepare stocks with enzymes in this solution.

   Achromopeptidase: 250 μg/mL or 7500 U/mL final concentration in enzyme lysis buffer.

   Lysozyme: 20 mg/mL final concentration in enzyme lysis buffer

   (Note: Only lysozyme is necessary for gram - cells)
3. Pellet bacterial culture in a micro centrifuge tube (5 min at 5,000 x g or 7500 rpm a micro centrifuge). Alternatively, colonies can be scraped from a plate.

   Resuspend cells in 180 μL of **enzyme lysis buffer**.
4. Incubate at 37°C for 30 min. Vortex occasionally.
5. Add 25 μl of **Proteinase K** stock solution and 200 μl of **Buffer AL** from the QIAamp kit. Mix by vortexing and incubate at 70°C for 30 min. Incubate at 95°C for an additional 30 min. Spin for a few seconds.

6. Add 210 µL of **100% ethanol** (not denatured ethanol) to the sample. Mix by vortexing.

7. Place a QIAamp spin column in a 2 ml collection tube (provided in kit). Carefully apply entire mixture to the column (including precipitate). Centrifuge at 6,000xg (8,000 rpm) for 1 min.

8. Place the spin column in a new 2 ml collection tube and discard the old tube containing the filtrate.

9. Add 500 µl of **Buffer AW** from the QIAamp kit. Centrifuge at 6,000xg (8,000 rpm) for 1 min. Place column in a new 2 ml collection tube.

10. Add another 500 µl of **Buffer AW** and centrifuge at full speed (14,000 rpm) for 3 min.

11. Place the spin column in an eppendorf tube. This is the tube that your pure DNA solution will be stored in (not provided in kit). Elute the DNA by adding 200 µl of **Buffer AE** preheated to 70°C. Incubate the column with **Buffer AE** for 2 min. Centrifuge at 6,000xg (8,000 rpm) for 1 min. Do this twice so that the final volume of **Buffer AE** in the eppendorf is 400 µl.

12. Determine yield and purity of DNA using the absorbance at 260 and 280 nm. Readings should fall between 0.3 and 1.0 to be accurate. A 1/10 dilution is recommended. Use **Buffer AE** as a diluent and to zero the spectrophotometer. The ratio of 260/280 should be 1.7 to 1.9 for pure DNA.

Extraction of DNA from soil samples

(UltraClean™ Soil DNA Isolation Kit Catalog # 12800-50)

Please wear gloves at all times

1. To the 2 mL Bead Solution tubes provided, add 0.25 – 1 g of soil sample. (For larger sample sizes up to 10 g, try using our Mega Prep Kit, catalog number 12900-10. For amounts of sample to process see Hints and Troubleshooting Guide).
1. Gently vortex to mix.
2. **Check Solution S1.** If **Solution S1** is precipitated, heat solution to 60°C until dissolved before use.
3. Add 60 µl of **Solution S1** and invert several times or vortex briefly.
4. Add 200 µl of **Solution IRS** (Inhibitor Removal Solution). Only required if DNA is to be used for PCR.
5. Secure bead tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.
6. Make sure the 2 mL tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds. **CAUTION:** Be sure not to exceed 10,000 x g or tubes may break.
7. Transfer the supernatant to a clean micro centrifuge tube (provided).
8. **Note**: With 0.25g of soil and depending upon soil type, expect between 400 to 450 µl of supernatant. Supernatant may still contain some soil particles.
9. Add 250 µl of **Solution S2** and vortex for 5 sec. Incubate 4°C for 5 minutes.
10. Centrifuge the tubes for 1 minute at 10,000 x *g*.
11. Avoiding the pellet, transfer entire volume of supernatant to a clean micro centrifuge tube (provided).

12. Add 1.3 mL of **Solution S3** to the supernatant (careful, volume touches rim of tube) and vortex for 5 seconds.
13. Load approximately 700 µl onto a spin filter and centrifuge at 10,000 x *g* for 1 minute. Discard the flow through, add the remaining supernatant to the spin filter, and centrifuge at 10,000 x *g* for 1 minute. Repeat until all supernatant has passed through the spin filter. **Note**: A total of three loads for each sample processed are required.
14. Add 300 µl of **Solution S4** and centrifuge for 30 seconds at 10,000 x *g*.
15. Discard the flow through.
16. Centrifuge again for 1 minute.
17. Carefully place spin filter in a new clean tube (provided). Avoid splashing any **Solution S4** onto the spin filter.
18. Add 50µl of **Solution S5** to the center of the white filter membrane.
19. Centrifuge for 30 seconds.
20. Discard the spin filter. DNA in the tube is now application ready. No further steps are required.

Mo-Bio recommends storing DNA frozen (-20°C). **Solution S5** contains no EDTA.

Qiagen RNeasy + SDS and homogenization (QHS)

(Qiagen RNeasy Catalog # 74104)

Wear gloves and be careful not to touch surfaces that might contain RNases

Add β-mercapto ethanol to **RLT buffer** (RLT buffer is provided with the Kit), this step needs to be done in the fume hood.

Add 10 µL of β-mercapto ethanol per 1 mL of **buffer RLT**, prepare only what you need because this is stable only for a month. (Better results are obtained if buffer is prepared fresh or is keep at the most a week)

Clean bench and pipettes with ethanol and with RNase erase

1. Prepare Lysozyme digestion buffer (30 mM Tris-Cl, 1 mM EDTA; pH 8.0) with 15 mg/mL lysozyme.
2. Add 100 µL of lysozyme digestion buffer, to sample (pellet).
3. Add 0.5 µL of 10% (weight/volume) SDS solution and mix well. Let stand at room temperature for 5 min.
4. Add 350 µL of **Buffer RLT** to the sample and vortex vigorously.
5. Pass lysate through a 25-gauge needle with 1-mL syringe 3-4 times (Homogenization).
6. Centrifuge for 30 sec
7. Transfer the supernatant to a clean tube
8. If there are visible precipitates centrifuge for 2 minutes at max speed
9. Add 250 µL of ethanol Mix by pipeting. Do not centrifuge
10. Apply the lysate containing ethanol; including precipitates to an RNeasy Mini column placed in a 2 mL collection tube, maximum loading volume is 700 µL.

11. Centrifuge for 15s at >10,000 rpm
12. Discard flow through
13. Add 700 µL **Buffer RW1** to the RNeasy column. Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through and collection tube.
14. Transfer column into a new 2ml collection tube
15. Pipet 500 µL **Buffer RPE** onto RNeasy column.
16. Centrifuge for 15s at >10,000 rpm
17. Discard flow through
18. Add another 500 µL **Buffer RPE** onto RNeasy column
19. Centrifuge for 2 min at >10,000 rpm
20. Transfer RNeasy column into a 1.5 mL collection tube. Pipet 30-50 µL RNase free water into the column centrifuge for 1 minute at >10,000 rpm (I usually do 40 µL)
21. Elution might be done a second time in another micro centrifuge tube. (I recommend doing this)

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(Qiagen RNeasy Catalog # 74104)

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15. Centrifuge for 15s at >10,000 rpm
16. Discard flow through
17. Add another 500 µL **Buffer RPE** onto RNeasy column
18. Centrifuge for 2 min at >10,000 rpm
19. Transfer RNeasy column into a 1.5 mL collection tube. Pipet 30-50 µL RNase free water into the column centrifuge for 1 minute at >10,000 rpm (I usually do 40 µL)
20. Elution might be done a second time in another tube. (I recommend doing this)

Qiagen RNeasy +SDS (QS)

(Qiagen RNeasy Catalog # 74104)

Wear gloves and be careful not to touch surfaces that might contain RNases

Add β-mercapto ethanol to RLT buffer (RLT buffer is provided with the Kit), this step needs to be done in the fume hood.

Add 10 µL of β-mercapto ethanol per 1 mL of buffer RLT, prepare only what you need because this is stable only for a month. (Better results are obtained if buffer is prepared fresh or is keep at the most a week)

Clean bench and pipettes with ethanol and with RNase erase

1. Prepare Lysozyme digestion buffer (30 mM Tris-Cl, 1 mM EDTA; pH 8.0) with 15 mg/ml lysozyme.
2. Add 100 µL of lysozyme digestion buffer, to sample (pellet).
3. Add 0.5 µL of 10% (weight/volume) SDS solution and mix well. Let stand at room temperature for 5 min.
4. Add 350 µL of **Buffer RLT** to the sample and vortex vigorously.
5. Centrifuge for 30 sec
6. Transfer the supernatant to a clean tube
7. If there are visible precipitates centrifuge for 2 minutes at max speed
8. Add 250 µL of ethanol Mix by pipeting. Do not centrifuge
9. Apply the lysate containing ethanol; including precipitates to an RNeasy Mini column placed in a 2 mL collection tube, maximum loading volume is 700 µL.
10. Centrifuge for 15s at >10,000 rpm
11. Discard flow through

12. Add 700 µl **Buffer RW1** to the RNeasy column. Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through and collection tube.
13. Transfer column into a new 2 mL collection tube
14. Pipet 500 µL **Buffer RPE** onto RNeasy column.
15. Centrifuge for 15s at >10,000 rpm
16. Discard flow through
17. Add another 500 µL **Buffer RPE** onto RNeasy column
18. Centrifuge for 2 min at >10,000 rpm
19. Transfer RNeasy column into a 1.5 mL collection tube. Pipet 30-50 µL RNase free water into the column centrifuge for 1 minute at >10,000 rpm (I usually do 40 µL)
20. Elution might be done a second time in another micro centrifuge tube. (I recommend doing this)

Micro to midi (M)

(Micro to Midi, Invitrogen Catalog # 12183-018)

Wear gloves and be careful not to touch surfaces that might contain RNases

Clean bench and pipettes with ethanol and with RNase erase

1. Lyse up to 1 x 109 pelleted bacterial cells in 100 µL of Lysozyme digestion buffer (30 mM Tris-Cl, 1 mM EDTA; pH 8.0) with 15 mg/mL lysozyme . Vortex the cell pellet until resuspended.
2. Add 0.5 µL of 10% SDS solution and mix well. Let stand at room temperature for 5 min.
3. Add 350 µL of freshly prepared RNA **Lysis solution** containing 3.5 µL of 2-mercaptoethanol. (Prepare this solution in the fume hood) Mix thoroughly.
4. Pass lysate through a 25-gauge needle with 1-mL syringe 3-4 times.

   b. Centrifuge the sample at 12,000 x g for 2 min at 25°C.

   c. Transfer the supernatant to a clean 1.5-mL tube and proceed to step 5.
5. Add 250 µL of ethanol to the sample. Mix by pipeting up and down 5 times.
6. Apply the sample to the RNA Spin Cartridge. Centrifuge at 12,000 x g for 15 s at 25°C. Discard the flow-through.
7. Add 700 µL of **Wash Buffer I** to the cartridge. Centrifuge at 12,000 x g for 15 s at 25°C. Discard the flow-through and tube. Place the spin cartridge into a clean RNA Wash Tube (2 mL tube).
8. Add 500 µL of **Wash Buffer II** (containing ethanol) to the cartridge. Centrifuge at 12,000 x g for 15 s at 25°C. Discard the flow-through.

9. Add 500 µL of **Wash Buffer II** (containing ethanol) to the cartridge. Centrifuge at 12,000 x g for 15 s at 25°C. Discard the flow-through. Centrifuge for 1 min to dry the membrane.

10. Remove the cartridge from the tube and place it in an RNA Recovery Tube.

11. Add 40 µL of RNase-free Water to the cartridge membrane and let stand 1 min. Centrifuge at 12,000 x g for 2 min at 25°C. Collect elusions in the same tube.

12. Store the RNA at -70°C. If you plan to use the RNA in a few hours, store on ice.

Micro to midi no SDS (MH)

(Micro to Midi, Invitrogen Catalog # 12183-018)

Wear gloves and be careful not to touch surfaces that might contain RNases

Clean bench and pipettes with ethanol and with RNase erase

1. Lyse up to 1 x 109 pelleted bacterial cells in 100 µL of Lysozyme digestion buffer (30 mM Tris-Cl, 1 mM EDTA; pH 8.0) with 15 mg/mL lysozyme . Vortex the cell pellet until resuspended.
2. Add 350 µL of freshly prepared RNA **Lysis solution** containing 3.5 µL of 2-mercaptoethanol. (Prepare this solution in the fume hood) Mix thoroughly.
3. Pass lysate through a 25-gauge needle with 1-mL syringe 3-4 times.

   b. Centrifuge the sample at 12,000 x g for 2 min at 25°C.

   c. Transfer the supernatant to a clean 1.5-mL tube and proceed to step 4.
4. Add 250 µL of ethanol to the sample. Mix by pipeting up and down 5 times.
5. Apply the sample to the RNA Spin Cartridge. Centrifuge at 12,000 x g for 15 s at 25°C. Discard the flow-through.
6. Add 700 µL of **Wash Buffer I** to the cartridge. Centrifuge at 12,000 x g for 15 s at 25°C. Discard the flow-through and tube. Place the spin cartridge into a clean RNA Wash Tube (2-mL tube).
7. Add 500 µL of **Wash Buffer II** (containing ethanol) to the cartridge. Centrifuge at 12,000 x g for 15 s at 25°C. Discard the flow-through.
8. Add 500 µL of **Wash Buffer II** (containing ethanol) to the cartridge. Centrifuge at 12,000 x g for 15 s at 25°C. Discard the flow-through. Centrifuge for 1 min to dry the membrane.

9. Remove the cartridge from the tube and place it in an RNA Recovery Tube.

10. Add 40 µL of RNase-free Water to the cartridge membrane and let stand 1 min. Centrifuge at 12,000 x g for 2 min at 25°C. Collect elusions in the same tube.

11. Store the RNA at -70°C. If you plan to use the RNA in a few hours, store on ice.

DNase treatment

DNAase amplification grade (Invitrogen Catalog # 18068-015)

Preparation of RNA Sample Prior to RT-PCR:

1. Prepare duplicate tubes if positive and negative reverse transcriptase (RNA) samples are to be used in the amplification reaction. Add the following to an RNase-free, 0.2 mL micro centrifuge tube on ice:

	µL	
RNA sample	8	up to 1ug
DNase 10X reaction buffer	1	
DNase I amp. grade	1	
DEPC-treated water	0	Adjust to 10ul
Total volume	10	

2. Incubate tube(s) for 15 min at room temperature.
3. Inactivate the DNase I by the addition of 1 µL of 25 mM EDTA solution to the reaction mixture.
4. Heat for 10 min at 65°C. The RNA sample is ready to use in reverse transcription, prior to amplification.

Reverse transcription

Omniscript (Qiagen Catalog # 205111) or Sensiscript RT (Qiagen Catalog # 205211)

Also needed: Random primers (Promega Catalog # C118A), RNase inhibitor (RNasin, Promega Catalog # N211A).

1. Incubate RNA 5 min at 65C to destroy possible hairpins
2. Place immediately on ice
3. Dilute RNase inhibitor to a final concentration of 10units/µL in 1X buffer RT
4. Prepare a master mix using the following table (do not add the RNA to the master mix)

   For one tube

Component	Volume/reaction (µL)	Final concentration
10X Buffer RT	2	1x
dNTP mix (5mM)	2	0.5mM each dNTP
Random primers (100uM)	2	10uM
RNase inhibitor	1	
Omniscript or Sensiscript R T	1	
RNase-free water	X	
RNA	X	
Total Volume	20	

5. Aliquot the master mix into 0.2 mL PCR tubes
6. Add RNA to each tube. The amount of RNA and water is adjustable in order to get to a 20 µL total volume.

When using sensiscript as the RT enzyme, the RNA concentration in the tube should be lower than 50 ng total RNA. When using omniscript the minimum RNA in the reaction should be 50 ng and the maximum amount of RNA to be used is 2 µg.

7) Incubate for 180 at 37C (In the PCR machine).