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Integrated application of genomic, biochemical and cultivation approaches to characterize 1,2-dichloropropane dichloroelemination in organohalide respiring Chloroflexi

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I am submitting herewith a dissertation written by Elizabeth Padilla entitled "Integrated application of genomic, biochemical and cultivation approaches to characterize 1,2-dichloropropane dichloroelemination in organohalide respiring Chloroflexi." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

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Integrated application of genomic, biochemical and cultivation approaches to characterize 1,2-dichloropropane dichloroelimination in organohalide-respiring *Chloroflexi*

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Dedication

To mom,
Love of my life,
For being my anchor
And always believing I could achieve great things.

To Roy and Dad,
For your support and love.

And to Arturo and Frank,
For making me the scientist that I am
And always showing me the way.

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Abstract

Chlorinated solvents are among the most encountered groundwater pollutants. These toxic compounds cause harm to ecosystem functioning and human health. 1,2-Dichloropropane (1,2-D) was used in a variety of industrial and agricultural applications until it was banned in the U.S. in the 1970s. Only a handful of bacteria have been described to reductively dechlorinate 1,2-D to innocuous propene and inorganic chloride, among these the *Dehalococcoides* (*Dhc*) strains RC and KS. In order to shed light into the genetic basis of 1,2-D dechlorination, efforts focused in identifying the gene encoding the enzyme system (i.e., reductive dehalogenase) responsible for 1,2-D to propene transformation. To accomplish this goal, a multiple lines of evidence approach combining gene cloning, transcriptional studies, and enzyme activity assays implicated the *dcpA* gene in 1,2-D reductive dechlorination in *Dhc* strains RC and KS. This gene was also identified in *Dehalogenimonas lykanthroporepellens* (*Dhgm*) strain BL-DC-9, another member of the organohalide-respiring *Chloroflexi* group, and also capable of growth with 1,2-D as electron acceptor. Propene-producing enrichment cultures were derived from a variety of environments and the presence of *dcpA* correlated with 1,2-D reductive dechlorination observed *in situ* and/or in microcosms. Nested PCR and qPCR assays were designed and validated to detected and quantify the gene in laboratory cultures and in environmental samples. These surveys shed light into the distribution of this gene in diverse environments including pristine environments. Genomic and bioinformatics tools explored the gene neighborhood of *dcpA* and revealed a genomic island shared between *Dhc* and *Dhgm* indicative of a horizontal gene transfer event. Metagenome analysis of consortia RC and KS enabled the draft genome assemblies of these two *Dhc* strains. This analysis revealed that the *Dhc* strain RC and strain KS harbor at least 34 and 31 reductive dehalogenase genes, including genes implicated in PCB reductive dechlorination. These findings reveal broad reductive dechlorination potential and emphasize that such dedicated dechlorinators (i.e., *Dhc* strain FL2) occur in pristine environments and are members of natural microbial assemblages that have not been exposed to anthropogenic contamination.

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List of Symbols and Abbreviations

1,1-DCA	1,1-dichloroethane
1,1,1-TCA	1,1,1-trichloroethane
1,1,2-TCA	1,1,2-trichloroethane
1,1,2,2-TeCA	1,1,2,2-tetrachloroethane
1,1-DCP	1,1-dichloropropane
1,2-DiBr-3-CP	1,2-dichloropropane
1,3-DCP	1,3-dichloropropane
1,2-DCP	1,2-dichloropropane
1,2-DCA	1,2-dichloroethane
1,2,3-TCP	1,2,3-trichloropropane
1-CP	1-chloropropane
2-CP	2-chloropropane
2,2-DCP	2,2-dichloropropane
2-Br-1-CP	1-bromo-1-chloropropane
6-FAM	6-carboxyfluorescein
aa	Aminoacid
ATSDR	Agency for Toxic Substances and Disease Registry
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
cDCE	*cis*-1,2-dichloroethene
CF	Chloroform
CP	Chloropropane
CRISPR,	Clustered regularly interspaced short palindromic repeats
CT	Carbon tetrachloride
DCA	Dichloroethane
DCM	Dichloromethane
Dhb	*Dehalobacter*
Dhc	*Dehalococcoides*
Dhgm	*Dehalogenimonas*
“Dhom”	“Dehalobium”
EPA	U.S. Environmental Protection Agency
GC	Gas chromatograph
HCl	Hydrochloric acid
HGT	Horizontal gene transfer
HPR	High plasticity region (in *Dehalococcoides* genomes)
JOE	6-carboxy-4', 5'-dichloro-2', 7'- dimethoxyfluorescein
MCL	Maximum Contaminant Limit
NAPL	Non-aqueous Phase Liquid
NaHCO3	Sodium Bicarbonate
NPL	National Priority List
NCBI	National Center for Biotechnology Information

PBDE	Polybrominated diphenyl ethers
PCBs	Polychlorinated biphenyls
PCDDs	Polychlorinated dibenzodifurans
PCE	Tetrachloroethene
PCR	Polymerase Chain Reaction
qPCR	Quantitative real-time PCR
RDase	Reductive dehalogenase
rdh	Reductive dehalogenase homologous gene
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	Ribosomal RNA genes
SERDP	Strategic Environmental Research and Development
sp.	Specie (singular)
spp.	Species (plural)
TAMRA	6-carboxy-tetramethylrhodamine
TCE	Trichloroethene
TCP	Trichloropropane
tDCE	*trans*-1,2-dichloroethene
VC	Vinyl chloride (monochloroethene)
VOC	Volatile organic compound
vol/vol	Volume per volume
µL	Microliter
µM	Micromolar

Chapter 1

Introduction

Thesis Rationale

Groundwater contamination by chlorinated ethenes (e.g. PCE, TCE, DCEs, VC) and chlorinated propanes (e.g. 1,2-D) is a major environmental problem in industrialized countries. In anoxic environments, chlorinated organics can be biologically transformed to less-chlorinated ethenes and, in some cases, completely dechlorinated to non-toxic end products (e.g. ethene, propene). *Dehalococcoides mccartyi* (*Dhc*) strains play a major role in the dechlorination of these compounds in the subsurface, and bioremediation may be the only viable means to reduce the environmental risk posed by many contaminated aquifers. Molecular tools have the potential to be used in monitoring and validation of intrinsic bioremediation by specifically linking microbial activity to the degradation process. Since *Dhc* strains that exhibit different dechlorination activities share highly similar 16S rRNA genes (and other housekeeping genes) the approach of looking at phylogenetic markers is insufficient to reliably evaluate the dechlorination potential at contaminated sites. Hence, additional targets that go beyond the 16S rRNA gene are needed to reliably predict whether sites that have *Dhc* populations can efficiently detoxify chlorinated ethenes and/or chlorinated propanes. Previous to this study no biomarker linked to 1,2-D detoxification had been described and there was an incomplete understanding about the environmental distribution if 1,2-D dechlorinating populations in nature hindering our knowledge about the cycle of halogenated compounds in nature. Moreover, there was no genome information available for the 1,2-D-dechlorinating *Dhc* strains RC and KS, and while the genome of the 1,2-D-respiring *Dehalogenimonas* sp. was available, the 1,2-D reductive dehalogenase in *Dhgm* was unknown and no horizontal gene transfer between *Dehalogenimonas* and *Dhc* sp. had been reported.

Goals and Research Objectives

The main objective of the proposed research was to discover a biomarker linked to 1,2-D detoxification, validate a tool to quantify the gene in environmental samples, describe the laboratory cultures and consortia linked to 1,2-D dechlorination, shed light into the ecology and distribution of 1,2-D dechlorinating populations in nature, study the possible mobility of RDase

genes between organohalide-respiring *Chloroflexi*, and determine the genomic features of *Dhc* strains RC and KS.

The major limitation for implementing enhanced bioremediation and monitored natural attenuation at the many sites polluted with chlorinated compounds is the lack of suitable assessment and monitoring tools. Therefore, the new assays described herein, provide new insights to engineers and practitioners on how to correlate 1,2-D detoxification with microbial activity during bioremediation processes. Additionally, the new tools provide new understanding to scientists on the genetic basis of 1,2-D dechlorination, and the ecology and distribution of the populations of interest. Finally, a set of policy recommendations are delineated, on contaminated site management, research and innovation needs and implementation of green and sustainable remedial practices.

To decipher the genomic basis of 1,2-D detoxification and further describe 1,2-D dechlorinating cultures the following objectives were addressed:

Objective 1: Identify the reductive dehalogenase (RDase) gene implicated in 1,2-D to propene dechlorination.

Objective 2: Develop PCR and qPCR assays for the 1,2-D to propene reductive dehalogenase (*dcpA*).

Objective 3: Establish microcosms and enrichment cultures that could dechlorinate 1,2-D to propene.

Objective 4: Investigate the environmental distribution of *dcpA* in pristine and contaminated environments.

Objective 5: Determine the genomes of *Dehalococcoides* (*Dhc*) strain RC and strain KS through high-throughput sequencing technologies.

Objective 6: Assemble, describe and annotate the genomes of *Dhc* strain RC and KS.

Objective 7: Investigate horizontal gene transfer events between *Dhc* and *Dhgm*.

Taking advantage of classic culturing methods, genomic approaches, cutting-edge sequencing technologies, enzyme assays, proteomics, and bioinformatic tools, the following objectives where accomplished and are described in Chapters 2-6. Chapter 7 discusses future directions on management of contaminated sites and the implementation of green and sustainable remediation approaches, bringing this thesis to a full circle from lab work to the field. In Chapter 8 the research findings of this work are briefly summarized and the status quo of the latest findings on RDase characterization and sustainable remediation practices/initiatives are discussed. Lastly, future recommendations and research directions are addressed.

Chapter 2

Literature Review

Chapter overview

This chapter comprises an overview on natural and anthropogenic sources of chlorinated solvents, their industrial use, toxicological profiles, as well as their means of disposal and environmental fate. Special attention is given to 1,2-dichloropropane, a hazardous pollutant regulated by the United States Environmental Protection Agency (EPA) and to metabolic processes carried out by anaerobic bacteria with potential for the bioremediation of this and other related chlorinated compounds. Emphasis is also given to current bioremediation practices, which include natural attenuation and enhanced bioremediation technologies such as bioestimulation and bioaugmentation.

Naturally occurring chlorinated chemicals

There are more than 5,000 naturally occurring organohalogen compounds containing bromine, chloride, fluorine, or iodine, which are produced by bacteria, fungi, plants, insects, marine organisms, and mammals (G W Gribble, 2000, 2003; Gordon W. Gribble, 2012; Öberg, 2002). Some of these biologically-generated halogenated compounds serve as hormones and pheromones in legume and ticks, respectively, while others are produced as chemical defenses in invertebrates and as signaling molecules in bacteria (Gordon W. Gribble, 2012). Different algal species have been documented to produce PCE, TCE and halogenated propanes (Abrahamsson, Ekdahl, Collén, Fahlström, & Pedersén, 1995; Gschwend, MacFarlane, & Newman, 1985). However, some are abiotically generated during volcanic eruptions, forest fires, or via geochemical processes, as in the case of organobromine and organochlorines, which are ubiquitous in marine sediments and soil, respectively (G W Gribble, 1998, 2000). Moreover, lightning strikes have been reported as additional emission sources of chlorinated compounds (G W Gribble, 2003).

The uncovering of natural organohalogens is a growing field of research, with approximately 100-200 compounds discovered yearly (G W Gribble, 2004). In the case of organochlorides, only a dozen of these chemicals were known to be naturally-produced by 1952. Nevertheless, by

2004 more than 2,400 were known (Gribble 2004). The amounts of chlorinated compounds produced in the natural environment can be substantial. Chlorinated phenols (CPs) have been reported to be produced in quantities as high as 40,000 tons per acre in Swedish peat bogs (De Jong, Field, Spinnler, Wijnberg, & De Bont, 1994). Moreover, it is estimated that volcanoes emit around 3,000,000 tons of HCl per year (Graedel, T . E., 1995). Examples of natural organohalogens encountered in terrestrial and marine environments are listed in Table 2.1. Despite their natural occurrence, chloroorganics are mainly associated with anthropogenic activity from the postindustrial age.

The history of anthropogenic chlorinated solvents usage and environmental pollution

The use and production of synthetic chemicals began during the Industrial Revolution (1760-1840). During that time elemental chlorine was discovered and the first patent for its use as a bleaching agent was developed (Deutsch, 1947). The manufacture of synthetic chemicals, at larger scales, in the U.S. started after World War I, when exports from Europe halted. Subsequently, the production of these compounds in the U.S. rapidly increased, going from 10,500 tons in 1921 to 316,500 tons in 1929, as reviewed by (H. Stroo, Leeson, & Ward, 2012).

The use of chlorinated solvents in the dry cleaning industry was implemented after 1962 as a safer alternative to the inherent threat of gasoline as a flammable agent. Shortly after, chlorinated compounds like tetrachloroethene (PCE) and trichloroethene (TCE) were adopted and widely used in the commercial (textile and dry cleaning) and military sectors due to their nonflammable, noncorrosive properties; in addition to their low boiling points and cleaning efficacies. These organic solvents have also been used as paint removers, degreasers, and components of industrial paint, lubricants and adhesives (ASTDR, 1993; ATSDR, 2010). Other chlorinated compounds like 1,2-dichropropane (1,2-D) have been extensively used in agriculture as soil fumigants and as commercial solvents in a variety of industrial and household products (Agency for Toxic Substances and Disease Registry (ATSDR), 1989). At the time, adequate treatment and disposal of industrial wastes, including chlorinated solvents, was not properly regulated resulting in the release of these chemicals into the environment.

Chlorinated solvents have been reported to be detrimental to the environment and human health leading to the development of regulations for their use and disposal (Ruder, 2006). The Clean Air Act formulated in 1970 by the U.S. EPA regulated emissions for TCE and PCE as these chemicals were suspected to be involved in ozone depletion and smog formation (Dimitriades, B., Gay Jr, B. W., Arnts, R. R., & Seila, 1983). In 1975, investigations carried by the U.S. National Cancer Institute showed that TCE caused cancerous tumors in mice (ASTDR, 1993), which also raised concerns on potential hazards posed by PCE and TCE daughter products: *cis*-1,2-dichloroethene (cDCE), *trans*-1,2-dichloroethene (tDCE), and vinyl chloride (VC). Also, toxicological studies in rats and mice showed that brief exposure to high concentrations of VC (100,000 to 400,000 ppm for 30 mins by inhalation) caused kidney, liver, and pulmonary hemorrhage (Lester, D., Greenberg, L. A., & Adams, 1963; Mastromatteo, E., Fisher, A. M., Christie, H., & Danziger, 1960). The toxicity of 1,2-D has also been documented in animals and humans (ATSDR, 1997; Pozzi C, Marai P, 1985). 1,2-D is mutagenic (*in vitro*) causing chromosomal damage. Oral intake in humans causes functional disorder to the liver and kidneys, while in rats long-term oral exposure led to liver tumors (ATSDR, 1997; Pozzi C, Marai P, 1985).

Therefore in 1977 the Clean Water Act listed chlorinated compounds like PCE, TCE, and 1,2-D as part of the U.S. National Priority List (NPL) of pollutants, and between 1979 and 1980 permissible levels for this chemicals were established. Today, compounds like PCE, TCE, and 1,2-D are no longer used in many industrial applications and their concentrations in drinking water are regulated by the U.S. EPA to 5 parts per billion (ppb); although drinking water standards at the state level may differ (Table 2.2). In Europe, levels of chlorinated solvents are regulated by the EU Water Framework Directive and the World Health Organization (WHO) guidelines. Federal regulated maximum contaminant levels (MCL) for various chlorinated solvents and their properties are listed in table 2.2. Nevertheless, poor handling and disposal of chlorinated solvents has left a legacy of contamination across the U.S. The government has listed these sites as a National Priority by declaring these locations as Superfund sites (i.e., locations within the U.S. and its territories that have been impacted by toxic chemicals and pose a thereat to human and ecosystem welfare). As of February 09, 2015, there were 1,754 declared Superfund sites (http://www.epa.gov/superfund/sites/npl/index.htm) (Table 2.3). Chlorinated

compounds are present in approximately 73% of these locations (Table 2.4). (http://cumulis.epa.gov/supercpad/cursites/srchsites.cfm). Despite these measures, their worldwide production and demand is substantial and the U.S. is the major producer as well as the top consumer (Fig. 2.1).

1,2-dichloropropane as a groundwater contaminant

1,2-Dichloropropane (1,2-D) is a halorganic compound extensively used in the past as a solvent and as a soil fumigant. Major trade names of 1,2-D- formulations as an agrochemical were: Vorlex®, Vidden-D®, Nemex®, Telone®, Nemafene®, Nemax®, Ditrapex®. These admixes also contained other toxic chemicals such as cis/trans-1,3-dichloropropene (1,3-DCP), 2,3-dichloropropene and chloropicrin (nitrochloroform) (*"1,2-dichloropropane" SIDS Initial Assessment Report for SIAM 17*, 2003). Although its use as a solvent and agrochemical is banned, an assessment report for 1,2-D performed by the International Organization for Economic Co-operation and Development (OECD) estimated an annual production of 350 kilotonnes (about 770 million pounds) in 2001 with the majority of 1,2-D produced in the U.S. (*"1,2-dichloropropane" SIDS Initial Assessment Report for SIAM 17*, 2003) Table 2.5. Various manufacturers of 1,2-D are listed in Table 2.6 (Greene, 2012). Additionally, EPA'sToxics Release Inventory reported that 102,478 pounds (close to 50 tonnes) of 1,2-D was disposed of, or released during 2013 in the U.S. (http://iaspub.epa.gov/triexplorer/tri_release.chemical Table 2.7. Because of its large-scale use in plant agriculture and its recalcitrant nature 1,2-D is widely distributed as a contaminant in groundwater and aquifers posing a threat to human health. Toxicological studies in rat models have demonstrated that after oral and inhalation exposure 1,2-D is rapidly absorbed and widely distributed within the body (Agency for Toxic Substances and Disease Registry (ATSDR), 1989). 12-D is a suspected carcinogen causing damage to the liver, brain, blood, lungs and kidneys at high exposure levels (Agency for Toxic Substances and Disease Registry (ATSDR), 1989; Pozzi C, Marai P, 1985). Consequently the Environmental Protection Agency has regulated its levels in drinking water to 5 ppb (http://water.epa.gov/drink/contaminants/index.cfm#List).

Although the presence of 1,2-D in the environment has been mainly attributed to anthropogenic activity, there is mounting evidence indicating the presence of naturally occurring processes constituting significant sources of halorganic compounds. For instance, for a long time it was considered that the presence of vinyl chloride in the environment was exclusive from human-related sources; but recent evidence has alluded to its natural production in soils by organic matter degradation (Keppler, Borchers, Pracht, Rheinberger, & Scholer, 2002). 1,2-D has been found in tobacco and tobacco smoke but its source as either a natural component, additive or as a product of combustion is still unclear. The natural environment is ruled by a plethora of processes that we can only start to unravel. Hence, the production of 1,2-D by organisms or naturally occurring abiotic processes cannot be disregarded. Based on the recent observations made for vinyl chloride (Keppler et al., 2002), it seem reasonable to hypothesize that natural chlorinated compounds could have a long history as substrates for microbial enzymes capable of carrying out the transformation of these compounds.

Remediation technologies

Nowadays, the use of chlorinated compounds is minimal and the levels in drinking water are regulated. Nevertheless, chlorinated ethenes and propanes are persistent pollutants widely distributed in groundwater resources. Due to their high density, chlorinated solvents are heavier than water and can travel deep into an aquifer creating what is known as Dense Non Aqueous Phase Liquid (DNAPLs). DNAPLs are immiscible and form a separate phase in the aquifer that is difficult to remediate (Stroo, Hans F., Marvin Unger, C. Herb Ward, Michael C. Kavanaugh, Catherine Vogel, Andrea Leeson, Jeffrey A. Marqusee, 2003). The restoration of contaminated sites has required the interaction between experts in the fields of environmental engineering, hydrogeology and microbiology in order to develop effective *in situ* (on-site) and *ex situ* (off-site) treatment strategies, as reviewed in (Brehm-Stecher & Johnson, 2004; H. F. Stroo et al., 2012; Van Deuren J, Lloyd T, Chhetry S, Liou R, 2002).

Among *ex situ* treatment strategies, injection and extraction (i.e. pump and treat) systems are commonly utilized for the treatment of contaminated groundwater. In this method water is

extracted from wells and treated *ex situ* by air stripping, catalytic oxidation or activated carbon absorption, while clean water is injected to the subsurface. Another technique used *ex situ* or *insitu* is cosolvent flushing which encompasses the application of water-soluble solvents (e.g. acetone or methanol) to help aid dissolve the DNAPLs. A similar method called *surfactant flushing* comprises the addition of compounds that act as detergents, which aid in the formation of micelles that can capture chlorinated solvents aiding in their solubility. *In situ* vapor extraction and bioventing can also be used by placing pumps in wells to remove the volatile contaminants, vaporize the DNAPLs or supply oxygen to induce biodegradation.

Other treatments available involve the alteration of physicochemical conditions at the site by *in situ* thermal treatment or *in situ* chemical processes. Examples of *in situ thermal treatment* includes the addition of hot air or water to increase temperatures in the subsurface up to 100 -120 degrees °C to volatilize and increase mobilization of the compounds of interest. *In situ* chemical processes include chemical oxidation and reductive reactions. Chemical oxidation involves the addition of compounds like Fenton's regent, permanganate, and ozone to transform contaminants into less toxic chemical species. In contrast, *reductive reactions* consists in the addition of zero valent iron [Fe(0)] to react with chlorinated ethenes (removing the chlorine atom) and resulting in the formation of Fe(II) and H_2 in the process.

The biodegradation of chlorinated solvents is also a feasible alternative that is frequently used as the first option for the treatment of polluted groundwater (Pandey, Chauhan, & Jain, 2009; H. Stroo et al., 2012). This method takes advantage of enzymes produced by microorganisms that can transform halorganic compounds to their benign form, through a thermodynamically favorable process known as organohalide respiration. In this process, anaerobic bacteria conserve energy by using organohalogens as electron acceptors, while oxidizing electron donors such as lactate and H_2 . Relative to other remedial methods, *in situ* bioremediation provides several advantages as it is less intrusive, typically produces minimal secondary waste, and is often less expensive. A more in-depth discussion on advantages and limitations of bioremediation is presented in Chapter 6 of this dissertation.

To conclude, the remedial strategy chosen must be implemented depending on the physico-

chemical conditions, magnitude of risks and history of contamination at the site. Very often more than one complementary method is necessary to achieve cleanup goals. In the next sections, an overview of microbial metabolism and viable and known bioremediation approaches for chlorinated solvents are explained.

Overview on microbial transformation of chlorinated compounds

Microbial metabolism encompasses a variety of catabolic (energy releasing) and anabolic (energy consuming) processes. Biochemical cellular processes where microorganisms derive energy (ATP) coupled to growth involve the transfer of electrons from a donor (food source) to an electron acceptor (equivalent to oxygen in aerobic respiration). Halogenated compounds can be biologically transformed via catabolic or cometabolic reactions, the latter provide no carbon or energy to the organism. In the other hand, catabolic reactions include (*i*) oxidation, where the halogenated compound serves as the electron donor (carbon source), and (*ii*) reductive dechlorination, where the halogenated compound serves as the electron acceptor. Reductive dechlorination is a thermodynamically favourable reaction that encompasses the removal of an halogen atom from an organic molecule and its subsequent replacement with hydrogen (Smidt & De Vos, 2004). In this reaction two protons are consumed, two electrons are concomitantly added to the organic compound, and H^+ and Cl^- are released (Figure 2.2). When the halogenated compound is used as terminal electron acceptor and the reductive dechlorination reaction is directly linked to the electron transport chain and energy conservation it is known as organohalide respiration (Figure 2.2).

Phylogenetically diverse taxa can co-metabolically transform chlorinated compounds under aerobic conditions. But co-metabolic processes are complex and challenging for bioremediation efforts (Ward, SH, CherryJA, Scalf, n.d.). Hurdles include the formation of toxic products, incomplete transformation of the contaminants of interest, and the need to supply the microorganisms with a substrate and oxygen for growth and energy. Even in oxidative metabolism reactions, addition of substrate is needed to induce the enzyme that will "accidentally" interact with the chlorinated compound.

Co-metabolic and growth-linked VC oxidation has been described, and apparently the microorganisms involved in these processes are widespread in nature (Coleman, Mattes, Gossett, & Spain, 2002b). In contrast, oxidation of cDCE under aerobic conditions is rare (Coleman, Mattes, Gossett, & Spain, 2002a). Since anaerobic conditions are usually encountered in chlorinated solvent-impacted sites, groundwater and subsurface environments, reductive dechlorination processes are of interest in remediation efforts.

Anaerobic bacteria use chlorinated compounds as terminal electron acceptors (i.e. via organohalide respiration) in a fashion analogous to oxygen in aerobic respiration in humans. Therefore, is it often said that dechlorinating bacteria can "breath" chlorinated solvents. In the past, the terms dehalorespiration, catabolic reductive dechlorination, chloridogenesis, among others, were used to describe the process by which bacteria derive energy from reductive dechlorination reactions. Nowadays, the term "organohalide respiration" is preferably used, which is more suitable for this particular energetic metabolism.

In the next section the organisms involved in the transformation of chlorinated compounds via reduction/oxidation of co-metabolic or catabolic reactions are discussed in more detail.

Aerobic transformation of chlorinated ethenes and propanes

PCE was long thought to be totally recalcitrant to biodegradation under aerobic conditions until *Pseudomonas stutzeri* OXI was described to dechlorinate PCE by a *toluene*-o-*xylene monooxygenase* (Ryoo, Shim, Barbieri, & Wood, 2000; Shim & Wood, 2000). Claims of PCE degradation were based on Cl^- released in the medium by *P. stutzeri* and by cloning and expression of the monoxygenase in *E. coli*. Later in 2006, Marco-Urrea et al. (2006) described how the white rot fungus *Trametes versicolor* transformed PCE to trichloroacetic elucidating a new mechanism for aerobic PCE transformation involving a cychrome P450 monooxygenase (Marco-Urrea, E., Pérez-Trujillo, M., Vicent, T., & Caminal, 2009).

TCE oxidation under aerobic conditions was first described in 1985 by Wilson and Wilson who

reported that methanotrophic bacteria could convert TCE to CO_2 (J. T. Wilson & Wilson, 1985). Methanotrophs use methane as their source of carbon and energy through the methane monooxygenase enzyme that can fortuitously interact with TCE and oxygen creating an epoxide molecule, which is latter transformed into CO_2. In *Rhodococcus* sp. AD45 a monoxygenase oxidizes cDCE to form a DCE epoxide; and a gluthatione-s-transferases (GST) has been implicated in the opening of the epoxide ring for further degradation (Van Hylckama Vlieg, J. E. T., G. J. Poelarends, A. E. Mars, 2000). The facultative aerobes *Sphingobium chlorophenolicum* and *Sphingobium japonicum* UT26 (formerly *Sphingomonas paucimobili*) are also able to dechlorinate compounds such as hexachlrobenzene thorough another GST-dependant enzyme denominated LinD (Miyauchi, K., Suh, S.K., Nagata, Y., and Takagi, 1998), see Figure 2.3 .

In 2002, Coleman et al., described *Polaromonas* sp. strain JS666, the first isolate capable to oxidize cDCE coupled to growth (Coleman et al., 2002a). Interestingly, this isolate can also transform VC, tDCE, TCE but only co-metabolically. A variety of taxa have been described to mineralize VC to CO_2 among, these *Actinomycetales* (Phelps, Malachowsky, Schram, & White, 1991), *Pseudomonas* (Verce & Freedman, 2000), *Nitrosomonas* (Vannelli, Logan, Arciero, & Hooper, 1990).

In the case of 1,2,3-TCP, there is no known natural metabolic aerobic degradation pathway. However, Bosma et al., (2002) expressed the haloalkane dehalogenase *(DhaA)* from *Rhodococcus* sp. m15-3 in *Agrobacterium radiobacter* strain AD1, making it able to use TCP as the sole carbon and energy source (Bosma, Damborsky, Stucki, & Janssen, 2002).

The aerobic cometabolic oxidation of 1,2-D by nitrifying and methanotropic bacteria has been documented (Oldenhuis, Vink, Janssen, & Witholt, 1989; Rasche, M. E., M. R. Hyman, 1990). The cometabolic transformation needs both oxygen and a carbon source supplied. This interferes with remediation processes in subsurface environments where anoxic conditions prevail, and the need to deliver co-substrates can be costly, making anaerobic reductive dechlorination the most viable solution.

Anaerobic transformation of chlorinated ethenes and propanes

PCE and TCE can be reduced co-metabolically by anaerobic microorganisms such as acetogens and methanogens. The homoacetogens *Acetobacterium woodii* strain WB1 and *Sporomusa ovata* are capable of dechlorinating PCE to TCE (Egli, Scholtz, Cook, Tschan, & Leisinger, 1988; Terzenbach & Blaut, 1994). Likewise, the ability to partially dechlorinate PCE to TCE has been demonstrated for the methanogens *Methanosarcina* sp. strain DCM and *Methanobacterium thermoautotrophicum* (Fathepure, Negu, & Boyd, 1987).

In contrast, via *organohalide respiration* (Fig. 2.2A) can bacteria conserve energy by using organohalogens as electron acceptors, while oxidizing electron donors such as lactate and H_2. *Desulfomonile tiedjei* strain DCB-1 was the first isolated anaerobic bacterium capable of organohalide respiration (Dolfing, 1990). This sulfate-reducing bacterium uses 3-chlorobenzoate as terminal electron acceptor and H_2 or formate as electron donor (Dolfing, 1990; Mohn & Tiedje, 1992). ATP is synthesized by coupling the reduction of the C – Cl of 3-chlorobenzoate to oxidation of hydrogen or formate, as shown in Fig. 2.4.

The reductive dechlorination pathway of PCE and TCE to non-toxic ethene is depicted in Figure 2.5. Dechlorination of PCE to TCE or cDCE has been described in bacterial isolates belonging to the Low G+C Gram-positives [e.g. *Dehalobacter restrictus* (*Dhb*)], ε-proteobacteria (e.g. *Sulfurospirillum multivorans*), δ-Proteobacteria (e.g. *Anaeromyxobacter dehalogenans, Geobacter lovleyi*) and the phylum *Chloroflexi* [*Dehalococcoides mccartyi* (*Dhc*) and "Dehalobium chlorocoercia", "Dhbm"]. Table 2.8 shows a more comprehensive list.

Various *Dhc* isolates and enrichments have been described to transform chlorinated ethenes to their benign, fully-dechlorinated form or to less, partially dechlorinated daughter products, as reviewed by Loeffler et al. (Löffler FE, Yan J, Ritalahti KM, Adrian L, Edwards EA, Konstantinidis KT, Müller JA, Fullerton H, Zinder SH, 2013). So far, dechlorination past DCE has been described only in two genera: *Dhc* and *Dehalogenimonas* (*Dhgm*) strain WBC-2 (Table 2.9).

Dhb, *Dhc* and *Dhgm* are restricted to organolilde respiration, thus have been described as "specialists" while the dehalogenating delta proteobacteria and the *Desulfitobacterium* spp. are considered "metabolic generalists" since they can use a broad spectrum of electron acceptors (e.g. sulfate, iron, nitrate, dimethylsulfoxide) and also chlorinated compounds (Richardson, 2013).

In the case of chlorinated propanes, members of the *Dhgm, Dhc, Dhb* and *Desulfitobacterium* genera have been implicated in the reductive dechlorination of 1,2-D (De Wildeman, Diekert, Van Langenhove, Verstraete, & De Wildeman S, Diekert G, Van Langenhove H, Verstraete, 2003; F E Löffler, Champine, Ritalahti, Sprague, Tiedje, et al., 1997; Moe WM, Yan J, Nobre MF, da Costa MS, Rainey, 2009; Kirsti M Ritalahti & Löffler, 2004; Schlötelburg et al., 2002). Three different reductive dechlorination mechanisms for 1,2-D have been proposed: hydrogenolysis, dehydrochlorination and dichloroelimination, see Figure 2.6 (F E Löffler, Champine, Ritalahti, Sprague, Tiedje, et al., 1997). In 1,2-D-dechlorinating *Dhc* pure and enrichment cultures, only transformation to propene has been observed (F E Löffler, Champine, Ritalahti, Sprague, Tiedje, et al., 1997; Moe WM, Yan J, Nobre MF, da Costa MS, Rainey, 2009; Padilla-Crespo et al., 2014; K. M. K. Ritalahti & Löffler, 2004) indicating that dichloroelimination is the major transformation mechanism. So far, incomplete reductive dechlorination leading to formation 1-chloropropapane (1-CP) and 2-chloropropapan (2-CP) have been observed only in microcosms and enrichments with sediments present, indicating the possibility of abiotic transformations (F E Löffler, Champine, Ritalahti, Sprague, Tiedje, et al., 1997; Padilla-Crespo et al., 2014).

Dhgm has also been reported to use a variety of vicinal chlorinated alkanes including 1,2,3-TCP. Dechlorination of 1,2,3-TCP by *Dhgm* strain BL-DC-9 involves the formation of allyl chloride which undergoes a series of abiotic transformations leading to allyl alcohol and compounds similar to those present in garlic odor (diallyl disulfide, diallyl sulfide, and allyl methyl sulfide) (Moe WM, Yan J, Nobre MF, da Costa MS, Rainey, 2009).

Dhc physiology and genome insights

Thirty years ago, chlorinated ethenes were though to be recalcitrant compounds, until the mid 80's when Bower and McCarty showed that anaerobic bacteria could degrade PCE and TCE to VC (Bouwer & McCarty, 1983). Since VC is highly toxic, efforts were focused to isolate a microorganism that could dechlorinate VC. *Dhc* strain 195 was the first isolate described to dechlorinate PCE to ethene using H_2 as electron donor; it was recovered from an anaerobic sewage digestor (Maymó-Gatell, Chien, Gossett, & Zinder, 1997) and phylogenetic analysis of its 16S rRNA gene showed that it belonged to the phylum *Chloroflexi*. Although cultures of *Dhc* strain 195 growing on PCE were able to dechlorinate VC to ethene, this last step was cometabolic and therefore slow. The first *Dhc* culture reported to dechlorinate VC coupled to growth was that of strain BAV1, originally isolated from a chloroethene-contaminated aquifer in Oscoda, Michigan (J He, Ritalahti, Yang, Koenigsberg, & Löffler, 2003). Since then, various *Dhc* strains (CBDB1, BAV1, GT, VS, FL2, MB, DCMB5, ANAS1, ANAS2, 11a 11a5, have been isolated from aquifer material or sediments and some of them can dechlorinate VC to ethene (Lee PK, Cheng D, West KA, Alvarez-Cphen L, 2013; Löffler FE, Yan J, Ritalahti KM, Adrian L, Edwards EA, Konstantinidis KT, Müller JA, Fullerton H, Zinder SH, 2013; Pöritz et al., 2013).

Dhc are strict anaerobes, non-motile, disc-shaped, small in size ($\leq$ 1 µm wide and 0.1-0.2 µm thick) and use only chlorinated or brominated compounds as electron acceptors (Löffler FE, Yan J, Ritalahti KM, Adrian L, Edwards EA, Konstantinidis KT, Müller JA, Fullerton H, Zinder SH, 2013). *Dhc* strains have a single 16S rRNA gene copy, which ranges from 98% to 100%, sequence identity between strains. Genome sequencing has revealed a small genome size (~1 Mb) among them, as well as a highly specialized metabolism lacking catabolic genes except those encoding reductive dehalogenation, which is consistent with physiological experiments. Multiple non-identical reductive dehalogenase genes are present in *Dhc* genomes, encoding for the catalytic enzymes involved in the halogen removal of the electron acceptors. *Dhc* also posses genes encoding for 5 putative hydrogenase complexes (Hym, Vhu, Hup, Hym, Hyc, and Ehc) involved in the oxidation of the electron donor, H_2. Due to the specialized metabolism of *Dhc* it

has been hypothesized that hydrogenases may be involved in the initial uptake of electrons from H_2 in their electron transport chain .

Dhc genomes are highly similar, (e.g. strain BAV1 and CBDB1, share a median nucleotide identity of >99% of their core genome) and the regions of variability (where rearrangement, indels and deletions have occurred) are localized in two High Plasticity Regions (HPRs) that flank the origin of replication (McMurdie et al., 2009). Despite being very similar at the genetic level, *Dhc* isolates differ substantially in their dechlorination potential, as shown in Table 2.9. *Dhc* are highly sensitive to oxygen, have slow growth rates, and require growth on liquid or semisolid media, which hinders the development of screening methods and genetic systems to manipulate these organisms.

The genetic basis and biochemistry of reductive dechlorination

Reductive dechlorination of chlorinated ethenes consists of the following pathway: PCE→TCE→DCE isomers→VC and finally ethene, where each step is catalyzed by RDases.

The overall reaction catalyzed by these enzymes is:

$$\mathbf{R-Cl + 2[H] = R-H + H^+ + Cl^-}$$

where R is the organic backbone.

Dhc RDases are encoded by an operon containing the *rdhA* and *rdhB* genes, which encode the catalytic unit and a highly hydrophobic membrane protein, respectively. The gene product of *rdhB* is believed to act as a membrane anchor of the catalytic unit. The operon also includes other genes designated as *rdhC-H* which that are thought to encode regulatory functions (Figure 2.7).

Most of *Dhc rdhA* genes are 1,300-1600 bp long and are proceeded by the *rdhB* gene (~100 bp). RDase genes are abundant in *Dhc* genomes (up to 36 in strain VS) and the majority are localized

in HPRs (Löffler FE, Yan J, Ritalahti KM, Adrian L, Edwards EA, Konstantinidis KT, Müller JA, Fullerton H, Zinder SH, 2013). It is hypothesized that multiple RDases gene copies give versatility to *Dhc* by expanding the range of halogenated compounds they can use as substrates; and "what they lack in overall metabolic diversity they make up for in RDase diversity" (Richardson, 2013).

Canonical RDase A variants require cobalamins as a cofactor. Other common features of RDaseA enzymes include: (1) a twin arginine (TAT) signal peptide motif (RRXFXK) indicative of periplasmic translocation; (2) amino acid sequence conservation at the C1-C5 regions; (3) a predicted cobalamin-binding (e.g. vitamin B_{12} or its derivates) domain; (4) the presence of two iron sulfur-binding motifs (e.g. CXXCXXCXXXCP) involved in electron transfer, and (5) the ability to associate with a small hydrophobic protein (RDaseB) that serves as a membrane anchor (Fig 2.1B) (Hölscher et al., 2004; Magnuson, Romine, Burris, & Kingsley, 2000; Smidt & De Vos, 2004). However, some RDases lack some of these characteristics (Hölscher et al., 2004; Hug et al., 2013).

The first *Dhc* RDase identified was the TCE RDase from *Dehalococcoides mccartyi* strain 195 (Magnuson, Stern, Gossett, Zinder, & Burris, 1998). This RDase reductively dechlorinates TCE, cDCE and 1,1-DCE at rates up to 12 μmol min^{-1} mg of $protein^{-1}$, while VC and tDCE were dechlorinated at substantially lower rates of 0.04 to 0.45 μmol min^{-1} mg of $protein^{-1}$ (Magnuson et al., 1998). Other RDases have been identified outside the *Dhc* genus, these include dehalogenases in *Desulfitobacterium* (Christiansen & Ahring, 1996; F E Löffler, Sanford, & Tiedje, 1996; Miller, Wohlfarth, & Diekert, 1998), *Dehalobacter* (Holliger et al., 1998), *Sulfurospirillum* (A Neumann, Scholz-Muramatsu, & Diekert, 1994), and *Desulfomonile* (Cole, Fathepure, & Tiedje, 1995). The reactions catalyzed by characterized RDases and the methods through which each enzyme was identified are described in Table 2.10.

Heterologous expression of the *pceA* genes of *Desulfitobacterium hafniense* Y51 and *Sulfurospirillum multivorans* in *E. coli* have been successful but the resulting RDase was inactive and in insoluble form (A Neumann, Wohlfarth, & Diekert, 1998; Suyama, Yamashita, Yoshino, & Furukawa, 2002). Recently the heterologous expression, of the PceA from *Dehalobacter*

restrictus in *E. coli* was achieved by fusing it with a trigger factor protein (H Sjuts, Fisher K, Dunstan MS, Rigby SE, 2012), the RDase was soluble but also remained inactive. The absence of chaperones involved in protein folding and proper corrinoids have been attributed to the inactivity of the expressed RDases. Recently, the PceA of *D. hafniense* Y51 was expressed in the non-dechlorinating bacterium *Shimwellia blattae* (Nelly, Kai, Svatoš, & Diekert, 2014). The resulting PceA was catalytically active, due to the coexpression of the respective chaperone PceT and adequate cofactor availability since *S. blattae* synthesizes cobamides *de novo.* Due to their high sensitivity to oxygen and low biomass of *Dhc* cultures heterologous expression has been challenging.

RDase structure elucidation is limited. Recent work succeeded in describing the structure of two RDases from *Nitratireductor Pacificus* (NprdhA) and the PceA from *S. multivorans* (Bommer, M. Kunze C, Fesseler J, Schubert T, Diekert G, 2014; Payne PK, Quezada PC, Fisher K, Dunstan MS, Collins FA, Sjuts H, Levy C & Rigby SEJ, 2015). NprdhA is an oxygen tolerant RDase capable of dechlorinating halogenated compounds as ortho-dibromophenol. It is more closely related to catabolic RDases than respiratory ones (e.g. PceA, TceA, etc.). The characterization of soluble and active NprdhA was achieved by heterologous recombination in *Bacillus megateriu* and the structure determined by electron paramagnetic resonance (EPR) and simulations (Payne PK, Quezada PC, Fisher K, Dunstan MS, Collins FA, Sjuts H, Levy C & Rigby SEJ, 2015). Results showed direct interaction of the halogenated substrate with cobalamin via a proposed halogen–cobalt bond formation.

The crystal structure of the PceA of *Desulfitobacterium hafniense* strain Y51 was determined by Bommer et al. 2014, which constitutes the first crystal structure from an anaerobic reductive dehalogenase (Bommer, M. Kunze C, Fesseler J, Schubert T, Diekert G, 2014). Crystallization was achieved by heterologous expression in *Shimwellia blattae* and also revealed that the PceA RDase has a nitroreductase fold, a characteristic not seen in other methyl transferases, with its closest resemblance to a B12 mammalian chaperone. The RDase was found to be dimeric, with short distances between the 4Fe-4S clusters to allow rapid electron transfer. The structure also revealed that the cobalamin-binding site (i.e. nor-pseudo-B12 binding core) is deeply buried in the dimer.

Finally, groundbreaking work was achieved at the time of finishing this dissertation when Parthasarathy et al. (2015), successfully overexpressed the VcrA of *Dhc* strain VS in *E. coli* (Parthasarathy A, Stich TA, Lohner ST, Lesnefsky A, Britt RD, 2015). The over expressed VcrA was not in soluble form, it was aggregated in inclusion bodies and required His-tag affinity chromatography under denaturation conditions for purification. The enzyme's activity was reconstituted by the addition of cobalamin and iron–sulfur cluster cofactors. VcrA is the first and only *Dhc* RDase successfully heterologously expressed and reconstituted to its active form. EPR results led to a proposed mechanism involving the reduction of the iron sulfur clusters by ferrodoxin/flavodoxin. Subsequently, the enzyme-bound cobalamin is reduced from Co(II) to Co(I). The electron transfer from Cob(I)alamin to the chlorinated substrate leads to Cl^- release and the formation of a vinyl radical. Up to date no crystal structure for a *Dhc* has been determined.

Dhc biomarkers: molecular biology tools for enhanced bioremediation

In isolation, *Dhc* cells have doubling times of up to 3 days; and can reach levels of 1.0 E+07 - 1.0 E+08 per mL. Although they can grow to such densities, the cultures have very low turbidity making it impossible to use optical density measurements to monitor growth. Isolation of nucleic acids from laboratory and environmental samples, followed by 16S rRNA gene targeted qPCR, is a reliable approach to enumerate *Dhc* cells (Kirsti M Ritalahti et al., 2006). The use and application of 16S rRNA gene-based assays have been linked to the presence of *Dhc* and the detoxification of chlorinated ethenes (Cupples, 2008; Fennell, Nijenhuis, Wilson, Zinder, & Häggblom, 2004; Hendrickson et al., 2002; Ise, Suto, & Inoue, 2011; Lendvay et al., 2003; Major et al., 2002; Kirsti M Ritalahti et al., 2006; Smits, Devenoges, Szynalski, Maillard, & Holliger, 2004; Van Der Zaan et al., 2010). Since *Dhc* isolates exhibiting different dechlorinating capabilities share 98-100% sequence identity at the 16S rRNA level, and other housing keeping genes are almost identical, phylogenetic assays give an incomplete picture of the dechlorinating potential of a culture or a contaminated site.

Gene targets linked to specific key metabolic reactions are promising biomarkers to better predict contaminant detoxification, thus aiding in the remediation decision-making process. RDases and hydrogenases are the central enzymes involved in *Dhc* metabolism. *Dhc* have multiple, non-identical RDase genes. In contrast, genes encoding for hydrogenase subunits can also exhibit high sequence identity (Table 2.11). Therefore, quantitative real-time PCR approaches have been designed to target RDase genes (Cupples, 2008; Padilla-Crespo et al., 2014; Kirsti M Ritalahti et al., 2006). These assays can be applied to DNA from samples of interest to give information of the presence of these genes but also to RNA, which correlates, in most of the cases, to active expression and dechlorinating activity. Consultants and regulatory agencies have recognized the value of molecular biology-based analysis for *in situ* bioremediation and natural attenuation (National Research Council, 2013; H. F. Stroo, Leeson, Shepard, Koenigsberg, & Casey, 2006; H. Stroo et al., 2012). Hence, these molecular analyses are commercially available (i.e., Microbial Insights Inc., SiREM Inc.) and are routinely used for site assessment and bioremediation monitoring (Major et al., 2002; H. Stroo et al., 2012). Tables 2.12-13 show phylogenetic and functional gene targets that have been described in the literature to detect organohalide-respiring bacteria.

The development and use of molecular biology tools to detect and quantify genes of interest are dependable of efforts focused on biomarker discovery. Early proteomics and reverse genetics efforts characterized the PCE RDase (PceA) in phylogenetically diverse bacteria, as well as the TCE RDase (TceA) in *Dhc* (as depicted in Table 2.10). Additional integrated approaches including transcriptional, enzymatic assays, expression and high throughput proteomic approaches have assigned function to additional *Dhc* RDases in (e.g. *vcrA, bvcA, dceA, cbrA*) without the need of a genetic system, heterologous expression or complete enzyme purification (Table 2.10).

The present study assigned function to the 1,2-D to propene RDase in *Dhc* strain RC and KS, and also in *Dhgm* strain BL-DC-9 (Padilla-Crespo et al., 2014). But still only a handful of *Dhc* RDases have assigned function while, hundreds of putative RDase genes homologues (from various taxa) linger in the databases awaiting for functional characterization. The current novel, and "about to be discovered" RDase biomarkers, combined with 16S rRNA gene-targeted

approaches, will help implement enhanced bioremediation or monitored natural attenuation at contaminated sites. Together, these efforts will assist in better allocation of resources, help in site restoration and closure, while unraveling the ecology and distribution of organohalide-respiring bacteria and the mobility of their genes in the environment.

References

"1,2-dichloropropane" SIDS Initial Assessment Report for SIAM 17. (2003). *UNEP PUBLICATIONS*. Switzerland.

Abrahamsson, K., Ekdahl, A., Collén, J., Fahlström, E., & Pedersén, M. (1995). The natural formation of trichloroethylene and perchloroethylene in seawater. (A. Grimwall & E. W. B. de Leer, Eds.) *Naturally-Produced Organohalogens*. The Netherlands: Kluwer Academic Publishers.

Adrian, L., Rahnenführer, J., Gobom, J., & Hölscher, T. (2007). Identification of a chlorobenzene reductive dehalogenase in *Dehalococcoides* sp. strain CBDB1. *Appl. Environ. Microbiol.*, *73*(23), 7717–7724.

Agency for Toxic Substances and Disease Registry (ATSDR). (1989). *Toxicological profile for 1,2-dichloropropane*. Atlanta, GA.: U.S. Department of Health and Human Services, Public Health Services.

Amos, B. K., Sung, Y., Fletcher, K. E., Gentry, T. J., Wu, W.-M. M., Criddle, Löffler, F. E. (2007). Detection and quantification of *Geobacter lovleyi* strain SZ: implications for bioremediation at tetrachloroethene- and uranium-impacted sites. *Applied and Environmental Microbiology*, *73*(21), 6898–6904. http://doi.org/10.1128/AEM.01218-07

ASTDR. (1993). Toxicological profile for tetrachloroethylene. Atlanta, GA: Agency for Toxic Substances and Disease Registry.

ATSDR. (1997). Toxicological profile for 1,1-dichloroethene. Atlanta, GA: Agency for Toxic Substances and Disease Registry, U. S. Public Health Service, U. S. Department of Health and Human Services.

ATSDR. (2010). Toxicological Profile for Chlorine, 269.

Bedard, D. L., Ritalahti, K. M., & Loffler, F. E. (2007). The *Dehalococcoides* population in sediment-free mixed cultures metabolically dechlorinates the commercial polychlorinated

biphenyl mixture Aroclor 1260. *Applied and Environmental Microbiology*, *73*(8), 2513–2521. http://doi.org/10.1128/aem.02909-06

Bommer, M. Kunze C, Fesseler J, Schubert T, Diekert G, D. H. (2014). Structural basis for organohalide respiration. *Science*, *346*(6208), 455–458.

Bosma, T., Damborsky, J., Stucki, G., & Janssen, D. B. (2002). Biodegradation of 1,2,3-trichloropropane through directed evolution and heterologous expression of a haloalkane dehalogenase gene. *Applied and Environmental Microbiology*, *68*, 3582–3587.

Bouwer, E. J., & McCarty, P. L. (1983). Transformation of 1- and 2-carbon halogenated aliphatic organic compounds under methanogenic conditions. *Applied and Environmental Microbiology*, *45*, 1286–1294.

Brehm-Stecher, B. F., & Johnson, E. A. (2004). Single-cell microbiology: Tools, technologies, and applications. *Microbiology and Molecular Biology Reviews*, *68*(3), 538–559. http://doi.org/10.1128/mmbr.68.3.538-559.2004

Bunge, M., Adrian, L., Kraus, A., Opel, M., Lorenz, W. G., Andreesen, J. R., … Lechner, U. (2003). Reductive dehalogenation of chlorinated dioxins by an anaerobic bacterium. *Nature*, *421*, 357–360.

Chen, J., Bowman, K. S., Rainey, F. A., & Moe, W. M. (2014). Reassessment of PCR primers targeting 16S rRNA genes of the organohalide-respiring genus *Dehalogenimonas*, 747–756. http://doi.org/10.1007/s10532-014-9696-z

Chow, W. L., Cheng, D., Wang, S., & He, J. (2010). Identification and transcriptional analysis of trans-DCE-producing reductive dehalogenases in *Dehalococcoides* species. *ISME J*, *4*(8), 1020–30. http://doi.org/10.1038/ismej.2010.27

Christiansen, N., & Ahring, B. K. (1996). *Desulfitobacterium hafniense* sp. nov., an anaerobic reductively dechlorinating bacterium. *International Journal of Systematic Bacteriology*, *46*, 442–448.

Cole, J. R., Fathepure, B. Z., & Tiedje, J. M. (1995). Tetrachloroethene and 3-chlorobenzoate dechlorination activities are co-induced in *Desulfomonile tiedje* DCB-1. *Biodegradation*, *6*, 167–172.

Coleman, N. V, Mattes, T. E., Gossett, J. M., & Spain, J. C. (2002a). Biodegradation of cis-dichloroethene as the sole carbon source by a b-Proteobacterium. *Applied and Environmental Microbiology*, *68*, 2726–2730.

Coleman, N. V, Mattes, T. E., Gossett, J. M., & Spain, J. C. (2002b). Phylogenetic and kinetic diversity of aerobic vinyl chloride-assimilating bacteria from contaminated sites. *Applied and Environmental Microbiology*, *68*, 6162–6171.

Cummings, D. E., Snoeyenbos-West, O. L., Newby, D. T., Niggemyer, A. M., Lovley, D. R., Achenbach, L. A., & Rosenzweig, R. F. (2003). Diversity of *Geobacteraceae* species inhabiting metal-polluted freshwater lake sediments ascertained by 16S rDNA analyses. *Microbial Ecology*, *46*, 257–269.

Cupples, A. M. (2008). Real-time PCR quantification of *Dehalococcoides* populations: methods and applications. *Journal of Microbiological Methods*, *72*(1), 1–11.

Cupples, A. M., Spormann, A. M., & McCarty, P. L. (2003). Growth of a *Dehalococcoides*-like microorganism on vinyl chloride and cis-dichloroethene as electron acceptors as determined by competitive PCR. *Applied and Environmental Microbiology*, *69*, 953–959.

Daprato, R. C., Löffler, F. E., & Hughes, J. B. (2007). Comparative analysis of three tetrachloroethene to ethene halorespiring consortia suggests functional redundancy. *Environmental Science and Technology*, *41*(7), 2261–2269.

De Jong, E., Field, J. A., Spinnler, H.-E., Wijnberg, J. B. P. A., & De Bont, J. A. M. (1994). Significant biogenesis of chlorinated aromatics by fungi in natural environments. *Applied and Environmental Microbiology*, *60*, 264–270.

De Wildeman, S., Diekert, G., Van Langenhove, H., Verstraete, W., & De Wildeman S, Diekert G, Van Langenhove H, Verstraete, W. (2003). Stereoselective microbial dehalorespiration with vicinal dichlorinated alkanes. *Appl. Environ. Microbiol.*, *69*(9), 5643–5647. http://doi.org/10.1128/AEM.69.9.5643

Dennis, P. C., Sleep, B. E., Fulthorpe, R. R., & Liss, S. N. (2003). Phylogenetic analysis of bacterial populations in an anaerobic microbial consortium capable of degrading saturation concentrations of tetrachloroethylene. *Canadian Journal of Microbiology*, *49*, 15–27.

Deutsch, Z. (1947). Alkali and chlorine industries. In E. Kirk RE, Othmer DF (Ed.), *Encyclopedia of chemical technology*.

Dimitriades, B., Gay Jr, B. W., Arnts, R. R., & Seila, R. L. (1983). Photochemical reactivity of perchloroethylene: a new appraisal. ,. *Journal of the Air Pollution Control Association*, *33*(6), 575–58.

Dolfing, J. (1990). Reductive dechlorination of 3-chlorobenzoate degrading methanogenic coculture. *Arch Microbiol.*, *149*, 102–105.

Dowideit, K, Scolz-Muramatsu H, Miethling-Graff, R, Vigelahn L, Freygang M, Dohrmann, AB, et al. (n.d.). Spatial heterogeneity of dechlorinating bacteria and limiting factors for in situ trichloroethene dechlorination revealed by analyses of sediment cores from a polluted site. *FEMS Microbiol. Ecol*, *58*, 538–549.

Duhamel, M., & Edwards, E. A. (2006). Microbial composition of chlorinated ethene-degrading cultures dominated by *Dehalococcoides*. *FEMS Microbiology Ecology*, *58*(3), 538–549. http://doi.org/doi:10.1111/j.1574-6941.2006.00191.x

Duhamel, M., & Edwards, E. A. (2007). Growth and yields of dechlorinators, acetogens, and methanogens during reductive dechlorination of chlorinated ethenes and dihaloelimination of 1 ,2-dichloroethane. *Environmental Science and Technology*, *41*(7), 2303–2310.

Duhamel, M., Mo, K., & Edwards, E. A. (2004). Characterization of a highly enriched *Dehalococcoides*-containing culture that grows on vinyl chloride and trichloroethene. *Applied and Environmental Microbiology*, *70*, 5538–5545.

Egli, C., Scholtz, R., Cook, A. M., Tschan, T., & Leisinger, T. (1988). Transformation of tetrachloromethane to dichloromethane and carbon dioxide by Acetobacterium woodii. *Applied and Environmental Microbiology*, *54*, 2819–2824.

El Fantroussi, S., Mahillon, J., Naveau, H., & Agathos, S. N. (1997). Introduction of anaerobic dechlorinating bacteria into soil slurry microcosms and nested-PCR monitoring. *Applied and Environmental Microbiology*, *63*, 806–811.

Fagervold, S. K., Watts, J. E. M., May, H. D., Sowers, K. R., & Carolina, S. (2005). Sequential reductive dechlorination of meta-chlorinated polychlorinated biphenyl congeners in sediment microcosms by two different chloroflexi phylotypes. *Applied and Environmental Microbiology*, *71*(12), 8085–8090. http://doi.org/10.1128/aem.72.2.1717.2006

Fathepure, B. Z., Negu, J. P., & Boyd, S. A. (1987). Anaerobic bacteria that dechlorinate perchloroethene. *Applied and Environmental Microbiology*, *53*, 2671–2674.

Fennell, D. E., Carroll, A. B., Gossett, J. M., & Zinder, S. H. (2001). Assessment of indigenous reductive dechlorinating potential at a TCE-contaminated site using microcosms, polymerase chain reaction analysis, and site data. *Environmental Science and Technology*, *35*, 1830–1839.

Fennell, D. E., Nijenhuis, I., Wilson, S. F., Zinder, S. H., & Häggblom, M. M. (2004). *Dehalococcoides ethenogenes* strain 195 reductively dechlorinates diverse chlorinated aromatic pollutants. *Environmental Science and Technology*, *38*, 2075–2081.

Fung, J. M., Morris, R. M., Adrian, L., & Zinder, S. H. (2007). Expression of reductive dehalogenase genes in *Dehalococcoides ethenogenes* strain 195 growing on tetrachloroethene, trichloroethene, or 2,3-dichlorophenol. *Applied and Environmental Microbiology*, *73*(14), 4439–45. http://doi.org/10.1128/AEM.00215-07

Glauser J, I. Y. (2008). Marketing research report on C2 chlorinating solvents. In *Chemical Economics Handbook* (pp. 12–17). Menlo Park, CA: SRI Consulting.

Graedel, T . E., K. W. (1995). Tropospheric budget of reactive chlorine. *Global Biogeochem. Cycles*, *9*, 47–48.

Greene, S. A. (2012). International Resources Guide to Hazardous Chemicals. In W. Andrew. (Ed.), .

Gribble, G. W. (1998). Chlorinated compounds in the biosphere, natural production. In R. A. Meyers (Ed.), *Encyclopedia of environmental analysis and remediation* (pp. 972–1035). John Wiley & Sons, Inc.

Gribble, G. W. (2000). The natural production of organobromine compounds. *Environmental Science and Pollution Research International*, *7*(1), 37–47.

Gribble, G. W. (2003). The diversity of naturally produced organohalogens. *Chemosphere*, *52*(2), 289–297.

Gribble, G. W. (2004). Natural organohalogens - occurrence, sources, quantities, natural function, and benefits. *European Chlorinated Science Dossier*, 1–77.

Gribble, G. W. (2012). Occurrence of Halogenated Alkaloids. *Alkaloids: Chemistry and Biology*, *71*, 1–165.

Grostern, A., & Edwards, E. A. (2006). Growth of *Dehalobacter* and *Dehalococcoides* spp. during degradation of chlorinated ethanes. *Applied and Environmental Microbiology*, *72*, 428–436.

Grostern, A, Edwards, E. A. (2009). Characterization of a *Dehalobacter* coculture that dechlorinates 1,2-dichloroethane to ethene and identification of the putative reductive dehalogenase gene. *Appl. Environ. Microbiol.*, *75*, 2684–2693.

Gschwend, P. M., MacFarlane, J. K., & Newman, K. A. (1985). Volatile halogenated organic compounds released to seawater from temperate marine macroalgae. *Science*, *227*, 1033.

H Sjuts, Fisher K, Dunstan MS, Rigby SE, L. D. (2012). Heterologous expression, purification and cofactor reconstitution of the reductive dehalogenase PceA from Dehalobacter restrictus. *Protein Expression and Purification*, *85*, 224–229.

He, J., Ritalahti, K. M., Aiello, M. R., & Löffler, F. E. (2003). Complete detoxification of vinyl chloride (VC) by an anaerobic enrichment culture and identification of the reductively dechlorinating population as a *Dehalococcoides* species. *Appl. Environ. Microbiol.*, *69*, 996–1003.

He, J., Ritalahti, K. M., Yang, K.-L., Koenigsberg, S. S., & Löffler, F. E. (2003). Detoxification of vinyl chloride to ethene coupled to growth of an anaerobic bacterium. *Nature*, *424*, 62–65.

He, J., Sung, Y., Krajmalnik-Brown, R., Ritalahti, K. M., & Löffler, F. E. (2005). Isolation and characterization of *Dehalococcoides* sp. strain FL2, a trichloroethene (TCE)- and 1,2-dichloroethene-respiring anaerobe. *Environmental Microbiology*, *7*(9), 1442–1450. http://doi.org/10.1111/j.1462-2920.2005.00830.x

Hendrickson, E. R., Payne, J. A., Young, R. M., Starr, M. G., Perry, M. P., Fahnestock, S., … Ebersole, R. C. (2002). Molecular analysis of *Dehalococcoides* 16S ribosomal DNA from chloroethene-contaminated sites throughout North America and Europe. *Applied and Environmental Microbiology*, *68*, 485–495.

Holliger, C., Hahn, D., Harmsen, H., Ludwig, W., Schumacher, W., Tindall, B., … Zehnder, A. J. B. (1998). *Dehalobacter restrictus* gen. nov. and sp. nov., a strictly anaerobic bacterium that reductively dechlorinates tetra- and trichloroethene in an anaerobic respiration. *Archives of Microbiology*, *169*, 313–321.

Holliger, C., Wohlfarth, G., & Diekert, G. (1999). Reductive dechlorination in the energy metabolism of anaerobic bacteria. *FEMS Microbiology Reviews*, *22*, 383–398.

Holmes, V. F., He, J., Lee, P. K. H., & Alvarez-Cohen, L. (2006). Discrimination of multiple *Dehalococcoides* strains in a trichloroethene enrichment by quantification of their reductive dehalogenase genes. *Applied and Environmental Microbiology*, *72*(9), 5877–5883. http://doi.org/10.1128/aem.00516-06

Hölscher, T., Krajmalnik-Brown, R., Ritalahti, K. M., Von Wintzingerode, F., Görisch, H., Löffler, F. E., & Adrian, L. (2004). Multiple non-identical putative reductive dehalogenase genes are common in "Dehalococcoides." *Applied and Environmental Microbiology*, *70*, 5290–5297.

Hug, L. a, Maphosa, F., Leys, D., Löffler, F. E., Smidt, H., Edwards, E. a, & Adrian, L. (2013). Overview of organohalide-respiring bacteria and a proposal for a classification system for reductive dehalogenases. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *368*, 20120322. http://doi.org/10.1098/rstb.2012.0322

Ise, K., Suto, K., & Inoue, C. (2011). Microbial diversity and changes in the distribution of dehalogenase genes during dechlorination with different concentrations of cis-DCE. *Environmental Science & Technology*, *45*(12), 5339–45. http://doi.org/10.1021/es104199y

Johnson, D. R., Lee, P. K. H., Holmes, V. F., Fortin, A. C., & Alvarez-Cohen, L. (2005). Transcriptional expression of the tceA gene in a *Dehalococcoides*-containing microbial enrichment. *Applied and Environmental Microbiology*, *71*(11), 7145–7151.

Keppler, F., Borchers, R., Pracht, J., Rheinberger, S., & Scholer, H. F. (2002). Natural formation of vinyl chloride in the terrestrial environment. *Environmental Science and Technology*, *36*(11), 2479–2483.

Krajmalnik-Brown, R., Hölscher, T., Thomson, I. N., Saunders, F. M., Ritalahti, K. M., Löffler, F. E., (2004). Genetic identification of a putative vinyl chloride reductase in *Dehalococcoides* sp. strain BAV1. *Appl. Environ. Microbiol.*, *70*(10), 6347–6351. http://doi.org/10.1128/AEM.70.10.6347

Lee PK, Cheng D, West KA, Alvarez-Cphen L, H. J. (2013). Isolation of two new Dehalococcoides mccartyi strains with dissimilar dechlorination functions and their comparative genomics via microarray analysis. *Enviromental Microbiology*, *15*(8), 2293–2305.

Lendvay, J. M., Löffler, F. E., Dollhopf, M., Aiello, M. R., Daniels, G., Fathepure, B. Z., … Adriaens, P. (2003). Bioreactive barriers: bioaugmentation and biostimulation for chlorinated solvent remediation. *Environmental Science and Technology*, *37*, 1422–1431.

Lester, D., Greenberg, L. A., & Adams, W. R. (1963). Effects of single and repeated exposures of humans and rats to vinyl chloride. *American Industrial Hygiene Association Journal*, *24*(3), 265–275.

Löffler, F. E., Champine, J. E., Ritalahti, K. M., Sprague, S. J., & Tiedje, J. M. (1997). Complete reductive dechlorination of 1,2-dichloropropane by anaerobic bacteria. *Applied and Environmental Microbiology*, *63*, 2870–2875.

Löffler, F. E., Champine, J. E., Ritalahti, K. M., Sprague, S. J., Tiedje, J. M., & Loffler, F. E. (1997). Complete reductive dechlorination of 1,2-dichloropropane by anaerobic bacteria. *Applied and Environmental Microbiology*, *63*(7), 2870–5.

Löffler, F. E., Sanford, R. A., & Ritalahti, K. M. (2005). Enrichment, cultivation and detection of reductively dechlorinating bacteria. *Methods in Enzymology*, *397*, 77–111.

Löffler, F. E., Sanford, R. A., & Tiedje, J. M. (1996). Initial characterization of a reductive dehalogenase from *Desulfitobacterium chlororespirans* Co23. *Appl. Environ. Microbiol.*, *62*, 3809–3813.

Löffler, F. E., Sun, Q., Li, J., & Tiedje, J. M. (2000). 16S rRNA gene-based detection of tetrachloroethene-dechlorinating *Desulfuromonas* and *Dehalococcoides* species. *Applied and Environmental Microbiology*, *66*(4), 1369–1374.

Löffler FE, Yan J, Ritalahti KM, Adrian L, Edwards EA, Konstantinidis KT, Müller JA, Fullerton H, Zinder SH, S. A. (2013). *Dehalococcoides mccartyi* gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation, belong to a novel bacterial class, *Dehalococcoidia c*lassis nov., order *Dehalococcoidales o*rd. nov. and famil. *Int. J. Syst. Evol. Microbiol.*, *63*, 625–635.

Loy A, Kuse K, Drake HL, W. M. (2004). Microarray and functional gene analyses of sulfate-reducing prokaryotes in low-sulfate, acidic fens reveal coocurrence of recognized genera and novel lineages. *Appl Environ Microbiol*, *70*, 6998–7009.

Magnuson, J. K., Romine, M. F., Burris, D. R., & Kingsley, M. T. (2000). Trichloroethene reductive dehalogenase from *Dehalococcoides ethenogene*s: sequence of *tceA* and substrate range characterization. *Applied and Environmental Microbiology*, *66*, 5141–5147.

Magnuson, J. K., Stern, R. V, Gossett, J. M., Zinder, S. H., & Burris, D. R. (1998). Reductive dechlorination of tetrachloroethene to ethene by a two-component enzyme pathway. *Applied and Environmental Microbiology*, *64*(4), 1270–1275.

Major, D. W., McMaster, M. L., Cox, E. E., Edwards, E. A., Dworatzek, S. M., Hendrickson, E. R., Buonamici, L. W. (2002). Field demonstration of successful bioaugmentation to achieve dechlorination of tetrachloroethene to ethene. *Environmental Science and Technology*, *36*(23), 5106–5116.

Marco-Urrea, E., Pérez-Trujillo, M., Vicent, T., & Caminal, G. (2009). Ability of white-rot fungi to remove selected pharmaceuticals and identification of degradation products of ibuprofen by Trametes versicolor. *Chemosphere*, *74*(6), 765–772.

Marzorati, M., De Ferra, F., Van Raemdonck, H., Borin, S., Allifranchini, E., Carpani, G., … Daffonchio, D. (2007). A novel reductive dehalogenase, identified in a contaminated groundwater enrichment culture and in *Desulfitobacterium dichloroeliminans* strain DCA1, is linked to dehalogenation of 1,2-dichloroethane. *Applied and Environmental Microbiology*, *73*(9), 2990–2999. http://doi.org/10.1128/aem.02748-06

Mastromatteo, E., Fisher, A. M., Christie, H., & Danziger, H. (1960). Acute inhalation toxicity of vinyl chloride to laboratory animals. *American Industrial Hygiene Association Journal*, *21 394-398*(5), (5),.

Maymó-Gatell, X., Chien, Y.-T., Gossett, J. M., & Zinder, S. H. (1997). Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. *Science*, *276*, 1568–1571.

McMurdie, P. J., Behrens, S. F., Müller, J. a, Göke, J., Ritalahti, K. M., Wagner, R., … Spormann, A. M. (2009). Localized plasticity in the streamlined genomes of vinyl chloride respiring *Dehalococcoides*. *PLoS Genetics*, *5*(11), e1000714. http://doi.org/10.1371/journal.pgen.1000714

Miller, E., Wohlfarth, G., & Diekert, G. (1998). Purification and characterization of the tetrachloroethene reductive dehalogenase of strain PCE-S. *Archives of Microbiology*, *169*, 497–502.

Miyauchi, K., Suh, S.K., Nagata, Y., and Takagi, M. (1998). Cloning and sequencing of a 2,5-dichlorohydroquinone reductive dehalogenase gene whose product is involved in degradation of γ-hexachlorocyclohexane by *Sphingomonas paucimobilis*. *J Bacteriol*, *180*, 1354–1359.

Moe WM, Yan J, Nobre MF, da Costa MS, Rainey, F. (2009). *Dehalogenimonas lykanthroporepellens* gen. nov., sp. nov., a reductively dehalogenating bacterium isolated from chlorinated solvent-contaminated groundwater. *Int. J. Syst. Evol. Microbiol.*, *59*(11), 2692–2697. http://doi.org/10.1099/ijs.0.011502-0

Mohn, W. W., & Tiedje, J. M. (1992). Microbial reductive dehalogenation. *Microbiology Reviews*, *56*, 482–507.

Müller JA, Rosner BM, Von Abendroth G, Meshulam-Simon G, McCarty PL, S. A., Müller, J. A., Rosner, B. M., Von Abendroth, G., Meshluham-Simon, G., McCarty, P., & Spormann, A. M. (2004). Molecular identification of the catabolic vinyl chloride reductase from

Dehalococcoides sp. strain VS and its environmental distribution. *Applied and Environmental Microbiology*, *70*(8), 4880–4888. http://doi.org/10.1128/AEM.70.8.4880

National Research Council. (2013). *Alternatives for Managing the Nation's Complex Contaminated Groundwater Sites*. *he National Academies Press, 2013.* Washington, DC.

Nelly, A. Mac, Kai, M., Svatoš, A., & Diekert, G. (2014). Functional Heterologous Production of Reductive Dehalogenases from *Desulfitobacterium hafniense* Strains. *Appl Environ Microbiol*, *80*(14), 4313–4322. http://doi.org/10.1128/AEM.00881-14

Neumann, A., Scholz-Muramatsu, H., & Diekert, G. (1994). Tetrachloroethene metabolism of *Dehalospirillum multivorans*. *Archives of Microbiology*, *162*, 295–301.

Neumann, A., Wohlfarth, G., & Diekert, G. (1996). Purification and characterization of tetrachloroethene reductive dehalogenase from Dehalospirillum multivorans. *Journal of Biological Chemistry*, *271*, 16515–16519.

Neumann, A., Wohlfarth, G., & Diekert, G. (1998). Tetrachloroethene dehalogenase from *Dehalospirillum multivorans:* cloning, sequencing of the encoding genes, and expression of the *pceA* gene in *Escherichia coli*. *Journal of Bacteriology*, *180*, 4140–4145.

Ni, S., Fredrickson, J. K., & Xun, L. (1995). Purification and characterization of a novel 3-chlorobenzoate-reductive dehalogenase from the cytoplasmic membrane of *Desulfomonile tiedjei* DCB-1. *Journal of Bacteriology*, *177*, 5135–5139.

Öberg, G. (2002). The natural chlorine cycle-fitting the scattered pieces. *Applied Microbiology and Biotechnology*, *58*, 565–581.

Oldenhuis, R., Vink, R. L. J. M., Janssen, D. B., & Witholt, B. (1989). Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. *Applied and Environmental Microbiology*, *55*, 2819–2826.

Padilla-Crespo, E., Yan, J., Swift, C., Wagner, D. D., Chourey, K., Hettich, R. L., … Löffler, F. E. (2014). Identification and environmental distribution of *dcpA*, which encodes the

reductive dehalogenase catalyzing the dichloroelimination of 1,2-dichloropropane to propene in organohalide-respiring Chloroflexi. *Applied and Environmental Microbiology*, *80*(3), 808–818.

Pandey, J., Chauhan, A., & Jain, R. K. (2009). Integrative approaches for assessing the ecological sustainability of in situ bioremediation. *FEMS Microbiology Reviews*, *33*(2), 324–375. http://doi.org/10.1111/j.1574-6976.2008.00133.x

Parthasarathy A, Stich TA, Lohner ST, Lesnefsky A, Britt RD, S. A. (2015). Biochemical and EPS-spectroscopic investigation into heterologously expressed vinyl chloride reductive dehalogenase (VcrA) from *Dehalococcoides mccartyi* strain VS. *J Am Chem Soc.*, *137*(18), 3525–3532.

Payne PK, Quezada PC, Fisher K, Dunstan MS, Collins FA, Sjuts H, Levy C, H. S., & Rigby SEJ, L. D. (2015). Reductive dehalogenase structure suggests a mechanism for B12-dependent dehalogenation. *Nature*, *517*(7535), 516–516.

Petrie, L., North, N. N., Dollhopf, S. L., Balkwill, D. L., & Kostka, J. E. (2003). Enumeration and characterization of iron(III)-reducing microbial communities from acidic subsurface sediments contaminated with uranium(VI). *Applied and Environmental Microbiology*, *69*, 7467–7479.

Phelps, T. J., Malachowsky, K., Schram, R. M., & White, D. C. (1991). Aerobic mineralization of vinyl-chloride by a bacterium of the order *Actinomycetales*. *Applied and Environmental Microbiology*, *57*(4), 1252–1254.

Pöritz, M., Goris, T., Wubet, T., Tarkka, M. T., Buscot, F., Nijenhuis, I., … Adrian, L. (2013). Genome sequences of two dehalogenation specialists - *Dehalococcoides mccartyi* strains BTF08 and DCMB5 enriched from the highly polluted Bitterfeld region. *FEMS Microbiology Letters*, *343*(2), 101–104.

Pozzi C, Marai P, P. R. (1985). Toxicity in man due to stain removers containing 1,2-dichloropropane. *British Journal of Industrial Medicine.*, *42*(11), 770–772.

Rahm, B. G., Morris, R. M., & Richardson, R. E. (2006). Temporal expression of respiratory genes in an enrichment culture containing *Dehalococcoides ethenogenes. Appl. Environ. Microbiol.*, *72*(8), 5486–5491. http://doi.org/10.1128/aem.00855-06

Rasche, M. E., M. R. Hyman, D. J. A. (1990). Biodegradation of halogenated hydrocarbon fumigants by nitrifying bacteria. *Appl. Environ. Microbiol.*, *56*, 2568–2571.

Reinhold A, Westermann M, Seifert J, von Bergen M, S. T., & G., D. (2012). Impact of vitamin B12 on formation of the tetrachloroethene reductive dehalogenase in *Desulfitobacterium hafniense* strain Y51. *Appl. Environ. Microbiol.*, *78*, 8025–8032. http://doi.org/http://dx.doi.org/10.1128 /AEM.02173-12.

Richardson, R. (2013). Genomic insights into organohalide respiration. *Current Opinion in Biotechnology*, *24*(1), 1–8.

Ritalahti, K. M., Amos, B. K., Sung, Y., Wu, Q., Koenigsberg, S. S., Löffler, F. E., & Lo, F. E. (2006). Quantitative PCR targeting 16S rRNA and reductive dehalogenase genes simultaneously monitors multiple *Dehalococcoides* strains. *Applied and Environmental Microbiology*, *72*(4), 2765–2774. http://doi.org/10.1128/AEM.72.4.2765

Ritalahti, K. M., & Löffler, F. F. (2004). Populations implicated in the anaerobic reductive dechlorination of 1,2-dichloropropane in highly enriched bacterial communities. *Appl. Environ. Microbiol.*, *70*(7), 4088–4095. http://doi.org/10.1128/AEM.70.7.4088

Ruder, A. M. (2006). Potential health effects of occupational chlorinated solvent exposure. In *Annals of the New York Academy of Sciences* (Vol. 1076, pp. 207–227).

Ryoo, D., Shim, H., Barbieri, P., & Wood, T. K. (2000). Aerobic degradation of tetrachloroethylene by toluene-o-xylene monooxygenase of *Pseudomonas stutzeri* OX1. *Nature Biotechnology*, *18*, 775–778.

Sanford, R. A., Q. Wu, Sung, Y., Thomas, S. H., Amos, B. K., & Prince, E. K. (2007). Hexavalent uranium supports growth of *Anaeromyxobacter dehalogenans* and *Geobacter*

spp. with lower than predicted biomass yields. *Environmental Microbiology*, *9*(11), 2885–2893.

Schaefer CE, Condee CW, Vainberg S, S. R. (2009). Bioaugmentation for chlorinated ethenes using *Dehalococcoides* sp.: Comparison between batch and column experiments. *Chemosphere*, *75*, 141–148.

Schlötelburg, C., Von Wintzingerode, C., Hauck, R., Von Wintzingerode, F., Hegemann, W., Göbel, U. B., & Schlötelburg C, Wintzingerode C, Hauck R, Wintzingerode F, Hegemann W, G. U. (2002). Microbial structure of an anaerobic bioreactor population that continuously dechlorinates 1,2-D. *FEMS Microbiology Ecology*, *39*(3), 229–237. http://doi.org/10.1111/j.1574-6941.2002.tb00925.x

Shim, H., & Wood, T. K. (2000). Aerobic degradation of mixtures of chlorinated aliphatics by cloned toluene-o-xylene monooxygenase and toluene o-monooxygenase in resting cells. *Biotechnology and Bioengineering*, *70*, 693–698.

Smidt, H., & De Vos, W. M. (2004). Anaerobic microbial dehalogenation. *Annual Review of Microbiology*, *58*, 43–73.

Smits, T. H. M., Devenoges, C., Szynalski, K., Maillard, J., & Holliger, C. (2004). Development of a real-time PCR method for quantification of the three genera *Dehalobacter*, *Dehalococcoides*, and *Desulfitobacterium* in microbial communities. *Journal of Microbiological Methods*, *57*, 369–378.

Stroo, H. F., Leeson, A., Marqusee, J. a, Johnson, P. C., Ward, C. H., Kavanaugh, M. C., … Unger, M. (2012). Chlorinated ethene source remediation: lessons learned. *Environmental Science & Technology*, *46*(12), 6438–47. http://doi.org/10.1021/es204714w

Stroo, H. F., Leeson, A., Shepard, A. J., Koenigsberg, S. S., & Casey, C. C. (2006). Monitored Natural Attenuation Forum: Environmental remediation applications of molecular biological tools. *Remediation Journal*, *16*(2), 125–137.

Stroo, H., Leeson, A., & Ward, C. (2012). *Bioaugmentation for Groundwater Remediation*.

Stroo, Hans F., Marvin Unger, C. Herb Ward, Michael C. Kavanaugh, Catherine Vogel, Andrea Leeson, Jeffrey A. Marqusee, and B. P. S. (2003). Remediating chlorinated solvent source zones. *Environmental Science & Technology, 37*(11), 224–230.

Sung. (2005). *Youlbong thesis*. *Environmental Engineering*.

Suyama, A., Yamashita, M., Yoshino, S., & Furukawa, K. (2002). Molecular characterization of the PceA reductive dehalogenase of *Desulfitobacterium* sp. Strain Y51. *Journal of Bacteriology*, *184*(13), 3419–3425.

Tang S, Chan WW, Fletcher KE, Seifert J, Liang X, Löffler FE, Edwards EA, A. L. (2013). Functional characterization of reductive dehalogenases by using blue native polyacrylamide gel electrophoresis. *Appl. Environ. Microbiol.*, *79*(3), 974–81. http://doi.org/10.1128/AEM.01873-12

Tang, S., & Edwards, E. A. (2013). Identification of Dehalobacter reductive dehalogenases that catalyse dechlorination of chloroform, 1,1,1-trichloroethane and 1,1-dichloroethane. *Trans. R. Soc. Lond. B. Biol. Sci*, *368*(1616), 20120318. http://doi.org/10.1098/rstb.2012.0318

Terzenbach, D. P., & Blaut, M. (1994). Transformation of tetrachloroethylene to trichlororethylene by homoacetogenic bacteria. *FEMS Microbiology Letters*, *123*, 213–218.

Thomas, S. H., Wagner, R. D., Arakaki, A. K., Skolnick, J., Kirby, J. R., Shimkets, L. J., … Löffler, F. E. (2008). The mosaic genome of *Anaeromyxobacter dehalogenans* strain 2CP-C suggests an aerobic common ancestor to the delta-proteobacteria. *PLoSONE. In Press*.

Thomas, S. H., Padilla-Crespo E, Jardine PM, Sanford RA, L. F. (2009). Diversity and distribution of *Anaeromyxobacter* strains in a uranium- contaminated subsurface environment with a nonuniform groundwater flow. *Appl. Environ. Microbiol.*, *75*, 3679–3687.

Van De Pas, B. A., Gerritse, J., De Vos, W. M., Schraa, G., & Stams, A. J. M. (2001). Two distict enzyme systems are responsible for tetrachloroethene and chlorophenol reductive dehalogenation in *Desulfitobacterium* strain PCE1. *Archives of Microbiology*, *176*, 165–169.

Van Der Zaan, B., Hannes, F., Hoekstra, N., Rijnaarts, H., De Vos, W. M., Smidt, H., & Gerritse, J. (2010). Correlation of *Dehalococcoides* 16S rRNA and chloroethene-reductive dehalogenase genes with geochemical conditions in chloroethene-contaminated groundwater. *Applied and Environmental Microbiology*, *76*(3), 843–850.

Van Deuren J, Lloyd T, Chhetry S, Liou R, P. J. (2002). Remediation Technologies Screening Matrix and Reference Guide, Version 4.0.

Van Hylckama Vlieg, J. E. T., G. J. Poelarends, A. E. Mars, and D. B. J. (2000). Detoxification of reactive intermediates during microbial metabolism of halogenated compounds. *Curr. Opin. Microbiol.*, *3*, 257–262.

Vannelli, T., Logan, M., Arciero, D. M., & Hooper, A. B. (1990). Degradation of Halogenated Aliphatic-Compounds by the Ammonia-Oxidizing Bacterium *Nitrosomonas europaea.* *Applied and Environmental Microbiology*, *56*(4), 1169–1171.

Verce, M. F., & Freedman, D. L. (2000). Modeling the kinetics of vinyl chloride cometabolism by an ethane-grown Pseudomonas sp. *Biotechnology and Bioengenering*, *71*(4), 274–285.

Wagner, A., Adrian, L., Kleinsteuber, S., Andreesen, J. R., & Lechner, U. (2009). Transcription analysis of genes encoding homologues of reductive dehalogenases in “Dehalococcoides” sp. strain CBDB1 by using terminal restriction fragment length polymorphism and quantitative PCR. *Applied and Environmental Microbiology*, *75*(7), 1876–84. http://doi.org/10.1128/AEM.01042-08

Wagner, D., Hug, L., Hatt, J., Spitzmiller, M., Padilla-Crespo, E., Ritalahti, K., … Loffler, F. (2012). Genomic determinants of organohalide-respiration in *Geobacter lovleyi*, an unusual member of the Geobacteraceae. *BMC Genomics*, *13*(1), 200.

Wang, S., Chng, K. R., Wilm, A., Zhao, S., Yang, K.-L., Nagarajan, N., & He, J. (2014). Genomic characterization of three unique *Dehalococcoides* that respire on persistent polychlorinated biphenyls. *Proceedings of the National Academy of Sciences of the United States of America*, (111), 12103–12108.

Ward, SH, CherryJA, Scalf, M. (Ed.). (n.d.). Aerobic cometabolism of chlorinated aliphatics hydrocarbons . In *Subsurface Restoration* (pp. 3373–3395). Chelsea, MI: Ann Arbor Press.

Watts, J. E. M., Fagervold, S. K., May, H. D., & Sowers, K. R. (2005). A PCR-based specific assay reveals a population of bacteria within the Chloroflexi associated with the reductive dehalogenation of polychlorinated biphenyls. *Microbiology (Reading, England)*, *151*(Pt 6), 2039–46. http://doi.org/10.1099/mic.0.27819-0

Wilson, J. T., & Wilson, B. H. (1985). Biotransformation of trichloroethylene in soil. *Applied and Environmental Microbiology*, *49*, 242–243.

Wu, Q., Thomas, S. H., Wagner, R., Rodriguez, G., Massol, A., Krishnani, K., Loeffler, F. E. (2007). Nitrous oxide respiration by facultative myxobacteria. *In Preparation.*

Yan, J., Rash, B. a, Rainey, F. a, & Moe, W. M. (2009). Detection and quantification of Dehalogenimonas and "Dehalococcoides" populations via PCR-based protocols targeting 16S rRNA genes. *Applied and Environmental Microbiology*, *75*(23), 7560–4. http://doi.org/10.1128/AEM.01938-09

Ye LD, Schilhabel A, Bartram S, Boland W, D. (2010). Reductive dehalogenation of brominated ethenes by Sulfurospirillum multivorans and *Desulfitobacterium hafniense* PCE-S. *Enviromental Microbiology*, *12*, 501–509.

Yoshida, N., Takahashi, N., & Hiraishi, A. (2005). Phylogenetic characterization of a polychlorinated-dioxin- dechlorinating microbial community by use of microcosm studies. *Applied and Environmental Microbiology*, *71*(8), 4325–4334.

Chapter 2 Appendix: Figures

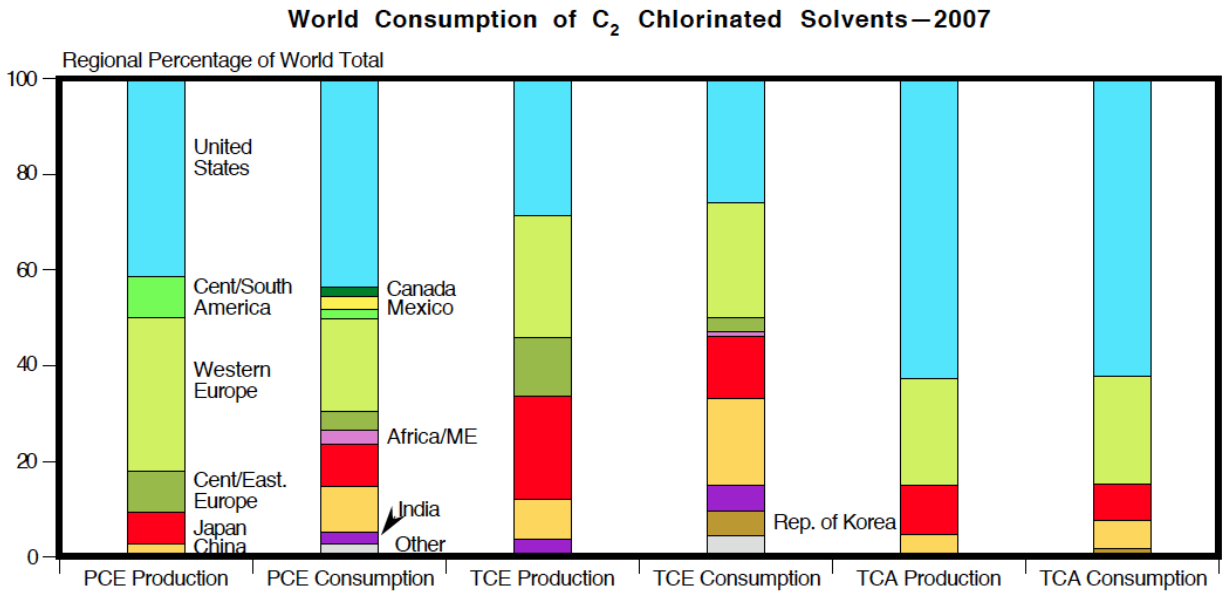


Figure 2.1. Worldwide production and consumption percentages of the three major chlorinate solvents (PCE, TCE and TCA). Figure from (Glauser J, 2008).

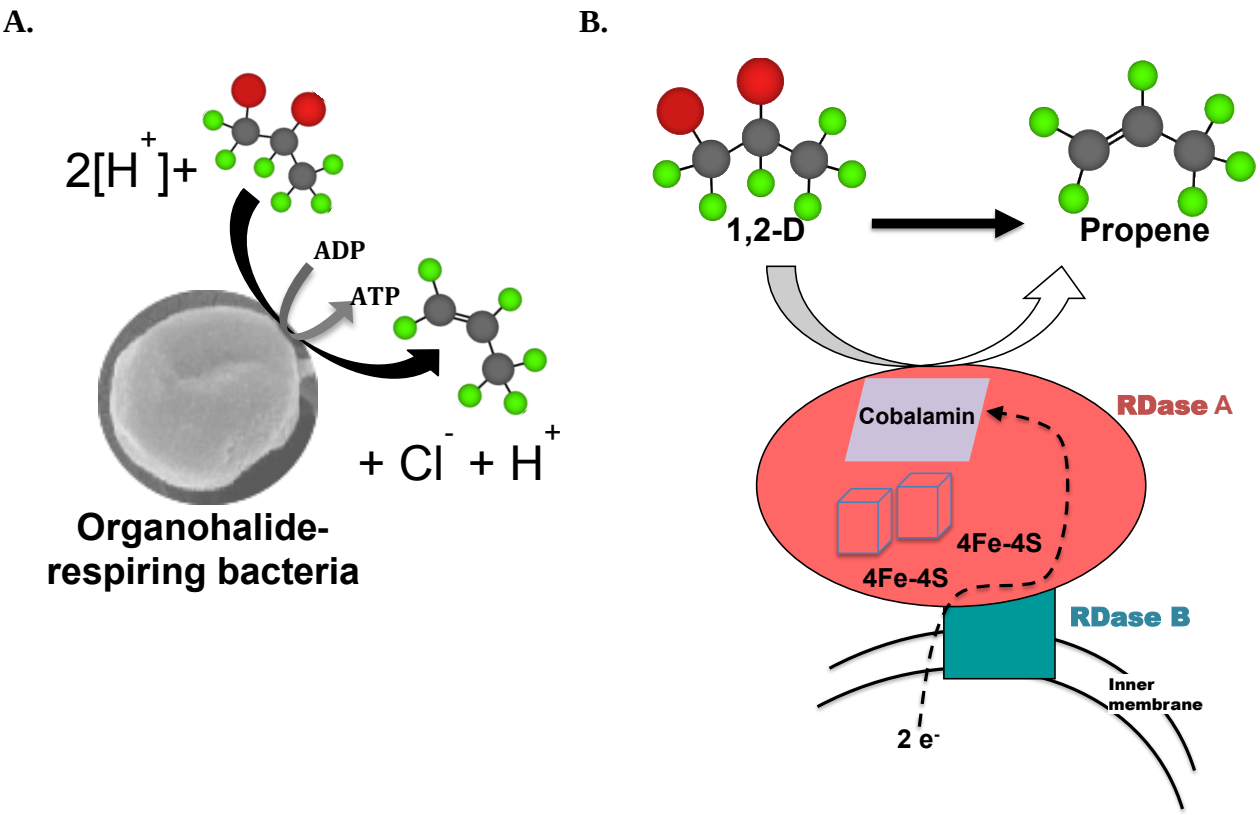


Figure 2.2. Physiology of organohalide respiration

A. Bacterial organohalide respiration. Bacteria conserve energy by using organohalogens as electron acceptors, while oxidizing electron donors such as lactate and H_2 (denoted as 2[H+]). The reaction leads to cleavage of at least one carbon-halide bond and involves a membrane-associated respiratory complex.

B. RDase complex as described by Holliger et al. (Holliger, Wohlfarth, & Diekert, 1999). Reducing equivalents available from lactate or H_2 (denoted as e^-) reduce the iron sulfur centers (4Fe-4S, blue squares), which in turn reduce the cobalt(III) of the corrinoid to cobalt(I). The reaction results in reduction of the organohalide substrate. The catalytic unit RDase A contains both iron-sulfur clusters and a cobamide cofactor at the active site while the RDase B anchors the catalytic unit to the inner membrane. Dotted lines depict the electron flow through the redox center.

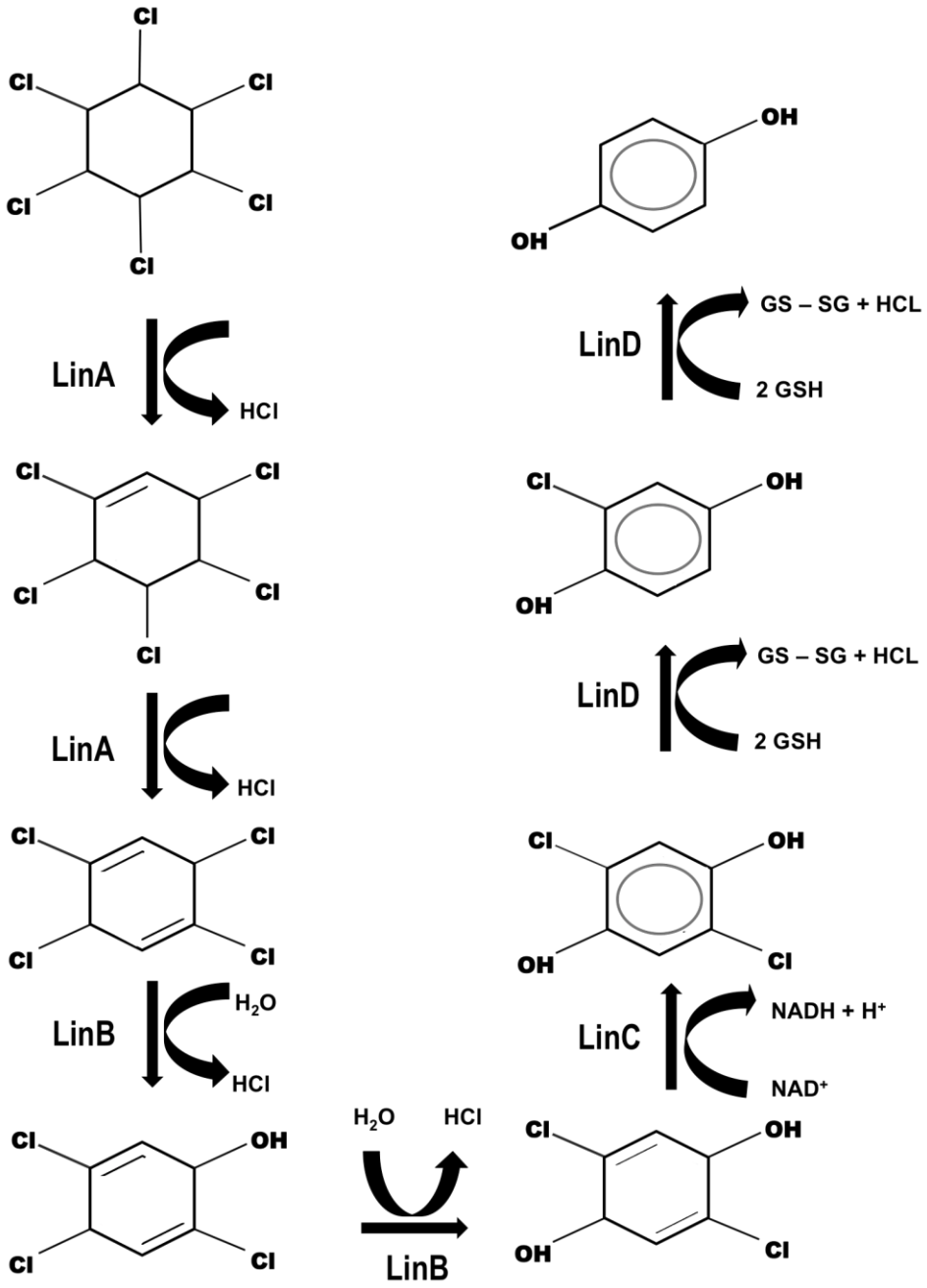


Figure 2.3 γ-hexachlorocylohexane dechlorination in *Sphingomonas paucimobilis* UT26. A GSH-dependent glutathione S-transferase (LinD) reductively dehalogenates 2,5 dichlorohydroquinone (2,5-DCHQ) to chlorohydroquinone and hydroquinone. GSH; glutathione, (reduced form), GS-SG; glutathione (oxidized form).

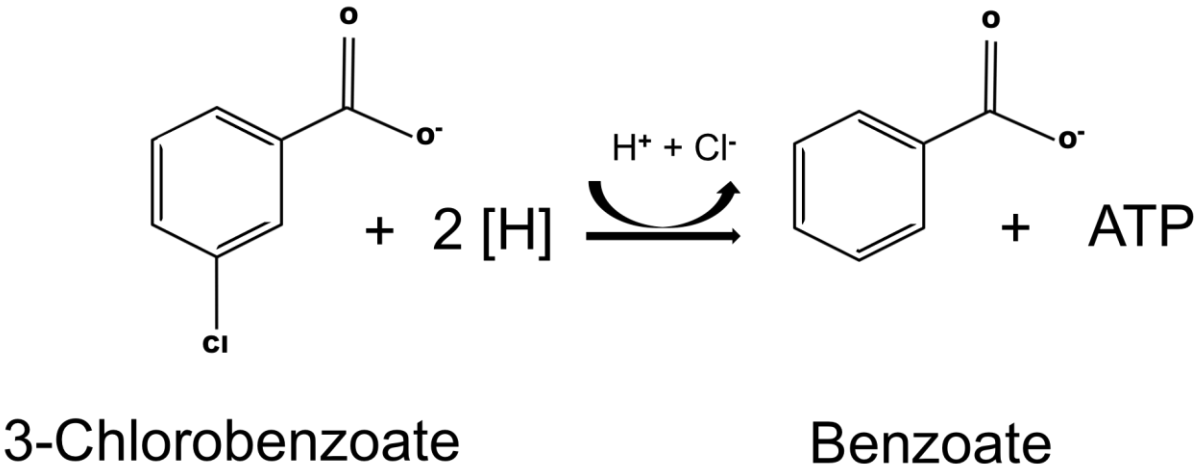


Figure 2.4. Reductive dechlorination of 3-chlorobenzoate (3-CB) by *Desulfomonile tiedjei*.

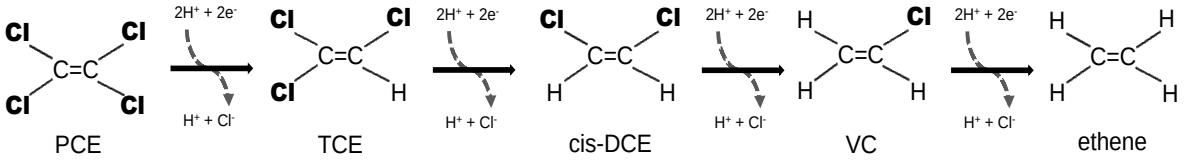
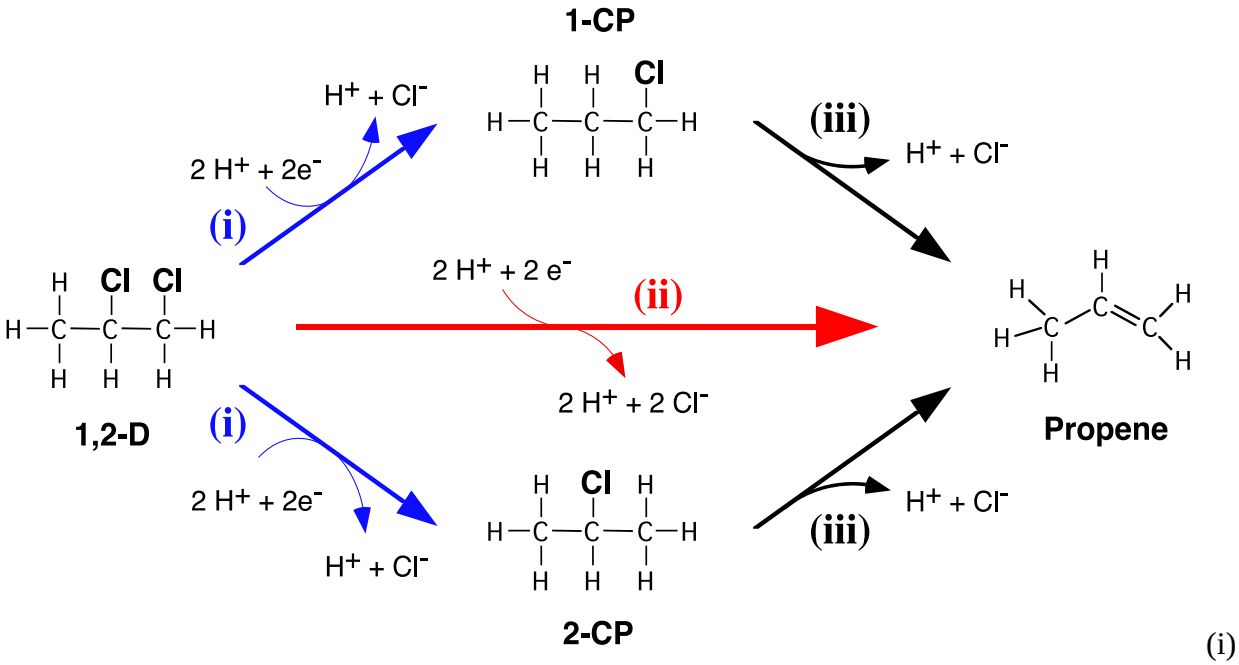


Figure 2.5. Reductive dechlorination pathway of chlorinated ethenes.



(i)

Figure 2.6. Anaerobic dechlorination pathway of 1,2-D in microcosms and enrichments as described by (F E Löffler, Champine, Ritalahti, Sprague, & Tiedje, 1997). Figure from Loeffler et al. (1997).(i) Hydrogenolysis of 1,2-D leading to monochlorinated propanes followed by (iii) Dehydrochlorination resulting in the formation of propene, and (ii) dichloroelimination. Hydrogenolysis and dehydrochlorination were only detected in microcosm with sediment material. In sediment-free cultures, 1,2-D was dechlorinated to propene without the intermediate formation of monochlorinated propanes (dichloroelimination). In the RC ad KS cultures only dichloroelimination is observed.

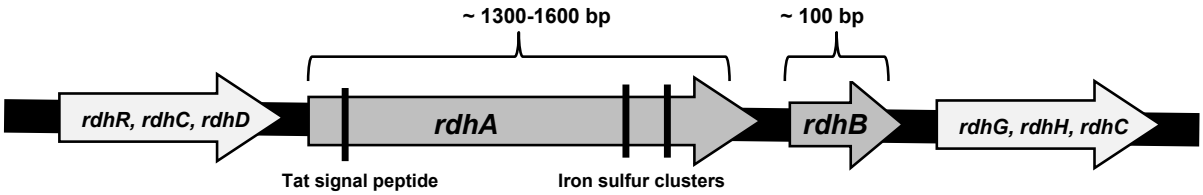


Figure 2.7. Schematic representation of a representative RDase gene operon.

Chapter 2 Appendix: Tables

Table 2.1 Sources, use, and production volumes and/or emissions of halogenated compounds.

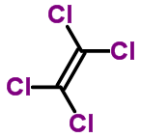
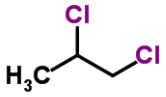
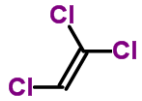
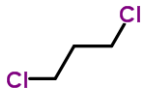
Chemical name (and synonyms)	Acronym, molecular formula and skeletal structure	Natural Source	Biogenic production volumes and/or emissions	Anthropogenic (past and current uses)	Antropogenic production volumes and/or emissions
Tetrachloroethene (Perchloroethylene, Perchloroethylene)	PCE C_2Cl_4 	Volcanic activity, barley, marine algae (reported in one temperate, subtropical, and tropical algae, and in one red microalga)	Production rates in algae were 0.0026 - 8.2 ng g^{-1} fresh weight h^{-1}	Solvent, dry cleaning, metal degreasing, chemical intermediate	Global production was 522,000 tonnes in 2010. In 2016, overall PCE production volume is anticipated to reach 567,000 tonnes.
1,2- Dichloropropane (Propylene dichloride)	1,2-D $C_3H_6Cl_2$ 	No known natural sources. [1]	N/A	Solvent, chemical intermediate, grain fumigant (as nematocide and insecticide)	In 2013,worldwide production was estimated to be 180,000 to 230,000 metric tons (400 to 510 million pounds). No longer used as fumigant.
Trichloroethene (Trichloroethylene)	TCE C_2HCl_3 	Volcanic activity, barley, marine sediments, marine invertebrates, marine mammals, foods marine algae (e.g. reported in one red microalga and in temperate, subtropical and tropical algae)	Production rates for trichloroethylene in algae varied between 0.022 - 3,400 ng g^{-1} fresh weight h^{-1}	Solvent, metal cleansing, chemical intermediate, dry cleaning,	Global consumption was 945 million lbs (around 472, 500 tons) in 2011. Production volume was 225 million lbs (112, 500 tonns) in 2012.
1,3-dichloropropane	1,3-DCP $C_3H_6Cl_2$ 	No known natural sources.	N/A	Soil fumigant, chemical intermediate (in 1971 an excess of over 1,285 tonnes of 1,3-dichloropropene-containing pesticides were used in CA.)	Recent production volumes are not available. Before 1978, about 25,000 tonnes were produced annually in the U.S. Estimated production in Europe in 1979 was 6-7 kilotonnes per year.

Table 2.1. (continued)

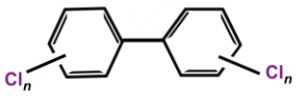
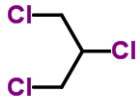
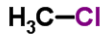
Chemical name (and synonyms)	Acronym, molecular formula and skeletal structure	Natural Source	Biogenic production volumes and/or emissions	Anthropogenic (past and current uses)	Antropogenic production volumes and/or emissions
Polychlorinated biphenyl	PCB $C1_2H_{10-n}Cl_n$ (where n = 1-10). 	Volcanic activity	NR	Insulating fluid, microscope oil, stabilizing additive	The estimated, cumulative production and consumption volumes (in millions of pounds) in the United States from 1930 to 1975 were: total production on, 1,400 (635 million kg); imports, 3 (1.4 million kg); domestic sales, 1,253 (568 million kg); and exports, 150 (68 million kg).
1,2,3-Trichloropropane (Allyl trichloride, Trichlorohydrin)	1,2,3-TCP $C_3H_5Cl_3$ 	No known natural sources	N/A	Pesticide, industrial solvent, paint remover, and cleaner, chemical intermediate	Less than 50,000 metric tons (110 million pounds) of are produced worldwide annually. In 2002, total U.S. production was estimated at between 453 to 4,530 metric tons (1 to 10 million pounds)
Chloromethane (methyl chloride)	CM CH_3Cl 	Volcanic activity, marine algae, diatoms, marine phytoplankton (e.g. *Synechocous* sp.), fungi, plants, coastal salt marshes, freshwater, peatlands sites, rice plantations fields, potato tubers, burning of grasslands and forested areas	Estimated annual global emissions [2]: *(1)* oceans to the atmosphere = 5 Tg per year *(2)* termites = 100 Gg y^{-1} of atmospheric $CHCl_3$. *(3) 910* Gg yr-1 produced by plants in Southeast Asia alone *(4)* wood rotting fungi = 150 Gg y-1 *(5)* salt marshes =170 Gg y-1	Refrigerant, foam-blowing agent and pesticide, silicones, agricultural chemicals, methylcellulose, quaternary amines and butyl rubber	In 2004, total global industry production was estimated at 2.7 billion pounds (1,200 metric tons)

Table 2.1. (continued)

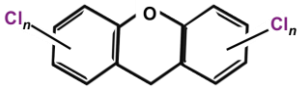
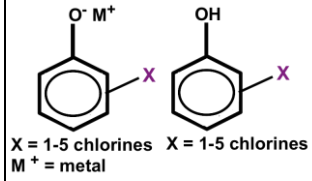
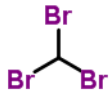
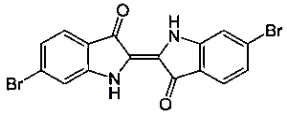
Chemical name (and synonyms)	Acronym, molecular formula and skeletal structure	Natural Source	Biogenic production volumes and/or emissions	Anthropogenic (past and current uses)	Antropogenic production volumes and/or emissions
Dioxins (Polychlorinated dibenzodioxins, PCDDs)	Dioxins $C1_2H_{8X}Cl_XO_2$, where x. 4 to 8 	Volcanic activity and forest fires	Forest and agricultural burning account for approximately 30 kg/year in the U.S.	Coal fired utilities, waste incineration, metal smelting, diesel truck, bleaching, pulp-bleaching, intermediary, herbicide	200 kg/year and 40 kg/year released in the U.S. by municipal waste incineration and hospital incinerators, respectively.
Chlorophenol	CP chlorophenol congeners 	Metabolites of microbes, fungi and lychens and sponges	40,000 tons of chlorinated phenols are produced by humic acid degradation process in Swedish peat bogs alone.	Pesticides, bleaching wood pulp, pesticides, herbicides, and disinfectants	Around 1975, the combined global production of all approached 200 million kg.
Brominated compounds	Organobromine compounds X-Br *e.g.* bromoform  *e.g.* dibromoindigo produced by snail 	Breakdown of organic matter, ubiquitous in marine sediments, algae (e.g *Asparagopsis taxiformis* has 80% by weight bromoform*)* sponge tissue (e.g. *Aplysina aerophob* produces bromophenol), other marine invertebrates (e.g. snails produces dibromoindigo), plants (e.g. broccoli and cabbage).	In soil, total Br concentrations can exceed 100 mg/kg and in marine sediments 5 – 40 mg/kg. Annual oceanic emissions are estimated to be 1–2 million tons and 56,000 tons of bromoform and bromomethane, respectively Production rates of brominated halomethanes and Maximun release rates of bromoform by macroalgae: 253 pmol g^{-1} wet algal weight.	Pesticides, textiles, foams, plastics	.

Table 2.1. (continued)

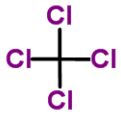
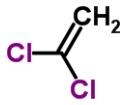
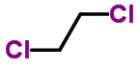
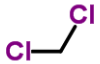
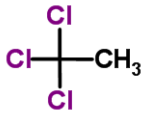
Chemical name (and synonyms)	Acronym, molecular formula and skeletal structure	Natural Source	Biogenic production volumes and/or emissions	Anthropogenic (past and current uses)	Antropogenic production volumes and/or emissions
Carbon tetrachloride	CT CCl_4 	Volcanic activity, marine algae (e.g. *Eucheuma denticulatum*), thermolysis, soil [3]	Volcanic emission rate accounts for .00341 Gg y^{-1} while biomasss combustion is estimated to be 3 Gg y^{-1}	Solvent, chlorofuorocabon production	In 2004 there was production capacity of 130 million pounds (around 65,000 tons) in the U.S.
1,1-Dichloroethene (1,1-dichloroethylene vinylidene dichloride)	1,1-DCE $C_2H_2Cl_2$ 	Volcanic activity	NR	Chemical intermediate, agrochemical.	Production in 1989 totaled 230 million pounds, 650 tons/year released to air in the U.S.
1,2-Dichloroethane; (Ethylene Dichloride)	1,2,-DCA $C_2H_4Cl_2$ 	No known natural sources.	N/A	Chemical intermediate and precursor, soil fumigant, lead scavenger	Global consumption in 2011was 46,238 metric kilotons, 102 billion pounds. U.S. production of 1,2-dichloroethane was about 14.5 million tonnes in 1994.
Dichloromethane (Methylene chloride)	DCM CH_2Cl_2 	Volcanic activity, algae, phytoplankton phylotypes.	0.64 3 10^6 t Cl per year for MeCl and 49 3 10^3 t Cl per year for CH_2Cl_2.62	Paint remover, foam agent, degreaser, solvent, propellant, pressure mediator in aerosols; fumigant and degreening agent	Total global industry capacity is approximately 1.1 billions pounds (520,000 metric tons).
Chloroform (Trichloromethane)	CF $CHCl_3$ 	Marine algae (e.g. *Porphyridium purpureum*), volcanic activity, fungi, trees, fruits, insects (e.g. termites), soil	Total global flux through the environment is approximately 660,000 tonnes per year, and about 90% of emissions are natural in origin.	Solvent, chlorofuorocabon production, chemical intermediate	In 2011, the global consumption of chloroform was estimated to be 1351 metric kilotonnes (770 million pounds).

Table 2.1. (continued)

Chemical name (and synonyms	Acronym, molecular formula and skeletal structure	Natural Source	Biogenic production volumes and/or emissions	Anthropogenic (past and current uses)	Antropogenic production volumes and/or emissions
1,1,1-trichloroethane (methyl chloroform, chlorothene)	1,1,1-TCA $C_2H_3Cl_3$ 	No known natural sources	N/A	Solvent, degreasing agent, chemical intermediate, aerosol formulations, adhesives, protective surface coatings, cutting oils, and printing inks.	As of 2007, it was commercially produced with an annual production volume of 78,439 tonnes in the United States.

NR; not reported

[1] Associated with tobacco and tobacco smoke but its source as either a natural component, additive or as a product of combustion is unclear

[2] Note: Studies have alluded to an unbalanced global flux between known sources and modeled sinks; therefore major unidentified source exists or current emission fluxes are overestimated

[3] Unclear if measurements in soil are from natural or anthropogenic sources

Useful unit conversions

- 1 tonne = 1 metric ton = 1,000 kg = 1 megagram (Mg) = 1.1023 tons (U.S.) = 2,204.6 pounds
- 1 ton (U.S.) = 0.907 metric ton = 2,000 pounds (U.S) = 907.18 kg
- 1 million pounds = 500 tons (U.S) = 453.59 metric tons
- 1 pound = 0.455 kg
- 1 kg = 2.20 lb
- 1 Tg (teragram) = 1.0 x 10^9 kg = 1000 Gg (gigagrams)

Sources:

http://www.chemspider.com/
http://www.ecd.bnl.gov/pubs/BNL66327.pdf
http://www.eurochlor.org/
http://www.inchem.org
http://www.atsdr.cdc.gov/ToxProfiles/
http://www.dow.com/productsafety/assess/finder.htm#B

Table 2.2. Chemical properties of chlorinated ethenes, propanes and propenes and their Maximum Contaminant Levels (MCL).

Compound	Molecular Weight (MW)	Density (g/mL)	Aqueous Solubility (mg/L)	K_h [a]	MCL [b] (ppb)
Chlorinated ethenes					
PCE	165.83	1.625	200	0.723	5[c]
TCE	131.39	1.462	1100	0.392	5[c]
cDCE	96.94	1.248	3500	0.167	70[d]
tDCE	96.94	1.257	6300	0.384	100
1,1-DCE	96.94	1.214	400	1.069	7
VC	62.49	– [e]	2700	1.137	2
Chlorinated propanes					
1-chloropropane	78.54	0.89	2720	0.536	– [f]
2-chloropropane	78.54	0.86	3100 [j]	0.716	– [f]
1,1-dichloropropane	112.99	1.13	2700 [j]	0.154	– [f]
1,2-dichloropropane	112.99	1.16	2800	0.115	5
1,3-dichloropropane	112.99	1.19	2750	0.0399	– [f]
2,2-dichloropropane	112.99	1.11	391 [j]	0.658	– [f]
1,2,3-trichloropropane	147.43	1.39	1750	0.014	– [g]
2-bromo-1-chloropropane	157.44	1.54	2240	0.216	– [f]
1,2-dibromo-3-chloropropane	236.33	2.1	1230 [i]	0.0061	0.2

Table modified after .

[a] K_h, Henry's law constants (dimensionless) at 25ºC (except K_h for 1,2-dibromo-3-chloropropane, which is at 20ºC) from http://www.syrres.com/esc/physdemo.htm.

[b] Maximum contaminant levels http://www.epa.gov/safewater/contaminants

[c] In NJ an MCL of 1 ppb has been established for PCE and TCE; while in FL an MCL of 3 ppb is in place for PCE and TCE.

[d] In CA an MCL of 6 ppb has been established for cDCE and 5ppb in NY.

[e] These compounds are gases at environmentally relevant temperatures.

[f] No EPA-mandated MCL.

[g] A federal MCL hasn't been established but the state of Hawaii has an MCL of 6 ppb, New York and Minnesota have state regulations of 5 ppb and 3 ppb, respectively.

Table 2.3. Number of federal and general sites for each status as of February 09, 2015.

Status	Non-Federal (General)	Federal	Total
Proposed Sites	43	4	47
Final Sites	1164	157	1321
Deleted Sites	369	17	386
		TOTAL SITES	**1754**

Source:
http://www.epa.gov/superfund/sites/query/queryhtm/npltotal.htm
http://www.epa.gov/superfund/sites/npl/

Table 2.4. Incidence of chloroorganic contaminants at the Superfund sites listed in the Comprehensive Environmental Response, Compensation and Liability Information System (CERCLIS) Public Access Database[a] .

Contaminant	Number of Superfund sites [b]
Chlorinated compounds	1261
Chlorinated ethanes and ethenes	938
Polychlorinated biphenyl	390
Chloropropanes	139
Tetrachloroethene	688
Trichloroethene	791
Dichloroethenes	696
Vinyl chloride	563
1,2-Dichloropropane	120

[a] CERCLIS data accessed on March 2015. The Superfund program is in the final stages of implementing a new information system, which will be available in April 2015. Therefore the public data displayed in their website is from the end of FY 2013.
Source: http://cumulis.epa.gov/supercpad/cursites/srchsites.cfm.

[b] Due to inconsistent naming of chemicals in the database, different queries were performed for the same compound, which included common acronyms and names. The resulting list of sites was cross-compared to remove duplicates. Queries searched: *Chlorinated compounds*: words containing "chloro" "chlori"; *Chlorinated ethanes and ethenes:* words containing" chloroethene", "chloroethylene", "chloroethane"; *Polychlorinated biphenyl:* PCB, PCBs, polychlorinated biphenyl; *Chloropropanes:* chloropropane; *Tetrachloroethene:* PCE, perchloroethene, perchloroethylene, tetrachloroethene, tetrachloroethylene; *Trichloroethene:* TCE, trichloroethylene, trichloroethene, trichloroethylene; *Dichloroethenes*: DCE, Dichloroethylene, Dichloroethene; *Vinyl chloride:* VC, vinyl chloride.

Table 2.5. Estimated regional production volumes of 1,2-D as of 2001.

Region	Estimated Percentage of Global Production
North America	64-69
Europe	19-25
Latin America	9-10
Japan	2-3

Source: http://www.inchem.org/documents/sids/sids/78875.pdf

Table 2.6. Manufacturers of 1,2-D as listed in the 2012 International Resources Guide to Hazardous Chemicals .

Manufacturer
Atofina (France)
BASF (Germany)
Bayer (Germnay)
Cyanamid Agro Products (USA)
Mitsui Chemical (Japan)
Shangai Chemical Reagent (China)
Shell Chemical (Netherlands)
Showa Denko (Japan)
Sigma-Aldrich Laborchemikalien (Germany)
Sithean Corporation (USA)

Table 2.7. Environmental releases for 1,2-dichloropropane in the U.S. [1]

Year	Pounds Released [2]
2013	102,507
2012	69,922
2011	67,523
2010	93,882
2009	92,366
2008	103,542
2007	115,714
2006	98,255
2005	132,585

[1] The EPA's Toxic Release Inventory (TRI) Explorer database was queried by year using the default data set of 2013 (released March 2015). The following criteria were selected: "Geographic Location: all locations" which cover all EPA regions, "Industry: all industries", "Chemical: 1,2-Dichloropropane".
(Source: http://iaspub.epa.gov/triexplorer/tri_release.chemical)

[2] TRI's "release" of a chemical refers to emissions in the air, land, water or underground, or placed in some type of land disposal.

Table 2.8. Bacteria capable of dechlorinating PCE and TCE.

Isolate	PCE Dechlorinating Activity
Dehalobacter restricus	PCE → cDCE
Desulfuromonas chloroethenica	PCE → cDCE
Dehalobium chlorocoercia	PCE → cDCE
Desulfuromonas michiganensis	PCE → cDCE
Sulfurospirillum multivorans	PCE → cDCE
Sulfurospirillum halorespirans	PCE → cDCE
Geobacter lovleyi	PCE → cDCE
Desulfitobacetrium sp. strain PCE-S	PCE → cDCE
Desulfitobacterium hafniense strain TCE1	PCE → cDCE
Desulfitobacterium hafniense strain Y51	PCE → cDCE
Anaeromyxobacter dehalogenans strain 2CP-C	PCE → cDCE
Desulfitobacterium hafniense strain JH1	PCE → TCE
Desulfitobacetrium sp. strain Viet1	PCE → TCE

Table 2.9. Substrates dechlorinated by *Dhc* and *Dhgm* isolates and enrichment cultures. The primary maintenance substrate (S) for each culture is indicated.

Culture Primary Substrate (S)	Dechlorination Activity	Chlorinated Substrates not used
Dhc mccartyi strain 195 (PCE)	PCE, TCE, cDCE, tDCE* 1,1-DCE → VC*, ethene 1,2-DCA→ ethene HCB →PeCB→TeCB, →TCB 1,2-dibromoethane, 1,2,3,4-tetrachlorodibenzodioxin 2,3,4,5,6- Pentachlorobiphenyl 1,2,3,4-tetrachloronaphthalene 2,3,6-trichlorophenol 1,2,3,4-tetrachloro-dibenzofuran 2,3-dichlorophenol 2,3,4-trichlorophenol polybrominated diphenyl ethers polychlorinated biphenyls (Aroclor 1260)	Monochlorophenols 2,3-dichlorodibenzo-p-dioxin 2,3,7,8- tetrachlorodibenzop-dioxin 2,3,4-trichlorophenol pentachlorophenol 2,4-, 2,5-, and 2,6-dichlorophenol (in summary, chlorophenols, that are NOT in the *ortho* position and are those where the chlorine substituent IS NOT present in the flanking *meta* position.)
Dhc mccartyi strain BAV1 (cDCE)	PCE*, TCE*, cDCE, tDCE, 1,1-DCE → VC, ethene 1,2-DCA, vinyl bromide	Chlorinated propanes, 1,1,1-TCA, 1,1-DCA and CA
Dhc mccartyi strain FL2 (TCE)	PCE*, TCE, cDCE, tDCE, → VC VC*→ ethene	1,1-DCE, 1,1,2-TCA, 1,1-DCA, 1,2-DCA, and CA
Dhc mccartyi strain GT (TCE)	TCE, cDCE, 1,1-DCE, VC → ethene	PCE, 1,2-DCA, tDCE, CA, 1,1-DCA, 1,1,1-TCA; 1,1,2-TCA, CT, 1,2-DCP; vinyl bromide, 1,1-dichloro-2,2-difluoroethene, 1,2-dichloro-1,2-difluoroethene; 2-chloro-1,1-difluoroethene; 1,1-difluoroethene
Dhc mccartyi strain CBDB1 (1,2,3-TCB, 1,2,4-TCB)	PCE, TCE → trans HCB, all TeCBs, 1,2,3-TCB and 1,2,4-TCB 2,3-DCP, 2,3,4-TCP Polychlorinated dioxins, polychlorinated biphenyls Aroclor 1260	DCEs, VC, 1,2-D, 1,2-DCA, 1,1-DCA
Dhc mccartyi strain MB (PCE)	PCE, TCE→ *trans*-1, 2-DCE	VC
Dhc mccartyi strains SG1, SG2, SG3 (Aroclor 1260, PCE)	PCB congeners in Aroclor 1260 PCE →TCE	TCE, cDCE, VC

Table 2.9. (continued)

Culture Primary Substrate (S)	Dechlorination Activity	Chlorinated Substrates not used
Dhc mccartyi strain BTF8 (in highly enriched culture)	1,2,3-trichlorobenzene	ND
Dhc mccartyi strain VS (VC)	TCE, cDCE, 1,1-DCE, VC→ ethene	ND
Dhc mccartyi strain DCMB5 (PCE)	1,2,3-TCB →1,3-DCB 1,2,3,4-tetra- and 1,2,4-trichlorodibenzo-p-dioxin, 1,2,3-trichlorobenzene	ND
Dhc mccartyi strain 11a	TCE, 1,1-DCE, cDCE, tDCE, and vinyl chloride (VC) →ethene	ND
Dhc mccartyi strain 11a5	TCE, 1,1-DCE, cDCE, tDCE →VC	VC
Dhc mccartyi strain *ANAS1*	TCE, 1,1-DCE, cDCE→VC	PCE, tDCE, VC
Dhc mccartyi strain *ANAS2*	TCE, 1,1-DCE, cDCE, VC→ethene	PCE, tDCE
Dehalogenimonas lykanthroporepellens strain BL-DC-9 (1,2,3-TCP)	1,2-DCP → propene 1,2-DCA→VC 1,1,2-TCA → VC 1,2,3-TCP→ Ally chloride 1,1,2,2-TeCA → cDCE and tDCE	1-CP, 2-CP, 1,2-dichlorobenzene, cDCE, tDCE, PCE, TCE, VC
Dehalogenimonas Alkenigignens IP3-3	1,2-DCA→ethene 1,2-DCP → propene 1,1,2-TCA → VC 1,2,3-TCP→ Ally chloride 1,1,2,2-TeCA → cDCE and tDCE	ND
Dehalogenimonas Alkenigignens SBP-1-	1,2-DCA→ethene 1,2-DCP → propene 1,1,2-TCA → VC 1,2,3-TCP→ ally chloride 1,1,2,2-TeCA → cDCE and tDCE	ND
Dehalogenimonas sp. in WBC2 consortium	tDCE →VC	ND
Dehalobium chlorocoercia DF-1	PCE, TCE→ tDCE HCB →PeCB→TeCB, →TCB	ND
Dehalobacter spp. strain RM* enrichment (DCM)	DCM → acetate	ND

Table 2.9. (continued)

Culture Primary Substrate (S)	Dechlorination Activity	Chlorinated Substrates not used
Geobacter lovleyi SZ (PCE)	PCE, TCE → cDCE	cDCE, VC, 1,2-DCA TCA, trifluoroacetic, tDCE, 1,1-DCE, CA, 1,1-DCA, 1,2-DCA, 1,1,1-TCA 1,1,2-TCA, 1,2-DCP
Geobacter sp. strain KB-1 (PCE)	PCE, TCE → cDCE	cDCE, VC, 1,2-DCA
Culture RC (contains one *Dhc* strain) (1,2-DCP)	1,2-DCP → propene	1,1,2-TCA, 1,2,3-TCP, 1,2-DCA, PCE, TCE, cDCE, tDCE, VC
Culture KS (contains one *Dhc* strain) (1,2-DCP)	1,2-DCP → propene	1,1,2-TCA, 1,2,3-TCP, 1,2-DCA, PCE, TCE, cDCE, tDCE, VC
Bio-Dechlor INOCULUM (BDI) (Contains *Dhc* strains BAV1, GT and FL2, *Geobacter lovleyi* strain SZ, and a *Dhb* sp. (PCE)	PCE → ethene TCE → ethene cDCE → ethene VC→ ethene 1,2-DCA → ethene 1,1-DCE → ethene	1-CP, 2-CP, 1,2-DCP 1,2,3-TCP, 1,1,1-TCA 1,1,2-TCA, 1,2,3-TCB, 1,2,4-TCB, CT, CF, DCM
Third Creek enrichments	PCE → ethene TCE → ethene cDCE → ethene VC → ethene 1,2-DCP → propene 1,2-DCA → ethene 1,1,2-TCA → ethene 1,1-DCA → chloroethane 1,1,1-TCA → chloroethane CF, DCM → nonchlorinated products	NA

*cometabolic

Table 2.10. Reductive dehalogenases with assigned function in *Dhc i*solates and enrichment cultures.

RDase gene	RDase	Bacteria	Catalyzing reaction	Methods	Reference
			***Dehalococcoides* sp.**		
pceA	PceA	strain 195	PCE/TCE → cDCE PCE → TCE	IV, LC, PAGE	(Magnuson et al., 1998)
tceA	TceA	strain 195, FL2	TCE → VC	IV, LC, PAGE	(Magnuson et al., 1998) (Jianzhong He, Sung, Krajmalnik-Brown, Ritalahti, & Löffler, 2005; Hölscher et al., 2004)
vcrA	VcrA	strains VS	DCEs → ethene	IV, LC, AA, GS	(Müller JA, Rosner BM, Von Abendroth G, Meshulam-Simon G, McCarty PL et al., 2004)
bvcA	BvcA	strain BAV1	DCEs, VC→ethene	TRA, IV, qPCR, PAGE, LC	(Krajmalnik-Brown et al., 2004; Tang S, Chan WW, Fletcher KE, Seifert J, Liang X, Löffler FE, Edwards EA, 2013)
mbrA	MbrA	strain MB	TCE → tDCE	TRA, qPCR, PAGE, MS	(Chow, Cheng, Wang, & He, 2010)
dcpA	DcpA	strains RC, KS	1,2-D → propene	PAGE, IV, qPCR, TRA, LC/MS,	(Padilla-Crespo et al., 2014)
cbrA	CbrA	strain CBDB1	1,2,3,4-TeCB → 1,2,4-TCB 1,2,3-TCB → 1,3-DCB	PAGE, IV, MS, TRA, T-RFLP, LC, LC/MS	(Adrian, Rahnenführer, Gobom, & Hölscher, 2007; A. Wagner, Adrian, Kleinsteuber, Andreesen, & Lechner, 2009)
pcbA	PcbA	strains CG1, CG4, and CG5	PCB congeners PCE →TCE	PAGE, MS, qPCR, ME, GS	(Wang, Chng, Wilm, et al., 2014)

Table 2.10. (continued)

RDase gene	RDase	Bacteria	Catalyzing reaction	Methods	Reference
			Other dehalogenating bacteria		
dcpA	DcpA	*Dhgm* strain BL-DC-9	1,2-D → propene	cPCR, BN-PAGE, IV, qPCR, LC/MS,	(Padilla-Crespo et al., 2014)
pceA	PceA	*Desulfito. hafniense* strain PCE-S	PCE/TCE → cDCE	IV, LC, AA	(Miller et al., 1998; Ye LD, Schilhabel A, Bartram S, Boland W, 2010)
pceA	PceA	*Desulfito. hafniense* strain PCE-1	PCE→TCE	IV, LC, AA	(Van De Pas, Gerritse, De Vos, Schraa, & Stams, 2001)
pceA	PceA	*Desulfito. hafniense* strain TCE-1	PCE/TCE → cDCE	IV, LC, AA	(Van De Pas et al., 2001)
pceA	PceA	*Desulfito. hafniense* strain Y51	PCE/TCE → cDCE	IV, LC, AA	(Suyama et al., 2002)
pceA	PceA	*Sulfurospirillum multivorans*	PCE/TCE → cDCE	IV, LC, AA	(A Neumann, Wohlfarth, & Diekert, 1996; Ye LD, Schilhabel A, Bartram S, Boland W, 2010)
pceA	PceA	*Geobacter lovleyi SZ*	PCE/TCE → cDCE	GS	(D. Wagner et al., 2012)
pceA	PceA	*Anaeromyxobacter dehalogenans* 2-CPC	PCE/TCE → cDCE	GS	(S. H. Thomas et al., 2008)
cfrA	CfrA	*Dhb* sp. CF	CF →DCM 1,1,1-TCA → 1,1-DCA	BN-PAGE, IV, LC/MS, ME	(Tang & Edwards, 2013)
dcrA	DcrA	*Dhb* sp. DCA	1,1-DCA → CA	BN-PAGE, IV, LC/MS, ME	(Tang & Edwards, 2013)
rdh1	Rdh1	*Dhb* sp.	1,2-DCA →ethene	TRA, qPCR	(Grostern, A, Edwards, 2009)

Table 2.10 (continued)

RDase gene	RDase	Bacteria	Catalyzing reaction	Methods	Reference
			Other dehalogenating bacteria		
	3-CIBI-RD	*Desulfomonile tiedjei strain DCB-1*	3-chlorobenzoate	LC, IV	(Ni, Fredrickson, & Xun, 1995)
dcaA	DcaA	*Desulfito. dichloroeliminans* strain DCA1	1,2-DCA → ethene	TRA, qPCR,	(Marzorati et al., 2007)

Desulfito; Desulfitobacterium
Dhb; Dehalobacter
Dhgm; Dehalogenimonas
CB; chlorobenze
IV; *in vitro* activity assays
CS; chromatography separation
PAGE; PAGE gel separation
cPCR; cloning with targeted primers on cDNA
qPCR; transcriptional analysis and quantification by qPCR
GS; Genome sequencing
TRFLP; Terminal Restriction Fragment Length Polymorphism on cDNA
MS; detection of peptides via mass spectrometry
LC; liquid chromatography separation
ME; metagenome
GENE; homology of gene sequence

Table 2.11. Identity matrix of hydrogenase genes in *Dhc* strains. Identities of 98-100% are in bold. (1) strain 195; (2) strain VS; (3) strain CBDB1; (4) strain BAV1, (5) strain GT

NiFe Hydrogenase (Hyc)	1	2	3	4	5
1. DET1571_HycE		93.2	85.4	84.9	84.9
2. DhcVS_1449	93.2		84.9	84.5	84.5
3. cbdbA1653	85.4	84.9		**99.2**	**99.2**
4. DehaBAV1_1317	84.9	84.5	99.2		**100**
5. DehalGT_1365_1366	84.9	84.5	99.2	**100**	
NiFe Hydrogenase (Hup)	1	2	3	4	5
1. det0110_Hup		90.8	89.2	89.2	89.1
2. DhcVS_120	90.8		90.7	90.8	90.5
3. DehalGT_0141	89.2	90.7		**98.7**	**99.8**
4. DehaBAV1_0258	89.2	90.8	**98.7**		**98.8**
5. cbdbA129	89.1	90.5	**99.8**	**98.8**	
NiFe Hydrogenase (Vhu)	1	2	3	4	5
1. DET0615_Vhu		88.4	88.4	88.3	87.8
2. DehalGT_0550	88.4		100	**99.7**	89.9
3. cbdbA597	88.4	**100**		**99.7**	89.9
4. DehaBAV1_0588	88.3	99.7	**99.7**		89.8
5. DhcVS_553	87.8	89.9	89.9	89.8	
NiFe Hydrogenase (Ech)	1	2	3	4	5
1. DET0867_EchEsubunit		91	87.1	87.1	87
2. DhcVS_770	91		86.9	86.9	86.9
3. DehalGT_0746	87.1	86.9		**100**	**99.4**
4. cbdbA850	87.1	86.9	**100**		**99.4**
5. DehaBAV1_0785	87	86.9	**99.4**	**99.4**	
Fe Hydrogenase (Hym)	1	2	3	4	5
1. DET0146_DET0147_Fe_HymB		92.8	91	42.7	90.9
2. DhcVS_154_155	92.8		92.3	43.8	92.3
3. cbdbA170-cbdbA171	91	92.3		42.7	**99.7**
4. DehaBAV1_0224_0225	42.9	44.1	42.8		42.7
5. DehalGT_0174_0175	90.9	92.3	**99.7**	42.9	

Table 2.12. Primers described in the literature and currently used as part of the molecular toolbox to detect 16S rRNA genes of *Dhc* and other dechlorinating bacteria.

Primer pair	Primer sequence (5'-3')	T_a (°C)	Product size (bp)	Assay	Ref
Dehalogenating *Chloroflexi*					
Chl348F Dehal884R	GAG GCA GCA GCA AGG AA GGC GGG ACA CTT AAA GCG	60	470	PCR, qPCR-SYBR chemistry, DGGE	(Fagervold, Watts, May, Sowers, & Carolina, 2005)
Dehalococcoides					
Dhc728, Dco728F, FL2F Dhc1164R, FL2R	AAG GCG GTT TTC TAG GTT GTC AC CGT TTC GCG GGG CAG TCT	58	436	PCR	(Frank E Löffler, Sun, Li, & Tiedje, 2000)
567F RpDHC1377, 1377R	CGG GAC GTG TCA TTC AAT AC GGT TGG CAC ATC GAC TTC AA	55	810	PCR	(Fennell, Carroll, Gossett, & Zinder, 2001)
Fp DHC 1 Rp DHC 692	GAT GAA CGC TAG CGG CG TCA GTG ACA ACC TAG AAA AC	62[a]	692	PCR	(Hendrickson et al., 2002)
Fp DHC 1 Rp DHC 1212	GAT GAA CGC TAG CGG CG GGA TTA GCT CCA GTT CAC ACT G	55	1212	PCR	(Hendrickson et al., 2002)
Fp DHC 1 Rp DHC 1377	GAT GAA CGC TAG CGG CG GGT TGG CAC ATC GAC TTC AA	55	1377	PCR	(Hendrickson et al., 2002)
Fp DHC 385 Rp DHC 806	GGG TTG TAA ACC TCT TTT CAC GTT AGC TTC GGC ACA GAG AG	68[a]	421	PCR	(Hendrickson et al., 2002)
Fp DHC 587 Rp DHC 1090	GGA CTA GAG TAC AGC AGG AGA AAA C GGC AGT CTC GCT AGA AAA T	66[a]	503	PCR	(Hendrickson et al., 2002)
Fp DHC 774 Rp DHC 1212	GGG AGT ATC GAC CCT CTC GGA TTA GCT CCA GTT CAC ACT G	55	438	PCR	(Hendrickson et al., 2002)

Table 2.12. (continued)

Primer pair	Primer sequence (5'-3')	T_a (°C)	Product size (bp)	Assay	Ref
Dehalococcoides					
Fp DHC 946 Rp DHC 1212	AGT GAA CCG AAA GGG AAA GGA TTA GCT CCA GTT CAC ACT G	55	266	PCR	(Hendrickson et al., 2002)
Dhc1200F Dhc1271R Dhc1240P	CTG GAG CTA ATC CCC AAA GCT CAA CTT CAT GCA GGC GGG TCC TCA GTT CGG ATT GCA GGC TGA A	58	65	PCR, qPCR-TaqMan chemistry	(Ritalahti et al., 2006)
Dhc-730F *Dhc*-1350R	GCG GTT TTC TAG GTT GTC CAC CTT GCT GAT ATG CGG	58	620	PCR	(Jianzhong He, Ritalahti, Aiello, & Löffler, 2003)
Dhc728F, FL2F, Dco728F Dhc1164R, FL2R	AAG GCG GTT TTC TAG GTT GTC AC CGT TTC GCG GGG CAG TCT	58	436	PCR	(Bunge et al., 2003)
DHE-for DHE-rev	AAG GCG GTT TTC TAG GTT CGT TTC GCG GGG CAG TCT	58	443	qPCR-SYBR chemistry	(Dennis, Sleep, Fulthorpe, & Liss, 2003; Yan, Rash, Rainey, & Moe, 2009)
Dhc728F, FL2F, Dco728F Dco944R	AAG GCG GTT TTC TAG GTT GTC AC CTT CAT GCA TGT CAA AT	58	216	qPCR-SYBR chemistry	(Smits et al., 2004)
FpDHC1, Dhc1f 1386r	GAT GAA CGC TAG CGG CG CCT CCT TGC GGT TGG CAC ATC	52	1380	PCR	(M Duhamel, Mo, & Edwards, 2004)
582f 728r	CTG TTG GAC TAG AGT ACA GC GTG ACA ACC TAG AAA ACC GCC TT	59	108	PCR, qPCR-SYBR chemistry	(M Duhamel et al., 2004; Yan et al., 2009)
FpDHC1, Dhc1f 259r	GAT GAA CGC TAG CGG CG CAG ACC AGC TAC CGA TCG AA	59	258	PCR, PCR-DGGE, qPCR-SYBR chemistry	(M Duhamel & Edwards, 2007; M Duhamel et al., 2004; Melanie Duhamel & Edwards, 2006; Hendrickson et al., 2002)

Table 2.12. (continued)

Primer pair	Primer sequence (5'-3')	T_a (°C)	Product size (bp)	Assay	Ref
Dehalococcoides					
DeF DeR	GCA ATT AAG ATA GTG GC ACT TCG TCC CAA TTA CC	55	1373	PCR	(Cupples, Spormann, & McCarty, 2003)
DHC66f DHC180r	GGT CTT AAG CAA TTA AGA TAG TG CAC CAA GCR CCT TRC GGC	60	114	PCR, qPCR	(Yoshida, Takahashi, & Hiraishi, 2005)
DHC793f DHC946r	GGG AGT ATC GAC CCT CTC TG CGT TYC CCT TTC TGT TCA CT	60	153	PCR, qPCR	(Yoshida et al., 2005)
DhcForward Dhc Probe DhcReverse	GGT AAT ACG TAG GAA GCA AGC G ACA TCC AAC TTG AAA GAC CAC CTA CGC TCA CT CCG GTT AAG CCG GGA AAT T	60	98	Taqman	(Holmes, He, Lee, & Alvarez-Cohen, 2006; Rahm, Morris, & Richardson, 2006)
Forward Reverse	GAAGTAGTGAACCGAAAGG TCTGTCCATTGTAGCGTG	NR	235	qPCR-SYBR chemistry	(Schaefer CE, Condee CW, Vainberg S, 2009)
FpDHC1, Dhc1f Dhc264r	GAT GAA CGC TAG CGG CG CCT CTC AGA CCA GCT ACC GAT CGA A	59	~260	PCR, qPCR-SYBR chemistry	(Grostern, A, Edwards, 2009; Hendrickson et al., 2002)
Dhc193f Dhc1048r	GGT TCA YTA AAG CCG YAA GG CCT GTG CAA RYT CCT GAC T	53	855	PCR	(Dowideit, K, Scolz-Muramatsu H, Miethling-Graff, R, Vigelahn L, Freygang M, Dohrmann, AB, n.d.)
Dehalogenimonas					
BL-DC-57f [b] BL-DC-1410r	GCA AGT CGA ACG GTC TCT CGC AGG TGT TAC CAA CTT TCA TGA C	63	1330	PCR	(Yan et al., 2009)
BL-DC-57f [b] BL-DC-1351r	GCA AGT CGA ACG GTC TCT CGC AAC GCG CTA TGC TGA CAC GCG T	63	1271	PCR	(Yan et al., 2009)

Table 2.12. (continued)

Primer pair	Primer sequence (5'-3')	T_a (°C)	Product size (bp)	Assay	Ref
BL-DC-117f [b] BL-DC-1020r	GTA ATA GGT AAG TAA CCT GCC CTT ATA GCT CCT GAC TTG ACA GGT GGA TC	63	911	PCR	(Yan et al., 2009)
BL-DC-142f BL-DC-796r [c]	GTG GGG GAT AAC ACT TCG AAA GAA GTG C ACC CAG TGT TTA GGG CGT GGA CTA CCA GG	63	661	PCR	(Yan et al., 2009)
BL-DC-142f [d] BL-DC-1020r	GTG GGG GAT AAC ACT TCG AAA GAA GTG C ATA GCT CCT GAC TTG ACA GGT GGA TC	63	885	PCR	(Yan et al., 2009)
BL-DC-142f [c] BL-DC-1243r	GTG GGG GAT AAC ACT TCG AAA GAA GTG C CCG GTG GCA ACC CAT TGT ACC GC	63	1093	PCR	(Yan et al., 2009)
BL-DC-183f [b] BL-DC-796r [b]	GGT GCT CTT TCA CAA GGA AGA GTA CTG ACC CAG TGT TTA GGG CGT GGA CTA CCA GG	63	620	PCR	(Yan et al., 2009)
BL-DC-610f [b] BL-DC-1020r	TCT CCC GGC TCA ACT GGG AGG GGT CAT CTG ATA GCT CCT GAC TTG ACA GGT GGA TC	63	439	PCR	(Yan et al., 2009)
BL-DC-610f [c] BL-DC-1243r	TCT CCC GGC TCA ACT GGG AGG GGT CAT CTG CCG GTG GCA ACC CAT TGT ACC GC	63	647	PCR	(Yan et al., 2009)
BL-DC-727f [b] BL-DC-1020r	GAA GGC GGT TTT CTA GGC CAW A ATA GCT CCT GAC TTG ACA GGT GGA TC	63	322	PCR	(Yan et al., 2009)
BL-DC-727f [b] BL-DC-1351r	GAA GGC GGT TTT CTA GGC CAW A AAC GCG CTA TGC TGA CAC GCG T	63	636	PCR	(Yan et al., 2009)
BL-DC-631f [c] BL-DC-796r	GGT CAT CTG ATA CTG TTG GAC TTG AGT ATG ACC CAG TGT TTA GGG CGT GGA CTA CCA GG	63	194	PCR, qPCR-SYBR chemistry	(Yan et al., 2009)
BL-DC-727f [b] BL-DC-982r	GAA GGC GGT TTT CTA GGC CAW A TCT AAC ATG TCA AGC CCT GGT G	63	294	PCR	(Yan et al., 2009)
BL-DC-142 [c] BL-DC-1351r	GTG GGG GAT AAC ACT TCGAAA GAA GTG C AAC GCG CTA TGC TGA CACGCG T	63	1199	PCR	(Chen, Bowman, Rainey, & Moe, 2014)

Table 2.12. (continued)

Primer pair	Primer sequence (5'-3')	T_a (°C)	Product size (bp)	Assay	Ref
Dehalogenimonas					
mod-BL-DC-1243f [c] BL-DC-1351r	GGY ACA ATG GGT TGC CACCGG AAC GCG CTA TGC TGA CACGCG T	63 / 68.2	127	PCR, qPCR EvaGreen chemistry	(Chen et al., 2014)
Dehalobium					
14F Dehal1265R	AGA GTT TGA TCC TGG CTC AG GCT ATT CCT ACC TGC TGT ACC	62	1215	PCR	(Watts, Fagervold, May, & Sowers, 2005)
Dehalobacter					
Deb179F Deb1007R	TGT ATT GTC CGA GAG GCA ACT CCC ATA TCT CTA CGG	53	828	PCR	(Schlötelburg et al., 2002)
Dre441F Dre645R	GTT AGG GAA GAA CGG CAT CTG T CCT CTC CTG TCC TCA AGC CAT A	58	225	PCR, qPCR-SYBR chemistry, DGEE	(Smits et al., 2004)
Dre441F Dre1013R\	GTT AGG GAA GAA CGG CAT CTG T CGA AGC ACT CCC ATA TCT	58	589	PCR	(Smits et al., 2004)
Dhb477f Dhb647r	GAT TGA CGG TAC CTA ACG AGG TAC AGT TTC CAA TGC TTT ACG G	63	~170	qPCR-SYBR chemistry	(Grostern & Edwards, 2006)
Anaeromyxobacter					
60F 461R	CGA GAA AGC CCG CAA GGG ATT CGT CCC TCG CGA CAG T	56.5	401	PCR	(Petrie, North, Dollhopf, Balkwill, & Kostka, 2003)
F112 R227	GTA ATC TGC CCT AGA GTC CGG A AGA GCG ATA GCT TGT GTA CAG AGG	60	115	qPCR-SYBR chemistry	(Sanford et al., 2007)
Ade399Fwd Ade466Rev	GCA ACG CCG CGT GTG T TCC CTC GCG ACA GTG CTT	60	67	qPCR-TaqMan chemistry	(Thomas, S. H., Padilla-Crespo E, Jardine PM, Sanford RA, 2009)

Table 2.12. (continued)

Primer pair	Primer sequence (5'-3')	T_a (°C)	Product size (bp)	Assay	Ref
Anaeromyxobacter					
2CP444Fwd 2CP513Rev	TCG CGA GGG ACG AAT AAG G CGG TGC TTC CTC TCG AGG TA	60	69	qPCR-TaqMan chemistry	(Thomas, S. H., Padilla-Crespo E, Jardine PM, Sanford RA, 2009)
Desulfitobacterium					
Dsb174F, Dd1 Dsb1373R, Dd2	AAT ACC GNA TAA GCT TAT CCC TAG CGA TTC CGA CTT CAT GTT C	55	1199	PCR	(El Fantroussi, Mahillon, Naveau, & Agathos, 1997)
Dsb460F, Dd3 Dsb1084R, Dd4	TCT TCA GGG ACG AAC GGC AG CAT GCA CCA CCT GTC TCA T	55	624	PCR	(El Fantroussi et al., 1997)
Dsb406F Dsb619R	GTA CGA CGA AGG CCT TCG GGT CCC AGG GTT GAG CCC TAG GT	58	213	PCR, qPCR-SYBR chemistry, DGGE	(Smits et al., 2004)
dsb434f dsb1299r	TAC TGT CTT CAG GGA CGA AC TGA GAC CAG CTT TCT CGG AT	60	865	PCR	(Dowideit, K, Scolz-Muramatsu H, Miethling-Graff, R, Vigelahn L, Freygang M, Dohrmann, AB, n.d.)
Dsb406F Dsb619R	GTA CGA CGA AGG CCT TCG GGT CCC AGG GTT GAG CCC TAG GT	55	213	PCR, DGGE	(Smits et al., 2004)
Dsb406F Dsb1373R, Dd2	GTA CGA CGA AGG CCT TCG GGT TAG CGA TTC CGA CTT CAT GTT C	55	967	PCR	(Smits et al., 2004)

Table 2.12. (continued)

Primer pair	Primer sequence (5'-3')	T_a (°C)	Product size (bp)	Assay	Ref
Desulfomonile					
Dsm59F, Dt1 Dsm1054R, Dt2	CAA GTC GTA CGA GAA ACA TAT C GAA GAG GAT CGT GTT TCC ACG A	55	995	PCR	(El Fantroussi et al., 1997)
Dsm205F, Dt3 Dsm628R, Dt4	GGG TCA AAG TCG GCC TCT CGA CG GCT TTC ACA TTC GAC TTA TCG	55	423	PCR	(El Fantroussi et al., 1997)
DSMON85F DSMON1419R	CGG GGT RTG GAG TAA AGT GG CGA CTT CTG GTG CAG TCA RC	62	1334	PCR	(Loy A, Kuse K, Drake HL, 2004)
Geobacter					
Geo564F [e] Geo840R	AAG CGT TGT TCG GAW TTA T GGC ACT GCA GGG GTC AAT A	57	276	PCR, qPCR-SYBR chemistry	(Bedard, Ritalahti, & Loffler, 2007; Cummings et al., 2003; Sanford et al., 2007)
Geo196F Geo999R	GAA TAT GCT CCT GAT TC ACC CTC TAC TTT CAT AG	53	820	PCR	(Sung, 2005)
Geo73f Geo485r	CTT GCT CTT TCA TTT AGT GG AAG AAA ACC GGG TAT TAA CC	59	412	qPCR-SYBR chemistry	(Melanie Duhamel & Edwards, 2006)
Geo196F Geo535R	GAA TAT GCT CCT GAT TC TAA ATC CGA ACA ACG CTT	50	357	PCR, qPCR-SYBR chemistry	(Amos et al., 2007)
Geo63F Geo418R	CAG GCC TAA CAC ATG CAA GT CCG ACC ATT CCT TAG GAC	62	1443	PCE	(Dennis et al., 2003)
Sulfurospirillum and Desulfuromonas					
Sulfuro114f Sulfuro421r	GCT AAC CTG CCC TTT AGT GG GTT TAC ACA CCG AAA TGC GT	59	307	qPCR-SYBR chemistry	(Melanie Duhamel & Edwards, 2006; F E Löffler, Sanford, & Ritalahti, 2005)

Table 2.12. (continued)

Primer pair	Primer sequence (5'-3')	T_a (°C)	Product size (bp)	Assay	Ref
Dsf205F, BB1F Dsf1020R, BB1R	AAC CTT CGG GTC CTA CTG TC GCC GAA CTG ACC CCT ATG TT	58	815		(Frank E Löffler et al., 2000)

T_a; Reported annealing temperature

* Optimized by .

[b] Only detect *Dhgm lykanthroporepellens* BL-DC-9, failed to detect *Dhgm alkenigignens* IP3-3 and *Dhgm* strain NSZ-14

[c] Detect *Dhgm lykanthroporepellens* BL-DC-9, *Dhgm alkenigignens* IP3-3 and *Dhgm* strain NSZ-14

[d] Detect both *Dhgm lykanthroporepellens* BL-DC-9 and *Dhgm alkenigignens* IP3-3, failed to detect *Dhgm* strain NSZ-14

[e] Reported to also amplify *Anaeromyxobacter* 16S rRNA genes

Table 2.13. Primers designed to detect and/or quantify RDase genes.

Primer pair	Primer sequence (5'-3')	Assay	Ref
pceA			
DET0318-484f DET0318-664r	ATGGTGGATTTAGTAGCAGCGGTC ATCATCAAGCTCAAGTGCTCCCAC	qPCR-SYBR chemistry	(Fung, Morris, Adrian, & Zinder, 2007)
pceAF pceAR	GGA GTG TAA TCC CGC TTT ATC AAT TTC CAC TGT TGG CCT TGT	qPCR-SYBR chemistry	(Reinhold A, Westermann M, Seifert J, von Bergen M & G., 2012)
tceA			
tceAF tceAR tceAP	ATCCAGATTATGACCCTGGTGAA GCGGCATATATTAGGGCATCTT TGGGCTATGGCGACCGCAGG	qPCR-Taqman chemistry	(Holmes et al., 2006; Johnson, Lee, Holmes, Fortin, & Alvarez-Cohen, 2005; Kirsti M Ritalahti et al., 2006)
tceA-500 F tceA-795R	TAATATATGCCGCCACGAATGG AATCGTATACCAAGGCCCGAGG	qPCR-SYBR chemistry	(Fung et al., 2007)
bvcA			
bvcAF – Bvc925F bvcAR – Bvc1017R bvcAP – Bvc977Probe	AAAAGCACTTGGCTATCAAGGAC CCAAAAGCACCACCAGGTC TGGTGGCGACGTGGCTATGTGG	qPCR-Taqman chemistry	(Daprato, Löffler, & Hughes, 2007; Krajmalnik-Brown et al., 2004; Kirsti M Ritalahti et al., 2006)
Forward Reverse Probe	GGTGCCGCGACTTCAGTT TCGGCACTAGCAGCAGAAATT TGCCGAATTTTCACGACTTGGATGAAG	qPCR-Taqman chemistry	(Holmes et al., 2006; Johnson, Lee, Holmes, Fortin, et al., 2005)
vcrA			
Forward Reverse	TGCTGGTGGCGTTGGTGCTCT TGCCCGTCAAAAGTGGTAAAG	qPCR-SYBR chemistry	(Müller JA, Rosner BM, Von Abendroth G, Meshulam-Simon G, McCarty PL et al., 2004)
Forward Reverse Probe	CTCGGCTACCGAACGGATT GGGCAGGAGGATTGACACAT CGCACTGGTTATGGCAACCACTC	qPCR-Taqman chemistry	(Holmes et al., 2006; Johnson, Lee, Holmes, Fortin, et al., 2005)
cbrA			
cbdbA84_f cbdbA84_r	CTTATATCCTCAAAGCCTGA TGTTGTTGGCAACTGCTTC	qPCR-SYBR chemistry	(A. Wagner et al., 2009)

Table 2.13 (continued)

Primer pair	Primer sequence (5'-3')	Assay	Ref
crfA			
cfrA-413f cfrA-531r	CCCGAACCTCTAGCACTTGTAG ACGGCAAAGC TTGCACGA		(Tang & Edwards, 2013)
dcrA			
dcrA-424f dcrA-533r	AGCACTCAGAGAGCGTTTTGC CAACGGCCCAGCTTGCAT	qPCR-SYBR chemistry	(Tang & Edwards, 2013)
mbrA			
mbrAF *mbrAR*	CCTGTAAACGACTCCCCAGA GGATTGGATTAGCCAGCGTA	qPCR-SYBR chemistry	(Chow et al., 2010)
pcbA1, pcbA4, pcbA5			
CG1-17F CG1-17R	CCGTCAATGGCACTCTGTTCCTTC TGCTGGCTTCATTCTCGAAGATCAG	qPCR-SYBR chemistry	(Wang, Chng, Wilm, et al., 2014)
CG4-1F CG4-1R	GGCACAGATGCCTCAAGGAACATAC TTGTCCGGCTGCTCCGTCAG	qPCR-SYBR chemistry	(Wang, Chng, Wilm, et al., 2014)
CG5-1F CG5-1R	TGACCAAGGATCTGGTGGAAGGTTG AGAAGCGCAATGCCTGAGTGATC	qPCR-SYBR chemistry	(Wang, Chng, Wilm, et al., 2014)

Chapter 3

Identification and environmental distribution of *dcpA*, which encodes the reductive dehalogenase catalyzing the dichloroelimination of 1,2-dichloropropane to propene in organohalide-respiring *Chloroflexi*

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Abstract

Dehalococcoides mccartyi (*Dhc*) strains KS and RC grow with 1,2-dichloropropane (1,2-D) as an electron acceptor in enrichment cultures derived from hydrocarbon-contaminated and pristine river sediments, respectively. Transcription, expression, enzymatic and PCR analyses implicated the reductive dehalogenase gene *dcpA* in 1,2-D dichloroelimination to propene and inorganic chloride. Quantitative real-time PCR (qPCR) analyses demonstrated *Dhc* cell increase during growth with 1,2-D and suggested that both *Dhc* strains carried a single *dcpA* gene copy per genome. *Dhc* strain RC and strain KS produced $1.8 \pm 0.1 \times 10^7$ and $1.4 \pm 0.5 \times 10^7$ cells per µmole of propene formed, respectively. The *dcpA* gene was identified in 1,2-D-to-propene-dechlorinating microcosms established with sediment samples collected from different geographical locations in Europe and North and South America. Clone library analysis revealed two distinct *dcpA* phylogenetic clusters, both of which the *dcpA* gene-targeted qPCR assay captured, suggesting the qPCR assay is useful for site assessment and bioremediation monitoring at 1,2-D-contaminated sites.

Introduction

1,2-dichloropropane (1,2-D) has been used in a variety of applications including as an industrial solvent, a lead scavenger in gasoline, and a fumigant to prevent root nematode damage to high value crops (Agency for Toxic Substances and Disease Registry (ATSDR), 1989). In addition, 1,2-D is a precursor in the production of other chlorinated solvents and is produced as a

byproduct in the manufacture of propylene oxide and epichlorohydrine (Nijhuis, Makkee, Moulijn, & Weckhuysen, 2006). 1,2-D is toxic and a suspected carcinogen and the U.S. Environmental Protection Agency (EPA) regulates its maximum concentration level (MCL) in drinking water to 5 µg/L (http://water.epa.gov/drink/contaminants/index.cfm#List). Today, 1,2-D is no longer used as a solvent and in soil fumigant applications; however, the 2010 EPA's Toxics Release Inventory reported that 1,2-D in excess of 90,000 pounds were disposed of or released in the U.S. (http://iaspub.epa.gov/triexplorer/tri_release.chemical). A 2006 study, conducted by the National Water-Quality Assessment Program (NAWQA) and lead by the U. S. Geological Survey (USGS), detected 1,2-D in 1.3% of aquifers samples throughout the U.S. (at an assessment level of 0.02 µg/L). In 18 of 723 (2.5 %) shallow groundwater samples adjacent to agricultural areas, 1,2-D was present at concentrations exceeding 0.2 µg/L. In some aquifer samples and domestic wells, 1,2-D was reported at levels above the 5 µg/L MCL (Zogorski JS, Carter JM, Ivahnenko T, Lapham WW, Moran MJ, Rowe BL, Squillace PJ, 2006). The NAWQA aquifer study detected fumigants in 10 - 30% of the aquifers sampled in areas where fumigant applications were common such as Oahu, Hawaii, and the Central Valley of California (Zogorski JS, Carter JM, Ivahnenko T, Lapham WW, Moran MJ, Rowe BL, Squillace PJ, 2006). Hawaii used more than 1.8 million pounds of fumigants in the 1970's to protect pineapple crops from root-parasitic nematodes (Honolulu & Hawaii., 1975). Today, 1,2-D use is controlled and new contamination minimized; however, 1,2-D has emerged as a pervasive groundwater contaminant and threatens environmental health and drinking water quality.

1,2-D is recalcitrant under oxic conditions but a few strictly anaerobic bacteria have been implicated in 1,2-D reductive dechlorination to non-toxic propene. These microorganisms use 1,2-D as terminal electron acceptor for organohalide respiration. Microbes involved in this process include a *Dehalobacter* identified in a mixed culture derived from river sediment (Schlötelburg C, Wintzingerode C, Hauck R, Wintzingerode F, Hegemann W, 2002), the bacterial isolate *Desulfitobacterium dichloroeliminans* strain DCA1 obtained from an industrial site impacted with 1,2-dichloroethane (1,2-DCA) (De Wildeman S, Diekert G, Van Langenhove H, Verstraete, 2003), *Dehalogenimonas lykanthroporepellens* (*Dhgm*) strains BL-DC-8 and BL-DC-9 and *Dehalogenimonas alkenigignens* strain IP3-3 isolated from groundwater collected at a Superfund site (Bowman, Nobre, da Costa, Rainey, & Moe, 2013; Moe WM, Yan J, Nobre MF,

da Costa MS, Rainey, 2009) and *Dhc* strains RC and KS derived from pristine and contaminated river sediment, respectively (F E Löffler, Champine, Ritalahti, Sprague, Tiedje, et al., 1997; Kirsti M Ritalahti & Löffler, 2004). *Dhc* strains RC and KS share up to 99.8% 16S rRNA gene sequence identity with *Dhc* strains of the Pinellas group, which cannot grow with 1,2-D (Kirsti M Ritalahti & Löffler, 2004). The incongruence between the 16S rRNA gene sequence and reductive dechlorination activity demonstrates the need for identifying 1,2-D dechlorination-specific biomarkers.

Reductive dechlorination reactions are catalyzed by reductive dehalogenases (RDases). *Dhc* genomes contain multiple RDase genes (e.g., 11 in strain BAV1, 36 in strain VS) (Löffler FE, Yan J, Ritalahti KM, Adrian L, Edwards EA, Konstantinidis KT, Müller JA, Fullerton H, Zinder SH, 2013). Few *Dhc* RDases have assigned function and all of them share common characteristics such as a Tat signal peptide close to the N-terminus, two iron sulfur clusters close to the C-terminus, and an adjacent B gene. To date, only a few RDases have been biochemically characterized and implicated in specific dechlorination reactions, and an RDase responsible for 1,2-D-to-propene dechlorination has not been identified. In this study, RDase transcript, cDNA clone libraries and expression analysis implicated DcpA in 1,2-D reductive dechlorination to propene. A *dcpA*-targeted qPCR approach correlated *dcpA* presence with 1,2-D reductive dechlorination activity indicating that *dcpA* serves as a biomarker for 1,2-D reductive dechlorination.

Methods

Microcosms and enrichment cultures.

Details on sample collection and site information are provided as Supplemental Material and given in Table 3.1. Microcosms were prepared using completely synthetic, reduced mineral salts medium following established procedures (Jianzhong He et al., 2002) with the following modifications: One g (wet weight) of solids were transferred to sterile 60-mL (nominal capacity) glass serum bottles inside an anoxic chamber (N_2/H_2, 97/3%, vol/vol),, lactate and vitamin B_{12}

were added to a total volume of 40 mL to achieve final concentrations of 5 mM and 50 μg per liter, respectively. Microcosms established with groundwater were initiated with 20 mL of groundwater plus 19 mL of medium. 1,2-D was added to each bottle to obtain a final aqueous phase concentration of 0.2 mM. All microcosms were prepared in duplicates and incubated statically, in the dark, at room temperature. After all of the 1,2-D was dechlorinated to propene, 3% inocula [vol/vol] were transferred to glass vessels containing fresh medium. After four consecutive transfers, all solids were removed. Propene and 1,2-D concentrations were monitored by manually injecting 0.1 mL headspace samples into a HP 7890 gas chromatograph (GC) equipped with a DB-624 column (60 m length, 0.32 mm diameter; film thickness of 1.8 μm nominal) and a flame ionization detector (FID) as described (Amos, Christ, Abriola, Pennell, & Löffler, 2007). To verify propene formation, additional GC measurements were made with an Agilent HP-PLOT/Q column (30 m length, 0.53 mm diameter; 40 um of film thickness), which resolves propene from other C_1-C_3 alkanes and alkenes.

DNA isolation.

The DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) was used to extract DNA from sediment-free cultures, with modifications to improve cell lysis (Kirsti M Ritalahti & Löffler, 2004). DNA from solid and groundwater samples used the MO BIO Power Soil DNA kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) and the PowerWater DNA Isolation Kit (MO BIO Laboratories Inc.), respectively, following the manufactures recommendations.

RNA isolation and preparation of cDNA libraries.

Biomass was collected from 10-20 mL of RC and KS culture suspensions, when 50-75% of the initial 1,2-D dose had been converted to propene. Cells were harvested by vacuum filtration onto a Durapore hydrophilic polyvinylidene fluoride membrane (25 mm diameter and 0.22 μm pore size) (Millipore, Billerica, MA). RNA extraction, DNase treatment, cDNA synthesis and purification were performed as described (K M Ritalahti, Cruz-García, Padilla-Crespo, Hatt, & Löffler, 2009). cDNA libraries were established with degenerate primers B1R and RR2F

targeting *Dhc* RDase genes (Krajmalnik-Brown et al., 2004). Primer walking procedures extended the partial *dcpA* and *dcpB* genes (see Supplemental Material for details).

Quantitative real-time PCR (qPCR)

Dhc and *Dhgm* 16S rRNA gene-targeted PCR assays followed established protocols (see Table 3.2 for primer information and references). The IDT DNA Primer Quest software (http://scitools.idtdna.com/Primerquest/) was used to design qPCR primers *dcpA*-1257F and *dcpA*-1449R with TaqMan probe *dcpA*-1426Probe to enumerate *dcpA* gene copies (Table 3.2). First, the primers were used with SYBR Green chemistry to recognize non-specific amplification and/or primer-dimer formation (Hatt & Löffler, 2011). Standard curves were generated with ten-fold serial dilutions (10^8 to 10^0 copies) of the partial *Dhc* strain KS *dcpAB* gene fragment cloned into the TOPO TA (Invitrogen) pCRII plasmid. To confirm target specificity, melting curves were obtained with genomic template DNA from *Dhc* strains RC and KS and *Dhgm* strain BL-DC-9.

Additionally, the qPCR amplicons were resolved in 1% agarose gels run at 120 volts for 30 min to assess amplicon size target-specific amplification. Reactions with sterile water (no-template DNA) and with genomic DNA from *Dhc* strain GT, which does not possess the *dcpA* gene, served as negative controls. After validation and optimization in SYBR Green qPCR, the primers were used with the TaqMan probe *dcpA*-1426 in assays as described (Kirsti M Ritalahti et al., 2006). All assays exhibited amplification efficiencies between 100 ± 10%; (i.e., slope within -3.6 and -3.1), consistency across replicate reactions, and linear standard curves (R^2 > 0.980) (ABI, 2005; Bustin et al., 2009). A quantification limit of 30 copies per reaction was determined based on fluorescence signals above the cycle threshold value of 0.2 within the first 38 PCR cycles measured in 20 replicate assays. The lowest value of the standard curve (3 copies per reaction) was the detection limit, and all non-template control assays fell below this value. The qPCR assay conditions for the reactions targeting the *Dhc* 16S rRNA gene have been published (Kirsti M Ritalahti et al., 2006). All known *Dhc* genomes possess one 16S rRNA gene copy and the enumeration of this gene is used to determine the *Dhc* cell numbers (Kirsti M

Ritalahti et al., 2006). The number of *dcpA* genes per *Dhc* genome was calculated by dividing the number of *dcpA* genes copies detected per mL of culture by the total number of *Dhc* 16S rRNA genes detected in the same culture volume.

For transcriptional analysis, cDNA generated from active 1,2-D-dechlorinating cultures served as template. All qPCR data were corrected for the loss (i.e., % recovery) of luciferase transcripts, which were used as an internal standard (Johnson, Lee, Holmes, Alvarez-Cohen, & V. F. Holmes, 2005) and *rpoB* transcripts were quantified as a measure of general metabolic activity as described (Fung, Morris, Adrian, & Zinder, 2007). The *rpoB* housekeeping gene is conserved in *Dhc* genomes and *rpoB* transcripts were quantified as a measure of general metabolic activity and for normalization (Fung, Morris). When applicable, *dcpA* transcript abundances were normalized to *rpoB* or to *dcpA* gene copy numbers. Starved cultures (i.e., cultures that had consumed all 1,2-D for at least 1 month) served as baseline controls for the transcriptional studies. All samples were analyzed in triplicate at two dilutions (1:10 and 1:100) using the ABI 7500 fast quantitative real-time PCR (qPCR) instrument (Applied Biosystems) and the reported values represent the average of at least three biological replicate cultures (i.e., six pseudo-replicates per sample, or 18 datasets for the three biological replicates).

Cloning dcpA sequences from environmental samples and phylogenetic analysis.

Primers *dcpA*-360F and *dcpA*-1449R were designed for standard PCR reactions to detect, clone and sequence *dcpA* genes from samples of interest (Table 3.2). These primers were designed based on alignments of the *dcpA* sequences retrieved from the cDNA libraries of *Dhc* strains RC and KS and the *dcpA* gene (Dehly_1524) of *Dhgm* strain BL-DC-9. *dcpA* clone libraries were established using DNA isolated from the original sediment and groundwater samples listed in Table 3.1. Environmental DNA samples were subjected to nested PCR and using an initial PCR amplification reaction with 2 μL of undiluted or 1:10 diluted template DNA with the degenerate primers B1R and RRF2 as described . Subsequently, a second (nested) round of PCR using the *dcpA*-specific primers *dcpA*-360F and *dcpA*-1449R was performed using 2 μL of the DNA solution obtained from the first round of amplification (see Figure 3.1 for approximate binding

sites for primers RRF2 and B1R as well as the *dcpA* specific primers). The expected amplicon size generated in nested PCR was 1,089 bp. The *dcpA*-specific PCR reactions consisted of (final concentrations) 1x PCR buffer, 2.5 mM of $MgCl_2$, 250 μM of each deoxynucleoside triphosphate (ABI), 250 nM of each primer, and 2.5 U of AmpliTaq polymerase (ABI). The following temperature program was used to amplify the *dcpA* gene: 94°C for 2 min 10 s (1 cycle); 94°C for 30 s, 56.0°C for 45 s, and 72°C for 2 min 10 s (30 cycles); and 72°C for 6 min. The *dcpA* amplicons were cloned in the pCRII TOPO vector and transformed into *E. coli* TOP'10 competent cells (TOPO TA cloning kit, Invitrogen) following the manufacturer's recommendations. The QIAprep Spin Miniprep kit (Qiagen, Valencia, CA, USA) was used for plasmid isolation, the inserts were sequenced using primers M13F and M13R (http://tools.invitrogen.com/content/sfs/manuals/nupage_tech_man.pdf), and the DNA nucleotide sequences were translated (http://web.expasy.org/translate/) and aligned using ClustalW (J. D. Thompson & Higgins, 1994) in MEGA version 5 (Tamura et al., 2011). Phylogenetic relationships were calculated from a total of 53 amino acid (aa) sequences using the neighbor joining tree method (Saitou & Nei, 1987) and evolutionary distances were computed using the number of differences method (Nei & Kumar, 2000). Branch support values were estimated with a bootstrap test (1,000 replicates) (Felsenstein, 1985). The 53 translated nucleotide sequences used in the phylogenetic tree comprised 48 partial *dcpA* sequences (~1 kb in length) obtained from environmental samples, the complete *dcpA* sequence of *Dhgm* strain BL-DC-9 RC, *Dhc* strain RC and strain KS, the translated nucleotide sequence of the pseudogene DET0162, and DET1538, encoding a putative RDase with unknown function (both identified on the genome of *Dhc* strain 195 (Seshadri et al., 2005). DET1538 served as the out-group for phylogenetic analyses.

Protein assays and BN-PAGE

Dhgm strain BL-DC-9 was used for *in* vitro enzyme assays, BN-PAGE and proteomics workflows because strain BL-DC-9 is a pure culture and its genome sequence is available. Strain BL-DC-9 harboring the *dcpA* gene was grown with 0.5 mM 1,2-D. Cells were harvested by centrifugation (10, 000 x g for 20 min at 4°C) and lysed by bead beating (Tang S, Chan WW,

Fletcher KE, Seifert J, Liang X, Löffler FE, Edwards EA, 2013). The crude extracts were subjected to Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) to detect reductive dechlorination activity in gel slices following electrophoretic separation. Activity assays with individual gel slices were performed as described (Tang S, Chan WW, Fletcher KE, Seifert J, Liang X, Löffler FE, Edwards EA, 2013) with minor modifications (see Supplemental Material). In the activity assays, the positive controls consited of 1 mL of pelleted cell culture suspended in assay buffer while negative control vials received not protein.

Two dimensional liquid chromatography-tandem mass spectrometry (2D LCMS/MS).

BN-PAGE enzyme assays were combined with proteomic workflows to identify RDase peptides present in the gel slices showing 1,2-D dechlorination activity. Identified peptides sequences were matched to the closed genome sequence of *Dhgm* strain BL-DC-9 (NC_014314.1) using MyriMatch Tabb, Fernando, & Chambers, 2007) along with the IDPicker software to translate spectra into peptide sequences (Holman, Ma, & Tabb, 2012). Detailed information on sample preparation for 2D-LC-MS/MS analysis and database parameters are available in the Supplemental Material.

Computational analyses.

The DcpA sequences were analyzed for secretory signal peptides using the TatP (www.cbs.dtu.dk/services/TatP) and SignalP (http://www.cbs.dtu.dk/services/SignalP/) programs. The Compute pI/Mw program (http://us.expasy.org/tools/pi_tool.html) was used to predict the molecular weight and isoelectric point of DcpA. The DcpB sequences were analyzed with TMMOD (http://www.liao.cis.udel.edu/website/servers/TMMOD/) to predict protein topology based on transmembrane motifs. The presence of corrinoid and ribosome binding sites, and a putative Dehalobox (a stretch of nucleotides that resemble the FNR-box that bind to the promoter for transcription initiation) (Smidt H, van Leest M, van Der Oost J, de Vos WM, 2000) were identified by visually inspecting the nucleotide sequences and by manually aligning the regions with known motifs.

Nucleotide sequence accession numbers.

The *dcpA* gene sequences from *Dhc* strain KS and RC are deposited in GenBank under the accession numbers JX826286 and JX826287, respectively. The 48 *dcpA* sequences obtained from environmental samples have the accession numbers JX913691-JX913735 and KC906160-KC906162.

Results

cDNA libraries identify the 1,2-D RDase gene

Using template cDNA derived from the RNA pool of the 1,2-D-grown cultures RC and KS, PCR with the degenerate RDase-targeted primers B1R and RR2F yielded amplicons of approximately 1,500 base pairs (bp) in length (Figure S3.1). No amplification occurred when RNA prior to the RT step was used as PCR template, confirming that all genomic DNA had been removed from the RNA pool (Figure S3.1). Among 200 *E. coli* clones screened from the B1R and RR2F amplicon-derived clone libraries of cultures RC and KS, 12 and 10, respectively, had vectors carrying cDNA fragments of the expected size of ~1,500 bp. Sequence analysis of the 10 KS cDNA library clones with an insert of the correct size revealed a single 1,486 bp long sequence. The sequence included the nearly complete RDase A and a partial RDase B gene indicating that these genes were co-transcribed. An open reading frame corresponding to the RDase B gene start was found 18 nucleotides downstream of the RDase A gene TAA stop codon, and included 35 nt of the adjacent RDase B gene. Analysis of the 12 RC cDNA library clones revealed eight inserts with the same 1,486 bp insert found in the KS cDNA library clones and four clones had a 1,589 bp-long insert. The 1,589 bp-long insert consisted of 1,472 bp of the partial RDase A gene, 19 nt of intergenic region and 98 bp of a partial RDaseB gene.

The 1,486 bp insert cloned from both *Dhc* strain RC and strain KS genes showed 90% sequence identity to a putative RDase gene (gene tag ID: Dehly_1524) found in the genome of the 1,2-D-dechlorinating *Dhgm* strain BL-DC-9. To rule out the presence of a *Dhgm*-type population in the RC and KS cultures, PCR with primers targeting the *Dhgm* 16S rRNA gene was performed;

however, DNA from cultures RC and KS failed to produce an amplicon confirming that *Dhgm* was not present in these cultures. The second 1,589 bp-long insert found in four RC clones was 99.9% similar to the putative RDase gene RCRdA02 (EU266045). This gene was almost identical (98-99%) to the *Dhc* RDase genes DehalGT_1352 (CP001924), cbdbA1638 (AJ965256), KSRdA02 (EU266035) and also demonstrated high similarity (96% nt identity) to FL2RdhA6 in *Dhc* strain FL2 (AY374250). Additionally, RCRdA02 shared 98-99% nt sequence identity with RDase genes retrieved from the KB-1 and TUT2264 dechlorinating mixed cultures (KB1RdhAB5; DQ177510 and TUT2264_rdhA2; AB362921) and from a contaminated site (FtL-RDase-1638; EU137843). At the nucleotide level, no gene in *Dhgm* strain BL-DC-9 genome shared similarity with RCRdA02.

Protein assays and LC-MS/MS analysis

After BN-PAGE separation of *Dhgm* strain BL-DC-9 proteins, a gel slice representing the section below the 75 kDa marker demonstrated 1,2-D-dechlorinating activity (Figure 3.2A, slice 4). Coomassie staining revealed that this gel slice comprised multiple polypeptides (i.e., several visible bands) near the 75-55 kDa size marker, and a major band of 45.5 kDA (Figure 3.2A). Subsequent SDS-PAGE separation of the proteins eluted from this gel piece exhibited protein bands with masses of 75, 63 and 50 kDa (Figure 3.2B, slice N1, N2 and N3).

LC-MS/MS analysis of the proteins separated by SDS PAGE identified 15 *Dhgm* strain BL-DC-9 proteins (Table 3.3, slices N2 and N3). In the N1 gel section, protein levels were too low for confident identification, while in the gel section around 50 kDa (i.e., slice N3), Dehly_0337 (annotated as a translation elongation factor Tu) and Dehly_1524 (annotated as RDase) were the dominant proteins based on peptide spectral abundances (Table 3.3). Other peptides associated with gel slice N3 included subunits of the nickel-dependent hydrogenases encoded by Dehly_0929 and Dehly_0726 (Table 3.3). Among all the protein fragments recovered from slices N2 and N3, the highest coverage and spectral counts belonged to Dehly_1407, a chaperonin GroEL protein (Table 3.3, slice N2). Dehly_1524 was the only RDase associated

with the gel slices (Table 3.3), corroborating that this enzyme catalyzes 1,2-D-to-propene dichloroelimination. The 1,2-RDase was designated DcpA, encoded by the *dcpA* gene.

Primer walking and characterization of dcpAB gene cassette.

Since the degenerate primer pair B1R and RRF2 did not amplify the complete *dcpA* and *dcpB* gene sequences, the entire *dcpAB* genes from strains *Dhc* RC and KS were obtained using primer walking approaches. The application of primer *dcp_up*120F and *dcpA*-1449R yielded ~1,569 bp-long PCR products and extended the sequence 89 bp upstream of the ATG start codon (Figure S3.2). The final and complete assembly of the *dcpA* gene sequences derived from *Dhc* strains RC and KS were nearly identical (99.8% nt sequence identity), and the translated protein sequence differed by only a single aa at position 85 (i.e., a serine (S) in strain RC is replaced by a leucine (L) in strain KS). Inspection of the region upstream of the start codon identified the putative ribosome-binding site (RBS or Shine-Dalgarno sequence) 'AGAGG' starting nine nucleotides upstream of the *dcpA* start codon and a putative Dehalobox was identified 67 nt upstream of *dcpA*. Primer walking procedures also extended *dcpB* through the TAA stop codon (an additional 184 bp). The final assembly of the *dcpB* gene of *Dhc* strains RC and KS revealed that both sequences shared 99% sequence identity and their corresponding proteins differed in only one aa where glutamine (Q) was replaced by a glutamic acid (E) in strain RC at position eleven (Figure S3.3). Therefore, the motif WYXW, conserved in other RDase B proteins, is present in the form WYEW in *Dhc* strain RC and in the form WYQW in *Dhc* strain KS and *Dhgm* strain BL-DC-9 (Figure S3.3). The final assembly of the *dcpAB* gene cassette of *Dhc* strains RC and KS consisted of a 1,455 nt (484 aa) long *dcpA* gene and a 219 nt (73 aa) long *dcpB* gene separated by an 18 nt spacer.

Computational characterization of DcpA and DcpB.

A Tat signal peptide RRDFMK starting at position nine with a predicted peptide cleavage site between the aa positions 30 and 31 was identified (Figure S3.4). The mature DcpA protein (i.e., after cleavage of the signal peptide) in *Dhc* strain RC and strain KS had a predicted isoelectric

point (pI) of 5.99 and a molecular weight of 50.8 kDa. The corrinoid-binding motif DxHxxG-x_{41-42}-SxL-x_{24-28}-GG found in several corrinoid-containing enzymes from prokaryotes (Ludwig & Matthews, 1997) was not present in DcpA but a putative corrinoid-binding sequence DHXG-X_{39}-S-X_{32}-G close to the C-terminus was identified (Figure S3.4). DcpA of both *Dhc* strain RC and strain KS share two identical iron sulfur cluster-binding motifs $FCX_2CX_2CX_3CP$ and CX_2CX_3C (Figure 3.1 and Figure S3.4).

The topology of the DcpB protein revealed two predicted transmembrane regions spanning from positions 12-32 and 41-61 (Figure S3.3). Additional characteristics included two inside loops (i.e., facing the cytoplasm) from aa positions 1-11 and 62-73, and one outside loop (i.e., facing the periplasm) from positions 33-40 (Figure S3.3). Furthermore, a putative RBS site 'AGAGG' for initiation of translation was detected in the small 18-nt intergenic region separating *dcpA* and *dcpB.*

dcpA Sequence similarity to other RDase genes.

The *dcpA* gene of *Dhc* strains RC and KS and *Dhgm* strain BL-DC-9 (Dehly_1524) shared 60% overall nt identity to pseudogene DET0162 identified on the genome of *Dhc* strain 195, and an even greater sequence identity of 66% (260 out of 395 nt) occurred near the 3' end. Pseudogene DET0162 is 1,464 nt long and has a point mutation that results in a premature stop codon leading to a truncated, 59 aa-long polypeptide. If completely translated, the gene would encode a putative RDase of approximately 486 aa in length with all the common RDase features, including the Tat RRDFMK motif near the N-terminus and the two $FCX_2CX_2CX_3CP$ and CX_2CX_3C iron sulfur cluster-binding motifs near the C-terminus. The 486 aa protein would have 45% aa identity to the DcpA of strain RC and strain KS, and 46% to the DcpA of strain BL-DC-9. Located 129 bp downstream of the 3' end of the pseudogene DET0162 is a characteristic B gene (DET0163). This B gene also shared 55 - 56% nt sequence identity (44 - 47% aa sequence identity) with the *dcpB* of *Dhgm* strain BL-DC-9 (Dehly_1525) and of *Dhc* strains RC and KS.

The *Dhc* DcpA proteins shared 92% sequence identity and 95% sequence similarity with the *Dhgm* strain BL-DC-9 DcpA protein (Dehly_1524) (Figure S3.5). DcpA shared no more than 34% aa sequence identity with other *Chloroflexi* RDases and other RDase sequences deposited in databases. Inspection of the recently closed genome of the 1,2-D-to-propene-dechlorinating *Desulfitobacterium dichloroeliminans* strain DCA1 (LMG P-21439) (http://www.ncbi.nlm.nih.gov/genome/?term=txid871963) revealed no RDase with greater than 18% aa sequence identity to DcpA.

***dcpA*-Targeted PCR and qPCR**

PCR with primers *dcpA*-360F and *dcpA*-1449R produced a single amplicon of the expected size of 1,089 bp when applied to genomic DNA from cultures RC and KS and *Dhgm* strain BL-DC-9. No amplicons were obtained with template DNA from *Dhc* strain GT, which cannot dechlorinate 1,2-D. qPCR standard curves generated with primers *dcpA*-1257F and *dcpA*-1449R using SYBR Green reporter chemistry exhibited linear amplification ranging from 1.7 to 1.7 x 10^8 16S rRNA gene copies per µL of template DNA (e.g., slope = -3.4, y intercept = 36.6 and R^2 = 0.999). Melting curve analyses of amplicons generated with genomic DNA from *Dhc* culture RC, culture KS and *Dhgm* strain BLDC-9 showed a single, symmetric peak (suggesting a single PCR product) with average melting temperatures (T_m) of 78.5±0.1, 79.2 ± 0.1, and 78.6 ± 0.1, respectively (Figure S3.6). TaqMan qPCR assays with the same primer pair combined with *dcpA*-1426Probe also exhibited linear amplification over the same range of template DNA concentrations (e.g., slope = -3.5, y intercept = 39.5 and R^2 = 0.997) (Figure S3.7).

TaqMan qPCR demonstrated that *Dhc* cell numbers increased during cultivation with 1,2-D as electron acceptor. Cultures RC and KS produced 1.8 ± 0.1 x $10^7$and 1.4 ± 0.5 x 10^7 *Dhc* cells per µmole of Cl^- released. The enumeration of *dcpA* and *Dhc* 16S rRNA genes in replicate cultures of *Dhc* strains RC and KS demonstrated that both gene targets occurred in similar abundances (in 1 mL of culture of *Dhc* strain KS 6.6 E7 ± 0.4 E7 16S rRNA genes and 6.2 E7 ± 0.4 E7 of *dcpA* genes were detected; while *Dhc* strain RC 6.1 E7 ± 0.2 E7 16S rRNA genes and 5.2 E7 ± 0.3 E7 *dcpA* genes were detected per mL of culture) indicating that the *Dhc* genomes harbored a single

dcpA gene copy The *dcpA*-targeted primers *dcpA*-1257F and *dcpA*-1449R were also used to quantify the *dcpA* transcript abundances in *Dhc* cultures RC and KS. The RT-qPCR results showed the upregulation of *dcpA* gene transcription in actively dechlorinating RC and KS cultures as compared to cultures that had consumed all 1,2-D (Figure 3.3).

Application of dcpA PCR and qPCR assays to microcosm and environmental samples.

Propene was detected in 1,2-D-amended microcosms established with five out of the 13 sample materials tested (Table 3.1). In contaminated sediments from Third Creek, TN, the *dcpA* gene increased from below the quantification limit to $5.6 \pm 0.1 \times 10^6$ copies per mL in propene producing sediment-free enrichment cultures. Nested PCR assays detected both *Dhgm* and *Dhc* 16S rRNA genes in the initial Third Creek sediment samples. qPCR assays indicated that *Dhc* and *Dhgm* populations also increased from below the quantification limit in the initial samples to $5.6 \pm 0.6 \times 10^6$ and $9.7 \pm 0.3 \times 10^3$ gene copies per mL of sediment-free enrichment culture, respectively.

Nested PCR detected the *dcpA* gene in all aquifer and sediment samples that yielded 1,2-D-dechlorinating microcosms. The only microcosms with positive *dcpA* detection but without propene formation were established with aquifer materials from the site at Barra Mansa, Rio de Janeiro, Brazil. The *dcpA* gene was not detected in site materials collected from the Waynesboro site and in Ft. Pierce groundwater samples collected outside of a 1,2-D plume. Consistent with the absence of the *dcpA* gene, the microcosms established with these materials failed to dechlorinate 1,2-D (Table 3.1). Interestingly, three of four wells collected inside the 1,2-D plume at the Ft. Pierce site tested positive for *dcpA*, consistent with the detection of propene at these well locations. Figure S3.8 depicts the PCR results from microcosm and environmental samples.

dcpA gene diversity.

The primer pair *dcpA*-360F and *dcpA*-1449R retrieved nearly complete (~1 kb) *dcpA* amplicons with template DNA extracted from seven distinct sample materials and cloning and sequencing efforts generated 48 *dcpA* sequences (Figure 3.4). Distance analysis of the DcpA sequences (total of 247 aa analyzed) and high bootstrap values indicated that the sequences formed two distinct phylogenetic clusters. Cluster 1 included 33 environmental DcpA sequences most similar (93-95%) to the *Dhgm* DcpA (*Dehly_1524*), and Cluster 2 comprised 15 DcpA sequences most similar (95-99%) to the DcpA of *Dhc* strains RC and KS. All but one of the 48 DcpA sequences contained both of the characteristic iron-sulfur cluster binding motifs ($FCX_2CX_2CX_3CP$ and CX_2CX_3C) (Figure S3.9). Overall, the translated environmental *dcpA* sequences differed by 5-7% from the DcpA of *Dhgm* strain BL-DC-9 and by 1-8% from the DcpA of *Dhc* strains KS and RC. Interestingly, the deduced DcpA sequences from different continents (i.e., Europe and South America) shared >98% sequence identity indicating that this RDase is either highly conserved or has been recently disseminated.

Discussion

Dhgm and *Dhc* populations have been implicated in 1,2-D-to-propene dechlorination(Moe WM, Yan J, Nobre MF, da Costa MS, Rainey, 2009; Kirsti M Ritalahti & Löffler, 2004). Since the *Dhc* 16S rRNA gene provides insufficient resolution to distinguish strains with the ability to transform 1,2-D from strains lacking this trait, a functional biomarker was sought to support site assessment (i.e., are 1,2-D-dechlorinating populations present?) and bioremediation monitoring (i.e., are 1,2-D-dechlorinating populations active?). An integrated approach combining transcription, expression, enzymatic and PCR approaches implicated *dcpA* in 1,2-D dichloroelimination to propene. The *dcpA* gene is co-transcribed with its associated *dcpB* gene indicating that polycistronic mRNA was generated, a feature shared among functional RDases of *Dhc* strains and other organohalide-respiring bacteria (e.g., *Desulfitobacterium dehalogenan*s, (Smidt H, van Leest M, van Der Oost J, de Vos WM, 2000). The putative RBS site preceding both the *dcpA* and the *dcp*B gene has been observed in other RDases (i.e., *cbrA, vcrA*) supporting

that bacterial polycistronic mRNA often possess internal RBS sites to allow translation initiation at multiple sites resulting in efficient protein biosynthesis(Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, 2000). The presence of transcriptional regulatory elements (e.g., a ribosomal binding site and a putative Dehalobox box upstream of the *dcpA* gene start codon) and the results of the experimental transcription studies (cDNA library, RT-qPCR) suggest that *dcpA* gene activity is regulated. qPCR revealed equal abundances of *dcpA* and *Dhc* 16S rRNA genes in cultures RC and KS suggesting that the *dcpA* gene occurs as a single chromosomal copy in *Dhc* strains RC and KS as well as in *Dhgm* strain BL-DC-9.

The *Dhc*- and *dcpA*-targeted qPCR assays linked propene production to *Dhc* growth. The abundances of *Dhc* strain RC and strain KS with 1,2-D as electron acceptor ($1.8 \pm 0.1 \times 10^7$ and $1.4 \pm 0.5 \times 10^7$ cells per mL, respectively) are similar to values reported for *Dhgm* (1.5×10^7 cells per mL) (Yan J, Rash BA, Rainey FA, Moe WM, 2009). Standard Gibbs free energy calculations indicate that 1,2-D dichloroelimination (the removal of two chlorine substituents from adjacent carbon atoms) yields less energy per chlorine atom released than stepwise hydrogenolysis. 1,2-D dichloroelimination is associated with a change in Gibbs free energy of -183 kJ/mol, which is less than the free energy change associated with stepwise hydrogenolysis (i.e., -131 kJ/mol per electron pair or 262.5 kJ/mol total). Theoretically, organisms capable of 1,2-D hydrogenolysis to a monochlorinated propane consume the same amount of H_2 but gain less energy compared to organisms catalyzing dihaloelimination; however, organisms capable of two-step 1,2-D hydrogenolysis to propane gain more energy but require the double amount of H_2. Therefore, under H_2-limiting conditions, 1,2-D dihaloelimination organisms may have an advantage over 1,2-D hydrogenolysis organisms because of the reduced H_2 requirement. All enrichment efforts to date have yielded cultures that directly transform 1,2-D to propene without the intermediate formation of monochlorinated propanes. The reasons why enrichment efforts with 1,2-D as electron acceptor only yielded cultures performing dichloroelimination are unclear but may have to do with deltaG, activation energy, kinetic barriers, the toxicity of monochlorinated propanes as dichloroelimination circumvents their formation, or biased cultivation conditions selecting against bacteria catalyzing stepwise reductive dechlorination (i.e., hydrogenolysis) reactions.

The gene most similar to *dcpA* is the pseudogene DET0162 from *Dhc* strain 195. This pseudogene may be a vestigial remnant of a functional gene that shared a common ancestor with *dcpA*. Interestingly, pseudogene DET0162 has a RBS site ("AGGAG"), and a possible Dehalobox, suggesting this gene is under regulatory control. The pseudogene has the alternate GTG start codon (instead of ATG), which is a less effective starting codon. Interestingly, transcriptional studies targeting all 19 RDases of *Dhc* strain 195 actively dechlorinating PCE or TCE demonstrated that *pceA* (encoding a PCE RDase), *tceA* (encoding a TCE-to-VC RDase) and DET0162 were the only RDase genes upregulated (Fung et al., 2007). Similar results were reported with PCE-dechlorinating mixed cultures containing *Dhc* strain 195, where pseudogene DET0162 and *tceA* were highly upregulated (Brian G Rahm et al., 2006). The pseudogene was also one of only two RDase genes (along with *pceA*) that were found to exhibit high transcript levels in cells grown with 2,3-dichlorophenol (Fung et al., 2007). Even though the transcript levels of the pseudogene significantly increased when PCE, TCE or 2,3-dichlorophenol were provided as electron acceptors to strain 195 cultures, the translated product was never detected in proteomics efforts (Fung et al., 2007), probably because of the stop codon and ensuing proteolysis. The original function of this pseudogene remains speculative. Recently, a nomenclature for RDases has been proposed and DcpA clusters close to the RD_OG20, this group is composed of BAV1_0104, cbdbA88 and DET0311; but this ortolog group shares only 31-33 % aa identity and no more than 45% similarity to DcpA.

RDase characterization has been challenging due to the difficulty obtaining biomass from *Dhc* and *Dhgm* pure cultures, the sensitivity of RDases to oxygen and the lack of a genetic system to heterologously express functional RDases. The utility of the BN-PAGE approach coupled with LC-MS/MS analysis to assign function to RDases has been demonstrated (Tang S, Chan WW, Fletcher KE, Seifert J, Liang X, Löffler FE, Edwards EA, 2013); however, functional assignment based solely on *in vitro* enzyme assays can be potentially misleading because the reduced corrinoid co-factor associated with the RDase can reductively dechlorinate compounds that are not RDase substrates (Anke Neumann et al., 2002). To assign function to an RDase capable of 1,2-D dichloroelimination, a multiple lines of evidence approach was employed, which combined qPCR and RT-qPCR analyses, BN-PAGE, enzyme activity assays, and SDS-PAGE followed by LC-MS/MS analysis. With genome sequence information of organohalide-respiring

bacteria rapidly increasing, this multiple lines of evidence approach will be useful to assign function to more RDase genes.

Protein assays combined with BN-PAGE and LC-MS/MS confirmed that DcpA catalyzed 1,2-D dichloroelimination to propene. The 1,2-D dechlorination activity was detected in gel slice 4 representing the 37-75 kDa size range. A recent study by Tang et al. found the main reductive dechlorination activity following BN-PAGE separation associated with the high molecular weight fraction near the 242 kDa size marker(Tang S, Chan WW, Fletcher KE, Seifert J, Liang X, Löffler FE, Edwards EA, 2013). RDases with assigned function range in size between 49.7 and 57.7 kDa, suggesting that the formation of protein complexes can occur, causing RDase protein(s) to associate with a higher than expected molecular size fraction. Interestingly, some of the peptides that co-migrated with DcpA (e.g., chaperonin GroEL, hydrogenase subunits, and the elongation translation factor Tu) have been shown to be abundant proteins in active *Dhc* cultures (Morris et al., 2006, 2007). The co-migration of RDases with GroEL and hydrogenases proteins in BN-PAGE is not unprecedented (Tang & Edwards, 2013), suggesting a possible association of these proteins. The interaction of RDases with the hydrogenases is intriguing because such complexes could enable direct electron transfer from the hydrogen-oxidizing hydrogenase to the RDase, which transfers the electrons to the chlorinated electron acceptor. Direct hydrogenase-RDase electron transfer would be consistent with the absence of electron carriers, which have not been identified in *Dhc.* The chaperonin GroEL may aid in the folding and stabilization of such a hydrogenase-RDase complex. EF-Tu (elongation factor thermo unstable) is a GTP-binding protein involved in protein translation and abundant in active bacterial cells (Furano, 1975), suggesting active protein biosynthesis occurring in 1,2-D-dechlorinating cells. Although RDases are the most direct indicators for reductive dechlorination reactions, general RDase-associated proteins may serve as additional biomarkers for monitoring metabolically active *Dhc* and *Dhgm* populations.

Recent studies have demonstrated that stress conditions (i.e., oxygen exposure, heat, starvation) influence RDase gene transcription and RDase transcript can be measured in *Dhc* cultures not exhibiting reductive dechlorination activity (Amos, Ritalahti, Cruz-Garcia, Padilla-Crespo, &

Löffler, 2008; Fletcher et al., 2011). These findings emphasize that *Dhc* RDase gene activity and/or transcript turnover underlies poorly understood regulatory controls. Nevertheless, transcriptional analysis applied to cultures amended with a growth-supporting chloroorganic substrate contributed to the identification of RDases including *dcpA* (this study), *bvcA* encoding a DCE and VC RDase (Krajmalnik-Brown et al., 2004) and *mbrA* encoding a PCE to *trans*-DCE RDase (Chow et al., 2010).

The analysis of the *Dhc* RC cDNA clone library revealed two active RDase genes, RCRdA02 and *dcpA*. *Dhgm* strain BL-DC-9 does not possess a RCRdA02 homolog but a highly similar *dcpA* gene. Further, gene RCRdA02 has nearly identical orthologs in *Dhc* strains that cannot grow with 1,2-D (i.e., strains CBDB1, FL2 and GT). These findings corroborate *dcpA* as a 1,2-D RDase and indicate that RCRdA02 is not directly involved in 1,2-D reductive dechlorination. Interestingly, the ortholog of RCRdA02 in strain FL2 (FL2RdhA6) was one of multiple RDase genes transcribed in strain FL2 cultures grown with TCE, *cis*-DCE (Waller, Krajmalnik-Brown, Löffler, & Edwards, 2005) and *trans*-DCE (unpublished results) indicating that this RDase gene is not induced by a specific chloroorganic substrate. In previous studies, the RCRdA02 homolog KB1RdhAB5 was transcribed in consortium KB-1 when exposed to TCE, *cis*-DCE, or VC (A S Waller, Krajmalnik-Brown, Löffler, & Edwards, 2005) while in the enrichment culture TUT2264, the RCRdA02 homolog TUT2264_rdhA2 was highly transcribed in cultures spiked with PCE (Futamata, Kaiya, Sugawara, & Hiraishi, 2009). Additionally, the gene FtL-RDase-1638 (99% nt identity to RCRdA02) was the most abundant transcript recovered in cDNA libraries established with RNA extracted from chlorinated ethene-contaminated groundwater (Lee, Macbeth, Sorenson, Deeb, & Alvarez-Cohen, 2008). Another RDase gene, DET1545, which is 86% identical to RCRdA02 (and its translated product 94% identical and 97% similar to RCRdA02) has also been documented to be highly expressed during the transition to stationary phase (B G Rahm & Richardson, 2008b) and in pseudo steady state (B G Rahm & Richardson, 2008a). Overall, these findings suggest that the RCRdA02 gene is constitutively transcribed in metabolically active *Dhc* strains. The quantification of RCRdA02 transcripts in environmental samples may serve as an indicator of general *Dhc* activity; however, RCRdA02 transcription appears not to be linked to specific reductive dechlorination reactions.

Previous studies investigated the environmental distribution of RDase genes with assigned functions (e.g., *vcrA, tceA*) in sample materials retrieved from aquifers contaminated with chlorinated ethenes (Krajmalnik-Brown et al., 2007; Müller JA, Rosner BM, Von Abendroth G, Meshulam-Simon G, McCarty PL, 2004). The recovered RDase sequences exhibited greater than 95% and 98% identity with known *tceA* and *vcrA* genes, respectively, even in samples collected from geographically distinct locations (Krajmalnik-Brown et al., 2007; McMurdie PJ, Hug LA, Edwards EA, Holmes S, 2011; Müller JA, Rosner BM, Von Abendroth G, Meshulam-Simon G, McCarty PL, 2004; Sung, Ritalahti, Apkarian, & Löffler, 2006). Similar results were found in this study, where *dcpA* sequences retrieved from geographically distinct samples shared >98% sequence identity to the *Dhc* strain RC and KS and the *Dhgm* BL-DC-9 *dcpA* sequences. Of course, it is expected that similar RDase gene sequences are recovered with PCR primers targeting conserved RDase gene motifs. Nevertheless, the primer pair *dcpA*-360F and *dcpA*-1449R amplified a 1,089 bp-long *dcpA* gene fragments with sequence variability between the conserved primer binding sites, and are useful to improve understanding of *dcpA* sequence variability. This approach revealed two *dcpA* clades, both of which were captured with the *dcpA*-targeted PCR approach described herein.

A multiple lines of evidence approach identified *dcpA* encoding the 1,2-D reductive dehalogenase that catalyzes the dichloroelimination of 1,2-D to propene in specialized organohalide-respiring *Chloroflexi*. The utility of *dcpA*-specific nested PCR and qPCR assays for the sensitive detection and enumeration of *dcpA* genes in environmental samples was demonstrated, and *dcpA* presence and abundance correlated with propene formation except in the samples from the Barra Mansa site in Brazil, where *dcpA* was detected but no 1,2-D dechlorination activity was observed in microcosms. This groundwater contained up to 7,860 µg/L chloroform (CF) as well as carbon tetrachloride (CT) and 1,1,1-dichloroethane (1,1,1-TCA), which were described as potent *Dhc* inhibitors (Melanie Duhamel et al., 2002). The microcosms did not produce methane, suggesting that CT, CF, and/or 1,1,1-TCA affected microbial activity, including the 1,2-D dechlorinating population(s). This observation suggests that 1,2-D bioremediation may require prior removal of inhibitory co-contaminants.

Although other 1,2-D RDases exist (e.g., *Desulfitobacterium dichloroeliminans* strain DCA1 does not harbor the *dcpA* gene but dechlorinates 1,2-D to propene), 1,2-D-respiring *Chloroflexi* appear to be major contributors to this activity at the sites investigated and the *dcpA*-targeted PCR assays augment the available toolbox for site assessment and bioremediation monitoring. Carbon stable isotope enrichment factors associated with 1,2-D dichloroelimination in cultures RC and KS harboring the *dcpA* gene have been determined (Fletcher KE, Löffler FE, Richnow HH, 2009) and a comprehensive molecular biological tools approach can now be applied to tackle 1,2-D-contaminated sites. The application of environmental molecular diagnostics promises to decrease cost and achieve cleanup goals faster leading to early site closures and realizing significant cost-savings to the site owner(s), which will ultimately determine the value of these molecular tools.

References

ABI. (2005). Applied Biosystems 7900HT fast real-time PCR system and 7300/7500 real-time PCR systems: chemistry guide 4348358, revision E. *ABI*.

Agency for Toxic Substances and Disease Registry (ATSDR). (1989). *Toxicological profile for 1,2-dichloropropane*. Atlanta, GA.: U.S. Department of Health and Human Services, Public Health Services.

Amos, B. K., Christ, J. A., Abriola, L. M., Pennell, K. D., & Löffler, F. E. (2007). Experimental evaluation and mathematical modeling of microbially enhanced tetrachloroethene (PCE) dissolution. *Environ. Sci. Technol.*, *41*(3), 963–970. http://doi.org/10.1021/es061438n

Amos, B. K., Ritalahti, K. M., Cruz-Garcia, C., Padilla-Crespo, E., & Löffler, F. E. (2008). Oxygen effect on *Dehalococcoides* viability and biomarker quantification. *Environmental Science and Technology*, *42*(15), 5718–5726.

Amos, B. K., Sung, Y., Fletcher, K. E., Gentry, T. J., Wu, W.-M. M., Criddle, C. S., Löffler, F. E. (2007). Detection and quantification of *Geobacter lovleyi* strain SZ: implications for bioremediation at tetrachloroethene- and uranium-impacted sites. *Applied and Environmental Microbiology*, *73*(21), 6898–6904. http://doi.org/10.1128/AEM.01218-07

Bowman, K. S., Nobre, M. F., da Costa, M. S., Rainey, F. A., & Moe, W. M. (2013). *Dehalogenimonas alkenigignens* sp. nov., a chlorinated-alkane-dehalogenating bacterium isolated from groundwater. *International Journal of Systematic and Evolutionary Microbiology*, *63*(PART4), 1492–1498.

Brown, S. D., Thompson, M. R., Verberkmoes, N. C., Chourey, K., Shah, M., Zhou, J., Thompson, D. K. (2006). Molecular dynamics of the *Shewanella oneidensis* response to chromate stress. *Mol. Cell. Proteomics.*, *5*(6), 1054–1071.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Wittwer, C. T. (2009). The MIQE guidelines: minimum information for publication of quantitative real-

time PCR experiments. *Clin Chem*, *55*(4), 611–22. http://doi.org/10.1373/clinchem.2008.112797

Chourey, K., Jansson, J., VerBerkmoes, N., Shah, M., Chavarria, K. L., Tom, L. M., … Hettich, R. L. (2010). Direct cellular lysis/protein extraction protocol for soil metaproteomics. *J. Proteome Res.*, *9*(12), 6615–6622.

Chow, W. L., Cheng, D., Wang, S., & He, J. (2010). Identification and transcriptional analysis of trans-DCE-producing reductive dehalogenases in *Dehalococcoide*s species. *ISME J*, *4*(8), 1020–30. http://doi.org/10.1038/ismej.2010.27

De Wildeman S, Diekert G, Van Langenhove H, Verstraete, W. (2003). Stereoselective microbial dehalorespiration with vicinal dichlorinated alkanes. *Appl. Environ. Microbiol.*, *69*(9), 5643–5647. http://doi.org/10.1128/AEM.69.9.5643

Duhamel, M., Wehr, S. D., Yu, L., Rizvi, H., Seepersad, D., Dworatzek, S., … Edwards, E. A. (2002). Comparison of anaerobic dechlorinating enrichment cultures maintained on tetrachloroethene, trichloroethene, cis-dichloroethene and vinyl chloride. *Water Res.*, *36*(17), 4193–4202.

Eng, J. K., McCormack, A. L., & Yates, J. R. (1994). An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J. Am. Soc. Mass. Spectrom.*, *5*(11), 976–989. http://doi.org/10.1016/1044-0305(94)80016-2

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, *39*(4), 783–791. http://doi.org/10.2307/2408678

Fletcher, K. E., Costanza, J., Cruz-Garcia, C., Ramaswamy, N. S., Pennell, K. D., & Löffler, F. E. (2011). Effects of elevated temperature on *Dehalococcoides d*echlorination performance and DNA and RNA biomarker abundance. *Environmental Science Technology*, *45*(2), 712–718.

Fletcher KE, Löffler FE, Richnow HH, N. I. (2009). Stable carbon isotope fractionation of 1, 2-dichloropropane during dichloroelimination by *Dehalococcoides p*opulations. *Environmental Science & Technology*, *43*(18), 6915–6919.

Fung, J. M., Morris, R. M., Adrian, L., & Zinder, S. H. (2007). Expression of reductive dehalogenase genes in *Dehalococcoides ethenogenes* strain 195 growing on tetrachloroethene, trichloroethene, or 2,3-dichlorophenol. *Applied and Environmental Microbiology*, *73*(14), 4439–45. http://doi.org/10.1128/AEM.00215-07

Furano, A. V. (1975). Content of elongation factor Tu in *Escherichia coli. Proc. Natl. Acad. Sci. U. S. A.*, *72*(12), 4780–4784.

Futamata, H., Kaiya, S., Sugawara, M., & Hiraishi, A. (2009). Phylogenetic and Transcriptional Analyses of a Tetrachloroethene-Dechlorinating "Dehalococcoides" Enrichment Culture TUT2264 and Its Reductive-Dehalogenase Genes. *Microbes and Environments*, *24*(4), 330–337. http://doi.org/10.1264/jsme2.ME09133

Hatt, J. K., & Löffler, F. E. (2011). Quantitative real-time PCR (qPCR) detection chemistries affect enumeration of the *Dehalococcoides* 16S rRNA gene in groundwater. *J. Microbiol. Methods.*, *88*(2), 263–270. http://doi.org/10.1016/j.mimet.2011.12.005

He, J., Ritalahti, K. M., Aiello, M. R., & Löffler, F. E. (2003). Complete detoxification of vinyl chloride (VC) by an anaerobic enrichment culture and identification of the reductively dechlorinating population as a *Dehalococcoides* species. *Appl. Environ. Microbiol.*, *69*, 996–1003.

He, J., Sung, Y., Dollhopf, M. E., Fathepure, B. Z., Tiedje, J. M., & Löffler, F. E. (2002). Acetate versus hydrogen as direct electron donors to stimulate the microbial reductive dechlorination process at chloroethene-contaminated sites. *Environmental Science and Technology*, *36*(18), 2945–3952.

Holman, J. D., Ma, Z.-Q., & Tabb, D. L. (2012). Identifying Proteomic LC-MS/MS Data Sets with Bumbershoot and IDPicker. *Current Protocols in Bioinformatics Editoral Board*

Andreas D Baxevanis et Al, *Chapter 13*, Unit13.17. http://doi.org/10.1002/0471250953.bi1317s37

Honolulu, H. epidemiologic studies program:, & Hawaii., U. of. (1975). *Hawaii epidemiologic studies program*. *Hawaii epidemiologic studies program.* (Annual Rep).

Johnson, D. R., Lee, P. K., Holmes, V. F., Alvarez-Cohen, L., & V. F. Holmes. (2005). An internal reference technique for accurately quantifying specific mRNAs by real-time PCR with application to the *tceA* reductive dehalogenase gene. *Applied and Environmental Microbiology*, *71*(7), 3866–3871.

Krajmalnik-Brown, R., Hölscher, T., Thomson, I. N., Saunders, F. M., Ritalahti, K. M., & Löffler, F. E. (2004). Genetic identification of a putative vinyl chloride reductase in *Dehalococcoides* sp. strain BAV1. *Appl. Environ. Microbiol.*, *70*, 6347–6351.

Krajmalnik-Brown, R., Hölscher, T., Thomson, I. N., Saunders, F. M., Ritalahti, K. M., Löffler, F. E., (2004). Genetic identification of a putative vinyl chloride reductase in Dehalococcoides sp. strain BAV1. *Appl. Environ. Microbiol.*, *70*(10), 6347–6351. http://doi.org/10.1128/AEM.70.10.6347

Krajmalnik-Brown, R., Sung, Y., Ritalahti, K. M., Saunders, F. M., Löffler, F. E., & Michael Saunders, F. (2007). Environmental distribution of the trichloroethene reductive dehalogenase gene (tceA) suggests lateral gene transfer among *Dehalococcoides. FEMS Microbiol. Ecol.*, *59*(1), 206–14. http://doi.org/10.1111/j.1574-6941.2006.00243.x

Lee, P. K., Macbeth, T. W., Sorenson, K. J. S., Deeb, R. A., & Alvarez-Cohen, L. (2008). Quantifying genes and transcripts to assess the in situ physiology of "Dehalococcoides" spp. in a trichloroethene-contaminated groundwater site. *Applied and Environmental Microbiology*, *74*(9), 2728–2739.

Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, D. J. (2000). Molecular definition of a gene. In *Molecular Cell Biology* (4th ed.). New York: W. H. Freeman.

Löffler, F. E., Champine, J. E., Ritalahti, K. M., Sprague, S. J., Tiedje, J. M., & Loffler, F. E. (1997). Complete reductive dechlorination of 1,2-dichloropropane by anaerobic bacteria. *Applied and Environmental Microbiology*, *63*(7), 2870–5.

Löffler FE, Yan J, Ritalahti KM, Adrian L, Edwards EA, Konstantinidis KT, Müller JA, Fullerton H, Zinder SH, S. A. (2013). *Dehalococcoides mccartyi* gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation, belong to a novel bacterial class, Dehalococcoidia classis nov., order Dehalococcoidales ord. nov. and famil. *Int. J. Syst. Evol. Microbiol.*, *63*, 625–635.

Ludwig, M. L., & Matthews, R. G. (1997). Structure-based perspectives on B12-dependent enzymes. *Annu. Rev. Biochem.*, *66*(1), 269–313.

McMurdie PJ, Hug LA, Edwards EA, Holmes S, S. A. (2011). Site-specific mobilization of vinyl chloride respiration islands by a mechanism common in *Dehalococcoides. BMC Genomics*, *12*(1), 287. http://doi.org/10.1186/1471-2164-12-287

Moe WM, Yan J, Nobre MF, da Costa MS, Rainey, F. (2009). *Dehalogenimonas lykanthroporepellens* gen. nov., sp. nov., a reductively dehalogenating bacterium isolated from chlorinated solvent-contaminated groundwater. *Int. J. Syst. Evol. Microbiol.*, *59*(11), 2692–2697. http://doi.org/10.1099/ijs.0.011502-0

Morris, R. M., Fung, J. M., Rahm, B. G., Zhang, S., Freedman, D. L., Zinder, S. H., & Richardson, R. E. (2007). Comparative proteomics of *Dehalococcoides* spp. reveals strain-specific peptides associated with activity. *Applied and Environmental Microbiology*, *73*(1), 320–326. http://doi.org/10.1128/aem.02129-06

Morris, R. M., Sowell, S., Barofsky, D., Zinder, S. H., Richardson, R. E., & Barovsky, D. (2006). Transcription and mass-spectroscopic proteomic studies of electron transport oxidoreductases in *Dehalococcoides ethenogenes. Environ. Microbiol.*, *8*(9), 1499–509. http://doi.org/10.1111/j.1462-2920.2006.01090.x

Müller JA, Rosner BM, Von Abendroth G, Meshulam-Simon G, McCarty PL, S. A. (2004). Molecular identification of the catabolic vinyl chloride reductase from *Dehalococcoides* sp . strain VS and its environmental distribution. *Appl. Environ. Microbiol.*, *70*(8), 4880–4888. http://doi.org/10.1128/AEM.70.8.4880

Nei, M., & Kumar, S. (2000). *Molecular evolution and phylogenetics*. *Molecular Phylogenetics and Evolution* (Vol. 25). New York, N. Y.: Oxford University Press. http://doi.org/10.1016/S1055-7903(02)00247-6

Neumann, A., Siebert, A., Trescher, T., Reinhardt, S., Wohlfarth, G., & Diekert, G. (2002). Tetrachloroethene reductive dehalogenase of *Dehalospirillum multivorans*: substrate specificity of the native enzyme and its corrinoid cofactor. *Arch Microbiol.*, *177*(5), 420–426.

Nijhuis, T. A., Makkee, M., Moulijn, J. A., & Weckhuysen, B. M. (2006). The production of propene oxide: catalytic processes and recent developments. *Ind. Eng. Chem. Res.*, *45*(10), 3447–3459. http://doi.org/10.1021/ie0513090

Rahm, B. G., Morris, R. M., & Richardson, R. E. (2006). Temporal expression of respiratory genes in an enrichment culture containing *Dehalococcoides ethenogenes*. *Appl. Environ. Microbiol.*, *72*(8), 5486–5491. http://doi.org/10.1128/aem.00855-06

Rahm, B. G., & Richardson, R. E. (2008a). Correlation of respiratory gene expression levels and pseudo-steady-state PCE respiration rates in *Dehalococcoides ethenogenes*. *Environmental Science and Technology*, *42*(2), 416–421.

Rahm, B. G., & Richardson, R. E. (2008b). *Dehalococcoides'* gene transcripts as quantitative bioindicators of tetrachloroethene, trichloroethene, and cis-1,2-dichloroethene dehalorespiration rates. *Environmental Science and Technology*, *42*(14), 5099–5105.

Ritalahti, K. M., Amos, B. K., Sung, Y., Wu, Q., Koenigsberg, S. S., Löffler, F. E., & Lo, F. E. (2006). Quantitative PCR targeting 16S rRNA and reductive dehalogenase genes

simultaneously monitors multiple *Dehalococcoides* strains. *Applied and Environmental Microbiology*, *72*(4), 2765–2774. http://doi.org/10.1128/AEM.72.4.2765

Ritalahti, K. M., Cruz-García, C., Padilla-Crespo, E., Hatt, J. K., & Löffler, F. E. (2009). RNA extraction and cDNA Analysis for quantitative assessment of biomarker transcripts in groundwater. In K. N. Timmis (Ed.), *Handbook of Hydrocarbon and Lipid Microbiology* (pp. 3671–3685). Springer, In review. http://doi.org/10.1007/978-3-540-77587-4_289

Ritalahti, K. M., & Löffler, F. F. (2004). Populations implicated in the anaerobic reductive dechlorination of 1,2-dichloropropane in highly enriched bacterial communities. *Appl. Environ. Microbiol.*, *70*(7), 4088–4095. http://doi.org/10.1128/AEM.70.7.4088

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*(4), 406–425.

Schlötelburg C, Wintzingerode C, Hauck R, Wintzingerode F, Hegemann W, G. U. (2002). Microbial structure of an anaerobic bioreactor population that continuously dechlorinates 1,2-dichloropropane. *FEMS Microbiol. Ecol*, *39*(3), 229–37. http://doi.org/10.1111/j.1574-6941.2002.tb00925.x

Seshadri, R., Adrian, L., Fouts, D. E., Eisen, J. A., Phillippy, A. M., Methe, B. A., … Heidelberg, J. F. (2005). Genome sequence of the PCE-dechlorinating bacterium *Dehalococcoides ethenogenes. Science*, *307*, 105–108.

Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V, & Mann, M. (2006). In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.*, *1*(6), 2856–60. http://doi.org/10.1038/nprot.2006.468

Smidt H, van Leest M, van Der Oost J, de Vos WM. (2000). Transcriptional regulation of the cpr gene cluster in ortho-chlorophenol-respiring *Desulfitobacterium dehalogenans*. *J. Bacteriol.*, *182*(20), 5683–5691.

Sung, Y., Ritalahti, K. M., Apkarian, R. P., & Löffler, F. E. (2006). Quantitative PCR confirms purity of strain GT, a novel trichloroethene (TCE)-to-ethene respiring *Dehalococcoides* isolate. *Appl. Environ. Microbiol.*, *72*, 1980–1987.

Tabb, D. L., Fernando, C. G., & Chambers, M. C. (2007). MyriMatch: highly accurate tandem mass spectral peptide identification by multivariate hypergeometric analysis. *Journal of Proteome Research*, *6*(2), 654–661. http://doi.org/10.1021/pr0604054

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology*, *28*, 2731–2739. http://doi.org/10.1093/molbev/msr121

Tang S, Chan WW, Fletcher KE, Seifert J, Liang X, Löffler FE, Edwards EA, A. L. (2013). Functional characterization of reductive dehalogenases by using blue native polyacrylamide gel electrophoresis. *Appl. Environ. Microbiol.*, *79*(3), 974–81. http://doi.org/10.1128/AEM.01873-12

Tang, S., & Edwards, E. A. (2013). Identification of *Dehalobacter* reductive dehalogenases that catalyse dechlorination of chloroform, 1,1,1-trichloroethane and 1,1-dichloroethane. *Trans. R. Soc. Lond. B. Biol. Sci*, *368*(1616), 20120318. http://doi.org/10.1098/rstb.2012.0318

Thompson, J. D., & Higgins, D. G. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix. *Nucleic Acids Res.*

Thompson, M. R., Chourey, K., Froelich, J. M., Erickson, B. K., VerBerkmoes, N. C., & Hettich, R. L. (2008). Experimental approach for deep proteome measurements from small-scale microbial biomass samples. *Analytical Chemistry*, *80*(24), 9517–9525.

Waller, A. S., Krajmalnik-Brown, R., Löffler, F. E., & Edwards, E. A. (2005). Multiple reductive-dehalogenase-homologous genes are simultaneously transcribed during

dechlorination by *Dehalococcoides*-containing cultures. *Applied and Environmental Microbiology*, *71*, 8257–8264.

Wu, W.-M., Carley, J., Fienen, M., Mehlhorn, T., Lowe, K., Nyman, J., Criddle, C. S. (2006). Pilot-Scale in situ bioremediation of uranium in a highly contaminated aquifer. 1. conditioning of a treatment zone. *Environ. Sci. Technol.*, *40*(12), 3978–3985. http://doi.org/10.1021/es051954y

Yan J, Rash BA, Rainey FA, Moe WM. (2009). Isolation of novel bacteria within the Chloroflexi capable of reductive dechlorination of 1,2,3-trichloropropane. *Environ. Microbiol.*, *11*(4), 833–843. http://doi.org/10.1111/j.1462-2920.2008.01804.x

Yu, N. Y., Laird, M. R., Spencer, C., & Brinkman, F. S. L. (2011). PSORTdb—an expanded, auto-updated, user-friendly protein subcellular localization database for Bacteria and Archaea. *Nucleic Acids Research*, *39*(Database issue), D241–D244.

Zogorski JS, Carter JM, Ivahnenko T, Lapham WW, Moran MJ, Rowe BL, Squillace PJ, T. P. (2006). Volatile organic compounds in the nation's ground water and drinking-water supply wells. *U.S. Geological Survey Circular 1292*, 112.

Chapter 3 Appendix: Tables

Table 3.1. Site materials used for microcosm setup to evaluate 1,2-D reductive dechlorination activity and analyzed for the presence of *Dhc* and *Dhgm* 16S rRNA gene and the *dcpA* gene.

Sample designation	Sample location	Sample type	Major reported contaminants	Date of collection	Dechlorination end products	*Dhc*	*Dhgm*	*dcpA*
Microcosms								
Third Creek, TRS1	Third Creek, Knoxville, TN	SE	PCE, TCE, 1,1,1-TCA	Feb. 2011	Propene	+	+	+
Third Creek, TRS2	Third Creek, Knoxville, TN	SE	PCE, TCE, 1,1,1-TCA	Feb. 2011	Propene	+	+	+
Third Creek, TRS3	Third Creek, Knoxville, TN	SE	PCE, TCE, 1,1,1-TCA	Mar. 2011	Propene	+	+	+
Neckar River	Stuttgart, Germany	SE	None	May, 2011	Propene	+	+	+
Trester	Stuttgart, Germany	Solids[a]	None	May, 2011	Propene	+	+	+
001-ST-SO, 2.7-2.9 m	Barra Mansa, Brazil	SE	Chloroform, CCl_4	Aug. 2010	-[b]	+	ND	+
002-ST-SO, 5.7-5.8 m	Barra Mansa, Brazil	SE	Chloroform, CCl_4	Aug. 2010	-[b]	+	ND	+
Way-MW13D-12J811	Waynesboro, GA.USA	GW	1,2-D and 1,2-DCA	Aug. 2010	-	ND	ND	ND
FP1-MW46, 22-26 m	Ft. Pierce, FL, USA	SE	None[c]	Aug. 2010	-	ND	ND	ND
FP2-MW49, 26-27 m	Ft. Pierce, FL, USA	SE	None[c]	Aug. 2010	-	+	+	ND
FP3-MW49, 46-47 m	Ft. Pierce, FL, USA	SE	None[c]	Aug. 2010	-	+	+	ND
FP4-MW47, 47-48 m	Ft. Pierce, FL, USA	SE	None[c]	Aug. 2010	-	+	ND	ND
FP5-MW49, 95-98 m	Ft. Pierce, FL, USA	SE	None[c]	Aug. 2010	-	ND	ND	ND
FP-MW33, 13-14 m	Ft. Pierce, FL, USA	GW	18,000 μg/L of 1,2-D	June, 2012	Propene	+	+	+
FP-MW26, 14-15 m	Ft. Pierce, FL, USA	GW	17,000 μg/L of 1,2-D	June, 2012	Propene	+	+	+
FP-MW20, 20-21m	Ft. Pierce, FL, USA	GW	810 μg/L of 1,2-D	June, 2012	Propene	+	+	+

Table 3.1 (continued)

Sample designation	Sample location	Sample type	Major reported contaminants	Date of collection	Dechlorination end products	*Dhc*	*Dhgm*	*dcpA*
DNA samples								
FP-MW-2S, 6-7 m	Ft. Pierce, FL, USA	Biobead	14,000 µg/L of 1,2-D	July, 2011	No Propene[d]	+	ND	ND
FP-MW-20, 20-21 m	Ft. Pierce, FL, USA	Biobead	810 µg/L of 1,2-D	Feb. 2011	Propene[d]	ND	ND	+
FP-MW-26, 14-15 m	Ft. Pierce, FL, USA	Biobead	17,000 µg/L of 1,2-D	Mar. 2011	Propene[d]	+	+	+
FP-MW-61, 20-21 m	Ft. Pierce, FL, USA	Biobead	140 µg/L of 1,2-D	May, 2011	Propene[d]	ND	+	+
FW-024	IFC site, Oakridge, TN	GW	Multiple	Feb. 2004	NT	+	ND	ND
FW-103	IFC site, Oakridge, TN	GW	Multiple	Feb. 2004	NT	+	+	+
FW-100-2	IFC site, Oakridge, TN	GW	Multiple	Aug. 2005	NT	+	ND	+
FW-100-3	IFC site, Oakridge, TN, USA	GW	Multiple	Feb. 2004	NT	+	ND	+

Legend: GW; groundwater SE; sediments NT; not tested ND; not detected

- ; No dechlorination in microcosm after 90 days incubation.

[a] ; Solid residues (trester) from wine making consisting mostly of grape skins.

[b] ; Small amounts of 1-CP or 2-CP were detected in live microcosm but also in negative controls.

[c] ; Ft. Pierce is contaminated with 1,2-D (up to 24,000 µg/L) but the sediments tested here were for wells outside plume area.

[d]; Dechlorination not tested on microcosm, data provided reflect field-site conditions; there was no propene detected in FP1-MW-

Table 3.2. Primers and probes used in this study.

Primer or probe	Primer sequence (5'- 3')	Target	Purpose
RRF2 [1]	SHMGBMGWGATTTYATGAARR	RDaseA RRDFMK motif	Amplification of RDase-like genes
B1R [1]	CHADHAGCCAYTCRTACCA	RDaseB WYEW motif	Amplification of RDase-like genes
dcpA-1257F[2]	CGATGTGCCAGCCATTGTGTCTTT	*dcpA* gene	*dcpA* gene quantification and primer
dcpA-1449R[2]	TTTAAACAGCGGGCAGGTACTGGT	*dcpA* gene	*dcpA* gene quantification, direct and
dcpA-1426Probe[2]	*FAM*-ACGTCATCTCAGATGAAGGCAGAGCT-*BHQ*	*dcpA* gene	*dcpA* gene quantification, direct and
dcpA-360F[2]	TTGCGTGATCAAATTGGAGCCTGG	*dcpA* qPCR	*dcpA* gene quantification, direct and nested PCR with primer *dcpA*-1449R, primer walking
Dhc-1200F	CTGGAGCTAATCCCCAAAGCT	*Dhc* 16S rRNA gene	*Dhc* 16S rRNA gene quantification
Dhc-1271R	CAACTTCATGCAGGCGGG	*Dhc* 16S rRNA gene	*Dhc* 16S rRNA gene quantification
Dhc-1240Probe	*FAM*-TCCTCAGTTCGGATTGCAGGCTGAA-*TAMRA*	*Dhc* 16S rRNA gene	*Dhc* 16S rRNA gene quantification
*Luci*F	TACAACACCCCAACATCTTCGA	Luciferase reference mRNA	Quantitation of internal standard
*Luci*R	GGAAGTTCACCGGCGTCAT	Luciferase reference mRNA	Quantitation of internal standard
Luci-probe	*JOE*-CGGGCGTGGCAGGTCTTCCC-*BHQ*	Luciferase reference mRNA	Quantitation of internal standard
rpoB-1648F	ATTATCGCTCAGGCCAATACCCGT	*Dhc rpoB* gene	*Dhc rpoB* gene quantification
rpoB-1800R	TGCTCAAGGAAGGGTATGAGCGAA	*Dhc rpoB* gene	*Dhc rpoB* gene quantification
Dhc-730F	GCGGTTTTCTAGGTTGTC	*Dhc* 16S rRNA gene	*Dhc* detection by PCR
Dhc-1350R	CACCTTGCTGATATGCGG	*Dhc* 16S rRNA gene	*Dhc* detection by PCR
BL-DC-631F	GGTCATCTGATACTGTTGGACTTGAGTATG	*Dhgm* 16S rRNA gene	*Dhgm* detection by PCR
BL-DC-796R	ACCCAGTGTTTAGGGCGTGGACTACCAGG	*Dhgm* 16S rRNA gene	*Dhgm* detection by PCR
dcp_up120F[2]	GCTCCTGGCAGAGCCGTCAGT	120 bp upstream of *dcpA*	Amplification and assembly of *dcpA*

[1] Abbreviations for degenerate nucleotides positions are as follows: R =A or G; K = G or T; M =A or C; S =C or G; W=A or T; Y =C or T; B =C, G, or T; D = A, G, or T; V =A, C, or G; H =A, C, or T

[2] Primer names are given based on their target position related to the *dcpA* start coordinates in *Dehalogenimonas lykanthroporepellens* BL-DC-9 sequenced genome.

Table 3.3. *Dhgm* strain BL-DC-9 proteins identified in gel slice 4 exhibiting 1,2-D-to-propene dechlorination activity following BN-PAGE. Gel slice 4 was further separated in SDS-PAGE into gel slices N1, N2, and N3. Proteins are listed in order of decreasing spectral counts; DcpA is indicated in bold letters. In the N1 gel section, proteins levels were too low for confident identification and not included in the table below.

Gel Slice	Gene ID	Protein Accession number	Protein Length	Sequence Coverage (%)	Distinct Peptides	Spectral Counts	Adjusted NSAF values	Protein Description
N2	Dehly_1407	YP_003759016	534	33.0	17	230	74691.4	chaperonin GroEL
	Dehly_0935	YP_003758558	543	9.0	5	12	3832.4	DAK2 domain fusion protein YloV
	Dehly_0744	YP_003758371	526	7.0	3	12	3956.2	D-3-phosphoglycerate dehydrogenase
	Dehly_1273	YP_003758885	610	6.7	3	13	3695.7	hypothetical protein
	Dehly_1425	YP_003759034	511	7.0	3	10	3393.6	phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase
	Dehly_1020	YP_003758642	557	3.9	2	10	3113.4	arginyl-tRNA synthetase
	Dehly_0812	YP_003758435	588	4.9	2	8	2359.4	formate--tetrahydrofolate ligase
	Dehly_1485	YP_003759090	500	4.4	2	5	1734.1	glutamine synthetase
	Dehly_0665	YP_003758293	555	4.3	2	4	1249.8	dihydroxy-acid dehydratase
	Dehly_0337	YP_003757978	400	5.5	2	3	1300.6	translation elongation factor Tu
	Dehly_0353	YP_003757993	515	6.6	2	2	673.5	carboxyl transferase
N3	Dehly_0337	YP_003757978	400	9.8	4	23	53241.2	translation elongation factor Tu
	Dehly_1524	**YP_003759128**	**482**	**6.6**	**3**	**14**	**26894.3**	**reductive dehalogenase**
	Dehly_0929	YP_003758552	423	6.9	2	3	6566.9	nickel-dependent hydrogenase large subunit
	Dehly_1407	YP_003759016	534	6.4	3	3	5201.9	chaperonin GroEL
	Dehly_0692	YP_003758320	437	3.9	2	2	4237.7	diaminopimelate decarboxylase
	Dehly_0726	YP_003758353	480	3.5	2	2	3858.1	nickel-dependent hydrogenase large subunit

***** NSAF stands for Normalized Spectral Abundance Factor (NSAF) and is used for quantitative proteomic analysis by taking the MS/MS spectral counts of a protein and dividing it by its length (number of amino acids) resulting in a spectral abundance factor (SAF). Following this, the SAF is normalized against the sum of all SAFs in the sample, resulting in the NSAF value. Adjusted NSAF values allow for direct comparison of a protein's abundance between individual runs and samples.

Chapter 3 Appendix: Figures

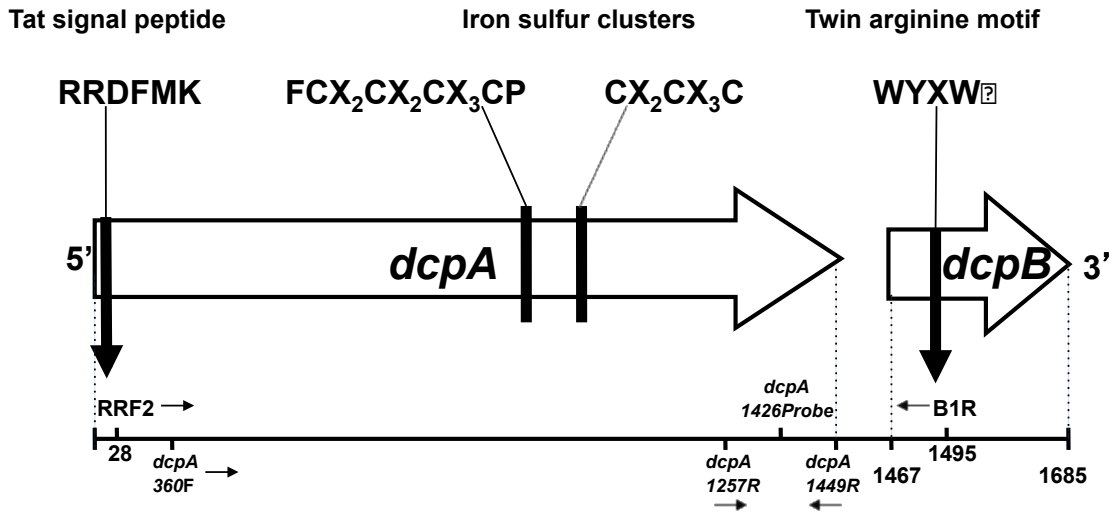


Figure 3.1. Arrangements of the *dcpA* gene and its corresponding *dcpB* genes in *Dhc* strain strains RC and KS. Approximate binding sites for the degenerate primers RRF2 and B1R as well as the *dcpA* specific primers designed in this study are indicated. Also shown are the characteristic dehalogenase features encoded by the *dcpA* gene which include the conserved Tat signal peptide RRXFXK at the N-terminus and two iron sulfur clusters closer to the C-terminus in the form of FCXXCXXCXXXCP (or $FCX_2CX_2CX_3CP$) and CXXCXXXC (or CX_2CX_3C). *dcpB* is located downstream of *dcpA* and encodes for a small highly hydrophobic protein with the conserved twin arginine motif in the form WYXW. The *dcpA* and *dcpB* gene (Dehly_1524 and Dehly_1523) in *Dhgm* strain BL-DC-9 also encodes for a dehalogenase with these common features.

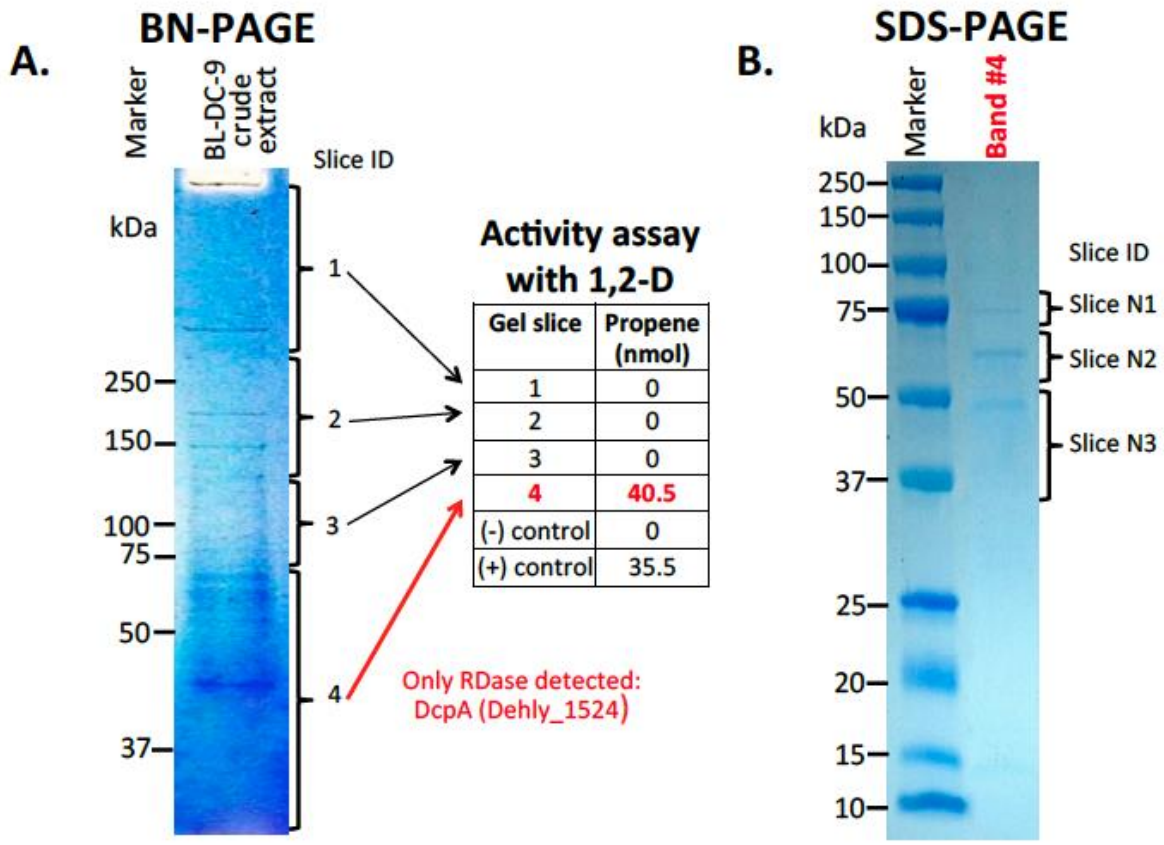


Figure 3.2. Activity assays completed on an unstained lane run in parallel to the BN-PAGE separation of crude extracts of *Dhgm* BL-DC-9 cells grown with 1,2-D. Panel A: Coomassie-stained BN-PAGE showing the predominant proteins and the gel sections that were subjected to dechlorination activity testing with 1,2-D. Propene formation was only observed in gel slice 4, which was subjected to SDS-PAGE (Panel B). Three bands were visualized by SDS-PAGE (Panel B) and gel slices (representing sections N1-N3) were further analyzed by LC-MS/MS. The Only RDase detected in Band 4 was DcpA (Dehly_1524)

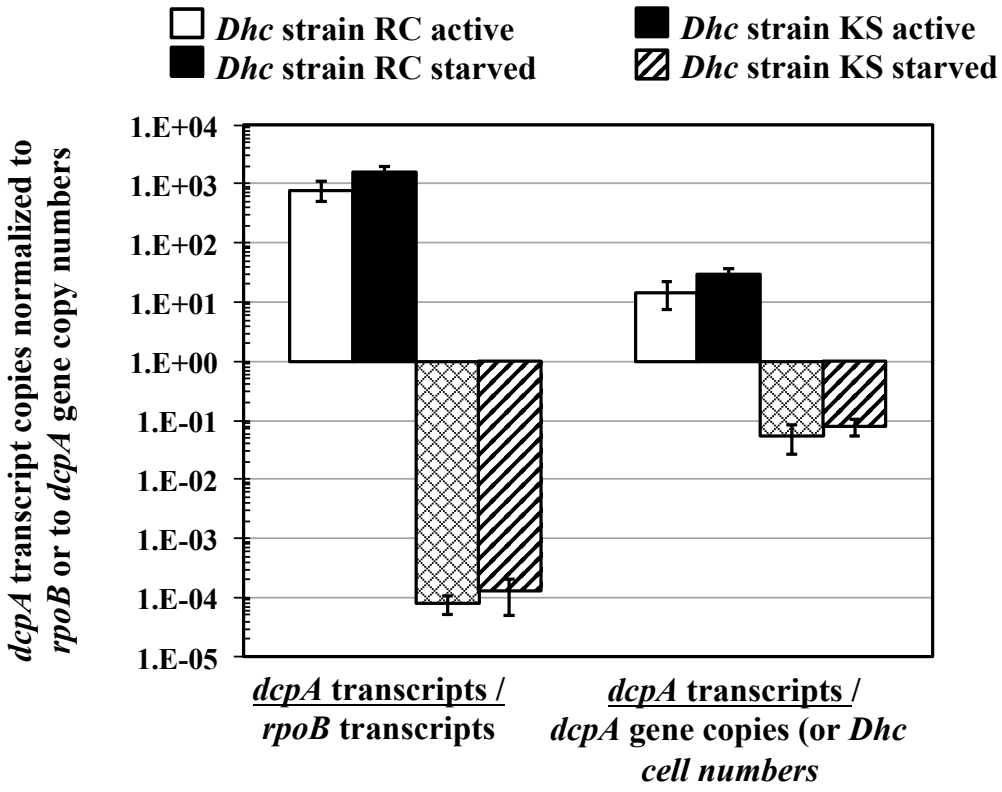


Figure 3.3. Relative transcript copy abundances in cells growing with 1,2-D. *dcpA* transcripts levels were normalized to *rpoB* or to *dcpA* gene copy numbers. Triplicate samples were analyzed and the reported values represent the average of at least three independent biological cultures. Error bars depict standard error. Negative numbers represent down regulated target genes, while positive numbers represented up regulated genes. A ratio near unity (close to 1) indicates an insignificant change in the number of *dcpA* transcripts per *rpoB* transcripts or *dcpA/Dhc* gene copy numbers.

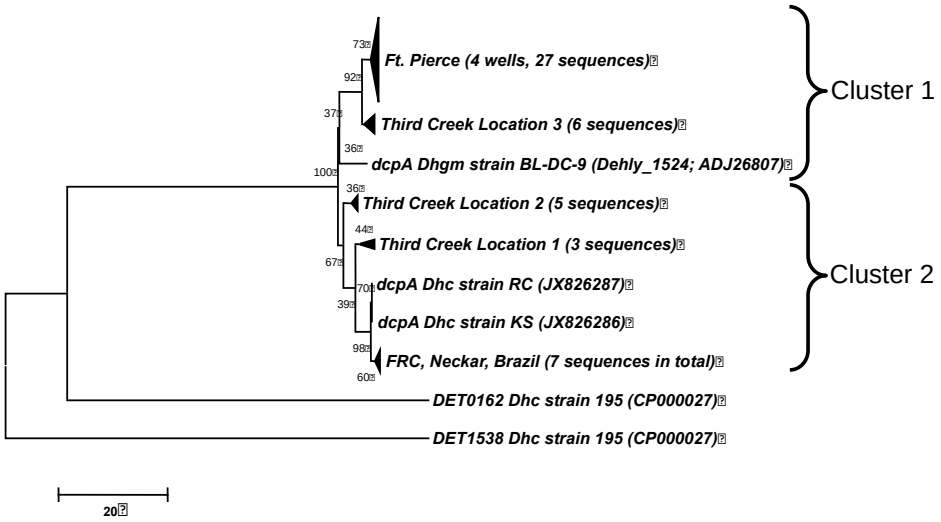


Figure 3.4. Phylogenetic tree of DcpA sequences. The neighbor-joining tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic relationships. All positions containing gaps and missing data were eliminated and a total of 247 aa positions were included in the final dataset. Evolutionary distances were computed using the number of differences method and are in the units of the number of *amino acid* differences per sequence. Samples that clustered together were grouped and numbers in parentheses indicate the number of sequences of each group. The RDase DET1538 of *Dhc* strain 195 served as an outgroup to root the tree. Cluster 1 shares highest aa sequence identity to DcpA of *Dhgm* strain BL-DC-9 (93-95%), while Cluster 2 comprises sequences with higher sequence identity to DcpA of *Dhc* strains KS and RC (95-99%).

Chapter 3 Appendix: Supplemental Methods

Sample collection and site description.

Sediment, aquifer and solids were collected in sterile glass or plastic containers from Third Creek, Knoxville, TN (three locations), Neckar River and an agricultural site near Stuttgart, Germany. The Third Creek site has a history of tetrachloroethylene (PCE), trichloroethylene (TCE), and 1,1,1-Trichloroethane (1,1,1-TCA) contamination. The Neckar River site has no history of chlorinated solvent contamination but is near urban and industrial areas. The agricultural site contained residues from the winemaking process (consisting mostly of grape skins). The samples from the municipality of Barra Mansa Brazil were from a contaminated mixed-waste site that contains up 7,860 µg/L of chloroform (CF) and 125 µg/L of carbon tetrachloride (CT) and 6.6 µg/L 1,1,1-TCA. Additional groundwater samples from a contaminated industrial site in Waynesboro (Georgia, USA) were collected. The Waynesboro site was a former facility that formulated pesticides and herbicides and is primarily contaminated with 1,2-dichloropropane (1,2-D) up to 30,000 µg/L, 1,2-dichloroethane (1,2-DCA) and alpha-, beta-, delta-, and gamma-hexachlorocyclohexane but other contaminants such as CF and CT are also present. Additional samples included sediments from a chlorinated solvent-contaminated site at Fort Pierce (Florida, USA), which is contaminated with mainly 1,2-D (up to 24,000 µg/L), 1,2-DCA and vinyl chloride; however, the solids provided for microcosm set up were collected outside the plume area. Additional Ft. Pierce DNA samples from wells inside the 1,2-D plume at the Ft. Pierce site were tested; the DNA was extracted from Bio-Sep® beads collected with Bio-Trap samplers (http://www.microbe.com/index.php/Bio-Trap-Samplers/bio-trap-samplers.html)

that were deployed in monitoring wells inside the plume at the Ft. Pierce site. All samples were delivered to the laboratory with overnight carrier and immediately processed or stored at 4°C for no more than 1 month. In addition, DNA was obtained from samples collected from an *in situ* uranium bioreduction pilot test plot in area 3 (wells FW104, FW103, FW100-2, and FW100-3) at the Integrated Field Research Challenge site at Oak Ridge National Laboratory (Amos, Sung, et al., 2007; W.-M. Wu et al., 2006)

Assembly of the dcpAB gene cassette by primer walking.

Because the genomes of *Dhc* strains KS and RC are not available, the primer *dcp_up120F* was designed to obtain the 5' end of the *Dhc dcpA* gene. The *dcp_up120F* primer design was based on the available genome information for *Dehalogenimonas lykanthroporepellens* (*Dhgm*) strain BL-DC-9, and targeted a region 120 bp upstream of the *dcpA* gene (Dehly_1524) start position. Combined with the primer *dcpA*-1449R, PCR products of ~1,569 bp were predicted. The PCR reactions consisted of 1 x PCR buffer, 2.5 mM $MgCl_2$, 250 µM of each deoxynucleoside triphosphate (ABI), primers (250 nM each), and 2.5 U of AmpliTaq polymerase (ABI). The following thermocycler temperature program was used for the amplification of the *dcpA* gene: 94°C for 2 min, 10 seconds (1 cycle); 94°C for 30 seconds, 56.0°C for 45 seconds, and 72°C for 2 min, 10 seconds (30 cycles); and 72°C for 6 min. Reactions with genomic DNA from *Dhgm* strain BL-DC-9 as template served as positive controls. Amplicons were separated on a 1% (wt/vol) agarose gel, stained with ethidium bromide (1 µg/mL), purified with the Qiagen PCR Purification kit (Qiagen, Valencia, CA, USA) and sequenced. Alignments of sequences using SeqMan II software (DNASTAR, Lasergene version 7) and ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) were used to assemble the missing 5' end and the *dcpA* start site coding sequence. In addition, the DNA Walking *SpeedUp*™ kit (Seegene, Seoul, South Korea) was used to extend the partial *dcpAB* sequence and amplify the entire *dcp*B gene. The procedure involved a series of consecutive PCR amplifications with primers targeting known sequence regions in combination with the kit's DNA walking-annealing control primers.

The internal primers *dcpA*-360F and *dcpA*-1257F (Figure S3.1) were used with *Dhc* culture RC and culture KS genomic DNA following the manufacturer's recommendations. The resulting chromosome walking PCR products were purified, sequenced and assembled with the SeqMan II software (DNASTAR, Lasergene version 7).

Protein assays and BN-PAGE for DcpA identification.

Dhgm strain BL-DC-9 was grown as described (Yan J, Rash BA, Rainey FA, Moe WM, 2009) in twenty 160 mL serum bottles containing 100 mL of medium and 0.5 mM of 1,2-D. The cultures received a second feeding of 1,2-D after the initial amount had been dechlorinated to propene. Cells were collected by centrifugation and suspended in sample buffer (50 mM Bis-Tris, 6 M HCl, 50 nM NaCl, 10% w/v glycerol, pH 7.2). The cell suspensions were subjected to lysis by adding a small amount of spherical, lead-free soda lime glass disrupter beads (0.1 mm, VWR, cat no. 101454-154) and agitating at maximum speed using a desktop vortex unit (Vortex Genie 2, Scientific Industries, Inc., Bohemia, NY) for 10 minutes, while periodically cooling the suspension in ice water. Following centrifugation, the extracts were subjected to Blue Native (BN) polyacrylamide gel electrophoresis (NativePAGE™ Novex® 4-16% Bis-Tris gels, 1.0 mm, Invitrogen, CA, part # BN1002BOX) and stained with the Fast Coomassie G-250 staining protocol (Invitrogen) to visualize proteins. The same samples were applied to multiple lanes, which were then used in the *in vitro* enzyme assays as well as for further analysis via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% Mini-PROTEAN® TGX™ Precast Gel, Bio-Rad, Inc. CA. part # 456-1044) for subsequent LC-MS/MS analysis. The excised gel segments were cut into smaller pieces and placed in 2 mL glass vials containing a mixture of 4 mM methyl viologen, 4 mM titanium III citrate, 0.5 mM 1,2-D and 100 mM Tris buffer, pH 7.4. The vials were sealed with Teflon-coated septum screw caps (Grace Davis & Discovery Sciences, part # 95020). Each vial received a small stir bar for continuous mixing inside an anoxic chamber at room temperature for 18 hours. Negative control incubations received no gel slice and the positive control consisted of 1 mL of cell suspension. The cells were collected by centrifugation (10, 000 x g for 20 min at 4°C) and suspended in 100 µL 100 mM Tris buffer, pH 7.4. Propene was quantified in headspace samples (0.1 mL) using a GC-FID

as described (Amos, Christ, Abriola, Pennell, & Löffler, 2007) except that an inlet split ratio of 1:1 was used. From the replicate lanes, the corresponding gel band was excised with a scalpel and placed in SDS elution buffer (100 mM Tris, pH 7.0; 0.1% (w/vol) SDS) and incubated at 4°C overnight to elute the proteins from the gel fragments. The eluted proteins were concentrated using a low-binding 10 kDa Microcon ultrafiltration unit (Millipore, Billerica, MA, USA) following the manufacturers recommendations. The concentrated samples were subjected to SDS-PAGE and Coomassie stained. Subsequently, visible bands were excised from the gels for LC-MS/MS analysis. The Precision Plus Protein Standards Kaleidoscope marker (cat #: 161-0375, Bio-Rad, Inc., CA) was used to determine the molecular weights of proteins in the SDS-PAGE gel.

Two dimensional-liquid chromatography-tandem mass spectrometry (2D-LC-MS/MS).

Coomassie-stained gel bands were excised from the SDS-PAGE gel and rinsed in HPLC-grade, degassed water (i.e., MilliQ water filtered through 0.02 micron filter and bubbled with Nitrogen for 30 minutes). In-gel digestion of proteins was performed as described (Shevchenko, Tomas, Havlis, Olsen, & Mann, 2006). Briefly, gel pieces were cut into small pieces and destained for 30 min in 100 mM ammonium bicarbonate/acetonitrile (1:1, vol/vol) at room temperature along with intermittent vortexing. Gel-enmeshed proteins were further subjected to reduction, alkylation and overnight trypsin digestion at 37°C as described (Shevchenko et al., 2006), and peptides were obtained in 100 µL of extraction buffer (5% formic acid/acetonitrile, 1:2 vol/vol). The extracted peptide mix (50 µL) was pressure loaded onto an in-house packed biphasic MuDPIT (Multi Dimensional Protein Identification Technology) column packed with ~3 cm of strong cation exchange (SCX) resin (Phenomenex, Torrance, CA) and ~5 cm of reverse phase (RP) C18 resin (Phenomenex, Torrance, CA). The sample column was connected to a 15 cm RP packed front column (New Objective, Woburn, MA) and analyzed via 2-D LC-MS/MS using three salt pulses (30, 60 and 100% of 500 mM ammonium acetate) followed by a 120 min elution gradient of 100% Solvent A (95% H2O, 5% acetonitrile, 0.1% formic acid to 60%) and Solvent B (30% H_2O, 70% acetonitrile, 0.1% formic acid). Peptide fragmentation data were collected using an LTQ or LTQ-Orbitrap (used in LTQ mode), operated in data-dependent mode and

under the control of the Xcalibur software (Thermo Scientific). The LTQ-Orbitrap was set to 30K resolution while rest of precincts on either instrument was maintained as described (Brown et al., 2006) (Chourey et al., 2010)(M. R. Thompson et al., 2008). The MS/MS data obtained were searched against *Dhgm* BL-DC-9 genome (NC_014314.1, downloaded from JGI, April 2012) using the SEQUEST algorithm (Eng, McCormack, & Yates, 1994). The resultant datasets were sorted using DTASelect (Tabb, McDonald, & Yates, 2002) set to following parameters: fully tryptic peptides only with ΔCN of at least 0.08 and cross-correlation scores (Xcorr) of at least 1.8 (+1), 2.5 (+2), and 3.5 (+3) (Brown et al., 2006)(M. R. Thompson et al., 2008). All chemicals for proteomic analysis were obtained from Sigma Chemical Co. (St. Louis, MO), trypsin was acquired from Promega (Madison, WI), formic acid (99%) was obtained from EM Science (Darmstadt, Germany) and HPLC-grade water and acetonitrile were purchased from Burdick and Jackson (Muskegon, MI).

Chapter 3 Appendix: Supplemental methods references

Amos, B. K., Christ, J. A., Abriola, L. M., Pennell, K. D., & Löffler, F. E. (2007). Experimental evaluation and mathematical modeling of microbially enhanced tetrachloroethene (PCE) dissolution. *Environ. Sci. Technol.*, *41*(3), 963–970. http://doi.org/10.1021/es061438n

Amos, B. K., Sung, Y., Fletcher, K. E., Gentry, T. J., Wu, W.-M. M., Criddle, C. S., … Löffler, F. E. (2007). Detection and quantification of *Geobacter lovleyi* strain SZ: implications for bioremediation at tetrachloroethene- and uranium-impacted sites. *Applied and Environmental Microbiology*, *73*(21), 6898–6904. http://doi.org/10.1128/AEM.01218-07

Brown, S. D., Thompson, M. R., Verberkmoes, N. C., Chourey, K., Shah, M., Zhou, J., … Thompson, D. K. (2006). Molecular dynamics of the *Shewanella oneidensis r*esponse to chromate stress. *Mol. Cell. Proteomics.*, *5*(6), 1054–1071.

Chourey, K., Jansson, J., VerBerkmoes, N., Shah, M., Chavarria, K. L., Tom, L. M., … Hettich, R. L. (2010). Direct cellular lysis/protein extraction protocol for soil metaproteomics. *J. Proteome Res.*, *9*(12), 6615–6622.

Eng, J. K., McCormack, A. L., & Yates, J. R. (1994). An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J. Am. Soc. Mass. Spectrom.*, *5*(11), 976–989. http://doi.org/10.1016/1044-0305(94)80016-2

Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V, & Mann, M. (2006). In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.*, *1*(6), 2856–60. http://doi.org/10.1038/nprot.2006.468

Tabb, D. L., McDonald, W. H., & Yates, J. R. (2002). DTASelect and Contrast: tools for assembling and comparing protein identifications from shotgun proteomics. *J. Proteome Res.*, *1*(1), 21–26.

Thompson, M. R., Chourey, K., Froelich, J. M., Erickson, B. K., VerBerkmoes, N. C., & Hettich, R. L. (2008). Experimental approach for deep proteome measurements from small-scale microbial biomass samples. *Analytical Chemistry*, *80*(24), 9517–9525.

Wu, W.-M., Carley, J., Fienen, M., Mehlhorn, T., Lowe, K., Nyman, J., … Criddle, C. S. (2006). Pilot-Scale in situ bioremediation of uranium in a highly contaminated aquifer. 1. conditioning of a treatment zone. *Environ. Sci. Technol.*, *40*(12), 3978–3985. http://doi.org/10.1021/es051954y

Yan J, Rash BA, Rainey FA, Moe WM. (2009). Isolation of novel bacteria within the Chloroflexi capable of reductive dechlorination of 1,2,3-trichloropropane. *Environ. Microbiol.*, *11*(4), 833–843. http://doi.org/10.1111/j.1462-2920.2008.01804.x

Chapter 3 Appendix: Supplemental Figures

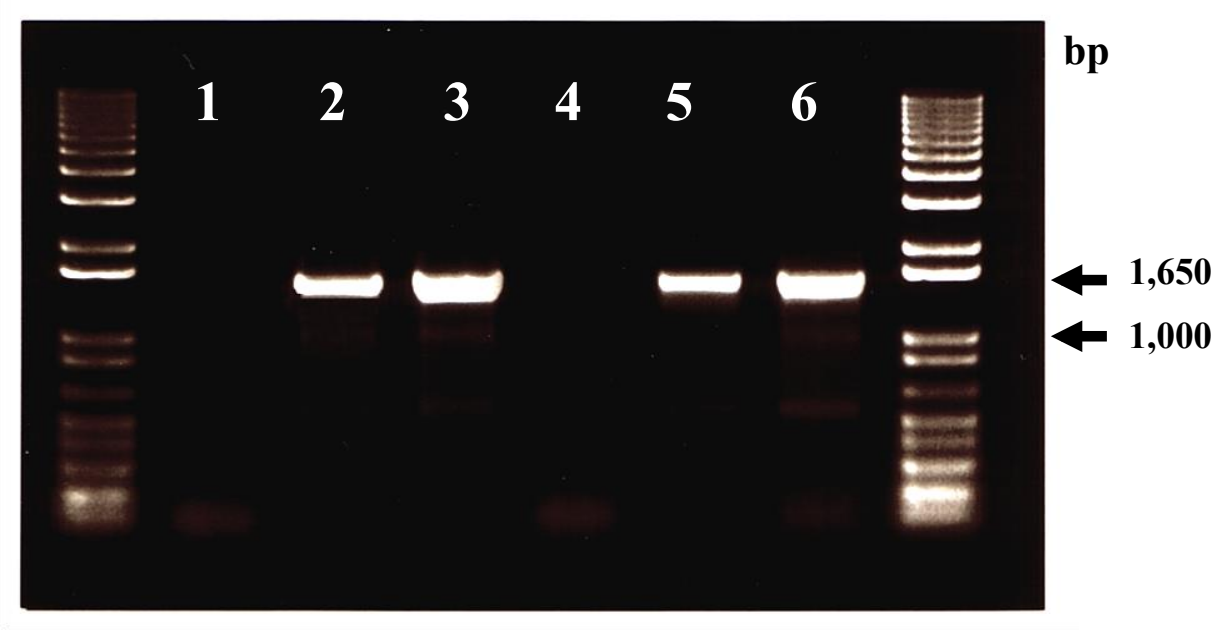


Figure S3.1. PCR amplification of the putative 1,2-D RDase gene for cDNA library construction. cDNA from 1,2-D-grown and propene producing RC and KS cultures was used as template with the degenerate primers RRF2 and B1R. Lanes 1-3 correspond to samples from culture RC and lanes 4-6 to samples from culture KS. Lanes 1 and 4 are "no reverse transcriptase reaction" controls to demonstrate the absence of genomic DNA in the RNA samples; lanes 2 and 5 show amplification using cDNA as template and lanes 3 and 6 correspond to positive controls performed with genomic DNA from the respective cultures. The 1Kb Plus Ladder from Invitrogen is shown.

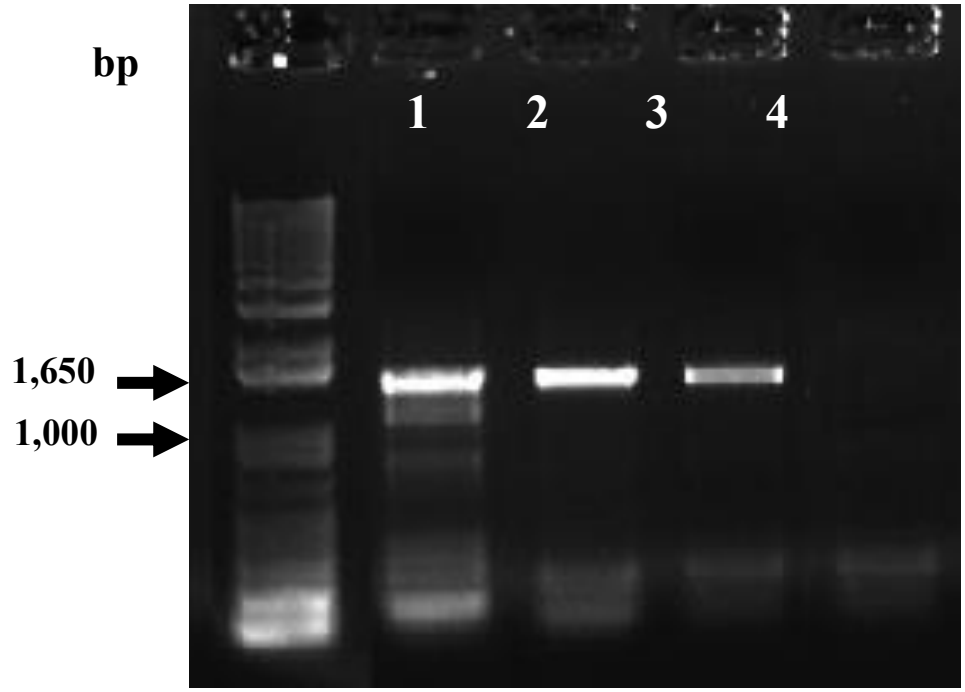


Figure S3.2. PCR amplicons obtained using the primers *dcp_up120F* and *dcp*-1449R. Lane 1 is a positive control that used genomic DNA from *Dhgm* strain BL-DC-9 as template for the PCR reaction. A PCR product of the expected size (~1569 bp) was also obtained using *Dhc* RC and KS genomic DNA as a templates (lanes 2 and 3, respectively). Lane 4 is a negative control that did not include any template DNA in the PCR reaction. A 1Kb Plus Ladder from Invitrogen is shown in the left lane.

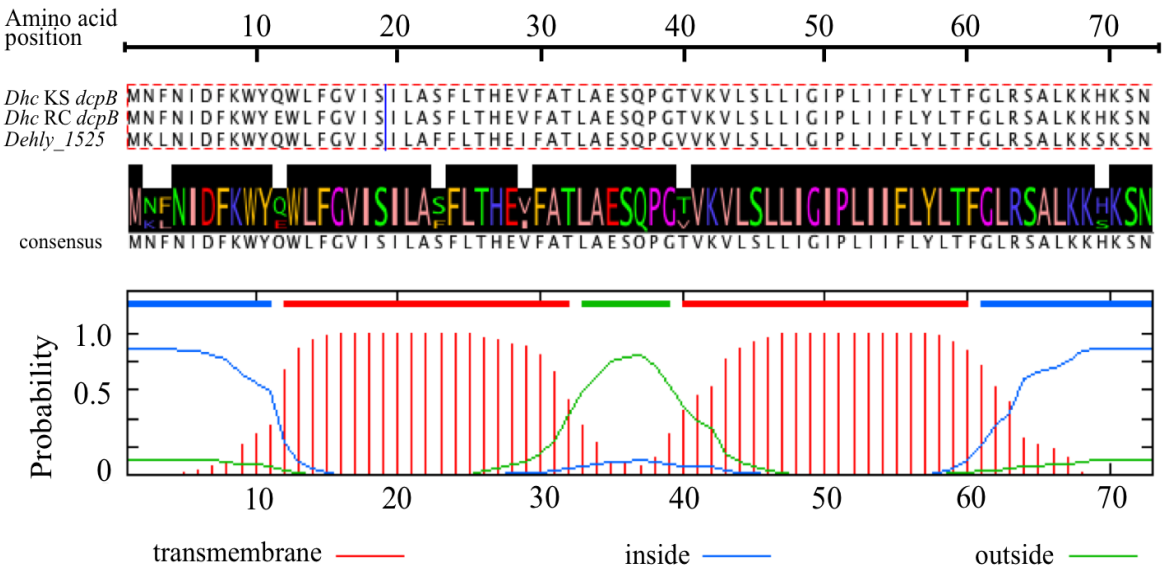


Figure S3.3. Characteristic DcpB features. The upper portion of figure 3 shows the translated amino acid sequence (73 aa long) of the *dcpB* gene of *Dhc* strains RC and KS and *dcpB* gene of *Dhgm* strain BL-DC-9 (Dehly_1525) aligned by ClustalW and visualized in Jalview. The lower portion of figure 3 represents the topology of this membrane anchoring protein predicted by TMMOD, a Hidden Markov model program used to predict where proteins span the cell membrane (http://www.liao.cis.udel.edu/website/servers/TMMOD/). The deduced topology of the DcpB protein revealed two transmembrane regions between positions 12-32 and 41-61. Additional characteristics include two inside loops (i.e., facing the cytoplasm) at amino acid positions 1-11 and 62-73, and one outside loop (i.e., facing the periplasm) from positions 33-40.

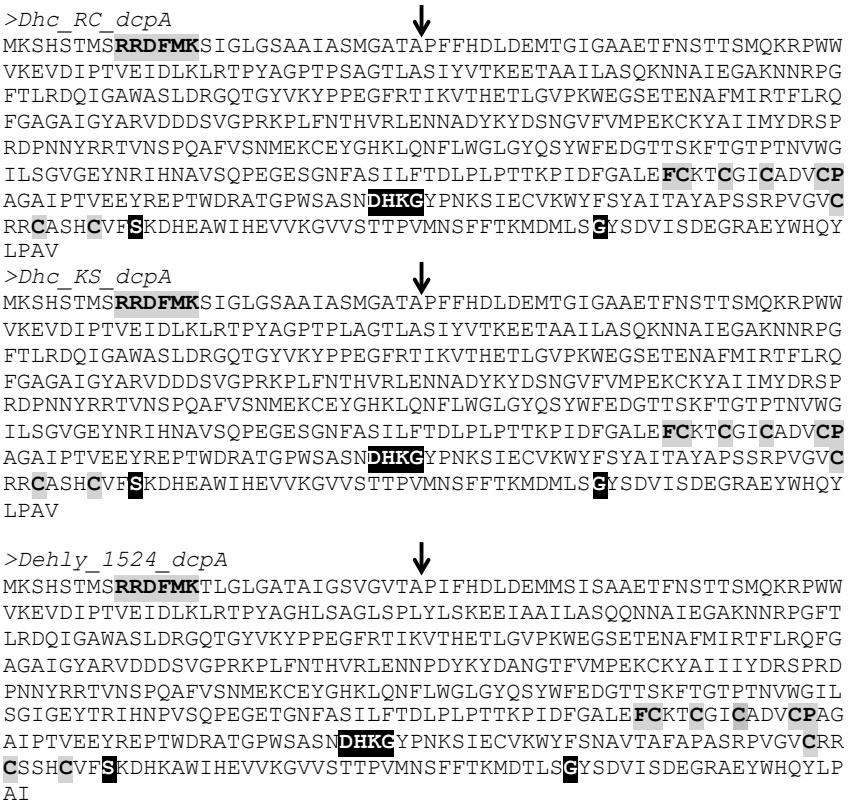


Figure S3.4. Deduced DcpA amino acid sequence of *Dhc* strain RC and KS and the DcpA of Dhgm strain BL-DC-9 (Dehly_1524). Highlighted are the Tat signal peptide motifs in the form RRDMFK present close to DcpA N-terminus. The arrow depicts the predicted leader peptide cleavage site. Also highlighted are the two iron sulfurs clusters in the form $FCX_2CX_2CX_3CP$ and CX_2CX_3C close to the C-terminus. In bold is a possible corrinoid-binding site in the form DHXG-X_{39}-S-X_{32}-G.

dcpA amino acid conservation (480 aa total)

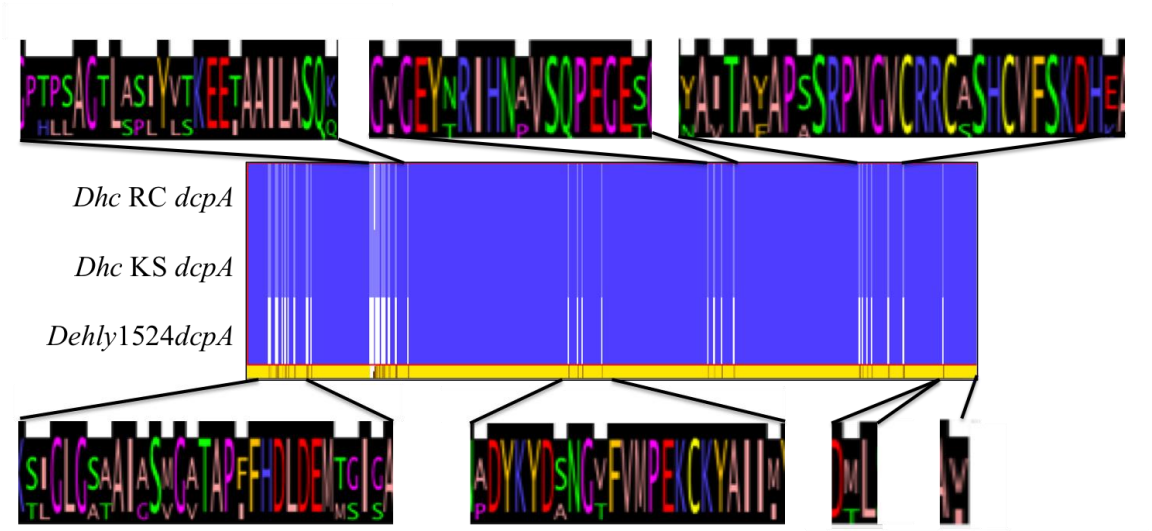


Figure S3.5. Aligned aa sequences depicting conservation sites between RC/KS *dcpA* translated sequences.

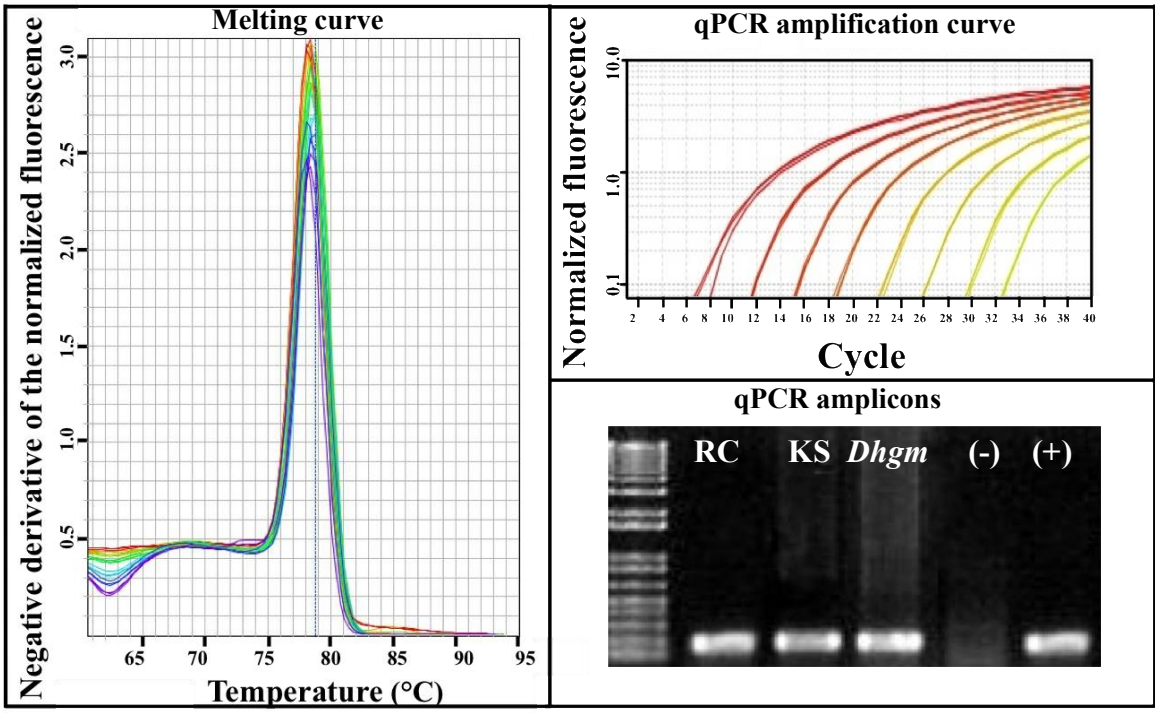


Figure S3.6. Evaluation of qPCR primers and assay conditions. The left panel shows the melting curve analyses performed with the SYBR Green assay with primers *dcpA*-1257F/1449R using genomic DNA of *Dh*c strain RC and strain KS and *Dhgm* strain BL-DC-9 as template. Shown in the top-right panel are examples of TaqMan amplification curves obtained for a 10-fold dilution series of template DNA with the *dcpA* gene-targeted primers *dcpA*-1257F/1449R and the *dcpA*-1426Probe, spanning a range of 1.7 to 1.7 x 10^8 gene copies per µL of template DNA. Additionally, *dcpA* TaqMan PCR amplification products were visualized by electrophoresis to confirm assay specificity (bottom-right panel). Lanes 1-3 are from reactions that used 10 ng of template DNA from *Dhc* strain RC, *Dhc* strain KS, and *Dhgm* strain BL-DC-9, respectively. Lane 4 is a no template control and lane 5 corresponds to a reaction that had 2 ng of plasmid DNA carrying the *dcpA* gene fragment. The 1kb Plus DNA Ladder (Invitrogen) was used.

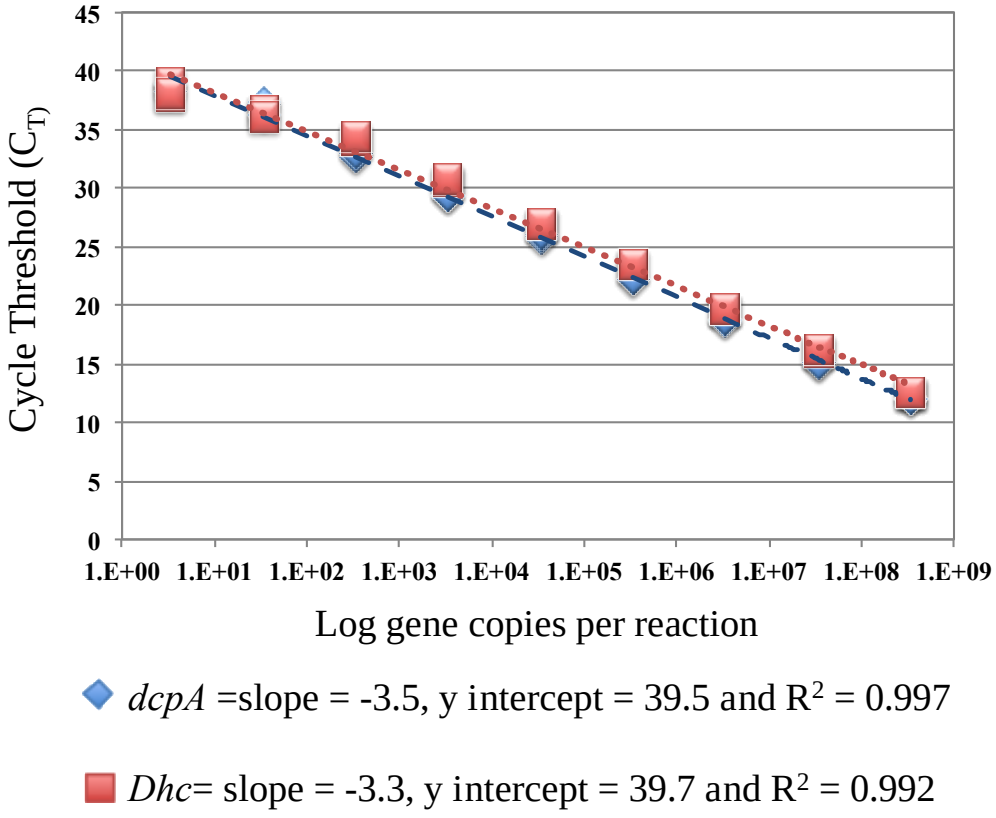


Figure S3.7. *dcpA* and *Dhc* 16S rRNA gene qPCR standard curves using 10-fold serial dilutions of plasmids with the inserts of interest. TaqMan qPCR assays were performed using partial plasmid DNA carrying the *dcpA* gene fragment of *Dhc* strain. Also included is the standard curve for *Dhc* 16S rRNA gene quantification (red squares). The standard curve shown has a dynamic range of 1.7 to 1.7 x 10^8 gene copies per µL of template DNA.

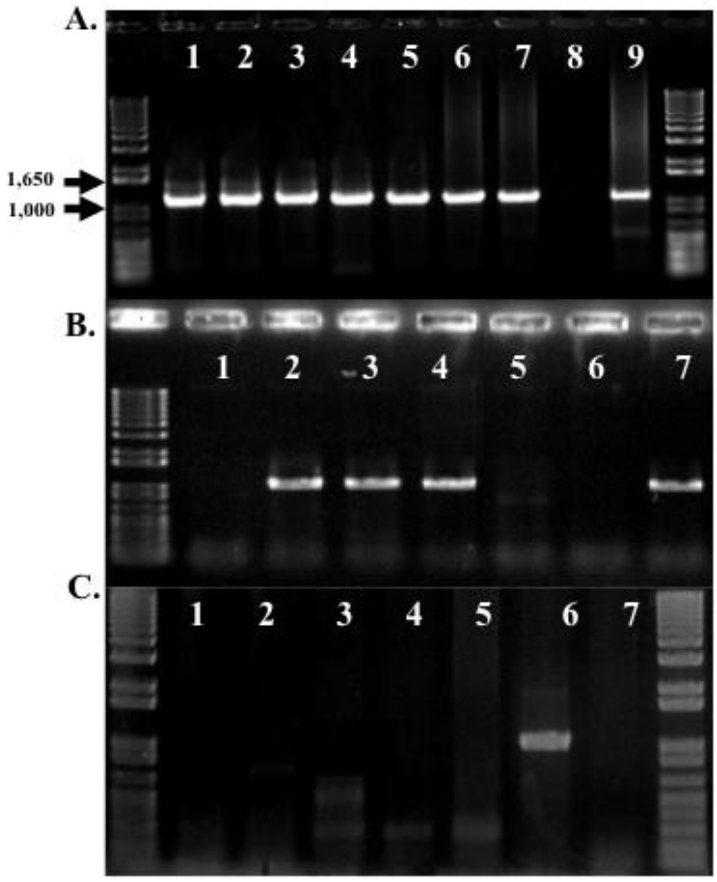


Figure S3.8. *dcpA*-targeted nested PCR results from microcosm and environmental samples. TRS1 TRS2 TRS3 Trester Neckar Brazil 1 Brazil 2, neg pos 1 % agarose gel of *dcpA* PCR products that have been stained with ethidium bromide**.** PCR was performed with the specific primers *dcpA*-360F, *dcpA*-1449R in 20-µL total volume reactions. Marker used on the left is 1kb Plus DNA Ladder (Invitrogen). For all samples five µl of PCR product were mixed with 1 µL of 6X loading dye and resolved in 1% (wt/vol) agarose gels and stained with ethidium bromide (1 µg/mL). Sample designation as follows: **Panel A:** TRS1, TRS2, TRS3, Trester, Neckar, Brazil 1 Brazil 2, negative control (PCR reaction with no template) and positive control 10 ng of genomic DNA of *Dehalococcoides mccartyi* (*Dhc*) RC. **Panel B:** Ft. Pierce Biobead DNA samples: FP-MW-2S, 6-7 m; FP-MW-20, 20-21 m; FP-MW-26, 14-15 m; FP-MW-61, 20-21 m; Waynesboro, negative and posotive control. **Panel C**. Ft. Pierce samples: FP1-MW46, 22-26 m; FP2-MW49, 26-27 m; FP3-MW49, 46-47 m; FP4-MW47, 47-48 m; FP5-MW49, 95-98 m Please refer to Table 3.1. for more comprehensive sample information**.**

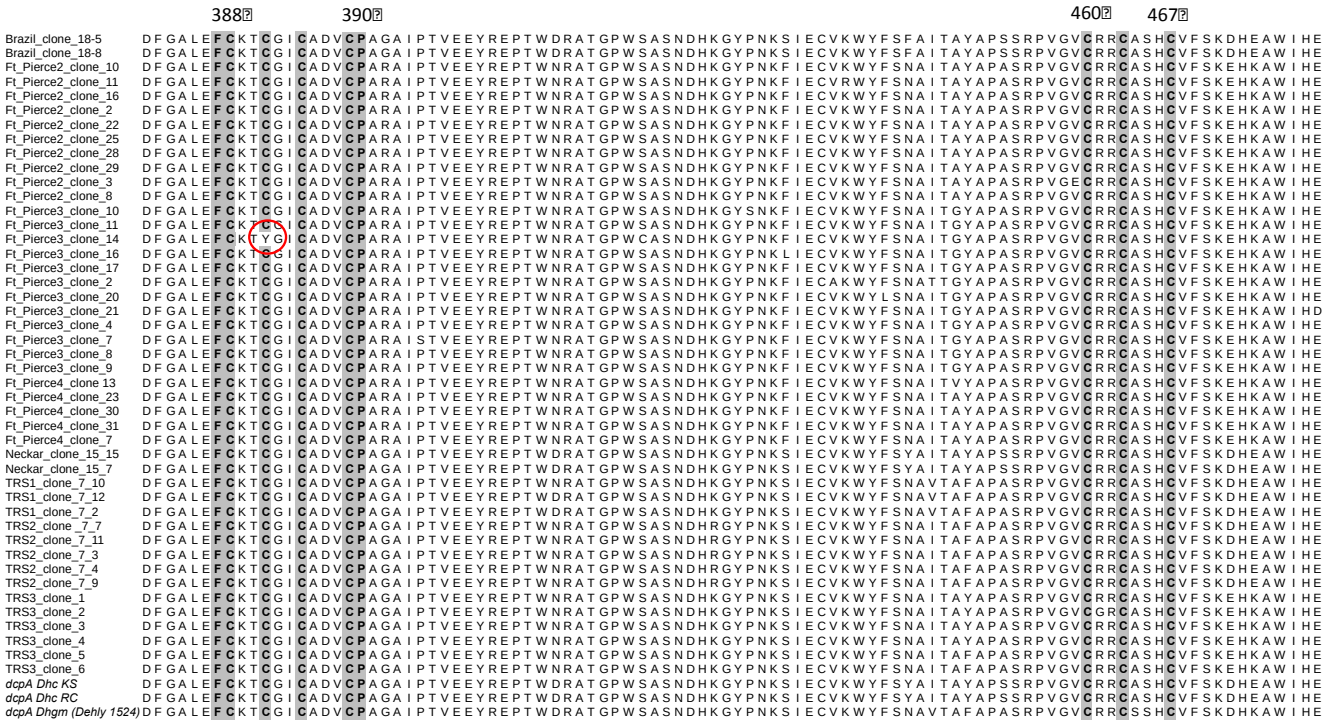


Figure S3.9. Amino acid alignment showing the conserved iron sulfur cluster motifs near the C-terminus of the environmental DcpA sequences. Also included are the *Dhc* RC and KS and the *Dhgm* DcpA sequences. Numbers on top represent approximate amino acid positions with respect to the methionine start site of the DcpA encoded on the genome of *Dhg* strain BL-DC-9. Only one environmental sequence does not possess this consensus motif (RED circle) perhaps representing a variant sequence or a sequencing error.

Chapter 4

Inter-genus reductive dehalogenase gene transfer between organohalide-respiring *Chloroflexi*

Abstract

Multiple, non-identical reductive dehalogenase (RDase) genes are common to genomes of organohalide-respiring *Chloroflexi*, and enable *Dehalococcoides mccartyi* (*Dhc*) strains, *Dehalogenimonas (Dhgm*) spp. and "Dehalobium chlorocoercia" ("Dhom") to couple the reductive dechlorination of halogenated organics to energy conservation. The discovery of *dcpA* in *Dhc* strains RC and KS led to the identification of a gene homolog with 90% nucleotide identity in *Dehalogenimonas lykanthroporepellens* strain BL-DC-9. Bioinformatic analyses and genomic comparisons provided evidence of gene synteny within the *dcp* operon and apparent horizontal gene transfer (HGT) between *Dhc* and *Dhgm*. Further examination revealed that features of a composite transposon flank the *dcp* operon. Various mechanisms for *dcpAB* dispersion are proposed, including transposition via 1S911 elements and XerD-mediated site-specific recombination. Two additional *Dhgm* RDase genes shared with *Dhc* were identified: Dehly_0275, sharing 72-73% nt identity (79% amino acid identity) with the putative *Dhc* RDases DhcVS_1336, DhcVS_96 and DehaBAV1_0284 encoded in the high-plasticity regions of *Dhc* strains BAV1 and VS, and Dehly_0283, which shares 68% aa similarity with RCRDA15 of *Dhc* strain RC. These results indicate that RDase gene exchange occurs across the distinct *Chloroflexi* genera *Dhc* and *Dhgm*. Inter-genus RDase gene HGT may play a major role in the dissemination of reductive dechlorination phenotypes and the acquisition of specific reductive dechlorination capabilities relevant for contaminant attenuation at sites impacted with chloroorganic contaminants.

Introduction

Horizontal gene transfer (HGT, also known as lateral gene transfer) is a major driving force in bacterial evolution by allowing the sharing of genetic material between organisms "separated by reproductive barriers" (Campbell, 2007). Known mechanisms of HGT include conjugation (involving cell-to-cell contact and mediated by plasmid, transposons and integrons), transduction (the mobility of DNA fragments mediated by a phage), transformation (the incorporation of exogenous "naked"-DNA by homologous recombination) and the more recently described gene transfer agents (GTAs) (defective phages that randomly package and transduce portions of host genomic DNA) (Zaneveld, Nemergut, & Knight, 2008).

Of interest for bioremediation is the dissemination of genes involved in the transformation of contaminants since this can affect the fate and longevity of these chemicals in the environment (Hickey, Chen, & Zhao, 2012; Sobecky & Coombs, 2009). Organohalide-respiring *Dehalococcoides mccartyi* (*Dhc*) strains transform toxic chlorinated compounds (e.g., chlorinated ethenes, chlorinated ethanes, chlorinated propanes, chlorobenzenes, PCBs) to their benign, or less chlorinated, products (Löffler FE, Yan J, Ritalahti KM, Adrian L, Edwards EA, Konstantinidis KT, Müller JA, Fullerton H, Zinder SH, 2013). *Dhc* strain RC and strain KS, *Dehalogenimonas* (*Dhgm*) *lykanthroporepellens* strain BL-DC-9 and *Dhgm alkenigignens, Desulfitobacterium dichloroeliminans* and members of the genus *Dehalobacter* have been described to dechlorinate 1,2-dichloropropane (1,2-D), a suspected carcinogen, to innocuous propene (Bowman et al., 2013; De Wildeman et al., 2003; F E Löffler, Champine, Ritalahti, Sprague, Tiedje, et al., 1997; Moe WM, Yan J, Nobre MF, da Costa MS, Rainey, 2009; Kirsti M Ritalahti & Löffler, 2004; Schlötelburg et al., 2002). *Dhc* possess multiple reductive dehalogenase (RDase) gene copies that encode for the key catalytic proteins involved in these dehalogenation reactions. The gene *dcpA* encoding the 1,2-dichloropropane-to-propene RDase has been identified in *Dhc* strains RC and KS and showed remarkable similarity (90% nucleotide identity) with an RDase gene present in the sequenced genome of *Dhgm lykanthroporepellens* strain BL-DC-9 (Padilla-Crespo et al., 2014). DcpA is a unique RDase with no more than 34% aa sequence identity to other RDases, but with 60% overall nt sequence identity to pseudogene DET0162 from *Dhc* strain 195 (Padilla-Crespo et al., 2014). Also, the *dcpB* gene (encoding for a

RDase anchoring protein) in strains RC and KS share high nucleotide similarity with Dehly_1525, including an identical 18 nt intergenic region separating *dcpA and dcpB* (Padilla-Crespo et al., 2014). *Dhgm* 16S rRNA genes share no more than 90% sequence identity with the known *Dhc* strains (Yan J, Rash BA, Rainey FA, Moe WM, 2009). The presence of this RDase gene in phylogenetically distinct organohalide-respiring genera suggests that a HGT event has occurred between *Dhc* and *Dhgm.*

The first indication of integrated elements present in *Dhc* came to light when the genome of strain 195 became available (Seshadri et al., 2005). Nine regions (corresponding to 13.2% of the total genome) were predicted to be integrated elements. Reagard et al. (2005) characterized these genomics regions of atypical oligonucleotide composition in strain 195 and revealed most RDases were located in these regions and suggested that *Dhc* RDases were acquired by HGT (Regeard, Maillard, Dufraigne, Deschavanne, & Holliger, 2005). In 2007, McMurdie et al. detected unusual codon bias in the vinyl chloride reductase gene *vcrA* and suggested that this RDase was horizontally acquired from a non-*Dhc* organism (McMurdie, Behrens, Holmes, & Spormann, 2007). A proposed site-specific mechanism for the dissemination of the RDase gene *vcrA* involving the *ssrA* gene, encoding a transfer messenger RNA, has been described (McMurdie PJ, Hug LA, Edwards EA, Holmes S, 2011). However, the *ssrA*-based mechanism only describes the sharing of the *vcrA* RDase gene and genetic material between *Dhc* strains and does not explain the origin and mobility of other RDase genes including *tceA, bvcA* and *dcpA*, or the presence of all integrated elements and atypical regions in *Dhc* genomes. The donors of these RDase genes remain a hypothetical "foreign host".

Previous observations indicated that 4.7% of the *Dhgm* strain BL-DC-9 genome is composed of insertion elements and that a prophage lies in the region from coordinates 1,604,159 to 1,672,879 (*dcpA* is outside this region), but additional genomic regions of putatively foreign DNA have not been identified. In this study, additional atypical regions in the genome of *Dhgm* strain BL-DC-9 were identified and the evolutionary history and genomic context of the RDase genes in these regions was investigated. The sharing of other RDases across the dechlorinating Chloroflexi, *Dhc, Dhgm* and "Dehalobium chlorocoercia" DF-1 ("Dhom') are discussed via phylogenetic analyses. Special focus was given to the genomic island (GI) around the *dcpA* gene in *Dhgm*

while the corresponding GI in *Dhc* strain RC and strain KS was revealed by PCR efforts. Two copies of IS911 transposase frame the *dcpAB* operon suggesting a composite transposon structure. Inverted repeats at the ends of the transposases indicate a target site. Furthermore, the presence of multiple IS911 elements in the *Dhgm* genome is discussed, especially those in the proximity of the *dcp* operon. Various HGT mechanisms for *dcpAB* mobilization are proposed; among these: dispersion the site-specific, and phage-like recombinase XerD and transposition mediated by the IS911 elements.

Methods

Molecular phylogenetic analysis of RDases.

To examine the phylogenetic association between *Dhgm* and *Dhc* RDases, the protein sequences of all putative RDaseA genes from the genomes of *Dhgm lykanthroporepellens* strain BL-DC-9 (NC_014314, 19 RDases in total), "Dhom" strain DF-1 and *Dhc* (including 16 and 14 sequences from strain RC and strain KS, respectively) were downloaded from GenBank. Translated *dcpA* sequences from the RC and KS strains were obtained as described . The gathered RDases sequences were queried with BLASTP and PSI-BLAST, and the matching sequences were obtained from GenBank. The sequences were aligned using the Clustal Omega software , and tree topology was inferred by the bootstrapped neighbor-joining method with branch lengths estimated by the maximum likelihood method .

DcpA amino acid (aa) sequences were aligned in Jalview and conservation scores for every site of the alignment were plotted in Excel (Excel 2003, Microsoft, Redmond, WA, USA). Jalview calculates a numerical index in a scale from 0 to 11 using the AMAS (Analysis of Multiply Aligned Sequences) method by measuring the number of conserved physico-chemical properties of the amino acids in each column in the alignment. Higher scores represent high conservation in the aa sites of the sequence of interest.

Computational analysis.

For the identification of atypical regions in *Dhgm lykanthroporepellens* strain BL-DC-9 genome (NC_014314.1), the IslandViewer (http://www.pathogenomics.sfu.ca/islandviewer/query.php) interface was used for the computational visualization of genomic islands (GI) (M. G I Langille & Brinkman, 2009). This tool incorporates several detection methods [e.g. IslandPath-DIMOB (Morgan G I Langille, Hsiao, & Brinkman, 2008) and SIGI-HMM (Waack et al., 2006) to examine common characteristics of foreign GIs for sequence composition bias and the presence of mobility genes. Another online tool, SeqWord Genome Browser (www.bi.up.ac.za/SeqWord/), allowed identification of atypical regions within a genome based on oligonucleotide usage (OU) (Ganesan, Rakitianskaia, Davenport, Tümmler, & Reva, 2008). The "interphase" calculates the statistical OU parameters defined as: (D)– distance between the OU patterns (calculated as a percentage of the sum of all possible distances between the OU patterns); PS – defined as pattern skew, is the distance between OU patters (D) in both the direct and the reverse strands of the same DNA molecule; RV– local relative oligonucleotide variance and GRV – global oligonucleotide variance (local variance normalized by the calculated nucleotide frequencies for the complete genome). The SeqWord web-browser was used to calculate the tetranucleotide usage (OU pattern: 4mer) and other parameters using a sliding window of 8 kbp genome fragments in 2 kbp steps. Genomic fragments deviating from the genome signature (with high RV, low D, and low PS values) where considered of HGT origin. Bacterial genomes typically have a low PS (indicating strand symmetry), whereas phage and viral elements were shown to have high strand asymmetry (high PS). For more in depth information on SeqWord OU pattern definitions and calculations see Ganesan et al. 2008 (Ganesan et al., 2008).

Furthermore, the average guanine cytosine (GC) content and genomic dissimilarity (δ*) of the *dcpA* GI (Dehly_1519-Dehly_1527, coordinates: 1493353 to 1499907) with respect to the *Dhgm* strain BL-DC-9 genome, was calculated using the web-based application delta-rho (http://deltarho.amc.uva.nl) using an non-overlapping window size of 10,000 bp or 10,500 (van Passel, Luyf, van Kampen, Bart, & van der Ende, 2005). A high δ* indicated a high discordance in the DNA nucleotide composition between the GI region and the genome of interest, and

fragments with a plot position of 90% or more δ* are indicative of HGT events. Statistical significance was determined by running a two-tailed *t*-test comparing the GC content and δ* values of the *dcpA* GI sequence to the overall average of the *Dhgm* genome.

The difference in codon usage (i.e., codon bias in a DNA sequence) was also used to detect foreign acquisition of genes. Codon adaptation indices (CAI) were calculated using the "CAI calculator software" (http://genomes.urv.es/CAIcal/). This interphase calculates the synonymous codon usage of a gene(s) of interest relative to a reference set of genes (Puigbò, Bravo, & Garcia-Vallvé, 2008). CAI values from the genes from the putative *dcpA* GI (Dehly_1518-1527) and Dehly_0269-0283 were computed using codon usage tables based on *Dhgm* strain BL-DC-9 and *Dhc* strain VS housekeeping genes (*n*=118 for *Dhgm* and *n*=142 for *Dhc*), and included genes encoding for ribosomal proteins, amino-acyl-tRNA-synthetases, chaperonins, oxidoreductases (e.g. ATP synthase F1, NADH:quinone, hydrogenases) among others. In this analysis, the genes from *Dhc* strain VS (whose genome possesses 36 RDase genes) were used as a proxy for the genomes of the 1,2 D-dechlorinating *Dhc* strains RC and KS, whose genomes sequences are not available). This proxy is justified by the fact that several sequenced *Dhc* genomes are more than 85% identical and share up to 100% similarity in housekeeping genes. Additionally, the E-CAI server (http://genomes.urv.es/CAIcal/E-CAI/) was used to calculate the expected CAI value for each gene, and to demonstrate that the CAI values are statistically significant the codon bias values are not due to sequence composition artifacts. CAI values were normalized (divided by) the expected CAI values (eCAI) using the Poisson method with a 99% interval confidence and 99% population coverage. Normalized CAI values with scores < 1.00 are indicative of codon bias and identified as putative foreign genes.

Synteny block construction and GI characterization.

Chromosomal regions identified as genomic islands were further analyzed in the KEGG Database (http://www.genome.jp/kegg/kegg1.html) to confirm their gene function, annotation and predicted motifs. The MaGe (Microbial Genome Annotation and Analysis Platform) interface was used to explore the gene organization in the GIs and to find synteny blocks to other

Dhc strains (https://www.genoscope.cns.fr/agc/microscope/home/index.php). Genes of interest were further analyzed by amino acid sequence and domain alignments using the GenBank, PFAM, COG and KEGG databases to identify orthologues, confirm their annotation and aid in identification of hypothetical genes. Homologous genes were aligned with ClustalW, and their % identity across the full length was reported. Genomic regions adjacent to the IS911 elements in the neighborhood of the *dcpA* GI were visually inspected for the presence of inverted repeats and compared with known IRL and IRR sequences of characterized IS911 elements in *E. coli.*

Source DNA and PCR primer design for dcpA genomic island discovery.

Cultures of *Dhgm* strain BL-DC-9 (isolated from a Superfund site near Baton Rouge, Louisiana, USA) and *Dhc* strain RC and KS (from the Red Cedar River near Okemos, Michigan, and the King Salmon River in Alaska, respectively) were grown as described (Moe WM, Yan J, Nobre MF, da Costa MS, Rainey, 2009; Padilla-Crespo et al., 2014; Kirsti M Ritalahti & Löffler, 2004). Replicate 100 mL-liquid cultures were harvested by vacuum filtration using a MoBio polyethersulfone filter membrane with 0.22 µm pore size (MoBio Laboratories Inc., Carlsbad, CA, USA, part number 14880-50-WF). The PowerWater DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) followed the manufacturer's recommendations and an additional step for improved cell lysis was included by heating the samples at 65°C for 10 minutes before the bead beading step.

Primers targeting the region surrounding the *dcpA* gene (Dehly_1524) were designed based on the genome of *Dhgm* strain BL-DC-9 (NC_014314.1). Primer pairs targeted the genes Dehly_1518 to Dehly_1531 and were tested under standard Phusion-enzyme PCR conditions (New England Biolabs, Table S4.1). The PCR reactions consisted of 1 U of the Phusion High Fidelity Polymeraze, 1x Phusion HF Buffer, 200 mM dNTPs, 100 pmol (or 0.5 µM forward primer and 0.5 µM reverse primer) each of primer, 3% (vol/vol) dimethylsulfoxide (DMSO) and 10 ng of template genomic DNA. Amplicons were purified with the Qiagen PCR Purification kit (Qiagen, Valencia, CA, USA) and cloned into the pCRII vector before transformation into chemically competent TOP-10 *E. coli* cells using the TOPO TA cloning kit (Invitrogen). Since

the Phusion enzyme produces blunt-end products and the TA cloning kit requires 3' A overhangs, an extra-step was required to adenylate the PCR products prior to cloning. The addition of 3´A-overhangs to the PCR products was performed by incubating the purified amplicons (no Phusion enzyme present) at 72°C for 15 min in the presence of 0.2 mM dATP, 1x GeneAmp PCR Buffer and 1U of AmpliTaq Polymerase (ABI). Cloned PCR products were not bigger that 2-3 kb, since the efficiency of the TOPO TA cloning protocol decreases when cloning inserts bigger than 3 kb. Plasmids with inserts of the correct size were sequences with vector-specific and internal sequencing primers (Table S4.1).

Results and Discussion

Phylogenetic analysis of dcpA.

The *Dhc* DcpA RDase shares 92% sequence identity with *Dhgm* RDase Dehly_1524. The analysis of the 484 aa positions illustrated that 98.7% (478/484 aa) are highly conserved (i.e., conservation scores > 7, Fig. 4.1) either identical or replaced with an aa with physico-chemical properties). The region with the least conservation is around the aa positions 80-90. The DcpA of *Dhc* strains RC and KS are true homologs (sharing common ancestry) to the DcpA RDase of strain BL-DC-9, as is apparent in the aa sequence bootstrapped maximum likelihood tree (Figures 4.2 and 4.3). The homology of the *dcpA* RDase gene across microorganisms that only share 90% nt identity at the 16S rRNA gene is evidence of an HGT event between these two genera of organohalide-respiring bacteria within the phylum Chloroflexi. Observations of plausible HGT across genus boundaries have been made, such is the case of the tetrachloroethene reductive dehalogenase (*pceA*) genes of the Gram-positive *Desulfitobacterium* and *Dehalobacter* groups (Maillard, Regeard, & Holliger, 2005).

At the nucleotide level, the most similar gene to *dcpA* is the pseudogene DET0160 identified on the genome of strain 195 (Padilla-Crespo et al., 2014). The fact that the *dcpA* gene in *Dhgm* is more similar to the *Dhc* RDase genes than to any other *Dhgm* RDase gene (Fig. 4.3) could be an indication that *Dhgm* acquired this gene from *Dhc*. In addition, the *dcpA* gene (Fig. 4.3) is part

of a larger cluster including DET0311, DehalBAV1_0104, cbdbA88, plus two "Dhom" strain DF-1 RDase gene sequences.

dcpA GI in the Dhgm genome.

Bioinformatics and computational analysis on the *Dhgm* strain BL-DC-9 genome predicted 16 putative GIs (Fig. S4.1). The *dcpA* gene (Dehly_1524) is located in one of these GI from genome coordinates 1493353 to 1499907. The *dcpA* GI is comprised of 9 genes (Dehly_1519 to Dehly_1527) and exhibits a high genomic dissimilarity value (δ*=69.9) based on dinucleotide frequency compared to the rest of the *Dhgm* genome (Fig. S4.2). Additionally, the % G+C content of the *dcpA* GI with 46.4% was well below the chromosomal average of 55% G+C (Fig. 4.3). The *dcpA* GI is an anomalous sequence, in comparison to 95% of the *Dhgm* genome fragments, which exhibit on average a lower δ* and a higher % G+C content (Fig. S4.4). Oligonucleotide usage analysis performed on the *Dhgm* strain BL-DC-9 genome showed that the *dcpA* GI has a dissimilar tetranucleotide usage pattern from the core genome. Specifically, the *dcpA* GI showed a high local pattern deviation and local relative variance, divergent from the global relative variance (Fig. 4.4). Further analysis of other oligonucleotide usage parameters also showed that the region possessed a low relative variance, significantly high local pattern deviation and moderate to high pattern skew when compared to the rest of the genome (Fig. 4.5).

Additionally, the codon usage for the genes surrounding *dcpA* in *Dhgm* shows a low normalized CAI (CAI/eCAI < 1.00) values further suggesting divergence following acquisition through HGT (Table S4.2). Together all of these properties are characteristic of HGT GIs, transposons, pseudogenes, phages and IS elements.

Oligonucleotide biases in *Dhc* genomes and in among RDase genes have been previously described (McMurdie et al., 2007; Regeard et al., 2005). Regeard *et al,* (2005) identified "atypical regions" in the genome of *Dhc* strain 195 (Regeard et al., 2005). These locations also depicted a GC composition different than the rest of the chromosome; reductive dehalogenase genes were located in these regions along with a high incidence of mobile elements (e.g.

transposase genes). Moreover, McMurdie *et. al.*, (2007) noted that RDases have a low GC content and that their third codon positions are biased to the T nucleotide (McMurdie et al., 2007).

The *dcpAB* genes in strain BL-DC-9 are sandwiched between a pair of genes annotated as "Transposase" (Dehly_1521 and Dehly_1528) and "Integrase catalytic unit" (Dehly_1522 and Dehly_1527) (Fig 4.6). These genes have similar size and arrangements of IS911 transposable elements, member of the large IS3 family (https://www-is.biotoul.fr//is/IS_infos/IS3_family.html). Further analysis confirmed that Dehly_1527-1528 and 1521-1522 posses all features present in IS3/911 insertions elements including: (1) an overlapping region between the genes 1521 (*orfA*) and 1522 (*orfB*) associated with a −1 programmed translational frameshift (induced by AG_4G motif), resulting in the expression of an OrfAB transposase (2) regions in the OrfA corresponding to helix-turn-helix (HTH) DNA-binding motifs similar to RuvC Holliday junction resolvases (Rousseau, Gueguen, Duval-Valentin, & Chandler, 2004), and a leucine zipper (LZ) motif transposase involved in regulation and protein oligomerization (Haren, Normand, Polard, Alazard, & Chandler, 2000; Haren, Polard, Chandler, & Paul, 1998) (3) a DDE motif in the OrfB for catalytic transposition closely related to retroviral integrases (Rousseau, Tardin, Tolou, Salomé, & Chandler, 2010). In figure 4.7 the distinctive features of 1S911 transposition noticed in Dehly_1521- Dehly_1522 are depicted (Rousseau et al., 2010). Furthermore, Dehly_1527-1528 and 1521-1522 are identical insertion sequences reading in opposite direction; a characteristic of composite transposons

Cloning and characterization of the genes encompassing the dcpA genomic island in Dhc strains RC and KS.

The *dcpA* GI in *Dhc* strains RC and KS was cloned using primer pair combinations targeting the upstream region of the *dcpA* gene (Dehly_1524) in *Dhgm* strain BL-DC-9. PCR reactions with primers 1520F and 1524R targeting for *Dhc* RC and KS genomic DNA amplified a smaller region of ~2.9 kb, in contrast to the 4 kb region in *Dhgm* (Fig. 4.8). The smaller amplicon obtained with template DNA from *Dhc* strains RC and KS was due to the absence of the

integrase (Dehly_1521) and transposase (Dehly_1522) genes (Fig. 4.8). Analysis of the 4 kb fragment of *Dhc* strains RC and KS contained a complete open reading frame (ORF) of 1,383 nt (467 aa) of a fused Dehly_1523 and Dehly_1520 gene. The *Dhgm* gene Dehly_1520 is annotated as a RDase gene yet it lacks common RDase features (i.e., no associated B gene and no iron sulfurs clusters) and a variant sequence (SNKDFMQ) instead of the conserved SRRXFLK twin-arginine motif. Dehly_1520 is also much smaller than a typical RDase (only 120 aa in length compared to the typical RDase size of ~ 450-500 aa). The Dehly_1520 protein has high sequence similarity in the N-terminal region sharing 32% aa identity and 57% similarity to RDases like DhcVS_99 (Fig. S4.5). By contrast, Dehly_1523 has two iron sulfur clusters in the form $CX_2CX_2CX_3CP$ and CX_2CX_3CP, which are commonly found near the C-terminus of RDases but is annotated as a "4Fe-4S ferredoxin". These observations suggest that the homologs to Dehly_1520 and 1523 are fused in *Dhc* strains RC and KS, comprising complete ORFs and are putative RDase genes. Dehly_1520 and Dehly_1523 may have been an intact gene in *Dhgm* strain BL-DC-9 that was interrupted by the insertion of the integrase and transposase genes Dehly_1521 and Dehly_1522, respectively. RDase gene interruption has been observed in *rdhA2* Dhaf_0696 of *Desulfitobacterium hafniense* strain DCB-2, which was interrupted by the insertion of the transpose gene *tra* (Kim et al., 2012).

PCR with the primer pair 1518F/1520R using template DNA of *Dhc* strain RC and KS resulted in an amplicon of 1.5 kb, larger than the expected 1 kb fragment expected based on the *Dhgm* genome sequence (Fig. 4.8). The difference in size suggested commonalities as well as differences in the gene composition and arrangement of this region; sequence analysis of the fragment revealed very high sequence identity to the region in *Dhgm* corresponding to genes 1519 and 1520. A homolog to the Xre (xenobiotic response element) transcriptional regulator Dehly_1519 was found in the *dcpA* GI in strains RC and KS (with 95 and 96% aa identity, respectively) (Fig. 4.8 and Fig. S4.5). Xre transcription regulators are a large family of transcription factors with a characteristic helix-turn-helix motif involved in DNA binding (Santos, Tavares, Thioulouse, & Normand, 2009). Characterized bacterial Xre family-like proteins include methylases, plasmid control proteins, restriction and modification systems, operator-promoter region in the metabolism of halogenated dibenzofurans and bacteriophage transcription control (i.e. in *B. subtilis* Xre genes repress the acquisition or transfer of integrative

and conjugative elements) (Tirumalai & Fox, 2013). MarR-type regulators have been implicated to modulate RDase gene expression; however, *xre* gene products may also function as DNA-binding response regulators in *Dhc* (A. Wagner et al., 2013).

Interestingly, in strains RC and KS, the primer pair 1518F/1520R amplified a 300-500 nt partial gene most similar to DET0157, which encodes a XerD-like phage integrase/site-specific recombinase in *Dhc* strain 195 (Fig. 4.8). DET0157 is located a few genes upstream of pseudogene DET0162, which shares a common ancestry with *dcpA* (Padilla-Crespo et al., 2014) and is located within an insertion element region, designated IE II (DET0155 to DET0169) (Regeard et al., 2005). The XerD integrase DET0157 shares a high degree of conservation with the C-terminus of XerD integrases in available *Dhc* genomes including those of strains GT, BAV1, VS, DCMB, BTF08, DCMB5, and CG1. No integrase with >36 % aa identity outside the *Dhc* genus was found in the databases (October 2014). XerD-like phage integrases are part of the superfamily of DNA breaking-rejoining enzymes involved in DNA binding and site-specific integration. This family of integrases includes the bacteriophage lambda integrase, the Cre recombinase from *E. coli* bacteriophage P1 and type IB topoisomerases. XerD enzymes are involved in mechanisms such as termination of chromosomal replication (in chromosome segregation), transposition of conjugative transposons, integration and excision of lysogenic bacteriophage genomes, and the stable control of circular replicons (plasmid) inheritance, which are possible mechanisms involved in foreign DNA insertion events in *Dhc*. In the neighborhood of this DET0162, other mobile elements including ISDet2 transposases, DET0165 (*orfA*) and DET0166 (*orfB*) were noted. Interestingly, DET0162 is preceded by a gene encoding a hypothetical protein (DET0161), which has as its closest match to a transposase from *Vibrio vulnificus* YJ016 (YP_180910). Both proteins share a helix-turn-helix domain characteristic of transferases involved in DNA binding, also alluding to possible HGT events (Grindley, Whiteson, & Rice, 2006; Nash, 1996). The assembled gene sequences around *dcpA* in *Dhc* strains RC and KS also had CAI values below the average (normalized CAI < 1.00) for *Dhc* housekeeping genes (*n*=142) (Table S3*)* further suggesting foreign origin. Lastly, primer sets targeting genes upstream of Dehly_1518 as well as downstream of *dcpA* (from Dehly_1527-1531) failed to produce amplicons indicating sequence divergence outside this region in strains RC and KS (Table S4.1).

Additional phylogenetic and genomic analysis of RDase genes in Dhc, Dhgm lykanthroporepellens and "Dhom" suggest HGT across genera.

Besides *dcpA*, three other RDase genes (Dehly_0275, Dehly_0274, Dehly_0283) where identified among the 16 putative GIs on the *Dhgm* strain BL-DC-9 genome (Fig. S4.1). Normalized CAI values, a comparative measure of codon usage, for the genes Dehly_0269-Dehly_0283 (genomic coordinates 258965 to 269792) were calculated. All the genes in this region had values below the average (i.e., normalized CAI < 1.00) for *Dhgm* housekeeping genes (n=118) suggesting a codon bias (Table S4.1).

Comparative analysis on RDases Dehly_0275 and Dehly_0283 revealed high sequence identities to RDases encoded on the genomes of *Dhc* strains VS and BAV1 (Figs. 4.2 and 4.3). Dehly_0275 shares 79% aa identity (across the entire length of the protein) with three *Dhc* RDases DhcVS_1336, DhcVS_96 and DehaBAV1_0284. The genes DhcVS_96 and DehaBAV1_0284 are orthologs (sharing 87% nt identity; 92% identity at the aa level) localized in the first of two high plasticity regions (HPR1) of *Dhc* strain VS and strain BAV1 . DhcVS_1336 is identical to RDase DhcVS_96 gene, and is localized in a genomic region within HPR2 that is specific to strain VS.

Additional examination of this gene neighborhood (Dehly_0269 - Dehly_0283) identified multiple genes associated with mobile elements. In this GI of 15 genes, transposase and integrase genes account for a third of the genes (5 out of fifteen) (Figure S4.6). The GI included two integrase catalytic units (Dehly_0279 and Dehly_0280) and three transposase genes of the IS3/IS911-type (Dehly_0272, Dehly_0278, Dehly_0281). These transposase match multiple gene copies (99-100% nt sequence identity) throughout the *Dhgm* genome (i.e. Dehly_0278 matches 10 genes that are 99-100% identical at the nt level), and the *Dhgm* genome is replete with mobile elements (i.e., it contains 74 transposase genes).

In the other hand, the transposase Dehly_0281 shares 57% aa identity with four IS3/IS911 transposases encoded on the genome of *Dhc* strain BAV1: DehaBAV1_0082, DehaBAV1_0293, DehaBAV1_0976, DehaBAV1_1326. In the genome of strain BAV1, these transposase genes

are 100% identical to each other, and all but one of these four transposase genes (DehaBAV1_0976) are located in HPR regions. Interestingly, this type of insertion element (IS3/IS911-type) is also present downstream the *pceABCT* operon of *Dehalobacter restrictus* and *Dehalobacter* sp. strain E1 (Maphosa, Van Passel, De Vos, & Smidt, 2012). Sequences encoding IS3/IS911 transposases appear to be common around RDases in *Dehalobacter* sp. CF (AFV06388.1) and *Dehalobacter* sp. DCA (AFV03401.1) as well as in *Dhc* genomes. Based on these findings, the insertion element IS3/911 and the *xerD* gene encoding a recombinase appear to be common in regions affected by HGT in organohalide-respiring bacteria.

Lastly, the putative RDase Dehly_0283 has high aa sequence identity (69%, 79% similarity) with the translated sequence of the RDase gene RCRDA15 (ABY28334.1) from *Dhc* strain RC (Figure 3). Other RDases encoded on the *Dhc* and *Dhgm* genomes shared less than 68% aa identity to Dehly_0283.

Other apparent HGT events between *Dhc* and "Dhom" are indicated in Figure 4.3. The RDases DET0311, cbdb_A88, DehaBAV1_0104 (from *Dhc* strain 195, CBDB1, BAV1, respectively) share 87% aa identity with the translated sequence of gene1_0603 from "Dhom" DF-1, while DhcVS_1320, shares 79% identity with the translated nucleotide of gene3_0607 from strain DF-1 (Fig 4.3). ORF4 on the draft genome (scaffold 0604) "Dehalobium chlorocoercia". In addition, DET0311 shared 87% aa identity with ORF1 on scaffold 0603 from "Dehalobium chlorocoercia". Given that housekeeping proteins in *Dhc* and "Dehalobium chlorocoercia" strain DF-1 share an average aa identity of 71%, cross-genus RDase similarities in groups *i* and *v* may represent lateral transfers between *Dhc* and "Dehalobium"

Other apparent HGT events between *Dhc* and "Dhom" are indicated in Figure 4.3. The RDases DET0311, cbdb_A88, DehaBAV1_0104 (from *Dhc* strain 195, CBDB1, BAV1, respectively) share 87% aa identity with the translated sequence of gene1_0603 from "Dhom" DF-1, while DhcVS_1320, shares 79% identity with the translated nucleotide of gene3_0607 from strain DF-1 (Fig 4.3).

The cluster containing DehaBAV1_0284, DhcVS_96, and DhcVS_1336 have two *Dhgm* sequences and two "Dhom*"* sequences at ancestral nodes, suggesting these sequences originated outside the *Dhc* genus. "Dhom" strain DF-1 gene59_1491 shares 91% identity and 95% similarity with the Venice lagoon clone CCA41202 obtained from PCB-contaminated sediment. Also "Dhom" strain DF-1 gene9_1501 shares 82% identity and 87% similarity with the marine sediment clone, BAI47846.

The red bracket in Figure 4.4, indicate the *Dhc* gene DhcVS_1342 with apparent origins outside of the *Dhc* group. This gene clusters most closely with Dehly_0910 from *Dhgm* and gene3_0607 from strain DF-1. Interestingly, DhcVS_1342 is downstream of the duplicated RDase gene DhcVS_1336 and the pair of IS3/IS911-transposase/integrase genes DhcVS_1332and DhcVS_1333. However, the 58% identity shared with its most closely related homologue (Dehly_0910) is too low to infer (recent) HGT. Moreover, Dehly_0910 is immediately upstream of essential nucleotide biosynthesis, which in general posses a low tendency for HGT. Other more recent apparent transfers between *Dhc* and "Dhom" are indicated in Figure 4.3: DET0311 from *Dhc mccartyi strain* 195 who shares 87% identity with gene1_0603 from "Dhom" strain DF-1 and DhcVS_1320 shares 79% identity with gene3_0607 from "Dhom" strain DF-1.

HGT and possible mechanism of insertion

RDase genes with assigned function include *tceA* (TCE RDase), *vcrA* (VC RDase), and *bvcA* (VC RDase) have all been shown to have features indicative of HGT (Krajmalnik-Brown et al., 2007; McMurdie PJ, Hug LA, Edwards EA, Holmes S, 2011). Regeard et al. found indications that 15 of the 18 RDase genes in in strain 195 (including *tceA*) were located in regions of putative HGT origin (Regeard et al., 2005). Furthermore, the *pceA* RDase gene (encoding a PCE-to-*cis*-DCE RDase) of *Geobacter lovleyi* strain SZ is located in an atypical region speculated to be a GI (D. Wagner et al., 2012). These findings suggest that RDase genes are prone to HGT.

The direction of HGT (donor versus recipient) and the mechanisms of insertion and mobility are unclear without experimental evidence, and correlations cannot be evaluated without bias because the databases consist primarily of *Dhc* RDases while only one *Dhgm* and "Dhom" genome are available. Recently, a site mobilization element *ssrA* (tmRNA, DhcVS_1281) was described in *Dhc* strain VS as an integration site for the VC RDase gene *vcrA*. The immediate upstream and downstream region of the *dcpA* gene in the *Dhgm* genome strain BL-DC-9 was inspected, but no *ssrA* signals were found. Further analysis revealed that a *ssrA* gene (tmRNA, Dehly_R0050) and *smpB* (Dehly_1478, encoding an SsrA-binding protein) are present in *Dhgm* strain BL-DC-9 but not in close proximity to *dcpA*. Moreover, the vicinity of *dcpA* does not share synteny with the integration modules described for *ssrA* GIs (McMurdie PJ, Hug LA, Edwards EA, Holmes S, 2011). The region surrounding *dcpA* does not consist of an *ssrA*-specific GI, indicating that multiple mechanisms are involved in RDase gene HGT.

The presence of IS911 elements in the *dcp* operon in *Dhgm* suggest that the RDase gene insertions in this genome are recent, while in *Dhc*, the genes essential for the transfer events may have been lost. Insertion elements of the IS3/911-type are characterized by having imperfect, terminal left and right inverted repeats (IRL and IRR) of ~36 bp, involved in binding and recognition of the flaking-end elements (Rousseau et al., 2010). Terminal imperfect inverted repeats of 34 bp in length were identified at the 5' and 3'ends of transposases; Dehly1521 - Dehly1522 and Dehly1527 - Dehly1526. The identified IRL and IRR, shared ~70% nt sequence identity between themselves and highly reassembled those in characterized IS911 elements in *E. coli* and (see Figure 4.6) for nucleotide sequence comparison of the terminal IRs). The pathway for IS911 transposition has been described by (Rousseau et al., 2010) and a proposed mechanism for its behavior in the region surrounding *dcpA* is depicted in Figure 4.10A. IS911 insertion has been shown to be both targeted (next to DNA sequences similar to *orfA*) and not targeted (with no need of DNA sequence homology) (Rousseau et al., 2007) and the transposition as a "copy-paste" mechanism (Duval-Valentin, Marty-Cointin, & Chandler, 2004). The genetics and mobile mechanism of IS911 transposition explains the high number and propagation of this element in the genome of *Dhgm* strain BL-DC-9. Thirty-tree copies of the IS911 elements are harbored in the genome. These are 99-100% identical and are located throught the genome in the exact arrangement as depicted in Figure 4.7 (66 overlapping genes predicted to encode a complete

transposase; OrfAB). Aside self-propagation as simple transposons (excision and reinsertion in the genome), identical elements flanking a series of genes can render the region mobile, a characteristic of composite transposons.

A possible scenario is that *dcpA* was originally in a *Dhc* host strain and the *dcpA* island was replicated and moved to *Dhgm* in an IS911 circle (or a plasmid containing the IS911 elements). Such a circular element was identified in the organohalide-respiring *Desulfitobacterium hafniense* strain TCE1, harboring the transposon (Tn-Dha1) carrying the *pceA* RDase gene (Maillard et al., 2005). Tn-Dha1 has two identical insertion sequences (belonging to the IS256 family) that flank the *pce* gene cluster (just like the IS3/911 elements surround the *dcpA* GI) and integrates by transposition and homologous recombination across the identical copies of the insertion sequences (Futagami, Yamaguchi, Nakayama, Goto, & Furukawa, 2006). Another case is the composite transposon Tn5280 of *Pseudomonas* sp. strain P51, which harbors a chlorobenzene dioxygenase and dehydrogenase gene cluster that is flanked by two almost identical (1 nt difference) insertion sequences IS1066 and IS106 (Van der Meer, Zehnder, De Vos, Van Neerven, & De Vries, 1991). The region around *dcpA* does reassemble that of composite transposon (Figure. 4.10) making transposition a putative mechanism for the mobilization of the *dcpA* island. No circular transposable element has been described in *Dhc* strains but recently the draft genome of *Dhc* strain SG1 suggests it carries a plasmid (the first *Dhc* reported to carry an extra chromosomal element) suggesting that RDase gene dissemination by integration or conjugation is possible (Wang, Chng, & Wu, 2014).

Another hypothesis is that *dcpA* moved via a phage infecting the cells, also involving the Xer-D phage-site specific recombinase. The application of PHAST (PHAge Search Tool), confirmed that complete, intact prophages are present in the genomes of *Dhc* strain 195 and strain BTF08, and incomplete phage or genomic regions with phage-like genes are present in the genomes of other *Dhc* as well as *Dhgm* (see Figure 5.7 in Chapter 5). Additionally, a complete phage genome was detected in a *Dhc* contig in the metagenome of the dechlorinating consortium KB-1 (Alison S. Waller et al., 2012), and phage-like particles have been detected in *Dhc* cultures (Dumas et al., 2008; K M Ritalahti, Helton, Fletcher, Wommack, & Löffler, 2007). GTAs could also be involved in transfer of RDase genes in *Dhc*, although direct evidence for this hypothesis is

lacking. Interestingly, scanning electron micrographs have shown *Dhc* cells with multiple extrusions and near small-round blebs (Kube et al., 2005; Kirsti M Ritalahti et al., 2006) of around ~50 nm, which is in size range (30–50 nm) of described GTAs (Lang, Zhaxybayeva, & Beatty, 2012). Currently, 70% of viral metagenomes are populated with gene sequences that share no similarity with those deposited in public databases (Lang et al., 2012); while approximately 30% of the protein coding genes in strain BL-DC-9 (Siddaramappa et al., 2012) and 19 % of *Dhc* core genome (Ahsanul Islam, Edwards, & Mahadevan, 2010) are annotated as uncharacterized hypothetical genes. Therefore it is difficult to draw further inferences on HGT mechanisms based on gene homology.

Final remarks

Organohalide-respiring *Chloroflexi* are of interest to bioremediation practitioners since strains of this genus have been shown to grow using a variety of chlorinated compounds as terminal electron acceptors including 1,2-D. The discovery of *dcpA* in *Dhc* strains RC and KS resulted in assigning function to the RDase in its nearest relative, *Dhgm* . *Dhc* and *Dhgm* share similar lifestyles and likely occupy similar environmental niches, which may facilitate HGT events between these organisms. The *dcpA* GI gives a selective advantage to *Dhgm* and *Dhc* by expanding their catabolic capabilities and providing the benefit of using 1,2 D as electron acceptor.

Acquisition of *dcpA* by *Dhgm* via HGT is supported by (1) high sequence similarities/identities between the *Dhc* and *Dhgm dcpA* genes, (2) localization of the *dcpAB* gene cassette in atypical genomic regions, as evidenced by % G+C content, di-nucleotide, tetra-nucleotide and codon biases in *Dhgm lykanthroporepellens* strain BL-DC-9, (3) the presence of mobilization genes encoding transposes and integrases upstream and downstream of *dcpA*, and (4) the finding that *Dhc* and *Dhgm* strains exhibit similar gene arrangements (i.e., gene synteny) in the gene neighborhood of *dcpA.*

Similar to *vcrA,* the *dcpA* gene shows an evolutionary history different from the genes in the core *Dhc* genome, with a plausible origin in a non-*Dhc* organism. Also, the donor might be not a

bacterium, but an independently replicating element such as a plasmid or a phage or transposon, common in subsurface environments (Burton, Day, & Bull, 1982; Eydal, Jägevall, Hermansson, & Pedersen, 2009; Kyle, Eydal, Ferris, & Pedersen, 2008; Ogunseitan O A, Tedford E T, Pacia D, Sirotkin K M, 1987). In addition, 16 GI were identified in *Dhgm lykanthroporepellens* strain BL-DC-9 genome, and these regions are of putative foreign origin and include *dcpA* and two other RDase genes that are shared with some *Dhc* strains, providing evidence of additional HGT events between *Dhc* and *Dhgm*. Understanding the mobility of RDase genes in the environment is relevant for elucidating the evolutionary history of organohalide respiration and for predicting a contaminated sites reductive dechlorination potential and trajectory in terms of meeting regulatory requirements.

References

Ahsanul Islam, M., Edwards, E. A., & Mahadevan, R. (2010). Characterizing the metabolism of Dehalococcoides with a constraint-based model. *PLoS Computational Biology*, *6*(8).

Bowman, K. S., Nobre, M. F., da Costa, M. S., Rainey, F. A., & Moe, W. M. (2013). *Dehalogenimonas alkenigignens* sp. nov., a chlorinated-alkane-dehalogenating bacterium isolated from groundwater. *International Journal of Systematic and Evolutionary Microbiology*, *63*(PART4), 1492–1498.

Burton, N. F., Day, M. J., & Bull, A. T. (1982). Distribution of Bacterial Plasmids in Clean and Polluted Sites in a South Wales River, *44*(5), 1026–1029.

Campbell, A. (2007). Phage integration and chromosome structure. A personal history. *Annual Review of Genetics*, *41*, 1–11.

Clamp, M., Cuff, J., Searle, S. M., & Barton, G. J. (2004). The Jalview Java alignment editor. *Bioinformatics*, *20*(3), 426–427.

De Wildeman, S., Diekert, G., Van Langenhove, H., Verstraete, W., & De Wildeman S, Diekert G, Van Langenhove H, Verstraete, W. (2003). Stereoselective microbial dehalorespiration with vicinal dichlorinated alkanes. *Appl. Environ. Microbiol.*, *69*(9), 5643–5647. http://doi.org/10.1128/AEM.69.9.5643

Dumas, M. D., Fletcher, K. E., Helton, R. R., Ritalahti, K. M., Löffler, F. E., & Wommack, K. E. (2008). Growth of *Dehalococcoides* Strain BAV1 in Conjunction with a Highly Infective Phage. *Abstract N-288. In Abstracts of the 108th General Meeting of the American Society for Microbiology, Boston, MA, June 1-5, 2008*.

Duval-Valentin, G., Marty-Cointin, B., & Chandler, M. (2004). Requirement of IS911 replication before integration defines a new bacterial transposition pathway. *The EMBO Journal*, *23*(19), 3897–906. http://doi.org/10.1038/sj.emboj.7600395

Eydal, H. S. C., Jägevall, S., Hermansson, M., & Pedersen, K. (2009). Bacteriophage lytic to Desulfovibrio aespoeensis isolated from deep groundwater. *The ISME Journal*, *3*(10), 1139–1147. http://doi.org/10.1038/ismej.2009.66

Futagami, T., Yamaguchi, T., Nakayama, S., Goto, M., & Furukawa, K. (2006). Effects of chloromethanes on growth of and deletion of the *pce* gene cluster in dehalorespiring *Desulfitobacterium hafniense* strain Y51. *Applied and Environmental Microbiology*, *72*(9), 5998–6003.

Ganesan, H., Rakitianskaia, A. S., Davenport, C. F., Tümmler, B., & Reva, O. N. (2008). The SeqWord Genome Browser: an online tool for the identification and visualization of atypical regions of bacterial genomes through oligonucleotide usage. *BMC Bioinformatics*, *9*, 333. http://doi.org/10.1186/1471-2105-9-333

Grindley, N. D. F., Whiteson, K. L., & Rice, P. A. (2006). Mechanisms of site-specific recombination. *Annual Review of Biochemistry*, *75*, 567–605.

Haren, L., Normand, C., Polard, P., Alazard, R., & Chandler, M. (2000). IS911 transposition is regulated by protein-protein interactions via a leucine zipper motif. *Journal of Molecular Biology*, *296*(3), 757–68. http://doi.org/10.1006/jmbi.1999.3485

Haren, L., Polard, P., Chandler, M., & Paul, Â. (1998). Multiple Oligomerisation Domains in the IS 911 Transposase : A Leucine Zipper Motif is Essential for Activity, 29–41.

Hickey, W. J., Chen, S., & Zhao, J. (2012). The *phn i*sland: A new genomic island encoding catabolism of polynuclear aromatic hydrocarbons. *Frontiers in Microbiology*, *3*(APR).

Kim, S.-H., Harzman, C., Davis, J. K., Hutcheson, R., Broderick, J. B., Marsh, T. L., & Tiedje, J. M. (2012). Genome sequence of *Desulfitobacterium hafniense* DCB-2, a Gram-positive anaerobe capable of dehalogenation and metal reduction. *BMC Microbiology*.

Krajmalnik-Brown, R., Sung, Y., Ritalahti, K. M., Saunders, F. M., Löffler, F. E., & Michael Saunders, F. (2007). Environmental distribution of the trichloroethene reductive

dehalogenase gene (*tceA*) suggests lateral gene transfer among *Dehalococcoides. FEMS Microbiol. Ecol.*, *59*(1), 206–14. http://doi.org/10.1111/j.1574-6941.2006.00243.x

Kube, M., Beck, A., Zinder, S. H., H., K., Reinhardt, R., & Adrian, L. (2005). Genome sequence of the chlorinated compound-respiring bacterium *Dehalococcoides* species strain CBDB1. *Nat. Biotechnol.*, *23*, 1269–1273.

Kyle, J. E., Eydal, H. S. C., Ferris, F. G., & Pedersen, K. (2008). Viruses in granitic groundwater from 69 to 450 m depth of the Äspö hard rock laboratory, Sweden. *The ISME Journal*, *2*(5), 571–574. http://doi.org/10.1038/ismej.2008.18

Lang, A. S., Zhaxybayeva, O., & Beatty, J. T. (2012). Gene transfer agents: phage-like elements of genetic exchange. *Nature Reviews Microbiology*.

Langille, M. G. I., & Brinkman, F. S. L. (2009). IslandViewer: An integrated interface for computational identification and visualization of genomic islands. *Bioinformatics*, *25*(5), 664–665.

Langille, M. G. I., Hsiao, W. W. L., & Brinkman, F. S. L. (2008). Evaluation of genomic island predictors using a comparative genomics approach. *BMC Bioinformatics*, *9*, 329.

Löffler, F. E., Champine, J. E., Ritalahti, K. M., Sprague, S. J., Tiedje, J. M., & Loffler, F. E. (1997). Complete reductive dechlorination of 1,2-dichloropropane by anaerobic bacteria. *Applied and Environmental Microbiology*, *63*(7), 2870–5.

Löffler FE, Yan J, Ritalahti KM, Adrian L, Edwards EA, Konstantinidis KT, Müller JA, Fullerton H, Zinder SH, S. A. (2013). *Dehalococcoides mccartyi* gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation, belong to a novel bacterial class, *Dehalococcoidia* classis nov., order *Dehalococcoidales* ord. nov. and famil. *Int. J. Syst. Evol. Microbiol.*, *63*, 625–635.

Maillard, J., Regeard, C., & Holliger, C. (2005). Isolation and characterization of Tn-Dha1, a transposon containing the tetrachloroethene reductive dehalogenase of *Desulfitobacterium hafniense* strain TCE1. *Environmental Microbiology*, *7*, 107–117.

Maphosa, F., Van Passel, M. W. J., De Vos, W. M., & Smidt, H. (2012). Metagenome analysis reveals yet unexplored reductive dechlorinating potential of *Dehalobacter* sp. E1 growing in co-culture with Sedimentibacter sp. *Environmental Microbiology Reports*, *4*(6), 604–616.

McMurdie, P. J., Behrens, S. F., Holmes, S., & Spormann, A. M. (2007). Unusual codon bias in vinyl chloride reductase genes of *Dehalococcoides* species. *Applied and Environmental Microbiology*, *73*(8), 2744–2747. http://doi.org/10.1128/aem.02768-06

McMurdie, P. J., Behrens, S. F., Müller, J. a, Göke, J., Ritalahti, K. M., Wagner, R., Spormann, A. M. (2009). Localized plasticity in the streamlined genomes of vinyl chloride respiring *Dehalococcoides*. *PLoS Genetics*, *5*(11), e1000714. http://doi.org/10.1371/journal.pgen.1000714

McMurdie PJ, Hug LA, Edwards EA, Holmes S, S. A. (2011). Site-specific mobilization of vinyl chloride respiration islands by a mechanism common in *Dehalococcoides*. *BMC Genomics*, *12*(1), 287. http://doi.org/10.1186/1471-2164-12-287

Moe WM, Yan J, Nobre MF, da Costa MS, Rainey, F. (2009). *Dehalogenimonas lykanthroporepellens* gen. nov., sp. nov., a reductively dehalogenating bacterium isolated from chlorinated solvent-contaminated groundwater. *Int. J. Syst. Evol. Microbiol.*, *59*(11), 2692–2697. http://doi.org/10.1099/ijs.0.011502-0

Nash, H. (1996). Site-specific recombination: integration, excision, resolution and inversion of defined DNA segments. In F. C. et al Neidhardt (Ed.), *Escherichia coli and Salmonella: Cellular and Molecular Biology* (pp. 2363–2376). Washington, D. C.: ASM Press.

Ogunseitan O A, Tedford E T, Pacia D, Sirotkin K M, S. G. S. (1987). Distribution of plasmids in groundwater bacteria. *J Ind Microbiol.*, 311–317.

Padilla-Crespo, E., Yan, J., Swift, C., Wagner, D. D., Chourey, K., Hettich, R. L., Löffler, F. E. (2014). Identification and environmental distribution of *dcpA*, which encodes the reductive dehalogenase catalyzing the dichloroelimination of 1,2-dichloropropane to propene in

organohalide-respiring Chloroflexi. *Applied and Environmental Microbiology*, *80*(3), 808–818.

Puigbò, P., Bravo, I. G., & Garcia-Vallvé, S. (2008). E-CAI: a novel server to estimate an expected value of Codon Adaptation Index (eCAI). *BMC Bioinformatics*, *9*, 65.

Regeard, C., Maillard, J., Dufraigne, C., Deschavanne, P., & Holliger, C. (2005). Indications for acquisition of reductive dehalogenase genes through horizontal gene transfer by *Dehalococcoides ethenogenes* strain 195. *Applied and Environmental Microbiology*, *71*(6), 2955–2961. http://doi.org/10.1128/AEM.71.6.2955

Ritalahti, K. M., Amos, B. K., Sung, Y., Wu, Q., Koenigsberg, S. S., Löffler, F. E., & Lo, F. E. (2006). Quantitative PCR targeting 16S rRNA and reductive dehalogenase genes simultaneously monitors multiple *Dehalococcoides* strains. *Applied and Environmental Microbiology*, *72*(4), 2765–2774. http://doi.org/10.1128/AEM.72.4.2765

Ritalahti, K. M., Helton, R. R., Fletcher, K. E., Wommack, K. E., & Löffler, F. E. (2007). Preliminary evidence for phage-mediated horizontal gene transfer in *Dehalococcoides. Presented at the 107th General Meeting of the American Society of Microbiology, Toronto, Canada, May 21-25, 2007*.

Ritalahti, K. M., & Löffler, F. F. (2004). Populations implicated in the anaerobic reductive dechlorination of 1,2-dichloropropane in highly enriched bacterial communities. *Appl. Environ. Microbiol.*, *70*(7), 4088–4095. http://doi.org/10.1128/AEM.70.7.4088

Rousseau, P., Gueguen, E., Duval-Valentin, G., & Chandler, M. (2004). The helix-turn-helix motif of bacterial insertion sequence IS 911 transposase is required for DNA binding. *Nucleic Acids Research*, *32*(4), 1335–44. http://doi.org/10.1093/nar/gkh276

Rousseau, P., Loot, C., Guynet, C., Ah-Seng, Y., Ton-Hoang, B., & Chandler, M. (2007). Control of IS911 target selection: how OrfA may ensure IS dispersion. *Molecular Microbiology*, *63*(6), 1701–9. http://doi.org/10.1111/j.1365-2958.2007.05615.x

Rousseau, P., Tardin, C., Tolou, N., Salomé, L., & Chandler, M. (2010). A model for the molecular organisation of the IS 911 transpososome, 1–8.

Santos, C. L., Tavares, F., Thioulouse, J., & Normand, P. (2009). A phylogenomic analysis of bacterial helix-turn-helix transcription factors. *FEMS Microbiology Reviews*, *33*(2), 411–29. http://doi.org/10.1111/j.1574-6976.2008.00154.x

Schlötelburg, C., Von Wintzingerode, C., Hauck, R., Von Wintzingerode, F., Hegemann, W., Göbel, U. B., & Schlötelburg C, Wintzingerode C, Hauck R, Wintzingerode F, Hegemann W, G. U. (2002). Microbial structure of an anaerobic bioreactor population that continuously dechlorinates 1,2-D. *FEMS Microbiology Ecology*, *39*(3), 229–237. http://doi.org/10.1111/j.1574-6941.2002.tb00925.x

Seshadri, R., Adrian, L., Fouts, D. E., Eisen, J. A., Phillippy, A. M., Methe, B. A., Heidelberg, J. F. (2005). Genome sequence of the PCE-dechlorinating bacterium *Dehalococcoides ethenogenes. Science*, *307*, 105–108.

Siddaramappa, S., Challacombe, J. F., Delano, S. F., Green, L. D., Daligault, H., Bruce, D., … Moe, W. M. (2012). Complete genome sequence of *Dehalogenimonas lykanthroporepellens* type strain (BL-DC-9T) and comparison to "Dehalococcoides" strains. *Standards in Genomic Sciences*.

Sobecky, P. A., & Coombs, J. M. (2009). Horizontal gene transfer in metal and radionuclide contaminated soils. *Methods in Molecular Biology (Clifton, N.J.)*.

Tirumalai, M. R., & Fox, G. E. (2013). An ICEBs1-like element may be associated with the extreme radiation and desiccation resistance of *Bacillus pumilus* SAFR-032 spores. *Extremophiles*, *17*(5), 767–774.

Van der Meer, J. R., Zehnder, A. J. B., De Vos, W. M., Van Neerven, A. R. W., & De Vries, E. J. (1991). Identification of a novel composite transposable element, Tn 5280, carrying chlorobenzene dioxygenase genes of *Pseudomonas* sp. strain P51. *Journal of Bacteriology*, *173*(22), 7077–7083.

Van Passel, M. W. J., Luyf, A. C. M., van Kampen, A. H. C., Bart, A., & van der Ende, A. (2005). Deltarho-web, an online tool to assess composition similarity of individual nucleic acid sequences. *Bioinformatics (Oxford, England)*, *21*(13), 3053–3055. http://doi.org/10.1093/bioinformatics/bti460

Waack, S., Keller, O., Asper, R., Brodag, T., Damm, C., Fricke, W. F., Merkl, R. (2006). Score-based prediction of genomic islands in prokaryotic genomes using hidden Markov models. *BMC Bioinformatics*, *7*, 142.

Wagner, A., Segler, L., Kleinsteuber, S., Sawers, G., Smidt, H., & Lechner, U. (2013). Regulation of reductive dehalogenase gene transcription in *Dehalococcoides mccartyi*. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *368*(1616), 20120317.

Wagner, D., Hug, L., Hatt, J., Spitzmiller, M., Padilla-Crespo, E., Ritalahti, K., Loffler, F. (2012). Genomic determinants of organohalide-respiration in *Geobacter lovleyi*, an unusual member of the Geobacteraceae. *BMC Genomics*, *13*(1), 200.

Waller, A. S., Hug, L. A., Mo, K., Radford, D. R., Maxwell, K. L., & Edwards, E. A. (2012). Transcriptional analysis of a *Dehalococcoides*-containing microbial consortium reveals prophage activation. *Applied and Environmental Microbiology*, *78*(4), 1178–1186.

Wang, S., Chng, K., & Wu, C. (2014). Draft genome sequence of polychlorinated biphenyl-dechlorinating *Dehalococcoides mccartyi* strain SG1, which carries a circular putative plasmid. *Genome Announcements*, *2*(5), 14–15. http://doi.org/10.1128/genomeA.00901-14.Copyright

Waterhouse, A. M., Procter, J. B., Martin, D. M. A., Clamp, M., & Barton, G. J. (2009). Jalview Version 2-A multiple sequence alignment editor and analysis workbench. *Bioinformatics*, *25*(9), 1189–1191.

Yan J, Rash BA, Rainey FA, Moe WM. (2009). Isolation of novel bacteria within the Chloroflexi capable of reductive dechlorination of 1,2,3-trichloropropane. *Environ. Microbiol.*, *11*(4), 833–843. http://doi.org/10.1111/j.1462-2920.2008.01804.x

Zaneveld, J. R., Nemergut, D. R., & Knight, R. (2008). Are all horizontal gene transfers created equal? Prospects for mechanism-based studies of HGT patterns. *Microbiology*.

Chapter 4 Appendix: Figures

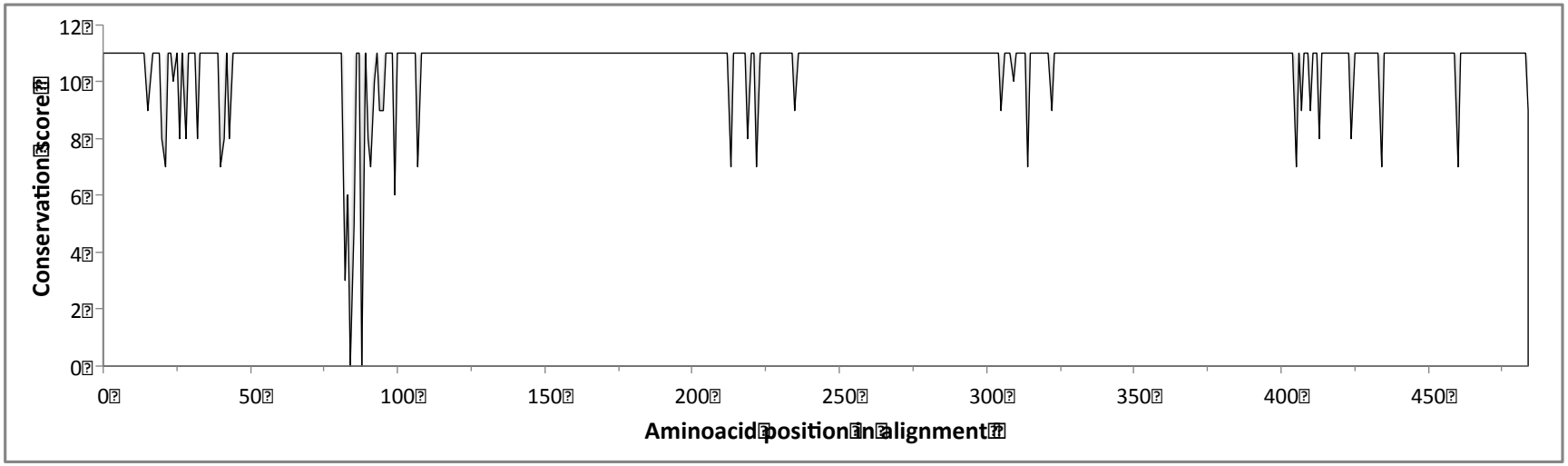


Figure 4.1. Amino acid sequence conservation of the set of *dcpA sequences* (*Dhc* strain RC/KS and *Dhgm*) illustrated with conservation scores obtained with JALVIEW. The translated *dcpA* sequences of *Dh*c RC/KS and *Dehly*_1524 were aligned with JALVIEW and the conservation scores extracted for every site of the alignment and plotted in EXCEL. In summary, Jalview calculates a numerical index in a scale from 0 to 11 using the AMAS (Analysis of Multiply Aligned Sequences) method (Livingstone et. al) and by measuring the number of conserved physico-chemical properties of each column in the alignment. Higher scores represent high conservation in the aa sites of the sequence of interest. Analysis of the 484 sites in the *dcpA* alignment illustrates that the vast majority (98.7%, 478/484) are highly conserved (with conservation scores above 7). The site with the least conservation is around the 80^{th}-90^{th} position. The overall results support the notion that DcpA is highly conserved between different bacterial genuses.

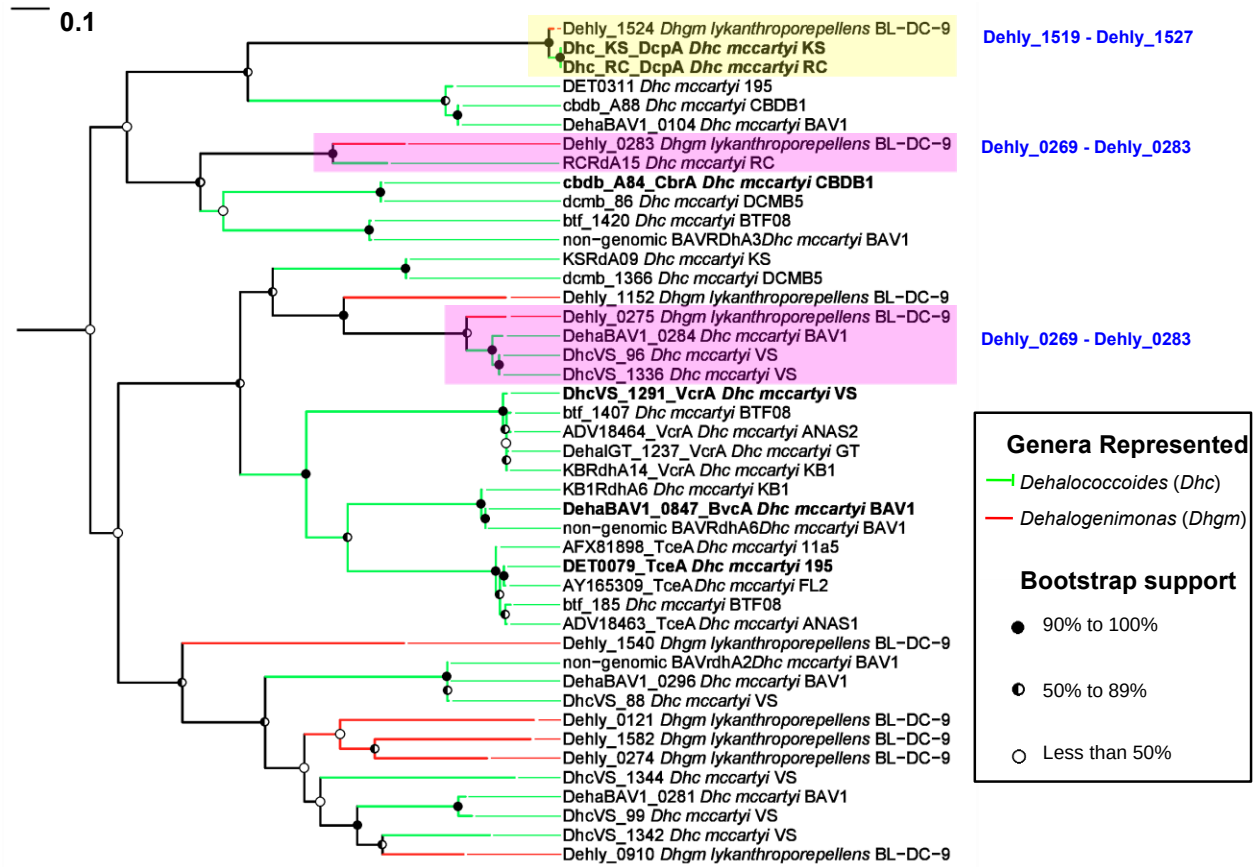


Figure 4.2. Phylogenetic relationships between predicted RDases from *Dhgm* strain BL-DC-9 and *Dhc mccartyi*. The yellow-highlighted region indicates Dehly_1524 from the *Dhgm* GI spanning Dehly_1519 to Dehly_1527 and related dichloropropane reductase RDases (DcpA) from *Dhc mccartyi* strains KS and RC. The magenta-highlighted clusters indicate *Dhgm* RDases from the genomic island spanning Dehly_0269 to Dehly_0283 and related putative RDases from *Dhc mccartyi* strains RC, BAV1, and VS. *Dhc mccartyi* RDases characterized by enzymological, proteomic, or reverse genetics studies are indicated in boldface.

Figure 4.3. Phylogenetic relationships of putative horizontal transfers across genera indicated for 174 predicted RDases from *Dehalogenimonas lykanthroporepellens* strain BL-DC-9 (*Dhgm), Dhc mccartyi* (*Dhc*), and "Dehalobium chlorocoercia" DF-1 ("Dhom'). The yellow-highlighted region indicates Dehly_1524 from the *Dhgm* GI spanning Dehly_1519 to Dehly_1527 and related dichloropropane reductase RDases (DcpA) from *Dhc mccartyi* strains KS and RC. The magenta-highlighted clusters indicate *Dhgm* RDases from the GI spanning Dehly_0269 to Dehly_0283 and related putative RDases from *Dhc mccartyi* strains RC, BAV1, and VS. The red bracket indicates the *Dhc mccartyi* gene, DhcVS_1342, with apparent origins outside *Dehalococoides*. More recent apparent transfers between *Dehalococcoides* and "Dehalobium" are indicated in magenta brackets: DET0311 from *Dhc mccartyi* 195 shares 87% identity with gene1_0603 from "Dehalobium" DF-1; DhcVS_1320 shares 79% identity with gene3_0607 from "Dehalobium" DF-1. *Dhc mccartyi* RDases characterized by enzymological, proteomic, or reverse genetics studies are indicated in boldface.

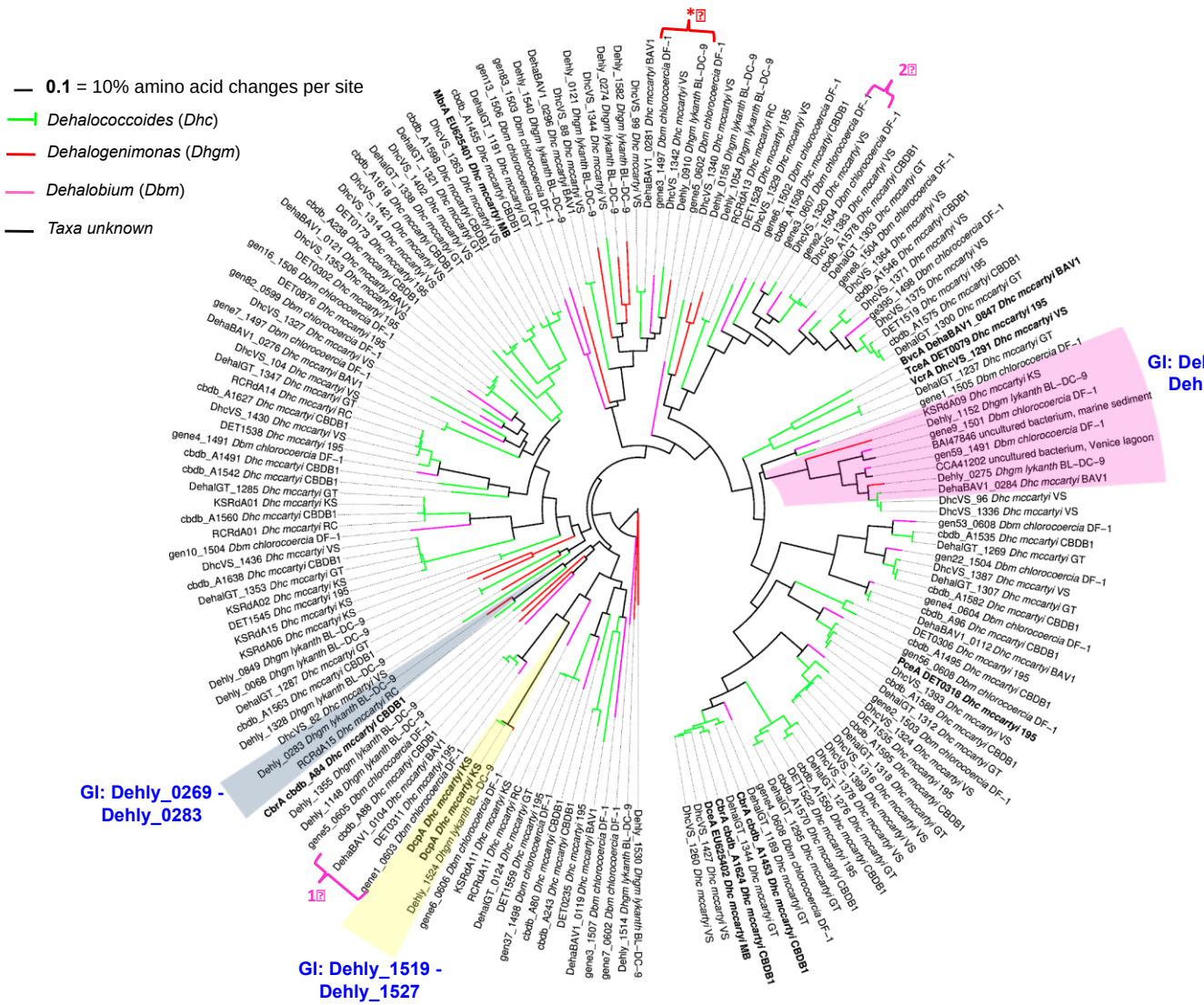


Figure 4.3. (continued)

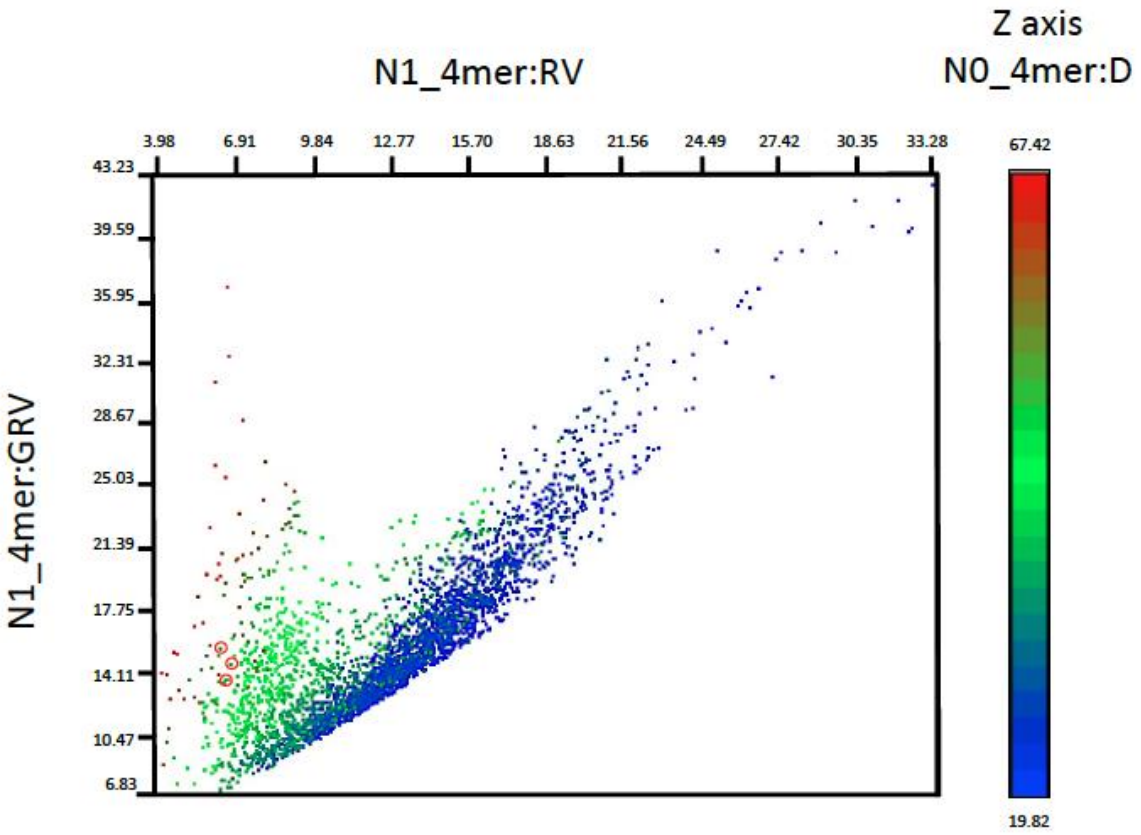


Figure 4.4 Identification of divergent regions in the genome of *Dehalogenimonas* lykanthroporepellens strain BL-DC-9 through oligonucleotide usage (RV, GRV, D). Genomic fragments dissimilar in tetranucleotide usage compared to the genomic average. x-axis: local relative variance of oligonucleotide usage (RV). y-axis: global relative variance of oligonucleotide usage (GRV). z-axis (color-coded): distance between two oligonucleotide usage patterns (D). Horizontally transferred regions generally deviate from the core genome yielding a high local pattern deviation [D] and a local relative variance divergent from the global relative variance). Positions where the *dcpA* GI is located are highlighted in red circles

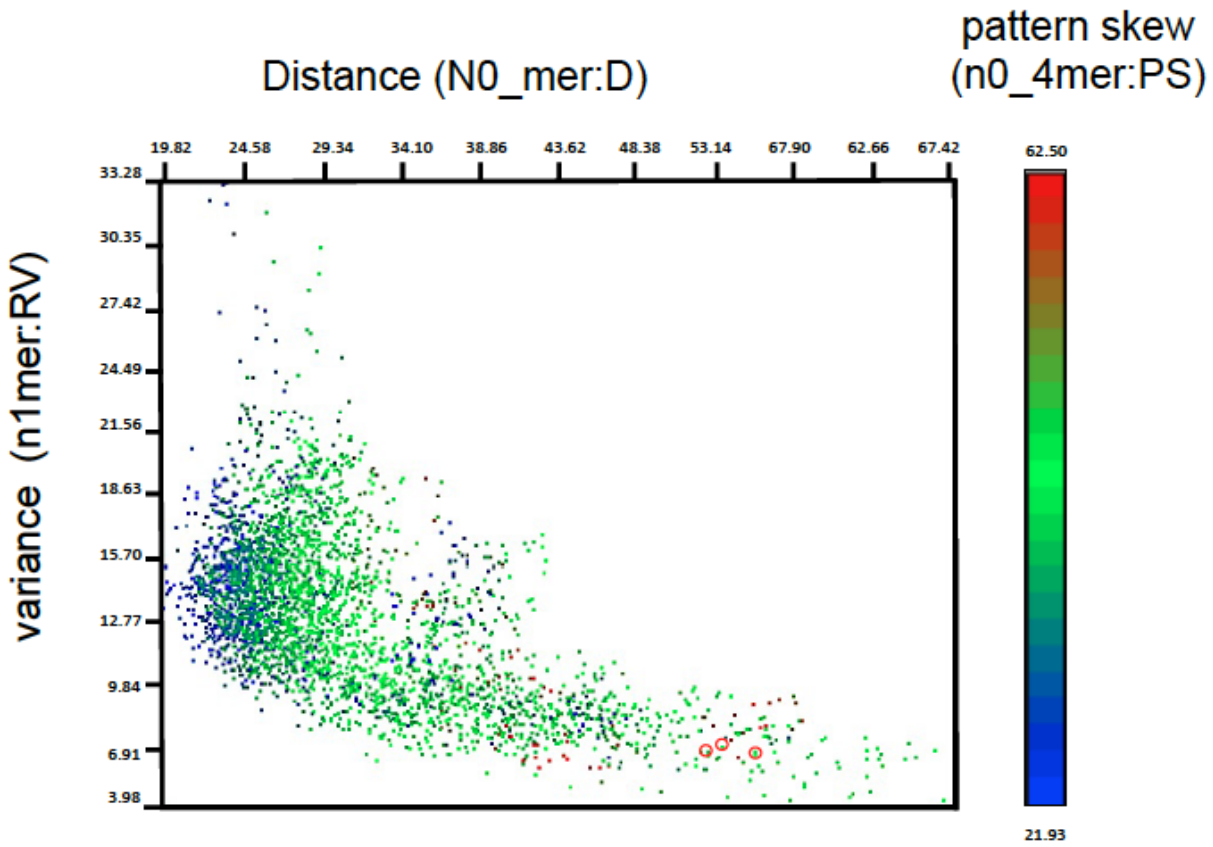


Figure 4.5. Identification of divergent regions in the genome of *Dehalogenimonas* lykanthroporepellens strain BL-DC-9 through oligonucleotide usage (RV, D, PS). Based on oligonucleotide usage parameters, *dcpA*'s GI posses the characteristics of HGT gene islands, transposons, pseudogenes phages and IS having a low relative variance (RV), significantly high local pattern deviation (D), and moderate to high pattern skew (PS).

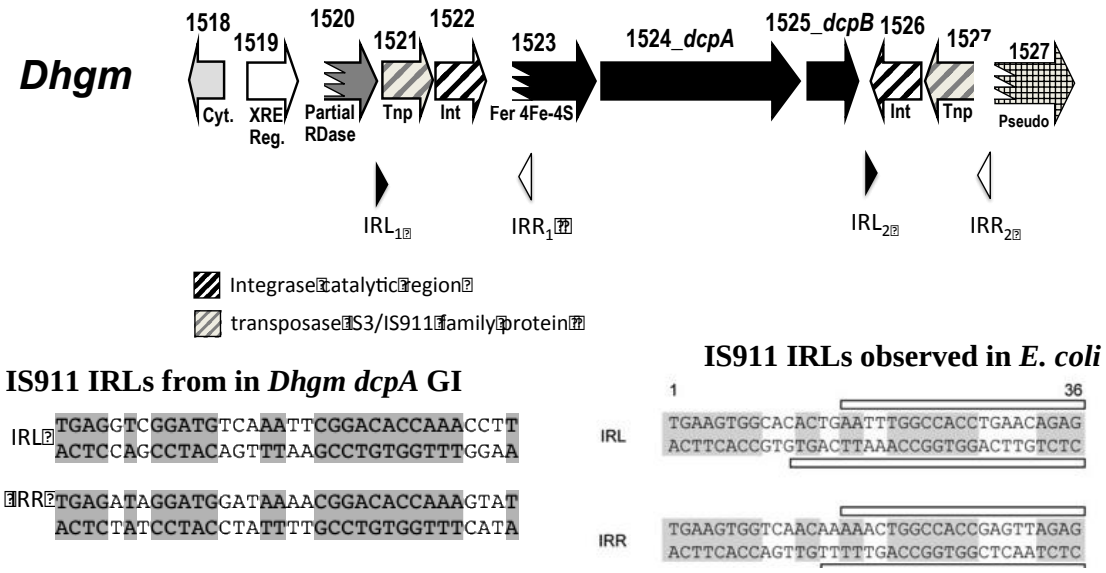


Figure 4.6. Genetic map *dcpA GI* in Dehalogenimonas strain BL-DC-9 genome. The positions of the following sequence elements are indicated: IS911 elements flanking the *dcpAB* genes that caused gene interruption and the location of 24-bp IRR and IRL bordering the right and left ends of the transposases. Nucleotide sequence alignment of terminal IRL and IRR of the IS911 elements in (A) *Dhgm dcpA* GI (B) in characterized IS911 elements in *E.coli*, as shown by (Rousseau et al., 2010). Conserved nucleotides are shown on a grey background.

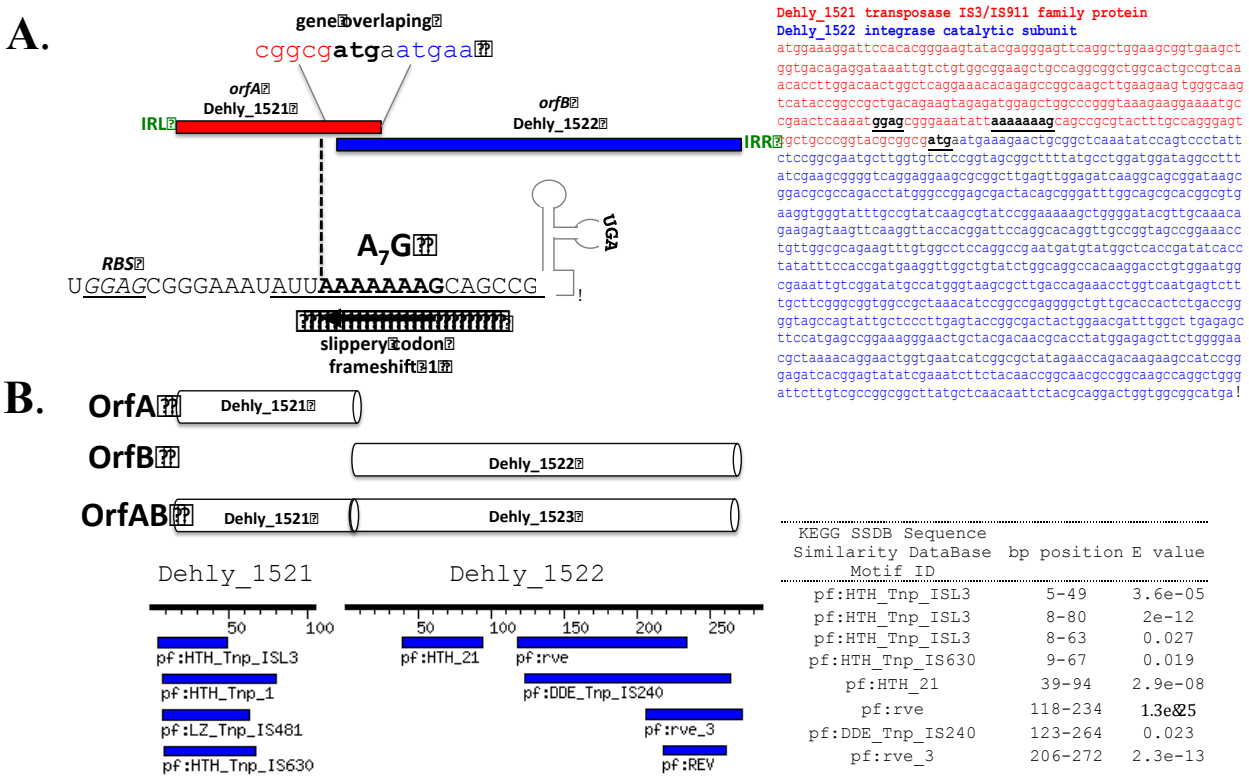


Figure 4.7. Genetic organization of Dehly_1521-1522: insertion sequence of the IS3/IS911 family. (A) The scheme represents the genetic organization of Dehly_1521 and 1522; both genes are in different reading frames (0 and -1, respectively) and overlap in a small region. The dotted line depicts the translational frame shift-site caused by the A_7G slippery codon. The RNA sequence involved in the frame shift is indicated. The shift (indicated as an arrow) occurs when the ribosome slides back one nucleotide to the left (-1). The termination codon for Dehly1521 (UGA) is depicted as well as Dehly_1522 putative ribosome bonding site (RBS) and its AUU start codon. Terminal inverted repeats located in intragenic regions at the left (IRL) and right side (IRR) of the transposase are shown. (B) Protein products of Dehly_1521-1522 are represented. In Dehly_1521 a α helix–turn– α helix (HTH) motif was detected and a leucine zipper (LZ), both involved in DNA binding. Dehly_1522 posses the catalytic DDE motif, a catalytic domain described to mediate sequence-specific strand cleavage in transposases. All of these are common features of the IS3/IS911-type and result in the full-length expression of the fused transposase OrfAB (Rousseau et al., 2010). The nearby pair of genes 1527-1628 have the same arrangement as depicted here

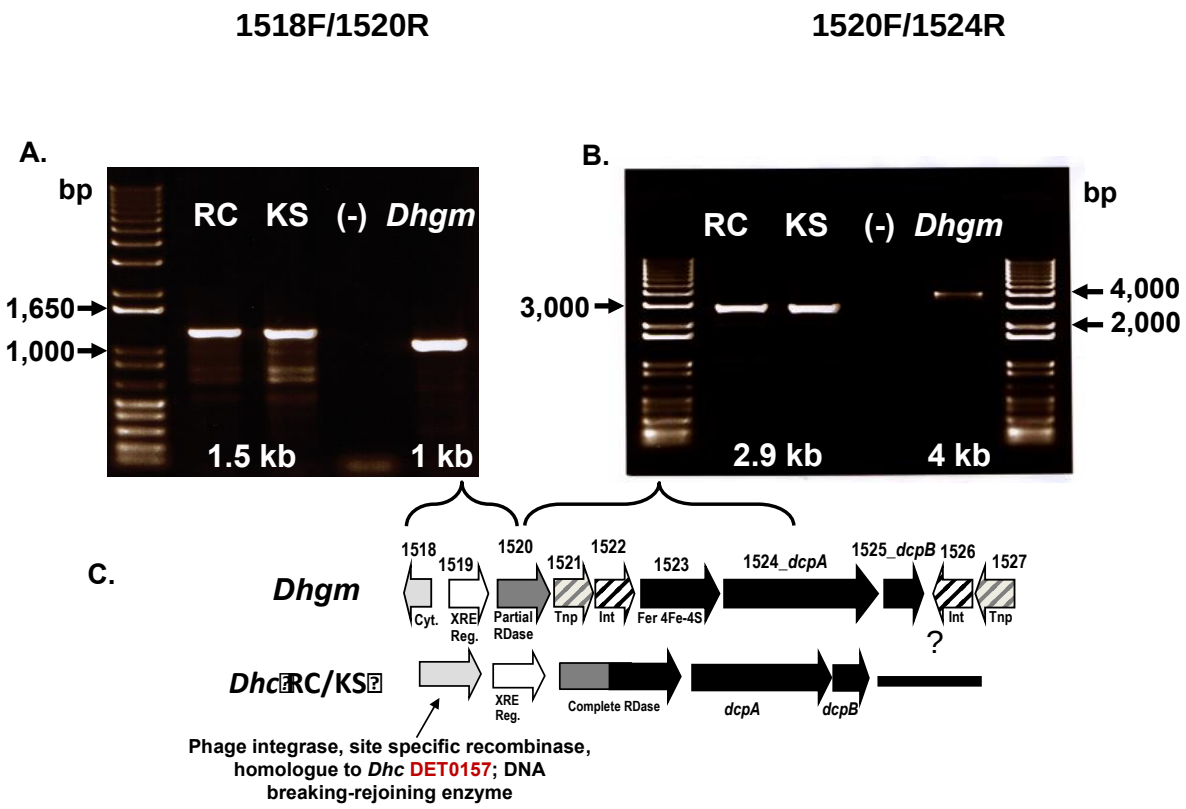


Figure 4.8. Elucidation of the genes encompassing the *dcpA* genomic island in *Dhc* strains RC and KS. PCR amplification of the region adjacent to *dcpA* using the primer pairs (A) 1518F/1520R and (B) 1520F/1524R and genomic template DNA from *Dhc* RC and KS cultures. In each gel picture the 1kb plus ladder (Invitrogen, left lane) was used as marker, a reaction with H_2O and no template DNA (lane 3) served as negative control, and *Dhgm* DNA (rightmost lane) was used as positive control to proof primer and reaction efficiency. Reaction with primers targeting genes downstream of *dcpB* failed to produce an amplicon when tested on DNA from RC and KS. (C) Organization of the *dcpA*B region as depicted in *Dhgm* genome and as elucidated in sequencing of the PCR amplicon produced with primers 1518F/1520R and 1520F/1524R on *Dhc* RC and KS gDNA.

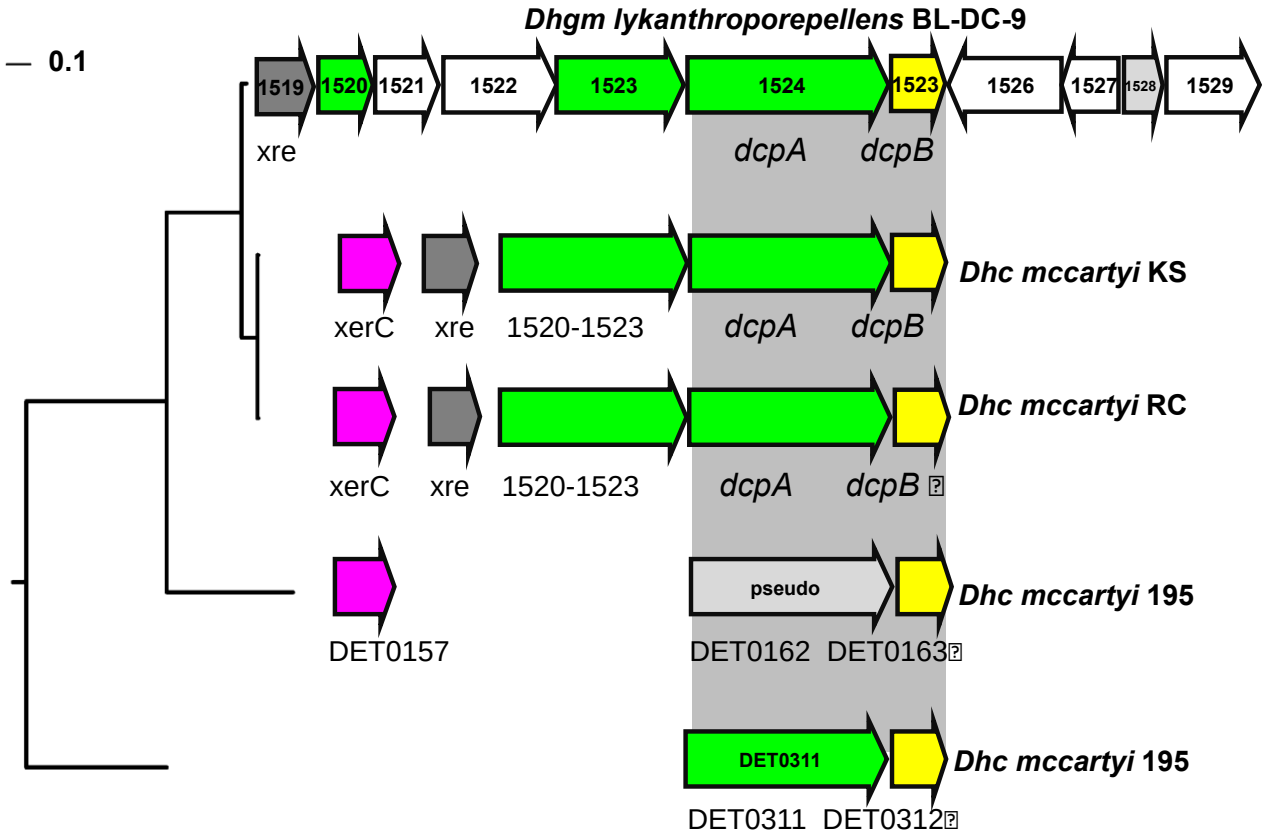


Figure 4.9. Phylogenetic relationships between *Dhc* KS and RC *dcpA* GIs and *Dhgm* BL-DC-9 GI with *Dhc mccartyi* putative RDase cluster. Phylogenetic tree was constructed in Phylip by maximum likelihood based upon concatenated aa alignments of RDaseA-RDaseB clusters indicated by gray shading. All bootstrap values at branches have 100% support out of 100 replicates and the scale bar in the upper left. DET0311-0312 was used as an outgroup.

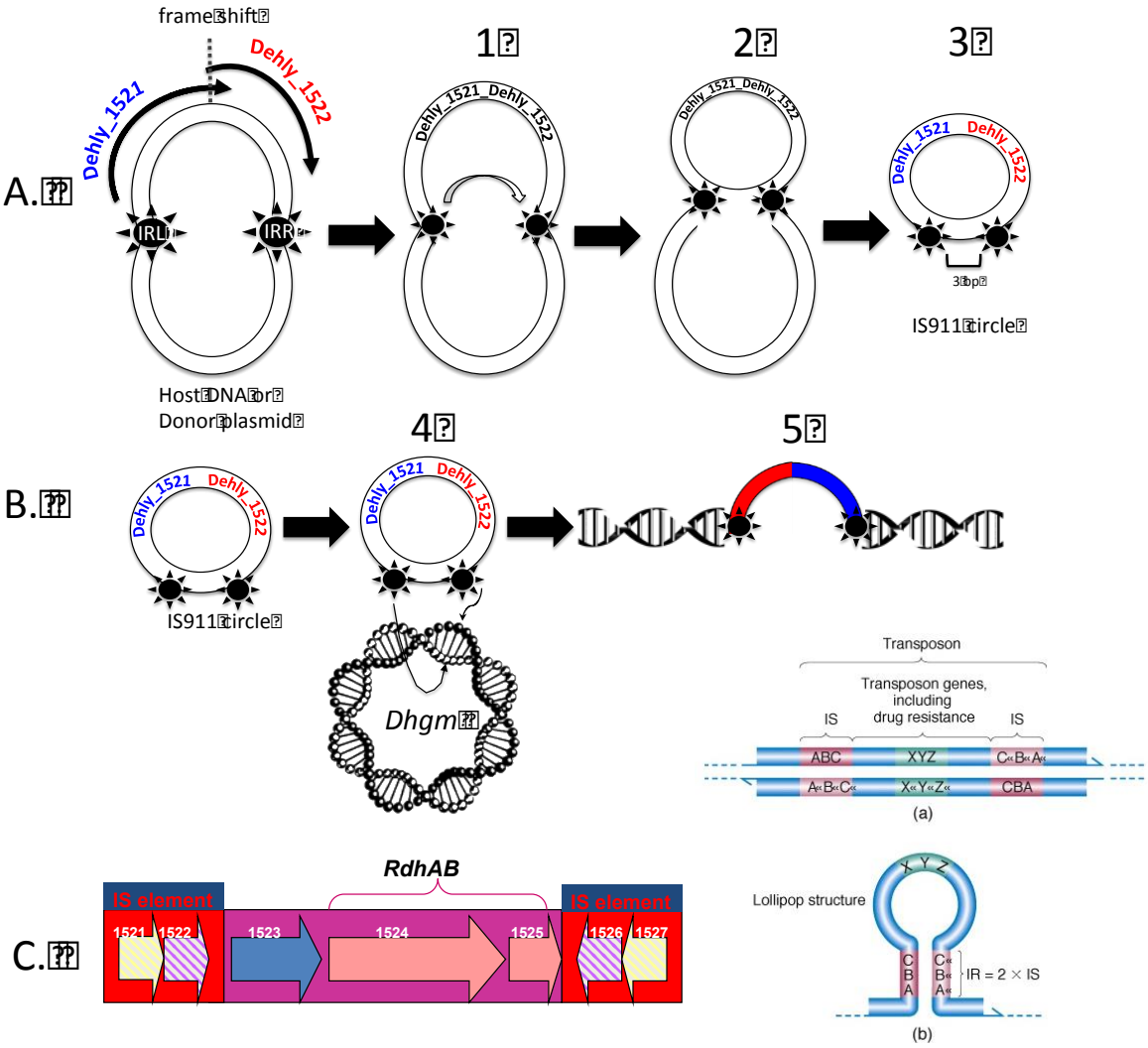


Fig 4.10. IS911 transposition in the *dcpA* GI. The 1S911 transposition mechanism is depicted as described by (Rousseau et al., 2010).

A. In summary, transposase OrfAB recognizes the IRRs leading them to form (1) a synaptic complex followed by (2) cleavage (hydrolysis) of one of the IRs to generate a 3′ OH group. The free ‘OH interacts (via nucleophilic attack) with 3 bp from the opposing end leading to the formation of a figure-eight structure (3). Following host-mediated secondstrand

synthesis and resolution, (3) a covalently closed circle (IS circle) is generated with two adjacent IRRs separated by 3bp.

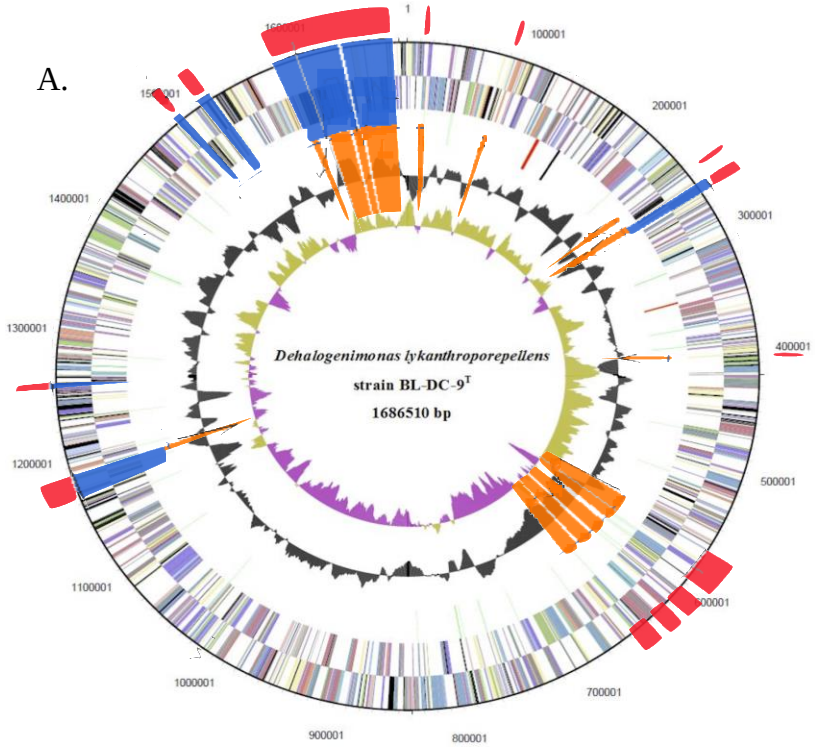
B. The mechanism of IS insertion is similar, involving a second a synaptic complex now encompassing the target DNA (e.g. *Dhgm* chromosome), the adjacent IRRs, and the transposase proteins (OrfAB). Briefly the IRR-IRL junction in the IS circle serve as a strong

promoter for the expression of the OrfAB proteins leading to (4) asymmetric single-strand cleavage following liberation of a 3′ OH’ nucleophile that attacks the opposing end, resulting in (5) the insertion into a host DNA.

C. Comparison of the *dcpA* region with a canonical composite transposon.

Chapter 4 Appendix: Supplemental Figures

A.



B.

Genome Coordinates Start	Genome Coordinates End	Size (bp)
10859	15236	4377
76596	80971	4375
244442	249405	4963
258965	268408	9443
412243	416439	4196
582841	610752	27911
617101	627792	10691
628314	632563	4249
636991	650480	13489
655368	671068	15700
1151134	1171359	20225
1243552	1249145	5593
1319049	1324192	5143
1493353	1499907	6554
1515618	1526318	10700
1580515	1672879	92364

Figure S4.1. Computational identification and visualization of genomic islands in *Dhgm* genome (NC_014314.1). Using IslandViewer, an integrated interface that integrates different genomic-island prediction methods, genomic islands were predicted in *Dhgm* genome (NC_014314.1). (A) On blue are regions identified by the IslandPath-DIMOB prediction method while on orange are regions identified by SIGI-HMM; both methods look for regions with abnormal sequence composition. In red are regions identified by at least one of the two methods. (B) Genomic coordinates of the putative GIs.

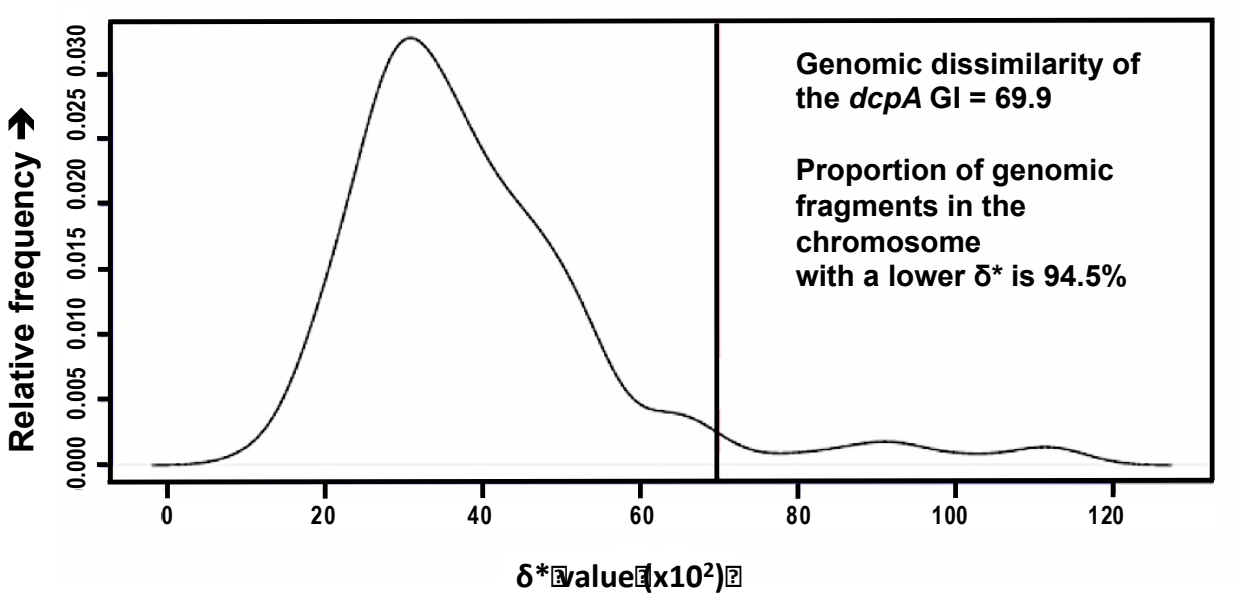


Figure S4.2. Compositional bias of dinucleotide frequency analysis using delta-rho WEB http://deltarho.amc.nl/cgi-bin/bin/index.cgi). For this analysis the genomic dissimilarity (the average dinucleotide relative abundance difference) of the *dcpA* genomic island and the *Dehly* chromosome was calculated. First the genome sequence is divided into non-overlapping fragments with a size equal to the length of the input GI sequence, the dinucleotide bias for each segment is calculated; after which a frequency distribution is made for both δ* scores. The δ* value of the genomic island is plotted vertically in the fragment distribution, indicating the proportion of genomic fragments with a lower δ* value. Marked with a solid line is the position of the GI indicating the 94.5 % of the genomic fragments in *Dehalogenimonas* have a lower δ*.

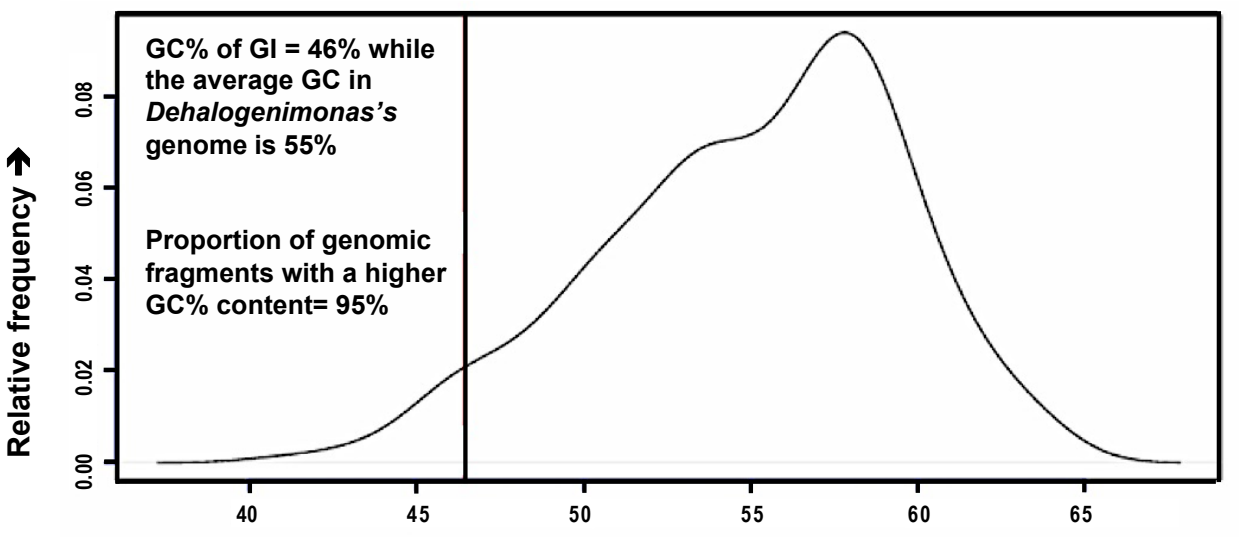


Figure S4.3 Comparison of GC content of the *dcpA* GI and that of the genome of *Dehalogenimonas lykanthroporepellens* strain BL-D-C9. Using delta-rho WEB (http://deltarho.amc.nl/cgi-bin/bin/index.cgi) the chromosome sequence of *Dehalogenimonas lykanthroporepellens BLDC9 strain* BLDC9 is divided into non-overlapping fragments with a size equal to the length of the input GI sequence. Then a frequency distribution is made for the GC% percentage scores of the genomic fragments. Marked with a solid line is the position of the GI indicating the 95% of the genomic fragments in *Dehalogenimonas* have a higher GC%.

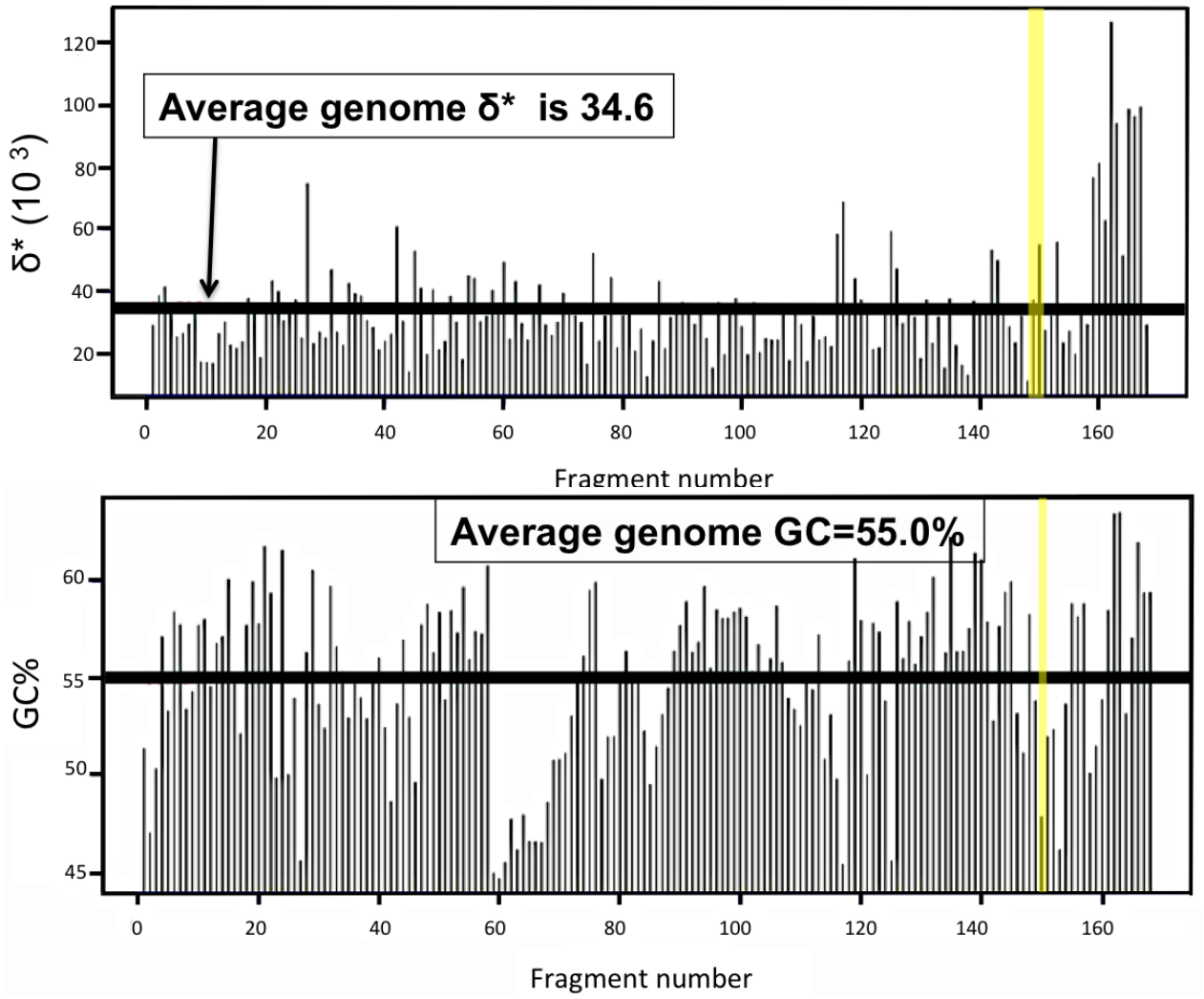


Figure S4.4. Visualization of the GC composition and the genomic signature of *Dehalogenimonas* BL-DC-9 using δρ-web (http://deltarho.amc.uva.nl.) and a window size of 10,000 bp. The graphs show the genomic dissimilarity (δ*) and GC percentage (bottom) distributions of *Dhgm.* strain BL-DC-9 genome the horizontal lines represent the respective average. Atypical areas on the genome have high δ or a GC that deviated from the average.

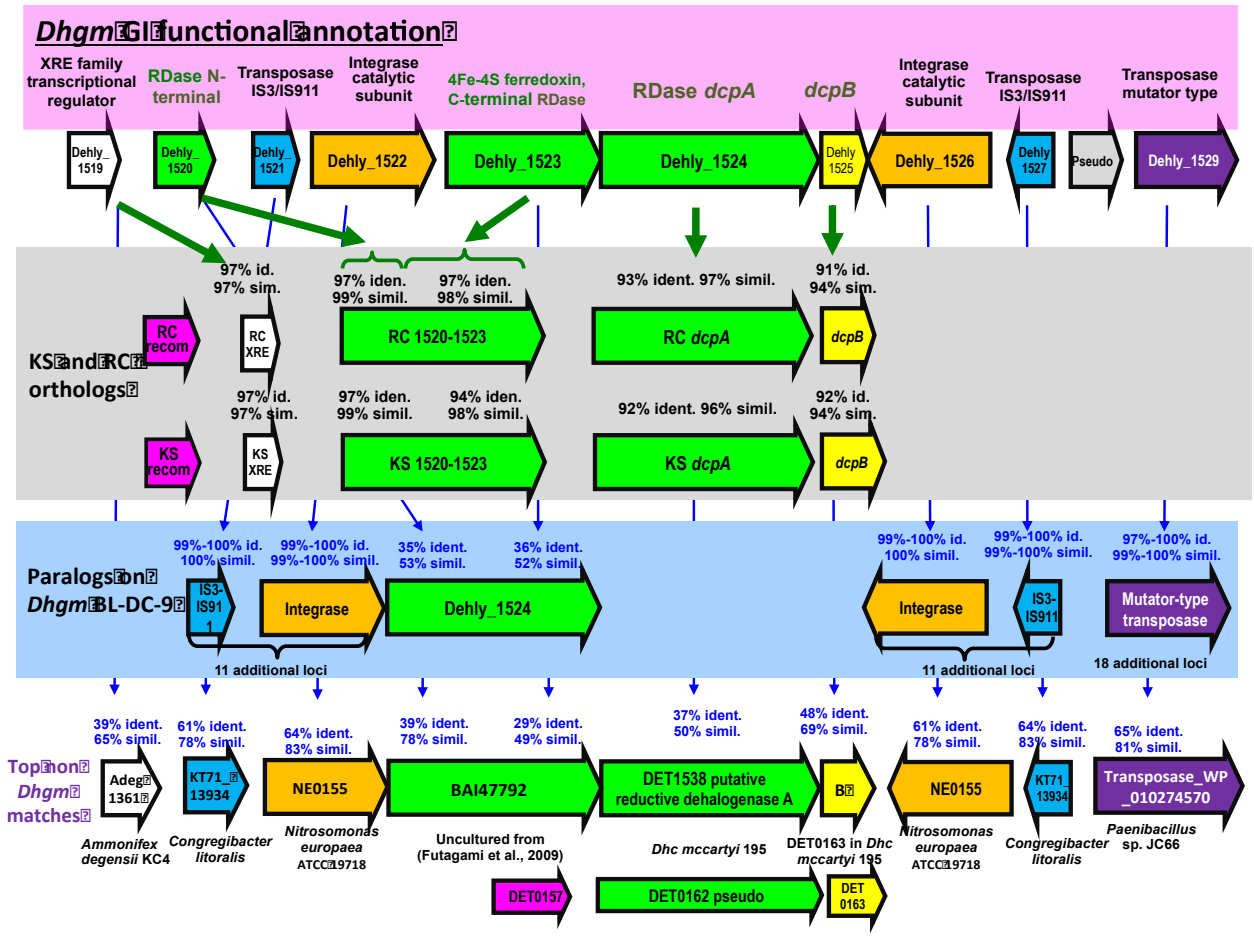


Figure S4.5. Protein sequence similarity between predicted *Dhgm* BL-DC-9 GI loci, Dehly_1519-Dehly_1529. Highest sequence similarity was found for predicted orthologs in *Dhc* strains KS and RC (second row, gray background). Paralogous matches in the databases are indicated in the third row (blue background), where the "split reductive dehalogenase", Dehly_1520 and Dehly_1523 shared their top BlastP match with the putative DcpA, Dehly_1524. The IS3/IS911-Integrase pair of the Dehly_1519-Dehly_1529 GI matched 11 duplicates throughout the BL-DC-9 genome, including within the GI itself. Similarly, the mutator-type transposase (Dehly_1529) matched 18 duplicates. *Dhgm* paralogs are also top database matches Dehly_1519-Dehly_1529 unless otherwise indicated. In contrast to the *Dhc*

KS and RC orthologs, top BlastP database matches outside the Dhgm BL-DC-9 genome (bottom row) tended to share markedly weaker similarity with Dehly_1519 – Dehly_1529.

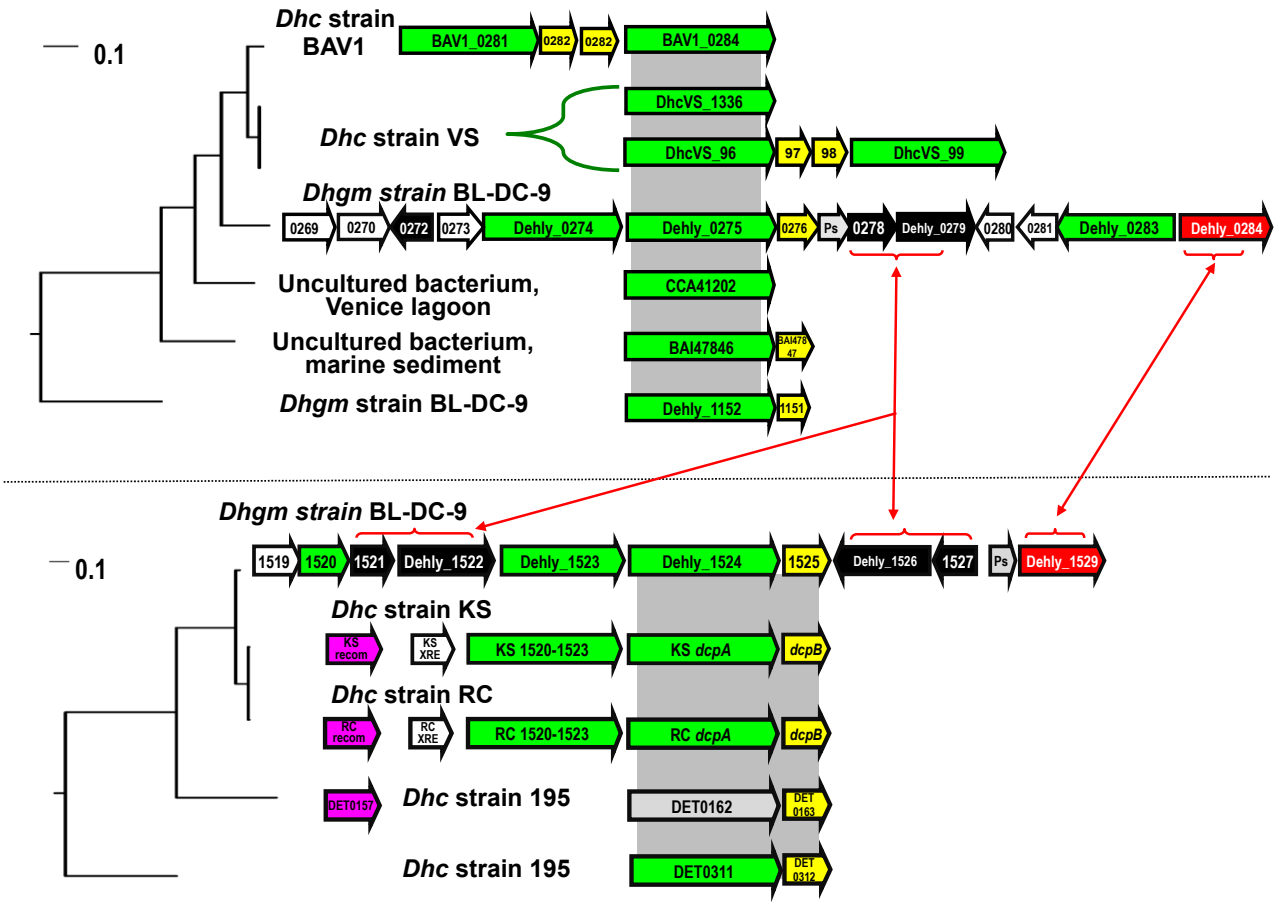


Figure S4.6. Combined trees showing the commonalities of certain insertion elements in *Dhc* and *Dhgm* GI. In black are the transposases (*orfA*) and integrases (*orfB*); in red the transposases mutator type.

Chapter 4 Appendix: Supplemental Tables

Table S4.1. Primers used to explore sequence similarities upstream and downstream of the *dcpA* gene in *Dhc* RC and KS.

Target locus	Primer Name	Annotated Target Gene Name	Primer sequence 5'->3'	Primer Length (bp)	Coordinates Gene Start	Coordinates Gene End	Gene Length (bp)	Primer Binding Coordinates	Comments
Dehly_1518	**1518F**	**Cytochrome c class I**	**TTCGGTCCCCAAAGTGCGGG**	**20**	**1493030**	**1492447**	**583**	**1492730…1492711**	**Used with primer 1520R**
Dehly_1520	**1520F**	**RDase**	**ACCAGATGTTGAGTGCATCGGAGT**	**23**	**1494014**	**1493625**	**389**	**1493748...1493725**	**Used with primer *dcpA*-1449R**
Dehly_1520	**1520R**	**RDase**	**TCGATTTCGACAGTAGGCACGTCG**	**19**	**1494014**	**1493625**	**389**	**1493800...1493777**	**Used with primer 1518F**
Dehly_1522	1522F	Integrase catalytic subunit	AGTTGGAGATCAAGGCAGCGGATA	24	1494383	1495243	860	1494510…1494533	Tested with primer *dcpA*-574R and failed to yield a product on RC and KS gDNA
Dehly_1523	**1523F**	**4Fe-4S ferredoxin**	**TGGCAGGCGACTGCGCATTT**	**20**	**1495262**	**1496284**	**1022**	**1495925…1495905**	**Sequencing primer**
Dehly_1524	***dcpA*-1257F**	**RDase**	**CGATGTGCCAGCCATTGTGTCTTT**	**23**	**1497940**	**1496492**	**1448**	**1497772...1497749**	**Sequencing primer**
Dehly_1524	***dcpA*-1449R**	**RDase**	**TTTAAACAGCGGGCAGGTACTGGT**	**23**	**1497940**	**1496492**	**1448**	**1497941…1497918**	**Used with primer 1520F**
Dehly_1524	***dcpA*-360F**	**RDase**	**TTGCGTGATCAAATTGGAGCCTGG**	**23**	**1497940**	**1496492**	**1448**	**1496875…1496852**	**Sequencing primer**
Dehly_1524	***dcpA*-574R**	**RDase**	**TCGTCATCCACTCTTGCATAGCCA**	**23**	**1497940**	**1496492**	**1448**	**1505449…1505426**	**Sequencing primer**
Dehly_1526	1526R	Integrase catalytic subunit	AGAATTGTTGAGCATAAGCCGCCG	24	1499047	1499907	860	1499070…1499093	Tested with primer *dcpA*-1257F and failed to yield a product on RC and KS gDNA

Table S4.1. (continued)

Target locus	Primer Name	Annotated Target Gene Name	Primer sequence 5'->3'	Primer Length (bp)	Coordinates Gene Start	Coordinates Gene End	Gene Length (bp)	Primer Binding Coordinates	Comments
Dehly_1527	1527F	Transposase IS3/IS911 family protein	TGGAAAGGATTCCACACGGGAAGT	24	1499904	1500221	317	1500220…1500197	Tested with primer 1531R and failed to yield a product on RC and KS gDNA
Dehly_1529	1529R	Transposase mutator type	ACATTACTGCGGCGCTTGATTTCC	24	1500745	1501959	1214	1501790…1501767	Tested with primer dcpA-1257F and failed to yield a product on RC and KS gDNA
Dehly_1531	1531R	LuxR transcriptional regulator	GGGGATGGACGGCTTTGCGA	20	1504069	1504752	684	1504543…1504524	Tested with primer dcpA-1257F and 1527F and failed to yield a product on RC and KS gDNA

Note: Primers on bold and shaded in grey successfully yielded a clean product that was cloned and sequenced. Primers were designed based on *Dhgm* strain BL-DC-9 genome (NC_014314).

Table S4.2. Dehly_1519-1527 genomic island codon adaptation index (CAI). Codon usage of the putative GI encompassing genes Dehly_1519-1527 in comparison to all *Dehalogenimonas* chromosomal genes using the codon adaptation index (CAI). Normalized CAI of < 1.00 indicates a possible laterally acquired gene and is scored below genomic expected CAI at a 1% level of significance (genomes.urv.es/CAIcal/E-CAI).

Description	Gene locus	Length (bp)	Normalized CAI
XRE family transcriptional regulator	Dehly_1519	249	0.92
reductive dehalogenase (partial)	Dehly_1520	390	0.87
transposase IS3/IS911 family protein	Dehly_1521	318	0.92
integrase catalytic region	Dehly_1522	861	0.91
4Fe-4S ferredoxin iron-sulfur-binding domain-containing protein	Dehly_1523	1023	0.84
reductive dehalogenase *(RDaseA)*	Dehly_1524	1449	0.90
reductive dehalogenase anchoring protein *(RDaseB)*	Dehly_1525	219	0.89
integrase catalytic region	Dehly_1526	861	0.92
transposase IS3/IS911 family protein	Dehly_1527	318	0.92

Table S4.3. *dcpA* genomic island codon adaptation index (CAI). Codon usage of *dcpA* and adjacent genes (elucidated by PCR amplification) in comparison to all *Dhc* housekeeping genes (*n*=142) using the codon adaptation index (CAI). Normalized CAI < 1.00 (red font) indicates a possible laterally acquired gene and is scored below genomic expected CAI at a 1% level of significance (genomes.urv.es/CAIcal/E-CAI).

Description	Length (bp)	Normalized CAI
KS_1519_XRE	249	0.97
KS_1520_and_1523_fused	1386	0.84
KS_1524_*dcpA*	1455	0.89
KS_1524_*dcpB*	219	0.97
RC_1519_XRE	249	0.97
RC_1520_and_1523_fused	1386	0.82
RC_1524_*dcpA*	1455	0.90
RC_1525_*dcpB*	219	0.98

Table S4.4. Dehly_0271-0283 genomic island CAI. Codon usage Dehly_0271-0283 in comparison to *Dehalogenimonas* strain BL-DC-9 housekeeping genes (*n*=118) using the codon adaptation index (CAI). Normalized CAI of < 1.00 indicates a possible laterally acquired gene and is scored below genomic expected CAI at a 1% level of significance (genomes.urv.es/CAIcal/E-CAI).

Description	Gene locus	Length (bp)	Normalized CAI (CAI/eCAI)
Puative PAS/PAC sensor protein (*rdhC*)	Dehly_0269	1422	0.89
LuxR family two component transcriptional regulator	Dehly_0270	813	0.92
Pseudogene	Dehly_0271	126	0.91
transposase_IS3/IS911_family_protein	Dehly_0272	306	0.91
hypothetical protein	Dehly_0273	285	0.85
reductive dehalogenase *(RDaseA)*	Dehly_0274	1419	0.90
reductive_dehalogenase *(RDaseA)*	Dehly_0275	1482	0.90
reductive dehalogenase anchoring protein *(RDaseB)*	Dehly_0276	276	0.84
Pseudogene	Dehly_0277	348	0.87
transposase_IS3/IS911_family_protein	Dehly_0278	318	0.92
integrase catalytic subunit	Dehly_0279	861	0.92
integrase catalytic subunit	Dehly_0280	330	0.96
transposase_IS3/IS911_family_protein	Dehly_0281	285	0.99
hypothetical protein	Dehly_0282	129	0.95
reductive_dehalogenase *(RDaseA)*	Dehly_0283	1431	0.84

Chapter 5

Genomic insights into 1,2-D organolide-respiration

Abstract

Dehalococcoides mccartyi (*Dhc*) strains RC and KS respire toxic 1,2-dichloropropane (1,2-D) to environmentally benign propene. The Ion Torrent semiconductor technology was used to sequence the draft genomes of these strains. The resulting draft genomes reflect *Dhc*'s adaptation to organohalide respiration and genes encoding multiple reductive dehalogenases (RDases), hydrogenase complexes, and corrinoid salvage and modification mechanisms were present. Additionally strain RC and KS exhibit the presence of various putative prophage regions and genes for arsenic resistance, features also observed in other *Dhc* strains. Interestingly, a copy of the recently identified polychlorinated biphenyl (PCB) RDase gene is present in the draft genomes implying that these strains can use PCB congeners as electron acceptors. The drafts may provide new insights into the adaptation of *Chloroflexi* to organohalide respiration.

Introduction

1,2-dichloropropane (1,2-D) is a potential carcinogen regulated by the Environmental Protection Agency to a MCL of 5 ppb in groundwater. *Dehalococcoides mccartyi* (*Dhc*) strains RC and KS conserve energy from dichloroelimination of 1,2-D to innocuous propene and inorganic chloride. This reductive detoxification process is of interest for bioremediation applications and restoration of contaminated sites impacted with polychlorinated propanes. The 1,2-D-dechlorinating *Dhc* strains RC and KS share high 16S rRNA gene sequence identity to *Dhc* strains that cannot grow on 1,2-D, and recent efforts have identified the reductive

dehalogenase (RDase) biomarker gene *dcpA* implicated in 1,2-D detoxification (Padilla-Crespo et al., 2014). Both strains were retrieved from geographically distinct locations, strain KS was derived from a BTEX-impacted site at the King Salmon River in Alaska while strain RC was derived from Red Cedar Creek sediments collected near Okemos, Michigan, USA with no previous reported contamination. Sequencing of these genomes will shed light into the uniqueness or common features between these strains and other *Dhc* strains unable to grow on 1,2-D. Furthermore, since 1,2-D is the only chlorinated compound that these strains have been successfully grown on, genome information may reveal the possibility of them using additional chlorinated compounds as substrates.

Methods

Cultures and DNA isolation

Cultures were grown with 1,2-D in reduced mineral salts medium as described (F E Löffler, Champine, Ritalahti, Sprague, & Tiedje, 1997; Padilla-Crespo et al., 2014; Kirsti M Ritalahti & Löffler, 2004) and genomic DNA (gDNA) was extracted from 100 mL of culture suspension containing 10^7 cells/mL using the Mo BIO PowerWater DNA isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA). RNA was removed by adding a mixture of 2.5 U of RNase A and 100 U of RNase T1 (catalog number AM2286; Invitrogen) following incubation at 37°C for twenty minutes. DNA concentrations were measured using a NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, DE) and quality assessed by gel electrophoresis.

Ion Torrent genome library preparation

The draft genomes were assembled from single-end DNA reads using the Ion Torrent Ion Torrent PGM sequencer (Life Technologies) with 200-bp chemistry. Genomic libraries of strains KS and RC were constructed using 100 ng of gDNAusing the Ion Xpress™ Plus Fragment Library Kit (4471269) following the User's Guide directions. All steps were performed using 1.5 mL LoBind tubes (Eppendorf, PN 022431021), which provide higher recovery rates by minimizing the interactions of nucleic acids with the tube surface wall, thus

avoiding sample loss. DNA shearing was performed by enzymatic fragmentation, by adding 5 µL of Ion Shear™ Buffer 10X, 10 µL of Ion Shear™ Enzyme-100 (Ion Shear™ Plus Reagents; PN 4471248) to a 100 ng of gDNA (diluted to 10 ng/µL). Nuclease free water was added up to a final volume of 50 µL. Reactions were mixed by gently pipetting up and down, incubated for 15 or 7 minutes at 37°C, and terminated with 5 µL of Ion Shear™ Stop Buffer. Samples were placed on ice and subsequently purified following the directions of the Agencourt AMPure XP Kit, PN A63881, Beckman Coulter, Brea, CA.). Briefly, the Ion Shear reaction mixture was combined with 99 µL of the Agencourt® AMPure® XP Reagent containing magnetic beads, incubated for 5 min at room temp, placed in a DynaMag magnetic bead stand (PN 12321D, Life Technologies) for 5 min, washed twice with 500 µL of 70% v/v ethanol and eluted with 25 uL of Low-TE buffer (PN 602-1155-010, Life Technologies). One microliter of the eluted sheared DNA was analyzed in the Bioanalyzer 2100 DNA High Sensitivity chips (Agilent Technologies, Palo Alto, CA) to check the size distribution of the resulting fragments. The fragmented DNA (~24 µL) was subjected to adaptor ligation by adding 10 µL of Ligase Buffer, 10 µL of Adapters mix, 1 µL of DNA Ligase (Xpress™ Fragment Library Kit, PN 4468987) and 54 µL of nuclease-free water. The mixture was pipetted up and down and incubated at room temperature for 30 mins. The ligations were purified and eluted in 30 µL of Low-TE using the Agencourt XP beads and magnetic rack as previously described. Size selection of the library was achieved using a Pippin Prep™ instrument (SAGE Science, Beverly, MA, USA), which allows for automated size selection and collection of DNA fragments. Fragment sizes of 180–210 bp were collected following the manufacturer's recommendations. Nuclease free water was added to the recovered fragments up to a final volume of 60 µL and purified with the Agencourt ® XP magnetic beads procedure as previously described but now eluted in 20 µL of Low-TE. The purified and size-selected DNA (20 µL) was amplified by mixing it with 50 µL of Platinum® High Fidelity PCR Super Mix and 5 µL of Library Amplification Primer Mix. Amplifications were carried with the following thermocycler parameters 72°C for 20 min and 95°C for 5 min, followed by 9 cycles of 95°C for 15s, 58°C for 15s and 72°C for 1 min. Subsequently, samples were once again purified with the Agencourt XP-PCR beads and eluted in 20 µL of Low-TE. Concentrations and size distribution of the final DNA library were determined using the Bioanalyzer 2100 DNA High Sensitivity chips (Agilent Technologies). Electropherograms were verified for indications of primer dimers (as a peak of ~75 bp, close to the lower marker), and concatemers, which are

artifacts of inefficient ligations and appear as peaks of high- molecular weight fragments ranging from ~300-500 bp in size. Each library was diluted to 2.8×10^8 DNA molecules in 18 μL, and used as templates for emulsion PCR according to the Ion Xpress™ Template Kit User Guide v2.0 (Life Technologies, PN 4469004). During this step the templates were bound to the Ion Sphere particles at the manufacturer's specified proportions and subjected to clonal amplification by emulsion PCR following the Template Kit User Guide's recommendations. After amplification, several washing steps broke the emulsion and beads with no template were removed by standard protocols. Ion sphere particles with amplified-bonded fragments were mixed with the Ion Torrent sequencing primers and polymerase (Ion PGM™ 200 Sequencing Kit, PN 4474004) and loaded onto Ion 316™ in the Ion Personal Genome Machine system (PGM; Life Technologies, Germany). Sequencing was performed following the protocol outlined in the user guide (4471999 Rev B, 13. Oct. 2011) and base-calling and preliminary alignments to the genome of *Dhc* strain 195 were performed by the Ion Torrent software suite (version 2.0.1)

Sequence assembly and annotation

A subset of 600,000 sequence fragments were randomly selected and different genomic sequence assembly strategies were tested: Newbler, which is based on the overlap-layout-consensus methodology, and three differet de Bruijn graph-based methods: Velvet, SOAP de novo and Ray. Various combined methods (e.g., Velvet and SOAP de novo contigs fed into Newbler) were also evaluated. The performance of the different assembling approaches was measured by: (1) the number of resulting contigs, (2) the length of contigs with a value of N50 (i.e., L50, a standard measure of quality in *de novo* assemblies that refers to the length of the smallest contig that covers at least 50% of the entire assembly), and (3) the maximum contig length. Following the trials with the different methods, consensus assemblies were obtained using Newbler version 2.6 (Roche) and reads aligned using Bowtie2 version 2.1.0 (Langmead & Salzberg, 2012). The genomeCoverageBed package of BEDTools version 2.17.0 (Quinlan & Hall, 2010) was used to calculate genome coverage statistics. The reads were aligned using BLAT (BLAST-like alignment tool) to the reference genomes of *Dhc* strains 195, BAV1, BTF08, CBDB1, DCMB8,

GT and VS (Kent, 2002). Alignments to the genome of DCMB5 produced the highest counts of over 3 million aligned reads, and this genome was used as reference for further analysis using the ABACAS software (Assefa, Keane, Otto, Newbold, & Berriman, 2009) to align, order, and orientate genomic regions. Protein coding sequences were predicted with the RAST annotation server version 4.0 (Aziz et al., 2008) with default settings and correction for frameshifts turned on. Further RDase identification and sequence comparison was performed using BLASTP and manually querying against NCBIs non-redundant sequence database (Altschul, Gish, Miller, Myers, & Lipman, 1990). Translated RDase gene sequences were aligned using CLUSTAL_W (J. D. Thompson, Gibson, & Higgins, 2002). RNA genes were annotated with RNammer version 1.2 (Lagesen et al., 2007) and transfer RNAs were predicted using tRNAscan-SE version 1.3.1 (Lowe & Eddy, 1997). Putative prophage genomic regions were identified using the PHAST Search Tool (http://phast.wishartlab.com/index.html).

Results and Discussion

DNA extractions from the RC and KS cultures yielded nucleic acid preparations of high molecular weight with 260/280 nm absorbance ratios of 1.8-1.9 (Figure 5.1). Enzymatic reactions with 100 ng total DNA incubated for 15 minutes resulted in fragmentation with a size distribution of 50-200 bp, with the majority of the fragments having a size of ~100 bp (Figure 5.2A). Since a 200-bp library was the goal, a second enzymatic shearing reaction was performed with an incubation time of only 7 minutes, which yielded a size distribution of 100-300 bp with the majority of fragments having the optimal size of ~200 bp (Figure 5.2B). The needed modification in the incubation time during the enzymatic shearing step may be due to the small size of *Dhc* genomes and/or the presence of small fragments already in the samples since the DNA extraction protocol consisted of bead beating (although gel electrophoresis confirmed the presence of mostly high molecular weight DNA).

After ligation, size selection and nick translation, the final Ion Torrent library preparation showed a peak of ~300 bp (Figure 5.3), the expected size for 200 bp libraries (adapters + insert

are expected to yield fragments of around 280–320 bp). The single narrow peak also indicated a clean preparation with no primer dimers or concatemers.

Sequencing of the RC and KS genomic libraries generated 4,501,960 and 3,640,282 reads with an average read length of 176.4 ± 65.2 bases and 148.0 ± 79.4 respectively (complete Ion Torrent run statistics are shown in Figures 5.4-5). Using the Ion Torrent software suite version 2.0.1, a preliminary alignment to the genome of *Dhc* strain 195 (CP000027.1) showed a 341 fold and 229 fold coverage with default settings (Figure 5.6). When using AQ20 values (a quality score that represents the length at which the error rate is 1% or less), a mean depth of genome coverage of 14.5 fold and 9.2 fold was achieved (Figure 5.7). The Newbler software package provided the best *de novo* sequence assembly resulting in the fewest numbers of total contigs, longer contigs and higher numbers of contigs with N50 values (Table 5.1).

The draft genomes of strains RC and KS were assembled into 23 and 15 contigs with estimated genome sizes of 1,509,214 and 1,485,739 bp, and G+C contents of 47.15 and 47.26 %, respectively (Table 5.2). These findings are in accordance with the average sizes and G+C contents of characterized *Dhc* genomes, which range from 1.34 to 1.47 Mbp and 47 to 48.9 %, respectively (Table 5.2). The maximum contig size had 643,701 and 647,595 bp and N50 values of 210,101 and 268,024 bp for the RC and KS genomes, respectively (Table 5.1). Genome coverage statistics showed that 99.53 and 99.70 % of nucleotide bases in the draft genomes had a read depth of >20 fold (which is a metric used to support reliable sequencing and variant detection). Moreover, an average read depth of 187.83 fold ± 123.73 and 76.5 fold ± 44.46 was reported for the genomes of strain RC and strain KS, respectively. This level of coverage is sufficient to make confident genotype calls (e.g., a 15-fold coverage was enough to call heterozygous single nucleotide polymorphism on the human genome) (Bentley et al., 2008; Nielsen, Paul, Albrechtsen, & Song, 2011).

A total of 1,610 and 1,626 putative protein-coding sequences (this is not counting the rra? genes) were identified for strains RC and KS, respectively. For both strains, 74% of the genes were annotated based on proteins with known biological functions and 26% were annotated as hypothetical proteins. Single copies of the 5S, 16S, and 23S ribosomal RNA genes shared 98-

100% identities to the respective genes on other *Dhc* strains, and a total of 46 genes encoding transfer RNAs were predicted, also similar to what has been observed for other *Dhc* genomes (Löffler FE, Yan J, Ritalahti KM, Adrian L, Edwards EA, Konstantinidis KT, Müller JA, Fullerton H, Zinder SH, 2013). The 16S rRNA gene is spatially separated from the 5S and 23S rRNA genes. Features characteristic of *Dhc*'s specialized organohalide-respiring energy metabolism are present in the draft genomes of strains RC and KS including genes encoding for the six hydrogenase complexes Hup, Hyp, Ech, Hyc, Hym, Vhu involved in hydrogen oxidation, which is the required electron donor (as described by Seshadri et al., 2005) (Table 5.3 and 5.4) and multiple putative reductive dehalogenase (RDase) genes that catalyze the reduction of the chlorinated substrates used as terminal electron acceptors.

In the draft genomes of strain RC and strain KS, 34 and 31 putative RDase genes were identified. Twenty-nine of the RDase genes in strain RC are shared with strain KS at an amino acid (aa) identity of 80% or higher (Table 5.5). Moreover, the encoded RDases share >92 % aa identity to other RDases in *Dhc* strains, and one RDase (DhcRC_1002; KS_1087) has an ortholog with 69-70% aa identity in *Dhgm* strain BL-DC-9 (Dehly_0283, YP_003757927). As expected, the *dcpA* gene previously cloned from strains RC and KS (JX826287, JX826286, respectively) is present in the draft genomes (DhcKS_1220; DhcRC_1554). This RDase gene is not found in other *Dhc* strains but is shared with the 1,2-D-respiring *Dhgm* strain BL-DC-9. Another putative RDase gene shared with strain BL-DC-9 (and not present in sequenced *Dhc* genomes) is found adjacent to *dcpA* (DhcRC_1553; DhcKS_1221). This RDase gene is a homologue of Dehly_1523 and, along with *dcpA*, is putatively part of a genomic island presumably acquired by HGT (Padilla-Crespo, E, Wagner D, n.d.)..

Gene homologues of *vcrA, bvcA, pceA, and mbrA* were not found in the draft genomes, which is in agreement with the inability of strains RC and KS to grow with chlorinated ethenes. The chlorobenzene (CB) RDase gene *cbrA* is absent while both genomes share an almost identical RDase gene (97-98 nt% identity, DhcRC_1592 and DhcKS_1618) to the recently described PCB RDase *pcbA4* (position 64056-65504 on the genome of *Dhc* strain CG4 (Wang, Chng, Wilm, et al., 2014). *Dhc* strains 195 and JNA, which also possess a RDase gene nearly identical to *pcbA4* dechlorinate the PCB mixture Aroclor 1260 (LaRoe SL, Fricker AD, 2014; Zhen, Du,

Rodenburg, Mainelis, & Fennell, 2014). Based on this finding, it is plausible that strains RC and KS can also respire PCB congeners. This observation has major implications for bioremediation practice at PCB-contaminated sites as discussed by Bedard (2014) (DL, 2014).

Similarly to other *Dhc* strains, the draft genomes of strains RC and KS also contain the *btu* gene operon encoding for the BtuCDF system involved in cobalamin uptake and transport, as well as genes involved in corrinoid scavenging and cofactor modifications (e.g., *cobT*, *cobU* and *cbiZ*) (Table 5.6). These genomic features are crucial for organohalide respiration since cobamides are required cofactors for RDases but *Dhc* cells lack *de novo* corrin ring synthesis genes. Strains RC and KS also possess several genes involved in arsenic resistance (Table 5.7), a feature described in strain DCMB5 . Further, the RC and KS genomes have as a cluster of CRISPR elements of the Cas- and Cse-types (Table 5.8), which are known to function as a defense mechanism against invading exogenous DNA sequences like phages and plasmids. Lastly, multiple putative prophage regions were found by PHAST analysis (Zhou, Liang, Lynch, Dennis, & Wishart, 2011) (Figure 5.7 and Table 5.9)

Conclusion

Several *Dhc* genomes have now been reported, only strains RC and KS have been described to grow with 1,2-D. The genomes of strains RC and KS share common *Dhc* genomic features but possess the RDase gene *dcpA* gene giving these strains the distinguishing 1,2-D-respiring phenotype. Although growth on PCBs have not been attempted with strain RC and strain KS cultures, their genome information suggest these *Dhc* strains use and PCBs as terminal electron acceptors (since they posses a nearly identical *pcbA4* RDase gene). Strain GT also has a RDase gene sharing 97% nucleotide identity (1,373 out of 1,412) with *pcbA4*. Therefore, along with the PCB-dechlorinating *Dhc* strains JN and 195, which also harbor a nearly identical *pcbA4,* strain GT may also grow with certain PCB congeners.

The finding that *pcbA4* is distributed among several *Dhc* strains is a relevant observation because the PCB RDase PcbA4 may also be involved in PCE to TCE and *cis*/*trans*-DCE reductive dechlorination. Although early RC enrichments could dechlorinate PCE to the DCE isomers

(with small amounts of TCE formed as an intermediate) this ability was lost in both the RC and KS cultures after 35 successive transfers in medium amended with 1,2-D (F E Löffler, Champine, Ritalahti, Sprague, & Tiedje, 1997; Kirsti M Ritalahti & Löffler, 2004). Interestingly, the PCE dechlorinating ability of strain CG4 (with a *trans*-DCE/*cis*-DCE ratio of 2.50-2.67) is very similar to that reported of an early RC enrichment culture by Löffler et al. (with a *trans*-DCE/*cis*-DCE ratio of 2.45) (F E Löffler, Champine, Ritalahti, Sprague, & Tiedje, 1997). The finding that the same RDase in strain CG4 is responsible for PCE and PCB dechlorination is puzzling since strain 195 (a strain that can also can dechlorinate PCB and has the *pcbA4* homologue DET1559 encoding a RDase that is 99% identical to the PCB RDase of strain CG4) also has a putative *pceA* gene (DET0138), which shares no similarity to the known PCB-dechlorinating RDases. In fact, the PceA RDase of strain 195 displays lower aa identity to RDases in *Dhc* genomes (<87 %) and was previously described to be "bifunctional" serving as a PCE and 2,3-dichlorophenol RDase (Fung et al., 2007). But both DET0138_*pceA* and DET1559_*pcbA4* were reported to be among the few RDase transcripts produced (along with *tceA* and the pseudogene DET0162 transcripts) in cultures of strain 195 grown with PCE. Morris et al. also detected peptides from both RDases encoded by DET0138 and DET1559 in the cell fractions of strain 195 in pure and mixed cultures grown with PCE (Morris et al., 2007). Therefore, it can be hypothesized that both RDases are involved in PCE dechlorination, while DET1559_PcbA4 is only responsible for growth on PCBs.

Biodegradation assays are needed to confirm the ability of *Dhc* strains RC and KS to respire PCB congeners, and new trials with PCE should be attempted to investigate if growth on PCE is restituted. Further molecular and proteomic approaches, along with substrate range characterization experiments, should assign function or redundancy to the high number of RDase in these strains only known to respire and grow on 1,2-D thus revealing more insights into 1,2-D dechlorination.

Nucleotide sequence accession numbers

The draft genome of *Dehalococcoides mccartyi* strain RC and KS was deposited in GenBank under accession numbers PRJNA230754 and PRJNA230755 respectively.

References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*, 403–410.

Assefa, S., Keane, T. M., Otto, T. D., Newbold, C., & Berriman, M. (2009). ABACAS: Algorithm-based automatic contiguation of assembled sequences. *Bioinformatics*, *25*(15), 1968–1969.

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., Zagnitko, O. (2008). The RAST Server: rapid annotations using subsystems technology. *BMC Genomics*, *9*, 75.

Bentley, D. R., Balasubramanian, S., Swerdlow, H. P., Smith, G. P., Milton, J., Brown, C. G., Smith, A. J. (2008). Accurate whole human genome sequencing using reversible terminator chemistry. *Nature*, *456*(7218), 53–59.

DL, B. (2014). PCB dechlorinases revealed at last. *Proc Natl Acad Sci U S A.*, *111*(33), 11919–20.

Fung, J. M., Morris, R. M., Adrian, L., & Zinder, S. H. (2007). Expression of reductive dehalogenase genes in *Dehalococcoides ethenogenes* strain 195 growing on tetrachloroethene, trichloroethene, or 2,3-dichlorophenol. *Applied and Environmental Microbiology*, *73*(14), 4439–45. http://doi.org/10.1128/AEM.00215-07

Kent, W. J. (2002). BLAT - the BLAST-like alignmen tool. *Genome Res.*, *12*(4), 656–64.

Lagesen, K., Hallin, P., Rødland, E. A., Stærfeldt, H. H., Rognes, T., & Ussery, D. W. (2007). RNAmmer: Consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Research*, *35*(9), 3100–3108.

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods*.

LaRoe SL, Fricker AD, B. D. (2014). *Dehalococcoides mccartyi* strain JNA in pure culture extensively dechlorinates Aroclor 1260 according to polychlorinated biphenyl (PCB) dechlorination Process N. *Environ Sci Technol.*, *48*(16), 9187–9196.

Löffler, F. E., Champine, J. E., Ritalahti, K. M., Sprague, S. J., & Tiedje, J. M. (1997). Complete reductive dechlorination of 1,2-dichloropropane by anaerobic bacteria. *Applied and Environmental Microbiology*, *63*, 2870–2875.

Löffler FE, Yan J, Ritalahti KM, Adrian L, Edwards EA, Konstantinidis KT, Müller JA, Fullerton H, Zinder SH, S. A. (2013). *Dehalococcoides mccartyi* gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation, belong to a novel bacterial class, Dehalococcoidia classis nov., order Dehalococcoidales ord. nov. and famil. *Int. J. Syst. Evol. Microbiol.*, *63*, 625–635.

Lowe, T. M., & Eddy, S. R. (1997). tRNAscan-SE: A program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Research*, *25*(5), 955–964.

Morris, R. M., Fung, J. M., Rahm, B. G., Zhang, S., Freedman, D. L., Zinder, S. H., & Richardson, R. E. (2007). Comparative proteomics of *Dehalococcoides* spp. reveals strain-specific peptides associated with activity. *Applied and Environmental Microbiology*, *73*(1), 320–326. http://doi.org/10.1128/aem.02129-06

Nielsen, R., Paul, J. S., Albrechtsen, A., & Song, Y. S. (2011). Genotype and SNP calling from next-generation sequencing data. *Nature Reviews. Genetics*, *12*(6), 443–451.

Padilla-Crespo, E., Yan, J., Swift, C., Wagner, D. D., Chourey, K., Hettich, R. L., Löffler, F. E. (2014). Identification and environmental distribution of *dcpA*, which encodes the reductive dehalogenase catalyzing the dichloroelimination of 1,2-dichloropropane to propene in organohalide-respiring Chloroflexi. *Applied and Environmental Microbiology*, *80*(3), 808–818.

Padilla-Crespo, E, Wagner D, L. F. (n.d.). Inter-genus reductive dehalogenase gene transfer between organohalide-respiring Chloroflexi. *In Preparation*.

Pöritz, M., Goris, T., Wubet, T., Tarkka, M. T., Buscot, F., Nijenhuis, I., Adrian, L. (2013). Genome sequences of two dehalogenation specialists - *Dehalococcoides mccartyi* strains BTF08 and DCMB5 enriched from the highly polluted Bitterfeld region. *FEMS Microbiology Letters*, *343*(2), 101–104.

Quinlan, A. R., & Hall, I. M. (2010). BEDTools: A flexible suite of utilities for comparing genomic features. *Bioinformatics*, *26*(6), 841–842.

Ritalahti, K. M., & Löffler, F. F. (2004). Populations implicated in the anaerobic reductive dechlorination of 1,2-dichloropropane in highly enriched bacterial communities. *Appl. Environ. Microbiol.*, *70*(7), 4088–4095. http://doi.org/10.1128/AEM.70.7.4088

Seshadri, R., Adrian, L., Fouts, D. E., Eisen, J. A., Phillippy, A. M., Methe, B. A., Heidelberg, J. F. (2005). Genome sequence of the PCE-dechlorinating bacterium **Dehalococcoides** *ethenogenes*. *Science*, *307*, 105–108.

Thompson, J. D., Gibson, T. J., & Higgins, D. G. (2002). Multiple sequence alignment using ClustalW and ClustalX. *Current Protocols in Bioinformatics / Editoral Board, Andreas D. Baxevanis [et Al.]*, *Chapter 2*, Unit 2.3.

Wang, S., Chng, K. R., Wilm, A., Zhao, S., Yang, K.-L., Nagarajan, N., & He, J. (2014). Genomic characterization of three unique *Dehalococcoides* that respire on persistent polychlorinated biphenyls. *Proceedings of the National Academy of Sciences of the United States of America*, (111), 12103–12108.

Zhen, H., Du, S., Rodenburg, L. A., Mainelis, G., & Fennell, D. E. (2014). Reductive dechlorination of 1,2,3,7,8-pentachlorodibenzo-p-dioxin and Aroclor 1260, 1254 and 1242 by a mixed culture containing *Dehalococcoides mccartyi* strain 195. *Water Research*, *52*, 51–62.

Zhou, Y., Liang, Y., Lynch, K. H., Dennis, J. J., & Wishart, D. S. (2011). PHAST: A Fast Phage Search Tool. *Nucleic Acids Research*, *39*(SUPPL. 2), W347–W352.

Chapter 5 Appendix: Figures

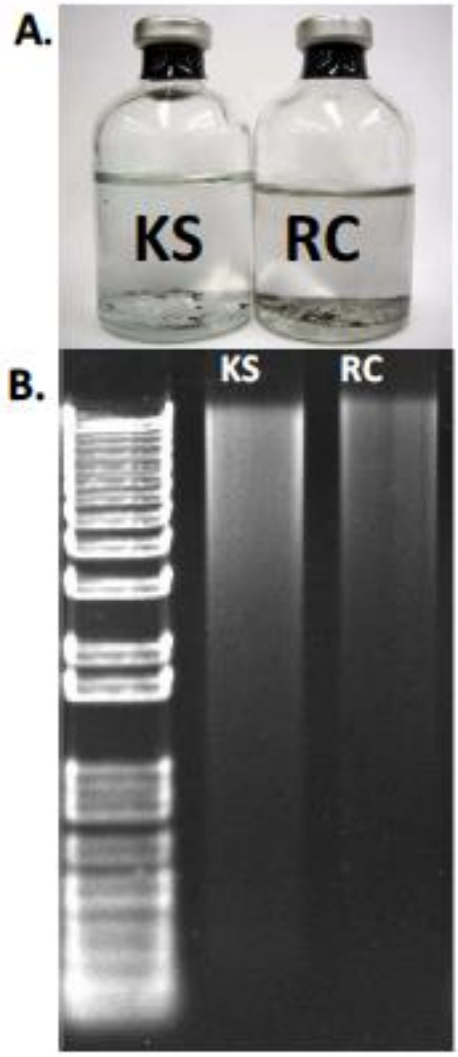


Figure 5.1. Genomic DNA extraction for Ion Torrent Genome Sequencing. RC and KS cultures were grown on defined anaerobic mineral salts medium with 5 mM lactate, and 0.2 mM 1,2-D. Genomic DNA was extracted from 100 mL of culture with the MoBio WaterKit. DNA was diluted to 10 ng/uL and ran into a 1% agarose gel.

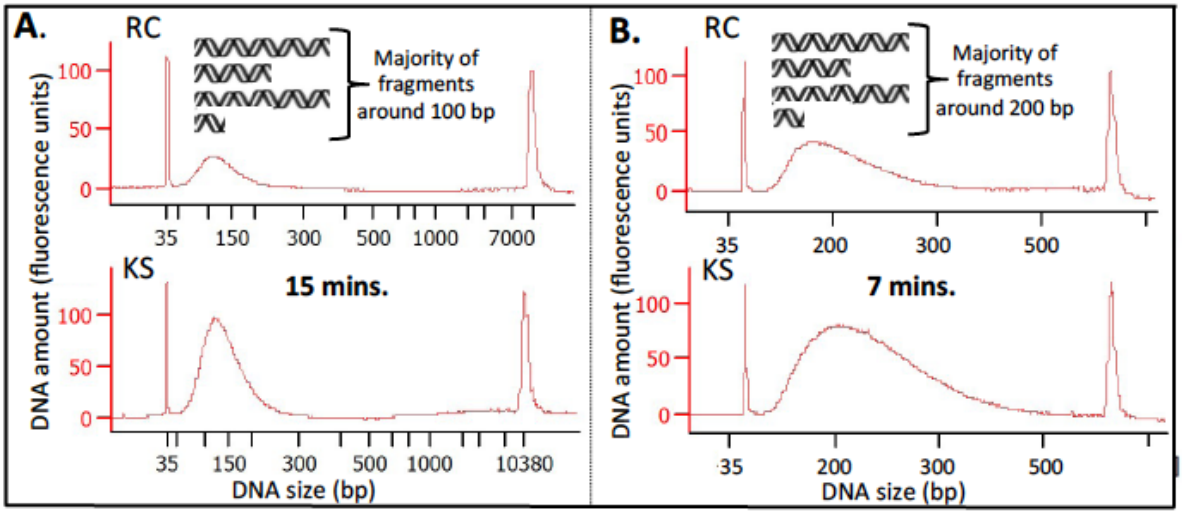


Figure 5.2. Bioanalyzer electropherograms of gDNA following enzymatic fragmentation. Reactions with 100 of ng total DNA incubated for 15 minutes resulted in excessive fragmentation with a size distribution of 50-200 bp (Panel A) with the majority having a size of ~100 bp. A second enzymatic shearing reaction was performed with an incubation time of only 7 minutes (Panel B) which yielded a size distribution of 100-300 bp with the majority of fragments having the optimal size of ~200 bp. Peaks at 35 and and 10380 bp indicate the low- and high-molecular weight markers. Agilent High Sensitivity DNA chips kit and chips were used.

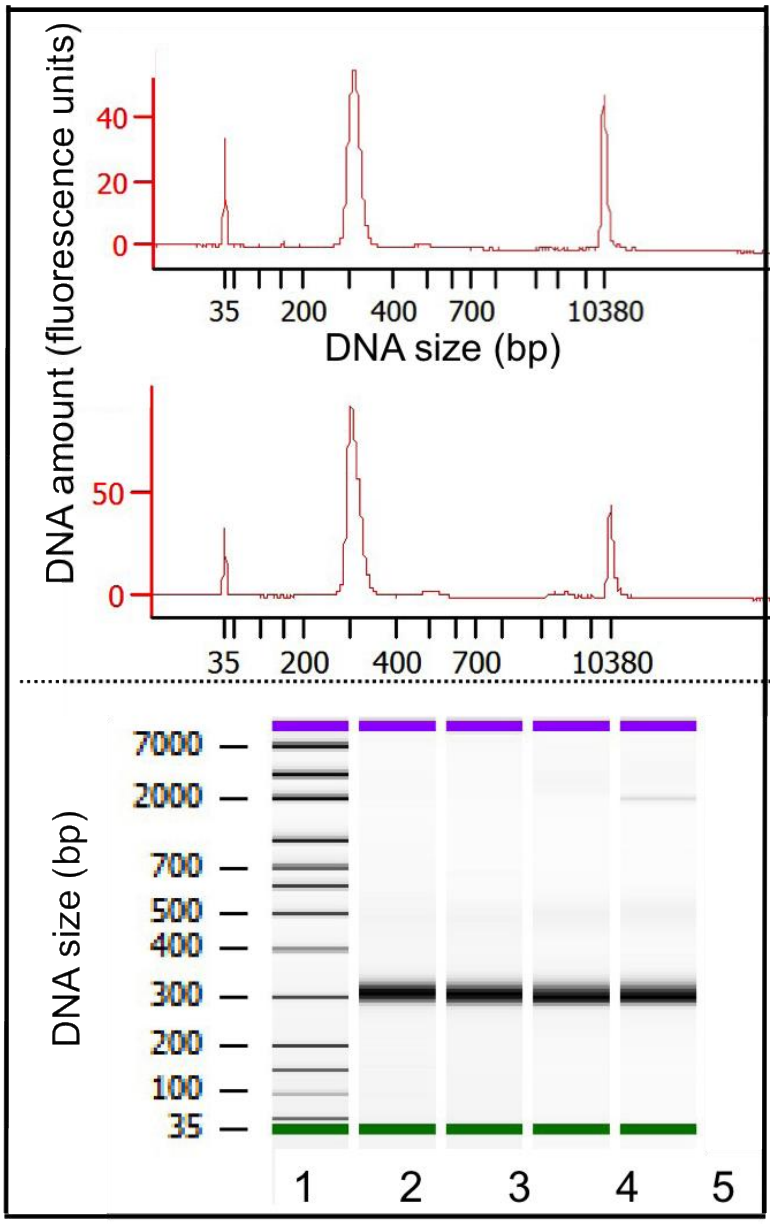


Figure 5.3. Final sequencing library as seen using the Bioanalyzer 2100 (Agilent, Technologies). The electropherogram and gel images show the size distribution of the DNA libraries of *Dhc* strains RC and KS prepared according to Ion Torrent's 200-bp chemistry. A single discrete peak of the expected size is depicted (ligated adapters cause the DNA fragments to migrate ~100 bp higher.) Peaks at 35 and and 10380 bp indicate the low- and high-molecular weight markers. Agilent High Sensitivity DNA chips kit and chips were used.

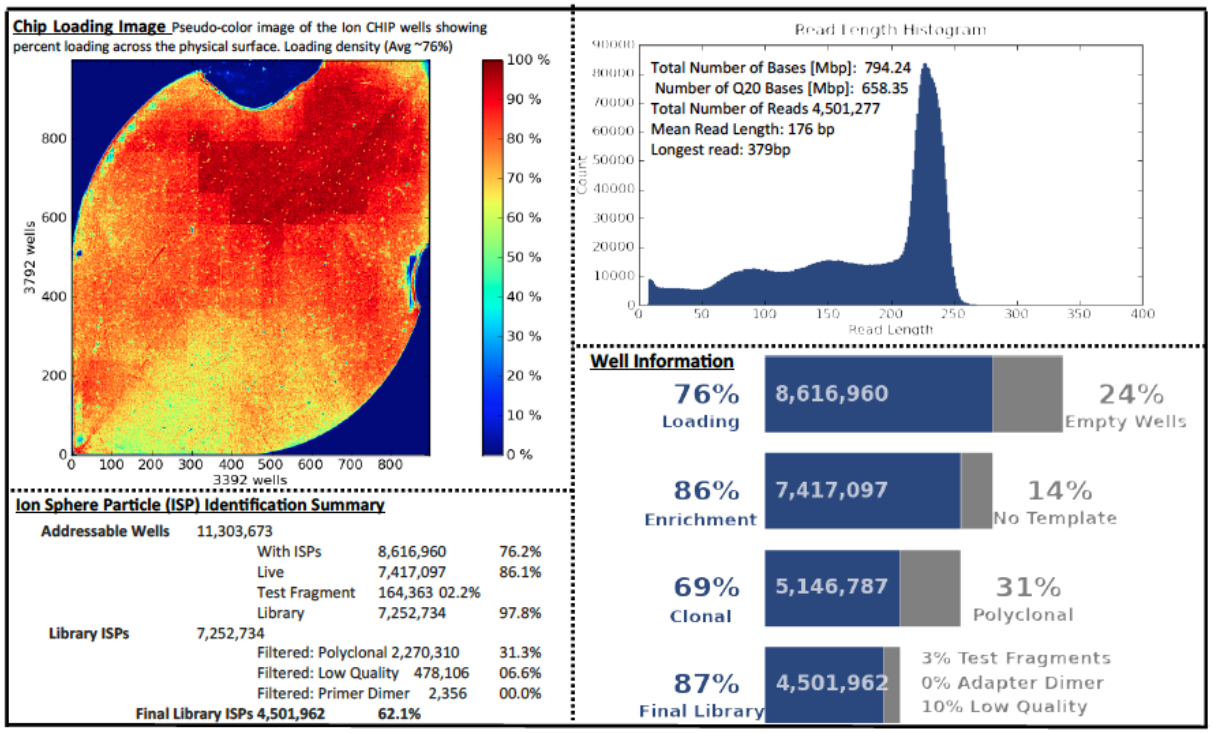


Figure 5.4. Ion Torrent sequencing run report for *Dhc* strain RC. Statistics and quality metrics for the 200-bp library and Ion Spheres particles (ISP) are shown. The average read length was 176 bp (close to the 200-bp goal). Seventy-six percent of the wells in the Ion 316 chip contained an ISP and only 14% of those wells contained a sphere with no DNA template. Thirty-one percent of the ISPs with a PCR template tethered to them were polyclonal (multiple DNA fragments were bounded and amplified in the surface of the bead). Finally, only 13% of the clonal ISPs were removed (due to low quality reads or because they had test fragments bound to them serving as controls) leaving a total 4,501,952 ISPs providing usable sequences for analysis.

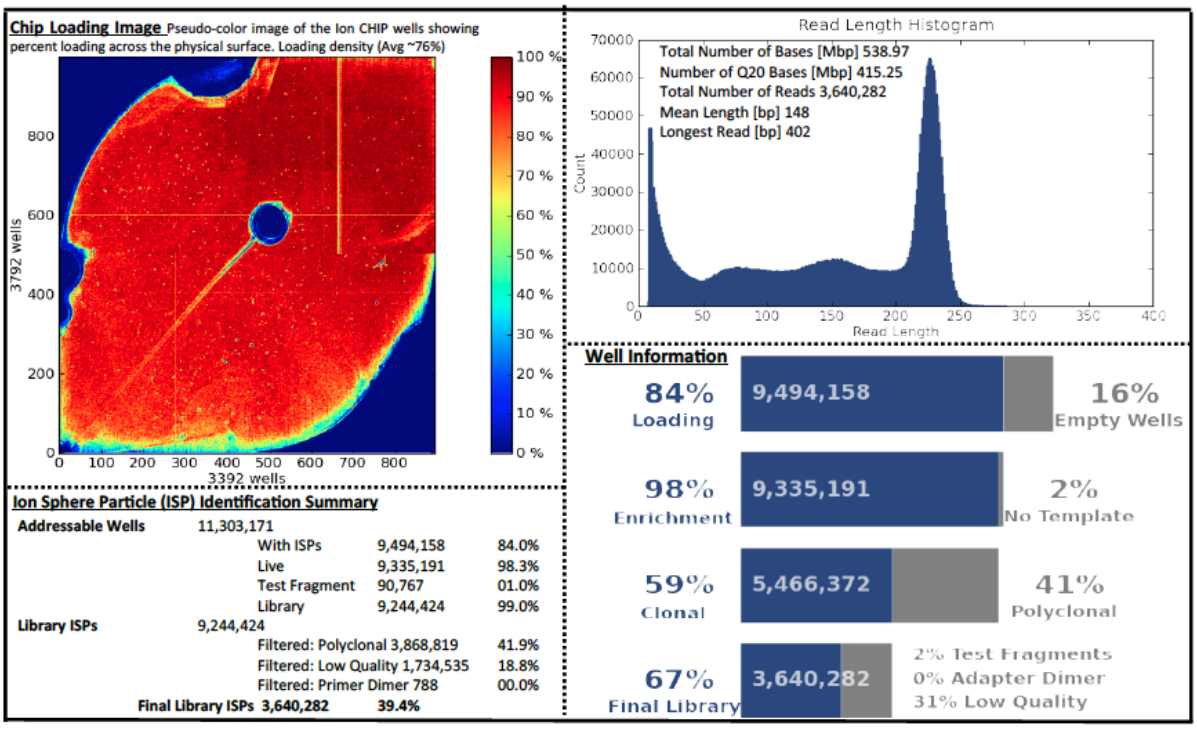


Figure 5.5 Ion Torrent sequencing run report for *Dhc* strain KS. Statistics and quality metrics for the 200-bp library and Ion Spheres particles (ISP) are shown. The average read length was 148 bp (close to the average 200-bp goal). Eighty four percent of the wells in the Ion 316 chip contained an ISP and only 2% of those wells contained a sphere with no DNA template. Forty-one percent of the ISPs with a PCR template tethered to them were polyclonal (multiple DNA fragments were bounded and amplified in the surface of the bead). Finally, 31% of the clonal ISPs were removed (due to low quality reads or because they had test fragments bound to them serving as controls) leaving a total 3,640,282 ISPs providing usable sequences for analysis.

Figure 5.6. Ion Torrent report metrics on reads aligned to *Dhc* strain 195. Filtered and trimmed reads (i.e. with no adapter sequences, excluding control/reference fragments and low quality reads) were aligned to the genome of *Dhc* strain 195 (CP000027.1)**.** The graphs on the left show the total aligned (in blue) and unaligned (in purple) bases by position. The average coverage depth of reference (228.9× and 341.3×) indicates the total aligned bases divided by the number of bases in the reference sequence. Alignment quality statistics are shown in the upper right tables. Two quality levels are depicted, the AQ20 quality level refers to values with an error rate of 1% or less, and "perfect" refers to the longest perfectly aligned segment. Mean coverage depth is the average number of times that a base was independently sequenced and aligned to the reference genome (e.g. 1× means that every base was sequenced and aligned, on average, once. 2× means that every base was sequenced and aligned, on average, twice). The raw accuracy graphs on the lower left, plot the percent accuracy for each position in the aligned sequences

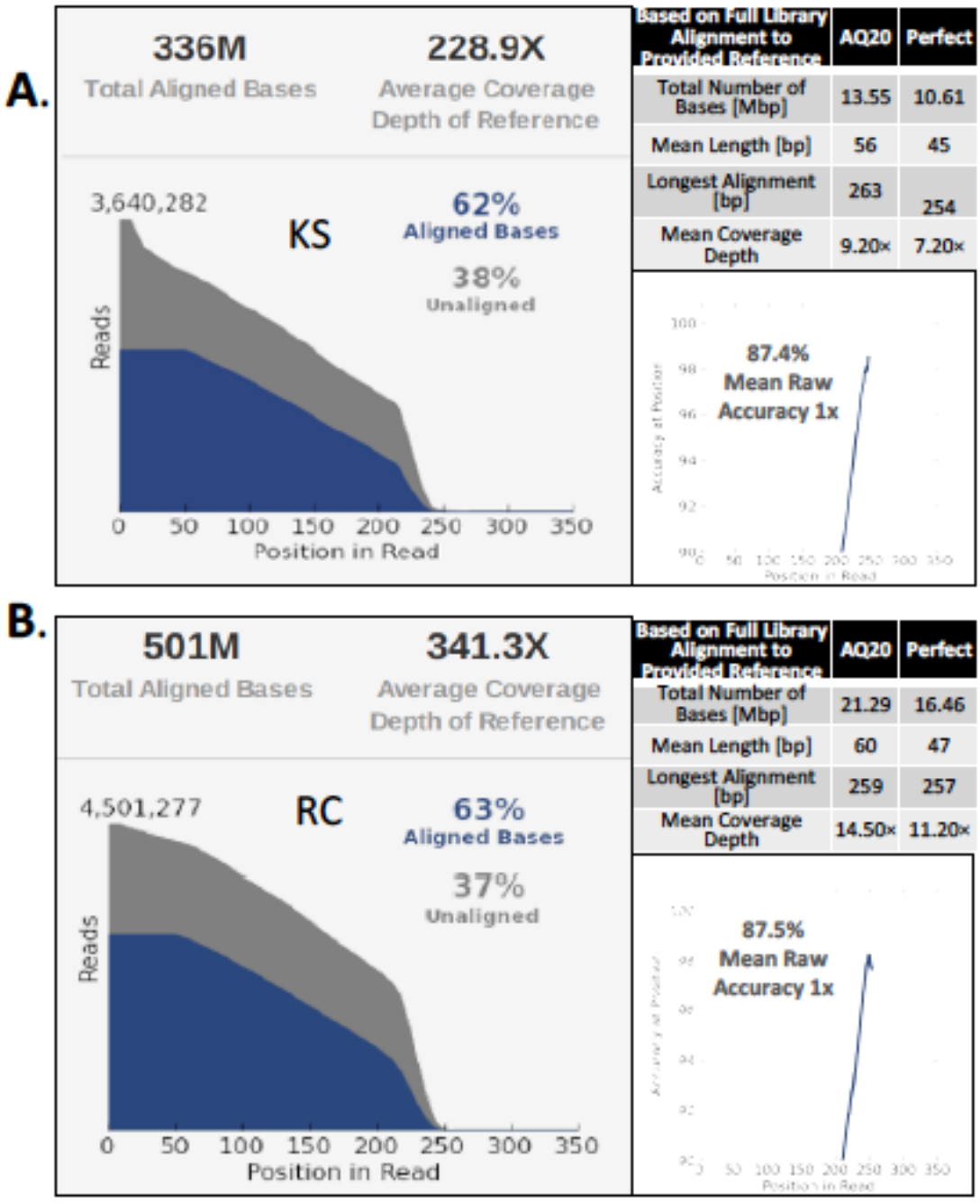
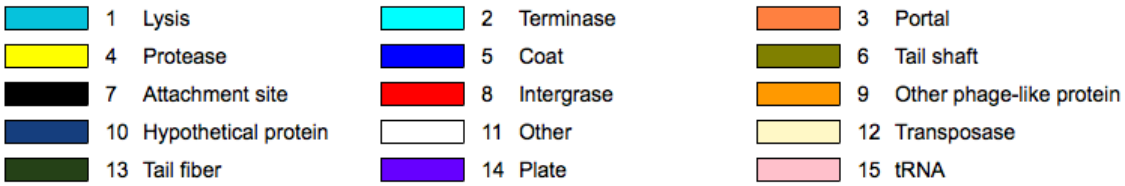


Figure 5.6. (Continued)

Figure 5.7. Putative prophage regions in *Dhc* strains. No prophage regions were identified in strains CG1, CY50, CT, VS, CBDB1, BAV1,UH007, DCMB5.

Figure Legend:



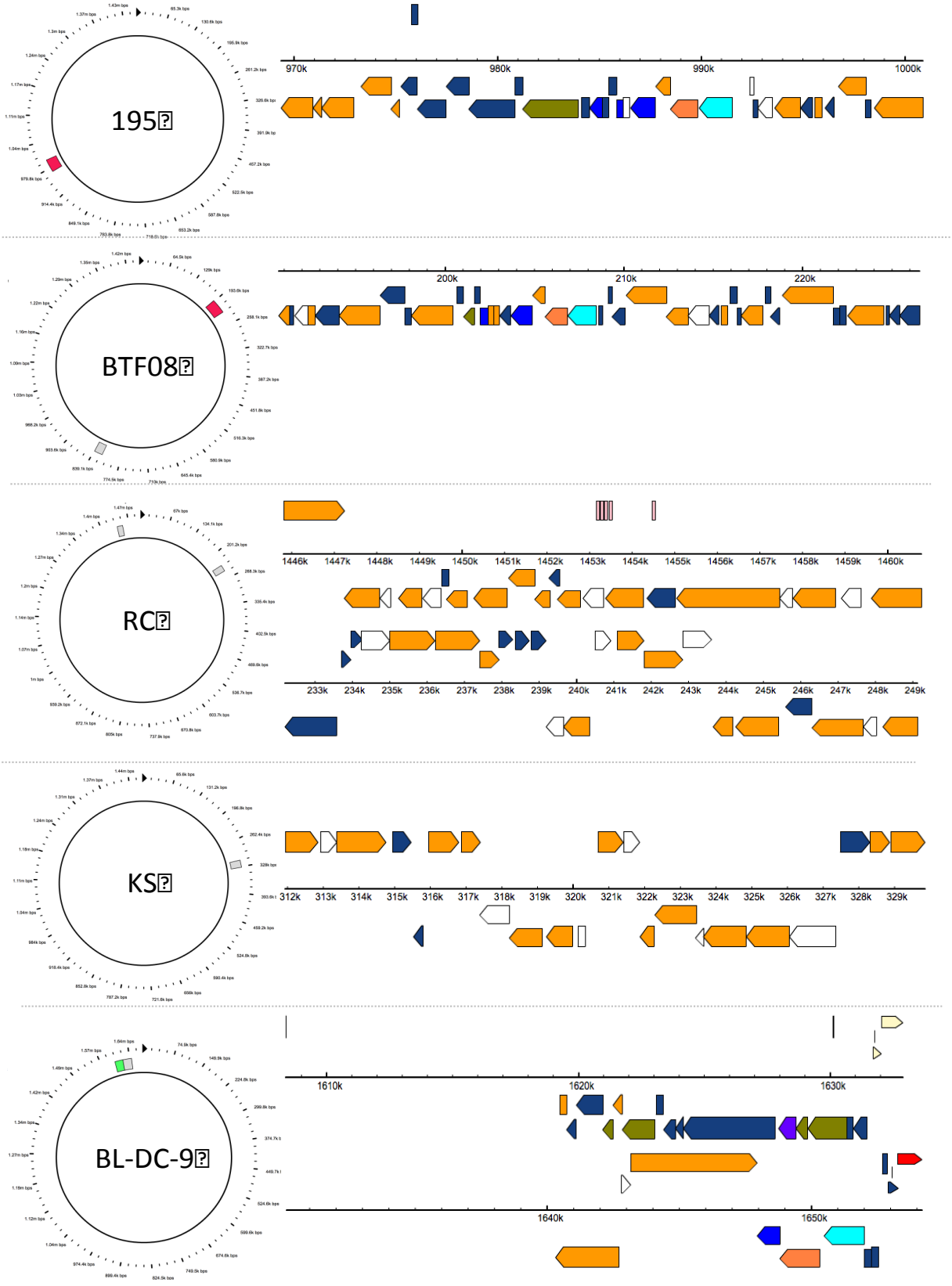


Figure 5.7. (continued)

Chapter 5 Appendix: Tables

Table 5.1. Performance of different de novo genome assembly tools.

Genome	Assembly method	N50	Number of contigs	Maximum contig length (bp)	Total bases in final assembly
KS	Velvet	859	6326	4240	1477523
	RAY	5499	503	20925	1692498
	Soapdenovo	109	30734	1283	2924569
	Velv_SOAP_Newb [1]	2198	566	11204	905320
	RAY_Velv_Newb [2]	60296	77	113991	1481443
	Newbler	268024	14	647595	1482308
RC	Velvet	2598	1705	10120	1475848
	RAY	847	5337	3629	2352638
	Soapdenovo	112	31359	1592	3109896
	Velv_SOAP_Newb [1]	5325	364	18112	1330869
	RAY_Velv_Newb [2]	10247	219	37685	1476130
	Newbler	210101	23	643701	1504103

[1] Contigs were first assembled with Velvet and Soapdenovo and subsequently entered to Newbler for re-assembly.

[2] Contigs were first assembled with Ray and Velvet and subsequently entered to Newbler for re-assembly.

Table 5.2. *Dehalococcoides mccartyi* sequenced genome statistics.

Organism	Genome Features										
	Type	**RefSeq**	**INSDC**	**Size (Mb)**	**GC%**	**Protein**	**rRNA[1]**	**tRNA**	**Gene**	**Pseudo**	**RDases**
***Dehalococcoides mccartyi* strains**											
RC	Draft genome	PRJNA230754 [2]	n/a	1.50	47.2	1,610	3	46	1,610	n/a	34
KS	Draft genome	PRJNA230755 [2]	n/a	1.49	47.3	1,626	3	46	1,626	n/a	31
CBDB1	Chr	NC_007356.1	AJ965256.1	1.40	47.0	1,458	3	47	1,510	1	32
BAV1	Chr	NC_009455.1	CP000688.1	1.34	47.2	1,371	3	46	1,436	14	10
DCMB5	Chr	NC_020386.1	CP004079.1	1.43	47.1	1,477	3	46	1,526	0	23
GT	Chr	NC_013890.1	CP001924.1	1.36	47.3	1,417	3	46	1,484	16	20
BTF8	Chr	NC_020387.1	CP004080.1	1.45	47.3	1,529	3	46	1,580	2	20
195	Chr	NC_002936.3	CP000027.1	1.47	48.9	1,580	3	46	1,642	11	17
VS	Chr	NC_013552.1	CP001827.1	1.41	47.3	1,439	3	46	1,491	1	36
GY50	Chr	NC_022964.1	CP006730.1	1.41	47.0	1,519	3	46	1,592	14	28
CG1	Chr	n/a	CP006949.1	1.41	46.9	n/a	3	49	1,557	n/a	35
CG4	Chr	n/a	CP006950.1	1.38	48.7	n/a	3	47	1,421	n/a	15
CG5	Chr	n/a	CP006951.1	1.37	47.2	n/a	3	n/a	1,413	n/a	26
***Dehalogenimonas lykanthroporepellens* strain BL-DC-9**											
BL-DC-9	Chr	NC_014314.1	CP002084.1	1.69	55.0	1,659	3	47	1,772	61	25

Table 5.3. Predicted hydrogenase complexes in the draft genome of *Dhc* strain RC.

Complex	Contig	Contig location (gene start)	Contig location (gene stop)	Strand	Function/Annotation	FIGfam protein family code	Comments
Hyc	contig00004	17603	17058	-	hydrogenase, group 4, HycG subunit, putative	FIG01304218	NiFe Hydrogenase, membrane-bound periplasmic, location in *Dhc* strain 195: DET1575-1570
	contig00004	19160	17584	-	hydrogenase, group 4, HycE subunit, putative	FIG082496	
	contig00004	20645	19164	-	Formate hydrogenlyase antiporter, MnhD subunit	FIG00004567	
	contig00004	21325	20645	-	Hydrogenase-4 component E	FIG00138953	
	contig00004	22270	21329	-	Formate hydrogenlyase subunit 4	FIG00133502	
	contig00004	24200	22267	-	hydrogenase, HycC subunit, putative	FIG143015	
Hyp	contig00008	21952	22248	+	[NiFe] hydrogenase nickel incorporation protein HypA	FIG00001182	NADH Hydrogenase, membrane-bound cytoplasmic, location in *Dhc* strain 195: DET0933-0923
	contig00008	22291	22944	+	[NiFe] hydrogenase nickel incorporation-associated protein HypB	FIG00001210	
	contig00008	22945	25236	+	[NiFe] hydrogenase metallocenter assembly protein HypF	FIG00001209	
	contig00008	25236	25451	+	[NiFe] hydrogenase metallocenter assembly protein HypC	FIG00003908	
	contig00008	25455	26550	+	[NiFe] hydrogenase metallocenter assembly protein HypD	FIG001208	
	contig00008	26513	27549	+	[NiFe] hydrogenase metallocenter assembly protein HypE	FIG003378	
Hup	contig00007	20892	20410	-	Hydrogenase maturation protease	FIG00621884	NiFe Hydrogenase, membrane-bound cytoplasmic, location in *Dhc* strain 195: DET0112-0109
	contig00007	22474	20895	-	[Ni/Fe] hydrogenase, group 1, large subunit	FIG132950	
	contig00007	23569	22505	-	[Ni/Fe] hydrogenase, group 1, small subunit	FIG01304860	
	contig00007	24368	23622	-	nickel-dependent hydrogenase, iron-sulfur cluster-binding protein	FIG01848221	
Hym	contig00007	54639	55104	+	NAD-reducing hydrogenase subunit HoxE	n/a	Fe Hydrogenase, membrane-bound cytoplasmic, location in *Dhc* strain 195: DET0148-0145
	contig00007	55106	56359	+	[Fe] hydrogenase, HymB subunit, putative	FIG00083472	
	contig00007	56347	58068	+	Periplasmic [Fe] hydrogenase large subunit	FIG00049433	
	contig00007	58108	58611	+	[Fe] hydrogenase, HymD subunit, putative	FIG01430996	

Table 5.3 (continued)

Complex	Contig	Contig location (gene start)	Contig location (gene stop)	Strand	Function/Annotation	FIGfam protein family code	Comments
Vhu	contig00001	394197	394670	+	[Fe] hydrogenase, HymA subunit, putative	FIG01303850	NiFe Hydrogenase, soluble cytoplasmic, location in *Dhc* strain 195: DET0616-0614
	contig00001	394670	396528	+	[Fe] hydrogenase, HymB subunit, putative	n/a	
	contig00001	396552	397273	+	Periplasmic [Fe] hydrogenase large subunit	FIG049433	
Ech	contig00001	511636	513580	+	Energy-conserving hydrogenase (ferredoxin), subunit A	FIG017701	NiFe Hydrogenase, membrane-bound cytoplasmic, location in *Dhc* strain 195: DET0868-0860
	contig00001	513577	514392	+	Energy-conserving hydrogenase (ferredoxin), subunit B	FIG00019322	
	contig00001	514427	514894	+	Energy-conserving hydrogenase (ferredoxin), subunit C	FIG00013663	
	contig00001	514887	515371	+	[Fe] hydrogenase, HymA subunit, putative	FIG031417	
	contig00001	515385	517307	+	[Fe] hydrogenase, HymB subunit, putative	FIG00083472	
	contig00001	517307	517900	+	NADH-ubiquinone oxidoreductase chain G (EC 1.6.5.3)	FIG00010922	
	contig00001	517903	519287	+	Energy-conserving hydrogenase (ferredoxin), subunit D	FIG096540	
	contig00001	519288	520366	+	Energy-conserving hydrogenase (ferredoxin), subunit E	FIG023953	
	contig00001	520385	520720	+	Energy-conserving hydrogenase (ferredoxin), subunit F	FIG00092760	

Table 5.4. Predicted hydrogenase complexes in the draft genome of *Dhc* strain KS.

Complex	Contig	Contig location (gene start)	Contig location (gene stop)	Strand	Function/Annotation	FIGfam protein family code	Comments
Hyc	contig00009	17895	17428	-	hydrogenase, group 4, HycG subunit, putative	FIG01304218	NiFe Hydrogenase, membrane-bound periplasmic, location in *Dhc* strain 195: DET1575-1570
	contig00009	19532	17954	-	hydrogenase, group 4, HycE subunit, putative	FIG082496	
	contig00009	21014	19565	-	Formate hydrogenlyase, MnhD subunit	FIG004567	
	contig00009	21694	21014	-	Hydrogenase-4 component E	FIG00138953	
	contig00009	22639	21698	-	Formate hydrogenlyase subunit 4	FIG00133502	
	contig00009	24568	22636	-	hydrogenase, HycC subunit, putative	FIG143015	
Hyp	contig00002	235264	235560	+	[NiFe] hydrogenase nickel incorporation protein HypA	FIG00001182	NADH Hydrogenase, membrane-bound cytoplasmic, location in *Dhc* strain 195: DET0933-0923
	contig00002	235603	236255	+	[NiFe] hydrogenase nickel incorporation-associated protein HypB	FIG001210	
	contig00002	236256	238545	+	[NiFe] hydrogenase metallocenter assembly protein HypF	FIG001209	
	contig00002	238545	238760	+	[NiFe] hydrogenase metallocenter assembly protein HypC	FIG00003908	
	contig00002	238764	239857	+	[NiFe] hydrogenase metallocenter assembly protein HypD	FIG001208	
	contig00002	239850	240857	+	[NiFe] hydrogenase metallocenter assembly protein HypE	FIG00003378	
Hup	contig00001	20568	20086	-	Hydrogenase maturation protease	FIG00621884	NiFe Hydrogenase, membrane-bound cytoplasmic, location in *Dhc* strain 195: DET0112-0109
	contig00001	22151	20571	-	[Ni/Fe] hydrogenase, group 1, large subunit	FIG01955975	
	contig00001	23246	22182	-	[Ni/Fe] hydrogenase, group 1, small subunit	FIG01304860	
	contig00001	24045	23299	-	nickel-dependent hydrogenase, iron-sulfur cluster-binding protein	FIG01848221	
Hym	contig00001	54780	56032	+	[Fe] hydrogenase, HymB subunit, putative	FIG083472	Fe Hydrogenase, membrane-bound cytoplasmic, location in *Dhc* strain 195: DET0148-0145
	contig00001	56020	57734	+	Periplasmic [Fe] hydrogenase large subunit HymC	FIG049433	
	contig00001	57860	58276	+	[Fe] hydrogenase, HymD subunit, putative	FIG01430996	
	contig00001	262704	262234	-	[Fe] hydrogenase, HymA subunit, putative	n/a	

Table 5.4 (continued)

Vhu	contig00001	480803	481275	+	[Fe] hydrogenase, HymA subunit, putative	FIG031417	NiFe Hydrogenase, soluble cytoplasmic, location in *Dhc* strain 195: DET0616-0614
	contig00001	481275	483145	+	[Fe] hydrogenase, HymB subunit, putative	n/a	
	contig00001	483158	483880	+	Periplasmic [Fe] hydrogenase large subunit HymC	FIG00049433	
Echo	Contig00001	598207	600150	+	Energy-conserving hydrogenase (ferredoxin), EchA	FIG017701	NiFe Hydrogenase, membrane-bound cytoplasmic, location in *Dhc* strain 195: DET0868-0860
	contig00001	600147	601046	+	Energy-conserving hydrogenase (ferredoxin), EchB	FIG00019322	
	contig00001	601132	601461	+	Energy-conserving hydrogenase (ferredoxin), EchC	FIG00013663	
	contig00001	601454	601939	+	[Fe] hydrogenase, HymA subunit, putative	FIG01303850	
	contig00001	602066	603872	+	[Fe] hydrogenase, HymB subunit, putative	FIG083472	
	contig00001	603872	604465	+	NADH-ubiquinone oxidoreductase chain G	FIG00010922	
	contig00001	604468	605852	+	Energy-conserving hydrogenase (ferredoxin), EchD	FIG096540	
	contig00001	605853	606931	+	Energy-conserving hydrogenase (ferredoxin), EchE	FIG023953	
	contig00001	606950	607285	+	Energy-conserving hydrogenase (ferredoxin), EchF	FIG00092760	

Table 5.5. Comparison of pairwise aminoacid identity of the RDases in the *Dhc* RC and KS draft genomes. The draft genome of strain RC has 32 putative reductive dehalogenases, these were compared to the 31 RDases found in strain KS. Bold KS IDs are RDases that are in duplicate just for table/comparison purposes).

RC Draft Genome ID	RC RDase protein length (aa)	KS Draft Genome ID	KS RDase protein length (aa)	Shared % aa Indentity
DhcRC_1535	429	DhcKS_1573	429	100
DhcRC_43	491	DhcKS_130	491	100
DhcRC_1055	475	**DhcKS_1120**	475	100
DhcRC_1018	482	DhcKS_1103	482	100
DhcRC_1010	508	**DhcKS_1096**	508	100
DhcRC_1006	469	DhcKS_1091	469	100
DhcRC_999	291	DhcKS_1085	463	100
DhcRC_946	465	DhcKS_1033	465	100
DhcRC_944	513	DhcKS_1031	513	100
DhcRC_935	505	DhcKS_1022	492	100
DhcRC_1554	484	DhcKS_1220	484	99.79
DhcRC_1002	417	DhcKS_1087	468	99.76
DhcRC_1553	472	DhcKS_1221	366	99.73
DhcRC_1389	509	DhcKS_3	510	99.41
DhcRC_45	407	DhcKS_132	407	99.26
DhcRC_38	514	DhcKS_125	514	99.22
DhcRC_1071	500	DhcKS_1587	500	99.2
DhcRC_1509	497	DhcKS_1547	484	99.17
DhcRC_998	482	DhcKS_1083	469	99.15
DhcRC_1453	455	DhcKS_67	455	98.9
DhcRC_987	453	DhcKS_1076	377	98.14
DhcRC_1601	581	DhcKS_1615	503	97.42
DhcRC_1592	285	**DhcKS_1618**	482	97.19
DhcRC_1023	504	DhcKS_1108	495	96.16
DhcRC_1062	491	DhcKS_1578	479	94.57
DhcRC_1384	531	DhcKS_1330	532	92.28
DhcRC_1531	525	DhcKS_1569	506	81.42
DhcRC_1593	199	**DhcKS_1618**	482	81.41
DhcRC_928	288	DhcKS_1015	264	80.3
DhcRC_990	213	DhcKS_1078	479	61.5
DhcRC_1034	503	**DhcKS_1120**	475	60.21
DhcRC_1055	475	DhcKS_1509	497	56.42
DhcRC_1509	497	DhcKS_1120	475	56.42
DhcRC_1030	494	**DhcKS_1096**	508	50.2

Table 5.6. Putative genes involved in the corrinoid salvage pathway and lower ligand modification in the draft genomes of *D. mccartyi* strain RC and KS.

Contig	Contig location (gene start)	Contig location (gene stop)	Strand	Function	Aliases	Figfam family protein
				Dhc KS		
contig00001	136142	136699	+	Cob(I)alamin adenosyltransferase	cobA	FIG00000641
contig00001	136696	137650	+	Adenosylcobinamide-phosphate synthase	cobD	FIG000689
contig00001	139085	139908	+	Cobalamine biosynthesis-related hypothetical protein CobX	cbiZ	n/a
contig00001	139905	141320	+	Vitamin B12 ABC transporter, B12-binding component BtuF	btuF	FIG00016684
contig00001	439993	441045	+	Vitamin B12 ABC transporter, B12-binding component BtuF	btuF	FIG00016684
contig00001	441101	442190	+	Vitamin B12 ABC transporter, permease component BtuC	btuC	FIG003124
contig00001	442202	443028	+	Vitamin B12 ABC transporter, ATPase component BtuD	btuD	FIG004481
contig00001	443018	443787	+	Cobalamine biosynthesis-related hypothetical protein CobX	cbiZ	n/a
contig00001	443790	444715	+	Adenosylcobinamide-phosphate synthase	cobD/cbiB	FIG000689
contig00001	446176	447235	+	Nicotinate-nucleotide--dimethylbenzimidazole phosphoribosyltransferase (EC 2.4.2.21)	cobT-2	FIG000824
contig00001	447275	448038	+	Cobalamin synthase	cobS	FIG006032

Table 5.6. (continued)

Contig	Contig location (gene start)	Contig location (gene stop)	Strand	Function	Aliases	Figfam family protein
				Dhc KS		
contig00001	448057	448589	+	Alpha-ribazole-5'-phosphate phosphatase (EC 3.1.3.73)	cobC	FIG000979
contig00001	448708	449273	+	Adenosylcobinamide-phosphate guanylyltransferase (EC 2.7.7.62)	cobU	FIG000764
contig00002	41897	42495	+	Cob(I)alamin adenosyltransferase (EC 2.5.1.17)	cobA3	FIG000641
contig00003	80401	80009	-	Adenosylcobinamide amidohydrolase (EC 3.5.1.90)	cbiZ	FIG00058905
contig00005	75547	76469	+	Adenosylcobinamide-phosphate synthase	cobD/cbiB	FIG000689
contig00005	76505	76627	+	Cob(I)alamin adenosyltransferase (EC 2.5.1.17)	cobA	FIG00000641
contig00005	76603	77052	+	Cob(I)alamin adenosyltransferase (EC 2.5.1.17)	cobA	FIG00000641
contig00020	42	248	+	Adenosylcobinamide amidohydrolase (EC 3.5.1.90)	cbiZ	FIG00058905
				Dhc RC		
contig00001	49480	50019	+	Cob(I)alamin adenosyltransferase (EC 2.5.1.17)	cobA	FIG00000641
contig00001	50143	50685	+	Adenosylcobinamide-phosphate synthase	cobD	FIG00000689
contig00001	50694	50990	+	Adenosylcobinamide-phosphate synthase	cobD	FIG00000689
contig00001	53244	54659	+	Vitamin B12 ABC transporter, B12-binding component BtuF	btuF	FIG00016684
contig00001	353384	354436	+	Vitamin B12 ABC transporter, B12-binding component BtuF	btuF	FIG00016684

Table 5.6. (continued)

Contig	Contig location (gene start)	Contig location (gene stop)	Strand	Function	Aliases	Figfam family protein
				Dhc RC		
contig00001	354492	355601	+	Vitamin B12 ABC transporter, permease component BtuC	btuC	FIG00003124
				Dhc RC		
contig00001	355594	356421	+	Vitamin B12 ABC transporter, ATPase component BtuD	btuD	FIG00004481
contig00001	356411	357180	+	Cobalamine biosynthesis-related hypothetical protein CobX	cbiZ	n/a
contig00001	357183	358109	+	Adenosylcobinamide-phosphate synthase	cbiZ	FIG00000689
contig00001	359622	360653	+	Nicotinate-nucleotide--dimethylbenzimidazole phosphoribosyltransferase (EC 2.4.2.21)	cobT-2	FIG000824
contig00001	360666	361430	+	Cobalamin synthase	cobS	FIG00006032
contig00001	361449	362032	+	Alpha-ribazole-5'-phosphate phosphatase (EC 3.1.3.73)	cobC	FIG000979
contig00001	362101	362667	+	Adenosylcobinamide-phosphate guanylyltransferase (EC 2.7.7.62)	cobU	FIG00000764
contig00002	38708	39253	+	Cob(I)alamin adenosyltransferase (EC 2.5.1.17)	cobA3	FIG00000641
contig00003	80415	80023	-	Adenosylcobinamide amidohydrolase (EC 3.5.1.90)	cbiZ	FIG00058905
contig00006	70197	71118	+	Adenosylcobinamide-phosphate synthase	cobD/cbiB2	FIG000689
contig00006	71154	71276	+	Cob(I)alamin adenosyltransferase (EC 2.5.1.17)	cobA1	FIG00000641
contig00006	71252	71701	+	Cob(I)alamin adenosyltransferase (EC 2.5.1.17)	cobA2	FIG00000641

Table 5.7. Putative genes involved in Arsenic resistance.

Contig	Contig location (gene start)	Contig location (gene stop)	Strand	Function
		***Dhc* strain KS**		
contig00001	53456	53868	+	Arsenate reductase
contig00001	614471	615640	+	Arsenic efflux pump protein
contig00002	65196	66258	+	Arsenical-resistance protein ACR3
contig00003	59592	60584	+	Arsenical-resistance protein ACR3
contig00007	23124	23465	+	Arsenate reductase
contig00007	23701	24767	+	Arsenical-resistance protein ACR3
		***Dhc* strain RC**		
contig00001	527906	529075	+	Arsenic efflux pump protein
contig00002	61953	63017	+	Arsenical-resistance protein ACR3
contig00007	53784	54196	+	Arsenate reductase
contig00007	53784	54196	+	Arsenate reductase
contig00005	36365	37430	+	Arsenical-resistance protein ACR3

Table 5.8. CRISPR locus in the draft genomes of *Dhc* RC and KS.

Contig	Contig location (gene start)	Contig location (gene stop)	Strand	Function
		***Dhc* strain KS**		
contig00003	34587	33658	-	CRISPR-associated protein Cas1
contig00003	35170	34587	-	CRISPR-associated protein, Cse3 family
contig00003	35613	35239	-	CRISPR-associated protein, Cas5e family
contig00003	37134	35851	-	CRISPR-associated protein, Cse4 family
contig00003	37375	37127	-	CRISPR-associated protein, Cse2 family
contig00003	39281	37650	-	CRISPR-associated protein, Cse1 family
contig00003	41966	39274	-	CRISPR-associated helicase Cas3, protein
		***Dhc* strain RC**		
contig00003	34544	33669	-	CRISPR-associated protein Cas1
contig00003	37146	35864	-	CRISPR-associated protein, Cse4 family
contig00003	37671	37139	-	CRISPR-associated protein, Cse2 family
contig00003	39292	37661	-	CRISPR-associated protein, Cse1 family
contig00003	41977	39285	-	CRISPR-associated helicase Cas3, protein

Table 5.9 Putative prophage genomic regions identified in *Dhc* strains and in *Dhgm* BL-DC-9. The genome of *Dhc* strains CG1, GY50, VS, CBDB1, DCMB5, BAV1 and UH007 were analyzed but no regions where identified.

Phage region	Region length	Phage completeness	Score	# of CDS	Region position in genome	Phage	Region GC %
Dhc							
195	31.5Kb	intact	110	32	969361-1000886	PHAGE_Geobac_E2	53
Dhc	35.8Kb	intact	100	40	190700-226538	Bacillus phagePBC1	52
BTF08	20.8Kb	incomplete	60	26	816956-837763	Natrialba_phage_PhiCh1	46
Dhc	16.9Kb	incomplete	10	22	232196-249122	PHAGE_Bacill_IEBH_NC_011167	49
RC	14.9Kb	incomplete	10	20	1445807-1460792	PHAGE_Ectoca_siliculosus_virus_1_NC_0026 87, PHAGE_Prochl_P_SSM2_NC_006883	48
Dhc						PHAGE_Bacill_IEBH_NC_011167,	
KS	17.9Kb	incomplete	10	22	311932-329850	PHAGE_Bacill_G_NC_023719	49
Dhgm						PHAGE_Aeromo_Aeh1_NC_005260,	
BL-	24.5Kb	questionable	80	17	1608371-1632888	PHAGE_Bacill_BCD7_NC_01951	63
DC-9	24Kb	incomplete	50	11	1630124-1654188	PHAGE_Bacill_G_NC_023719	57

Legend:

REGION: the number assigned to the region
REGION LENGTH: the length of the sequence of that region (in bp)
COMPLETENESS: a prediction of whether the region contains a intact or incomplete prophage based on the above criteria
SCORE: the score of the region based on the above criteria,. If the region's total score is less than 70, it is marked as incomplete; if between 70 to 90, it is marked as questionable; if greater than 90, it is marked as intact.
#CDS: the number of coding sequence
REGION_POSITION: the start and end positions of the region on the bacterial chromosome
PHAGE: the phage with the highest number of proteins most similar to those in the region
GC%: the percentage of GC nucleotides of the region

Chapter 6

Towards a clean and sustainable future: Green technologies, restoration and management of 1,2-D contaminated sites.

This chapter is based on a previous "White Paper" prepared for the Congressional Hispanic Caucus Institute (CHCI). It is reproduced in part with permission from the Elizabeth Padilla-Crespo and CHCI. http://www.chci.org/doclib/2014421233527696-2014STEMGraduateSummitWhitePaper-ElizabethPadilla-Crespo.pdf?trail=201548144630

Abstract

Poor handling and disposal of hazardous substances have left a legacy of contamination in sites all across the United States that affect human and ecosystem welfare. The U.S. Environmental Protection Agency (EPA) addresses these contaminated sites under the Comprehensive Environmental Response Compensation and Liability Act (CERCLA) commonly known as the "Superfund Program." Currently chlorinated contaminants are present in approximately 70% of these sites. TheThe management and cleanup of these impacted areas is a matter of national security and environmental justice as it is estimated that one in four Americans live near a Superfund site and that minorities, particularly Hispanics are more likely to live near affected areas. Green technologies such as bioremediation and sustainable practices represent a solution to treat and restore these sites; but several factors including scientific and regulatory considerations hinder the implementation of these technologies. Changes in environmental regulations, better management of the Superfund sites and the creation of initiatives that promote collaboration between academia and federal agencies should be made to safeguard the livelihood of U.S. citizens and enhance the restoration of contaminated sites.

Introduction

Environmental degradation is a threat in industrial and developing countries due to population growth, increased use of resources, and a legacy of poor handling and disposal of hazardous substances. The Comprehensive Emergency Response Compensation and Liability Act (CERCLA, 42 U.S.C. §§ 9601-9675) is the federal statute that addresses uncontrolled and

abandoned contaminated sites and requests these areas be investigated, evaluated and ultimately restored (S. US EPA, n.d.-a). This law gives authority to the EPA to compel responsible parties to perform cleanups at impacted sites and also establishes a trust fund to finance restoration of orphan sites, where no responsible party exists. After an evaluation by our federal government, locations that represent a high threat to human and ecosystem welfare are declared as "Superfund sites" and the worst cases are listed in the *National Priority List* (NPL). CERCLA was enacted in 1980 under the Reagan Administration after the discovery of toxic waste impacting sites such as Love Canal in New York and Times Beach in Missouri. Today the Superfund (or NPL) has become one of the nation's largest government programs, and as of January 24, 2014, there were 1,372 proposed or declared NPL sites (S. US EPA, n.d.-l) (Table 6.1 and Figure 6.1)

Superfund and chlorinated solvents impacted sites can be treated with various remedial technologies that can include physical methods (e.g., removal of the hazardous substances by excavation or incineration), application of biological processes (e.g., biodegradation of a particular waste by microorganisms, plants or fungi), and chemical treatments (e.g., the addition of certain compounds to induce chemical reactions that would transform hazardous compounds to inert or less toxic compounds). *Sustainable remediation* (also referred as green remediation) can be defined as remedial methods used to treat and restore areas considering all environmental effects of technology implementation; maximizing the environmental and human welfares, while minimizing cost and the use of limited resources. Cleanup strategies that involve the application of biological processes to achieve detoxification, cleanup and restoration of contaminated sites are of particular interest since these are often considered environmentally friendly, and are widely accepted by the scientific community. Special attention has been given to *in situ bioremediation* approaches (the use of microorganism at the site which can degrade the contaminants of interest at the site) since it has the potential to be a non-intrusive, non-waste generating and cost efficient natural method. However, there are four factors that hinder implementation of this green technology in Federal Superfund sites. These include: (1) scientific and regulatory aspects that limit the implementation of these technologies; (2) the current state of the CERCLA statue and management of the Superfund sites which have no clear language for implementation of sustainable practices; (3) the absence of incentives to promote the use

(implementation) of green technologies over other strategies; and (4) lack of collaboration between agencies, practitioners and academia.

This paper will review the history of the Superfund Program and the CERCLA statue, the current management of contaminated sites and describe how bioremediation as well as other sustainable approaches represent feasible and attractive cleanup methods to treat these locations. Emphasis will be given on addressing why the conditions of these areas are important to minority and Hispanic communities and the regulatory aspects of the program that could be amended regarding site management, contaminant removal actions, research and innovation and implementation of green sustainable remedial practices. Lastly, a set of recommendations is delineated to enhance the restoration of these sites.

Communities affected by contaminated sites

The EPA estimates that one in four Americans live within three miles of a toxic waste contaminated site and around 10 million children under the age of 12 live within four miles of a Superfund site (S. US EPA, n.d.-i, n.d.-k).. A study focused on evaluating 50 Superfund sites across the United States revealed between 205,349 and 803,100 people live within one mile of these areas. Furthermore, this study revealed these sites are in neighborhoods whose household incomes are below the national average. Moreover, 60% of the U.S. Census tracts in these regions comprised 40% or more racial or ethnic minorities (Steinzor, Clune, Progress, & Reform, 2006). Other studies have also shown that Blacks, Hispanics and low-income individuals are more likely (i.e., positively associated) to live near Superfund and NPL locations (Burwell-Naney et al., 2013; Stretesky & Hogan, 1998; S. M. Wilson et al., 2012). Executive Order 12898 entitled "Environmental Justice for Low Income & Minority Population" is intended to protect individuals and communities against unfair treatment due to color, race or nationality with respect to environmental policies, laws and regulations. However, a study conducted by O'Neil (2007) indicated that, since the enactment of EO 12898 in 1994, "marginalized and poor populations are less likely to benefit from a cleanup program such as Superfund despite their

overrepresentation in proximity to environmental hazards" (O'Neil, 2007). Another study by Anderton et al. (1997) also concluded that areas with a higher percentage of minorities are less likely to receive NPL status, thus delaying the cleanup process (Anderton, Oakes, & Egan, 1997).

The Commonwealth of Puerto Rico, a U.S. territory with a 99% Hispanic population, serves as an example of a minority community impacted with hazardous waste by having more than 150 contaminated sites (Padilla, Irizarry, & Steele, 2011) (Figure 6.2). Remarkably, as of January 24, 2014, Puerto Rico has 16 NPL sites; the same as states like Tennessee, Georgia and Utah; and more than states like Oregon, Delaware and Oklahoma which have 14, 13 and 7 NPL sites, respectively (S. US EPA, n.d.-e).. It is suspected that exposure to contaminants may contribute to the premature birth incidence in Puerto Ricans, which is among the highest in the United States (Ghasemizadeh et al., 2012; Padilla et al., 2011). Overall, these studies and occurrences reflect that racial and ethnic minorities (especially Hispanics) are among the most vulnerable and at-risk communities when it comes to Superfund sites, suggesting a case of inequality and environmental justice.

History of CERCLA

In the 1970s, the Love Canal in upstate New York, made headlines in what is considered to be "one of the most appalling environmental tragedies in American history" (Beck, 1979). Located near the Niagara Falls, this body of water and its adjacent community suffered the consequences of 21,000 tons of toxic waste that were disposed by a nearby chemical industry since the 1940s. The hazardous waste (which included pesticides such as DDT, carcinogenic solvents and heavy metals) was lined with clay and buried under the canal. The chemical company that owned the area sold it to the city for one dollar and included a warning about the chemical wastes buried and a disclaimer absolving the industry of any future liability. But on 1976, the waste was exposed after record-breaking rainfall; nearby vegetation started to die, corroding barrels were exposed to the surface, chemicals leached forming toxic puddles and a fouling smell covered the

residential area. In the years that followed, astonishing levels of miscarriages and stillbirths were recorded, and 56% of the children born between 1974–1978 had at least one birth defect. For two years, the local community battled to prove the industrial waste buried in the area was responsible for the citizens' illnesses, and finally their united efforts and mobilization brought attention at the state and federal level. On August 2, 1978, the New York State Health Department declared the site in a state of emergency and more than 800 families were relocated. Five days later, President Jimmy Carter declared a federal state of emergency in the Love Canal's surrounding areas and later allocated federal funds to remediate the area. This was the first time that federal monies were used to assist in a man-made disaster. The Love Canal incident was a "wake up call" creating awareness on the dangers of public exposure to toxic waste and the need to compel the parties liable for the incidents. Consequently, extensive House and Senate committee hearings were held during 1979, which led to Congress enacting CERCLA in 1980. On September 1, 1983, the Love Canal was added to the NPL list and the chemical company that generated the hazardous waste was found liable of the disaster and negligent in the way it handled the waste and sale of the area. Although the company had followed all U.S. applicable laws at the time of disposing the waste, the EPA sued the company for $129 million under a retroactive liability provision underlined in CERCLA (section 107) and the families were compensated for their properties. In, 2004 after great efforts, over $400 million dollars, and after 21 yrs. of its inception as an NPL, the Love Canal was clean enough to be taken off the Superfund list.

Management of Superfund Sites

EPA's Office of Solid Waste and Emergency Response (OSWER) in Washington, D.C oversees the Superfund Program. A representation and summary of the phases and milestones for a site cleanup under CERCLA are illustrated in Figure 6.3. In aggregate, the Act requires a *preliminary site assessment* to identify if the environment poses or not a threat to human health, and identifies sites where possible response actions are needed. These include removal actions (e.g., immediate control of the spread of hazardous substances during a spill) and remedial

activities (e.g., prolonged monitoring and ultimate restoration). If the environment and sites are considered a threat, further investigation is required and a *site inspection* is made to determine the nature and extent of the contamination and the potentially responsible parties. The information collected in these first two phases will be evaluated and sites will be given a score from 0 to 100 using the "Hazard Ranking System" (as stated in section 105(a)(8)(B) of CERCLA, as amended). Sites with a score of 28.50 or higher are eligible for listing as NPL. The NPL serves as an informational and management tool indicating which sites are priorities for cleanup, as they pose a high treat to the community, only sites in the NPL list can use federal funds for cleanup. After a site is listed as an NPL a *remedial investigation* and *feasibility study* are concurrently conducted for more detailed investigations on site contamination and exposure. At this phase, treatability studies of alternatives methods for treatment are considered and evaluated. Once the assessments and investigations are complete, a document identifying the treatment procedure to be used at the site is made public, this is known as a *"record of decision".*

Consequently, *remedial designs* and *remedial actions* are followed, which involve the design and implementation of the site cleanup strategies. The EPA designates sites as *"construction complete"* when any type of construction or containment activity at the site has been completed or when the site qualifies for NPL deletion. Complex sites with ongoing cleanup activities that require long term treatment and monitoring are overseen by *"post construction completion activities".* During this time, *five-year reviews* are requested to evaluate implementation and performance at sites where hazardous substance levels are higher than permitted. These reviews receive recommendations from the EPA, and aim to help determine whether the remedies in use protect human health and the environment. Finally, sites can be deleted from the NPL when the EPA, in conjunction with the State, considers that no further response action is needed to protect human and ecosystem health.

Remediation Technologies used for site restoration

Remediation technologies are techniques applied to impacted sites to achieve environmental restoration. Contaminated water, soil or sediments can be treated on site (*in situ*) or they can be removed and transferred to a different location for disposal or treatment (*ex situ*). Methods for restoration include biological, physical methods, chemical treatments, among others. *Ex situ* approaches that involve the *excavation* and removal of large quantities of water or soil are not ideal, transporting the hazardous materials imposes additional risk, cost and environmental impacts by adding to fossil fuel consumption and green house (CO_2) emissions. *Incineration* is subjected to technology-specific regulations and handling requirements because certain materials can only be incinerated offsite, while others produce ashes that require further stabilization impacting applicability and cost. Another remedy referred to as *"pump and treat"* is also considered an expensive, slow and energy-intensive technology. This process requires groundwater to be extracted out of the subsurface with vacuums pumps and then transferred to vessels where either chemical reagents are added for treatment or materials like activated carbon are used to absorb the contaminants. The addition of *chemical additives for treatment in situ* (to neutralize or precipitate the contaminants in place) can also be costly and considered a major capital investment, as the synthesis or purchase of these additives can be expensive and can create hazardous products that need subsequent disposal.

Conversely, the application of biological treatment using microorganisms (*bioremediation*) can be used to degrade chlorinated compounds at impacted sites. Bacteria have thrived over three billion years on this planet having evolved effective mechanisms to gain energy by utilizing a wide variety of substrates, including hazardous chemicals. Microbes can use materials like gasoline, diesel and other hydrocarbons as "food" (carbon source) while others can "respire" compounds like carcinogenic chlorinated solvents, pesticides and radioactive waste (uranium) similarly as we humans respire oxygen (F E Löffler & Edwards, 2006). An excellent example of microbiological processes aiding during environmental disasters was during the BP Deepwater Horizon, where indigenous marine bacteria degraded the oil plume to nearly undetectable levels within a few weeks of the spill (Hazen et al., 2010). Furthermore, bioremediation has proven

effective in other massive spills like the Exxon Valdez in the coast of Alaska and the Gulf War oil spill (Atlas & Hazen, 2011; Bragg, Prince, Harner, & Atlas, 1994; Höpner & Al-Shaikh, 2008; T. D. Thomas, Ellwood, & Longyear, 1979).

Analysis

Highlights and limitations of the Superfund Program

The Superfund Program has been considered the "world's most advanced hazardous waste program in the world" (Macey, 2007), and its significance is portrayed in the Love Canal's story. A study led by researchers at the Massachusetts Institute of Technology deduced that Superfund cleanups had reduced the incidence of congenital anomalies by 20 - 25 percent in the U.S. (Currie, Greenstone, & Moretti, 2011). Furthermore, since its inception, it has lowered the risks for cancer and poisoning of many citizens by reducing their exposure to hazardous substances. CERCLA has also increased knowledge on how to deal (planning and response) of accidents and established the Agency for Toxic Substances and Disease Registry (ATSDR) which nowadays conducts health surveys, assessment, surveillance and toxicological studies associated with exposure of hazardous chemicals. ATSDR also focuses on disseminating information via education and outreach initiatives and managing accessible databases of toxics incidents, and chemical profiles of substances of concern. By the end of FY 2013, the Superfund Program had controlled or reduced human exposure to contamination in 1,389 NPL sites, controlled groundwater contamination in 1,091 NPL sites and completed a cumulative total of 92,282 remedial assessments since the program's creation in 1980 (S. US EPA).

Superfund cleanups also have positive economic benefits. Mastromonaco (2001) showed the impact on property values in residences near a Superfund site; by looking at houses within 3 km of a site. Results indicated that houses increased in value by 7.3 percent after cleanups were completed (Mastromonaco, 2011). Restored areas can also serve as sources of revenue, recreation and job creation; an example of this is the Anaconda Co. NPL site in Montana. At this site cleanup and restoration has included the removal of heavy metals in contaminated water and

soil and the re-vegetation of more than 250 acres. EPA's coordinated efforts led to the creation of a park, trails and a golf course, which have then increased the commercial and residential growth in the area (S. US EPA, n.d.-f).

The Superfund program has also been the center of many environmental debates, long scrutiny and criticism. One of the aspects is that sites take too long time to remediate (8-11 years in average but many linger in the NPL for decades, like the Love Canal) and residual contamination remains at least 126,000 sites (National Research Council, 2013). Additional limitations include: insufficient information on sites that have been delisted but still have residual contamination, and the absence of resources (e.g., databases) to compare remedial technology performances across sites (National Research Council 2013). Recently, the situation with deleted sites has been referred as THE PARADOX OF "CLOSED" SITES. The EPA defines **site closure** as when "no further Superfund response is necessary to protect human health and the environment" and EPA's site closure guidelines include the intent to "provide an approach for conducting five-year reviews" (S. US EPA, n.d.-c). However, the EPA states that a five-year review is only required when hazardous contaminants are left in place in levels higher than the current safety standards (S. US EPA, n.d.-b). This contradiction in the current definition and language may confuse stakeholders as "operation and maintenance of a remedy may continue for many decades after closure" (National Research Council, 2013) . Other concern is that the **construction complete** milestone may be misleading to many, as it does not necessarily means that restoration is completed or the levels of hazardous substances are safe. The presence of emerging contaminants (those substances that have not historically been considered as hazardous), can impose an additional problem when evaluating the level of contamination at a site. These chemicals, which are not yet regulated and their toxicity not yet completely understood, are a problem that could affect the HRS (Hazard Ranking System) score given to a site and its inception or deletion as an NPL. Controlling "the unknown" is a challenge, plus it may lead to the selection and implementation of inadequate remedial responses.

A GAO report for FY 2010 investigated four Superfund sites deleted from the NPL. The investigation revealed gaps and errors in EPA's long-term monitoring reports and residual

contamination at these sites that was previously unknown. The same GAO investigation also highlighted the following weakness in EPA's Superfund management: (1) not completing the performance evaluations of Superfund contractors, (2) not managing efficiently the recommendations of Five-Year Reviews (84 percent of the review recommendations were overdue), (3) fines and penalty billings were not consistently recorded, and (4) errors in internal receipts and/or expenditures totaling about $2.5 million were discovered. Since site assessment, investigation and clean up can cost up to be hundreds of millions of dollars (S. US EPA, n.d.-j), it is imperative that EPA's oversight and management is strong and consistent.

Bioremediation as a possibility for Superfund remedial actions: principles and case studies

Bioremediation approaches include **natural attenuation,** a process that involves no intervention, letting natural occurring microbial communities degrade the contaminants, and **enhanced bioremediation** practices that require the application of procedures to promote the removal or containment of the hazardous substances. The methods of **bioestimulation** and **bioaugmentation** are among the most used *in situ* enhanced bioremediation procedures. During **bioestimulation**, injection of amendments are applied at the site to promote growth and activity of indigenous microbes; additions can include nutrients, oxygen (to promote the specific growth of aerobic microorganisms) and vegetable oil and molasses that serve as hydrogen supply to promote growth of anaerobic microbial populations. At times the site does not harbor the microorganisms capable of degrading or transforming the contaminants; in these cases **bioaugmentation** can be implemented by introducing microbial populations that are not native to the site but that can carry out the desired reactions (Philp & Atlas, 2005).

Bioremediation has the advantage of being a more cost effective technology (Philp & Atlas, 2005; Saaty & Booth, 1994; Wijensinghe, Knapp, Taylor, & Carman, 1992). As reviewed by Megharaj et al. (2011) bioremediation technologies are 80-90% cheaper than other approaches that rely merely on chemical or physical methods, and have been successfully applied in more than 400 areas in the United States (Megharaj, Ramakrishnan, Venkateswarlu, Sethunathan, &

Naidu, 2011). For example, the cost of cleaning 120 km of shoreline after the Exxon Valdez spill using biological methods resulted in less than a day's cost of performing physical washing (Philp & Atlas, 2005).. Furthermore, it has been estimated that applying non-biological approaches to remediate the current listed waste sites across the United States would cost around $750 billion (in a time frame of 30 years); while with bioremediation the cost would be an order of magnitude less, only $75 billion (Pimentel et al., 1997). Finally, Hunter-Cevera (1998) projected that worldwide bioremediation approaches would cost $14 billion compared to $135 billion per year if other technologies were used (Hunter-Cevera, 1998).

As previously stated, microbial-mediated bioremediation represents an alternative to degrade waste to benign products or to immobilize inorganic contaminants such as heavy metals and radionuclides. In the case of toxic chlorinated solvents, a recent review of 32 sites indicated that contaminant levels were reduced by 60-90% when *in situ* bioremediation approaches were implemented (H. F. Stroo et al., 2012). Another example is that of *Anaeromyxobacter dehalogenans,* a bacterium that can respire uranium. Although uranium cannot be biologically degraded or removed *Anaeromyxobacter* sp. can transform it, by reducing it from insoluble and mobile U(VI) to insoluble U(IV) oxide. This microbial reduction holds significant promise, as the insoluble uranium can then be contained in groundwater, preventing it from reaching aquifers and posing a human health risk. Bioestimulation can be achieved in these cases by the addition of acetate (diluted vinegar) to the subsurface, which will promote growth of uranium-reducing bacteria. Pilot studies have shown that this technique has a reduced cost when compared to pump and treat (U.S. Nuclear Regulatory Commission, 2008).

Finally, two particular case studies of **sustainable *in situ* bioremediation** are listed below showing the benefits and potential of the technology.

A) In a chlorinated solvent contaminated site in Cape Canaveral (Kennedy Space Center), bioremediation and bioestimulation were implemented with approaches to minimize the environmental footprint caused by the clean-up remedy. Treatment was optimized in various ways including the use of solar powered units for water recirculation and strategic and careful

selection of additives (for microbial growth) to avoid the need of multiple interventions. This strategic and greener approach resulted in less CO_2 equivalents released than technologies like pump and treat, air sparge and multiphase extraction (Daprato, R.C., J. Langenbach, R. Santos-Ebaugh, R. Kline, n.d.).

B) Another case study is a DuPont site contaminated with approximately 10 million tons of toxic waste. At this location excavation, stabilization and bioremediation were considered, and after evaluation, bioremediation was selected. Compared to excavation, bioremediation imposed a lesser disturbance to the nearby community and represented a reduction of 2.5 million tons of CO_2.

Shortcomings of Bioremediation and possibilities on other Sustainable approaches

Although bioremediation is a promising technique it may not be applicable to all sites. Some of drawbacks of bioremediation are listed below:

1. Not all contaminants are biodegradable, example of these are 1,4-diozane and chloroform, which are recalcitrant chemicals.
2. The process is sensitive to the geochemical conditions at the site, and changes could lead to incomplete detoxification. Inhibitory conditions like certain chemicals, pH, temperature can inhibit biodegradation, these can be adjusted but can result in higher cost.
3. The response of biological systems cannot always be predicted which can lead to a longer restoration time.
4. Constant monitoring is needed to quantify the rate of biodegradation, and ensure that the right densities and levels of the organisms of interest are present.
5. Preliminary pilot studies and laboratory experiments are encouraged before the complete site is treated. These trials help in evaluating the feasibility of treatment but require time and funding to conduct the investigations.

Therefore, the nature of contamination and conditions at the site may require a different

technology than bioremediation to restore the area, or a combined approach including biological, physical and/or chemical remediation methods. For example, after evaluating several methods to restore a landfill with vast soil contamination, excavation resulted in the most viable and sustainable approach. In this case excavation resulted in a third less CO_2 emissions than other approaches considered, lesser time and a more cost efficient method. Another case is the California Gulch Superfund NPL Site in Leadville, Colorado, an area impacted by past mining activities. At this Superfund site, an intelligent and strategic plan of remediation was implemented designed to minimize environmental disturbances. Excavation and offsite disposal was avoided and soil was treated *in situ*, therefore reducing air emissions associated with equipment work and transportation. Natural amendments present at the site (compost consisting of agricultural and forestry byproducts) were used for soil treatment instead of using synthetic materials (Kathleen S. Smith Katherine Walton-Day & Pietersen, 2012; S. US EPA, n.d.-g). . Efforts were coordinated with Colorado's Division of Natural Resources, Colorado Department of Public Health and Environment, U.S. Fish and Wildlife Service and the U.S. Bureau of Land Management.

Hence, it is clear that when evaluating treatment options, site-specific circumstances may not lead to bioremediation as the appropriate choice, but it is important that the techniques implemented are still cost-effective and also sustainable. This optimal decision-making process follows President Obama's Executive Order (EO) 13514, "Federal Leadership in Environmental, Energy, and Economic Performance" issued on October 5, 2009. EO 13514 calls for energy reduction, awareness of green technologies and practices "to establish an integrated strategy towards sustainability in the Federal Government". Following the EO, the EPA and Interstate Technology & Regulatory Council (ITRC) have published guideline documents for green remediation practices that provide an overview of the subject, current efforts and best practices (ITRC, n.d.; S. US EPA, n.d.-h). These documents aim to "educate and inform state regulators and other stakeholders in the concepts and challenges" but currently there is no regulation that specifically governs the green sustainable process. Actually the ITRC report clearly states how "There is no industry-wide consensus on the definitions of the term "green and sustainable"; therefore, discussions on this area may not be addressing consistent concepts." The EPA has

defined green remediation as "the practice of considering all environmental effects of remedy implementation and incorporating options to minimize the environmental footprints of cleanup actions" (S. US EPA, n.d.-h); but this narrow definition leaves behind social and economic aspects. Bardons et al. (2011) as well as a recent NRC report have indicated the importance of considering those aspects as elements of sustainable development but yet "ethical and equity considerations, indirect economic costs and benefits, and employment and capital gain (among others) are not explicitly provided for in any cleanup statute or existing programs" (Bardos, Harries, & Smith, 2011).

Research, education and innovation needs

It is the impression of many that there is a lack of collaboration between academia, the private sector (consultants, practitioners) and regulators (government) when it comes to Superfund sites. A recent National Academies report alluded to the impression that federal research funding for groundwater remediation has "generally declined over the past decade" (National Research Council, 2013). This is not surprising since overall research investments by federal agencies has declined in the last years due to budget cuts and financial constraints (NSF, n.d.). During 1996-2011 the National Institute of Environmental Health Sciences (NIEHS) awarded funding of approximate $500-800 millions on research related to groundwater remediation (National Research Council, 2013). But NIEHS gives funding to projects investigating the exposure and impacts of contaminants on human health; it does not funds research on contaminant removal or the implementation of remedial technologies. During the same time period, the EPA only awarded approximately $14 million; while most of the applied research on groundwater remediation in this area was funded by the Department of Defense (DoD) and the Department of Energy (DOE) with $315 and $138 million, respectively (National Research Council, 2013).

The DoD supports field demonstrations, application and validation of technologies under their Environmental Security and Technology Certification Program (ESTCP) (DOD, n.d.-a). DoD

also funds basic research and development under the Strategic Environmental Research and Development Program (SERDP) (DOD, n.d.-b). These programs are unique, providing funding to academia, the private and federal sector on a peer review, competitive basis. ESTCP is operated solely under DoD while SERDP operates in partnership with DOE and EPA, but EPA's support in recent years has been minimal. Programs like SERDP and ESTCP have opened many doors, for example vinyl chloride (a proven carcinogen and pervasive groundwater contaminant) was thought to be recalcitrant to biotic degradation. After much dedicated research, SERDP-funded investigators proved that bacteria can use this compound for growth and nowadays specific tools are addressed to monitor these microbes in vinyl chloride-contaminated sites during remediation efforts (Jianzhong He et al., 2003; F E Löffler & Edwards, 2006). Therefore, more research is needed to decipher the toxicity, environmental fate, and removal of toxic chemicals, including those considered recalcitrant, unregulated or not yet completely understood. This is imperative as sites become more complex by the presence of emerging and multiple contaminants. Furthermore, research efforts need to encompass the molecular basis of chemicals under controlled laboratory studies, but also their behavior at bigger scales (e.g., pilot and field studies); it is in these scenarios where interdisciplinary efforts are most needed for righteous remedy implementation. Investigations leading to discovery and innovation of novel remediation procedures are crucial, but there's also constant need for optimization of classical environmental engineering as more efficient and sensitive instruments are developed.

Research on remediation technologies can also turn a negative environmental incident into an opportunity for development. For example, if an investigator wants to find a bacterium capable of breaking down chloroform, having access to samples from a chloroform-contaminated site increases his chances of finding it, since natural processes (evolution and natural selection) would have already selected for microorganisms with those capabilities. In other words, scientists thrive the opportunity to work with "unusual" samples that will give rise to discoveries, while the industry and community benefits from the applications of these findings for site restoration. Without funding, researchers cannot address the needs of practitioners at the sites, and correspondingly, practitioners and regulators will not keep pace with the latest cutting-edge technologies gestated at the laboratories of universities and research centers. For example, a

responsible party or contractor may consider and choose more conservative methods instead of the latest "state to the art" sustainable and green technology due to misinformation and pressure, as EPA and CERCLA penalties are strict and can result up to $37,500 for each day of non-compliance. The isolation and miscommunication of "different professional and scientific cultures" is detrimental leading to stagnant practices and much longer cleanups of NPL sites.

Final Policy Recommendations:

To promote bioremediation practices and sustainable technologies:

- Government and private sector need to reach a consensus on the definition of sustainability and clarify the role of green remediation. Once a consensus is reached, specific regulations for green sustainable remediation can be drafted.
- Amend CERCLA to include sustainability criteria on remedy selection. The language should specify that after evaluation of all parameters, the remedy selected is not only efficient and cost effective but also sustainable.
- Create legislation that provides incentives to companies that voluntarily select and implement more sustainable remedial approaches, including bioremediation.

To foster education, research and development across all sectors especially with academia:

- Establish a Research Program (lead by the EPA) that gives grants on a competitive basis to remediate projects at Superfund sites. For a proposal to get awarded it needs to show a united effort between the private sector, regulators, and academia.
- The SERDP-ESTCP Program should be a model to follow by other institutions to foster collaborations and dialogue to better link science with practice (e.g., promoting institutional innovations to develop and improve techniques for environmental monitoring assessment).
- Create more education and training programs for regulators and practitioners. It is the impression of many academics (who are in more close contact to the state-of-the-art,

cutting edge techniques) that regulators (government) and practitioners simply don't know enough about the technologies, hindering the selection and implementation of bioremediations practices.

To improve management of contaminated sites:

- Track sites after they are deleted from the NPL, especially when those have indicatives of residual contamination.
- Require liable parties and contractors to file reports exclusively on their green remediation practices, environmental impact, remedial action procurement, cost and energy use.
- During the decision-making and remedy selection process, the anticipated land use (after restoration) should be considered.
- Follow an "adaptive management" that will allow decisional flexibility in the clean up process, as discussed by Cannon (2005).
- Firmly state that the remedy selected needs to serve not only the natural environment but also the surrounding community.
- Enforce oversight and adequate performance documentation to the contractors and liable parties.
- The creation of databases that can be used to compare the effectives of different technologies at different sites.

To improve strategies for sustainable remediation implementation:

- Establish incentives to promote the selection and implementation of bioremediation and other sustainable approaches (e.g., reduce fines for those liable parties that choose green remediation over other technologies).
- Standardize and reach a consensus on the metrics used to measure green and sustainable remediation actions (e.g., CO_2 emissions, use of renewable energy, environmental impact footprint, and community job creation).

- Reduce the use of natural resources (e.g., water), maximize use of renewable sources while considering social and economic impact. Identify innovative and optional uses for onsite materials and byproducts otherwise considered waste.
- Educate and train the local workforce (especially low income communities and minorities) on remedial technologies. This opens doors for quicker cleanups, job creation and better social relationships leading to community empowerment.

References

Anderton, D L, J M Oakes, and K L Egan. 1997. “Environmental Equity in Superfund. Demographics of the Discovery and Prioritization of Abandoned Toxic Sites.” *Evaluation Review* 21 (1): 3–26. doi:10.1177/0193841X9702100101.

Atlas, Ronald M, and Terry C Hazen. 2011. “Oil Biodegradation and Bioremediation: a Tale of the Two Worst Spills in U.s. History.” *Environmental Science & Technology* 45 (16): 6709–6715.

Bardos, Paul, Nicola D Harries, and Jonathan W N Smith. 2011. “Applying Sustainable Development Principles to Contaminated Land Management Using the SuRF-UK Framework.” *SuRF-UK Applying Sustainable Development Principles March 2011 - Bardos et Al. Applying Sustainable Development Principles - Spring 2011.pdf* (August 2007): 77–100. doi:10.1002/rem.

Beck, Edkardt C. 1979. “Love Canal Tragedy.” *EPA Journal*, January 1. doi:10.1177/004057368303900411.

Bragg, J R, R C Prince, E J Harner, and R M Atlas. 1994. “Effectiveness of Bioremediation for the Exxon Valdez Oil Spill.” *Nature* 368 (6470): 413–418.

Burwell-Naney, Kristen, Hongmei Zhang, Ashok Samantapudi, Chengsheng Jiang, Laura Dalemarre, LaShanta Rice, Edith Williams, and Sacoby Wilson. 2013. “Spatial Disparity in the Distribution of Superfund Sites in South Carolina: An Ecological Study.” *Environmental Health : a Global Access Science Source* 12 (1) (January): 96. doi:10.1186/1476-069X-12-96.

Currie, Janet, Michael Greenstone, and Enrico Moretti. 2011. “Superfund Cleanups and Infant Health.” *American Economic Review*. doi:10.1257/aer.101.3.435.

Daprato, R.C., J. Langenbach, R. Santos-Ebaugh, R. Kline, and T.A. Peel. “Green Remediation via an Enhanced In Situ Bioremediation Solar Powered System.” In *Proceedings of the*

Seventh International Conference Remediation of Chlorinated and Recalcitrant Compounds. Monterey, California.

DOD. 2014a. “About ESTCP.” Accessed February 25. http://www.serdp.org/About-SERDP-and-ESTCP/About-ESTCP.

———. 2014b. “About SERDP.” Accessed February 25. http://www.serdp.org/About-SERDP-and-ESTCP/About-SERDP.

“FY_2013_SF_EOY_accomp_sum_FINAL.pdf.” 2014. Accessed February 25. http://www.epa.gov/superfund/accomp/pdfs/FY_2013_SF_EOY_accomp_sum_FINAL.pdf.

Ghasemizadeh, Reza, Ferdinand Hellweger, Christoph Butscher, Ingrid Padilla, Dorothy Vesper, Malcolm Field, and Akram Alshawabkeh. 2012. “Review: Groundwater Flow and Transport Modeling of Karst Aquifers, with Particular Reference to the North Coast Limestone Aquifer System of Puerto Rico.” *Hydrogeology Journal* 20 (8): 1441–1461. doi:10.1007/s10040-012-0897-4.

Hazen, Terry C, Eric A Dubinsky, Todd Z DeSantis, Gary L Andersen, Yvette M Piceno, Navjeet Singh, Janet K Jansson, et al. 2010. “Deep-Sea Oil Plume Enriches Indigenous Oil-Degrading Bacteria.” *Science (New York, N.Y.)* 330 (6001): 204–208. doi:10.1126/science.1195979.

He, Jianzhong, Kirsti M Ritalahti, Michel R Aiello, and Frank E Löffler. 2003. “Complete Detoxification of Vinyl Chloride (VC) by an Anaerobic Enrichment Culture and Identification of the Reductively Dechlorinating Population as a Dehalococcoides Species.” *Appl. Environ. Microbiol.* 69: 996–1003.

Höpner, T, and KA Al-Shaikh. 2008. “Shoreline Bioremediation after the 1991 Gulf War Oil Spill.” *Protecting the Gulf’s Marine Ecosystems*. 265–272.

Hunter-Cevera, J C. 1998. “The Value of Microbial Diversity.” *Current Opinion in Microbiology* 1 (3): 278–285. doi:10.1016/S1369-5274(98)80030-1.

ITRC. 2014. “Green and Sustainable Remediation Documents.” Accessed February 25. http://www.itrcweb.org/Guidance/ListDocuments?TopicID=9&SubTopicID=15.

Kathleen S. Smith Katherine Walton-Day, Karin Olson Hoal Rhonda L Driscoll, and Kevin Pietersen. 2012. “Pre- and Post-Remediation Characterization of Acid-Generating Fluvial Tailings Material.” *9th International Conference on Acid Rock Drainage* (Usepa 2005).

Löffler, F E, and E A Edwards. 2006. “Harnessing Microbial Activities for Environmental Cleanup.” *Current Opinion in Biotechnology* 17: 274–284.

Macey, Gregg. 2007. “Introduction: The Promises and Pitfalls of Adaptive Site Stewardship,” In *Reclaiming the Land: Rethinking Superfund Institutions, Methods, and Practices*, edited by Jonathan Macey, Gregg, Cannon.

Mastromonaco, Ralph. 2011. “Hazardous Waste Hits Hollywood: Superfund and Housing Prices in Los Angeles.” *SSRN Electronic Journal* (January 1). doi:10.2139/ssrn.1926467.

Megharaj, Mallavarapu, Balasubramanian Ramakrishnan, Kadiyala Venkateswarlu, Nambrattil Sethunathan, and Ravi Naidu. 2011. “Bioremediation Approaches for Organic Pollutants: a Critical Perspective.” *Environment International* 37 (8): 1362–1375. doi:10.1016/j.envint.2011.06.003.

National Research Council. 2013. “Alternatives for Managing the Nation’s Complex Contaminated Groundwater Sites.” *He National Academies Press, 2013.* Washington, DC.

NSF, US National Science Foundation. 2014. “NSF Study Shows Declines in Federal Funding for Research and Development.” Accessed February 25. http://www.nsf.gov/news/news_summ.jsp?cntn_id=130156.

O’Neil, Sandra George. 2007. “Superfund: Evaluating the Impact of Executive Order 12898.” *Environmental Health Perspectives* 115 (7): 1087–1093.

Padilla, IY, C Irizarry, and K Steele. 2011. “Historical Contamination of Groundwater Resources in the North Coast Karst Aquifer of Puerto Rico.” *Dimension* 25 (3).

Padilla, I.Y., C. Irizarry and K. Steele. 2011. “Historical Contamination of Groundwater Resources in the North Coast Karst Aquifer of Puerto Rico.” *Dimensions* 25, (3): 7–12.

Philp, JC, and RM Atlas. 2005. “Bioremediation of Contaminated Soils and Aquifers.” *Bioremediation, ASM Press, Washington, DC*.

Pimentel, David, Christa Wilson, Christine McCullum, Rachel Huang, Paulette Dwen, Jessica Flack, Quynh Tran, Tamara Saltman, and Barbara Cliff. 1997. “Economic and Environmental Benefits of Biodiversity.” *BioScience* 47 (11): 747–757. doi:10.2307/1313097.

“Puerto Rico Testsite for Exploring Contamination Threats (PROTECT): Dynamic Transport and Exposure Pathways of Contaminants in Karst Groundwater System.” 2014. Accessed February 25. http://civil.uprm.edu/lab_ambiental/research/protect.html.

Saaty, RP, and SR Booth. 1994. “In Situ Bioremediation: Cost Effectiveness of a Remediation Technology Field Tested at the Savannah River Integrated Demonstration Site.” *Los Alamos National Laboratory, Los Alamos.*

Steinzor, Rena I., Margaret Clune, Center for American Progress, and Center for Progressive Reform. 2006. *The Toll of Superfund Neglect: Toxic Waste Dumps & Communities at Risk*. Center for Progresive Reform.

Stretesky, Paul, and Michael J. Hogan. 1998. “Environmental Justice: An Analysis of Superfund Sites in Florida.” *Social Problems*. doi:10.1525/sp.1998.45.2.03x0169m.

Stroo, Hans F, Andrea Leeson, Jeffrey a Marqusee, Paul C Johnson, C Herb Ward, Michael C Kavanaugh, Tom C Sale, et al. 2012. “Chlorinated Ethene Source Remediation: Lessons Learned.” *Environmental Science & Technology* 46 (12): 6438–47. doi:10.1021/es204714w.

Thomas, Terence D, Derek C Ellwood, and V Michael C Longyear. 1979. “Change from Homo- to Heterolactic Fermentation by Streptococcus Lactis Resulting from Glucose Limitation in Anaerobic Chemostat Cultures.” *Journal of Bacteriology* 138 (1): 109–117.

U.S. Nuclear Regulatory Commission. 2008. “Technical Basis for Assessing Uranium Bioremediation Performance.” *Office of Nuclear Regulatory Research*. http://purl.access.gpo.gov/GPO/LPS106258.

US EPA, OSWER, Office of Superfund Remediation and Technology Innovation. “Report and Product Descriptions | Superfund Information Systems | US EPA.”

US EPA, Superfund. “CERCLA Overview | Superfund | US EPA.”

———. 2014b. “US EPA National Priorities List (NPL).” Accessed February 24. http://www.epa.gov/superfund/sites/npl/.

———. “Superfund for Students and Teachers | Superfund | US EPA.”

———. “Superfund’s 25th Anniversary: Capturing the Past, Charting the Future | Superfund | US EPA.”

———. “Proposed National Priorities List (NPL) Sites - by State | National Priorities List (NPL) |.”

———. “Region 8 Montana Superfund Redevelopment Program Sites.”

———. “Comprehensive Five-Year Review Guidance | Superfund | US EPA.”

———. “Close Out Procedures for National Priorities List Sites | Superfund | US EPA.”

———. “Superfund Provides Communities with Significant Human Health, Environmental and Economic Benefits.”

———. “Sites in Reuse in Colorado.”

———. “Superfund & Green Remediation.”

———. “NPL Map (NPL).”

Wijensinghe, A.M., R.B. Knapp, R.T. Taylor, and L.M. Carman. 1992. “Preliminary Feasibility and Cost Analysis of the in-Situ Microbial Filter Concept” (March 20).

Wilson, Sacoby M., Herb Fraser-Rahim, Edith Williams, Hongmei Zhang, LaShanta Rice, Erik Svendsen, and Winston Abara. 2012. “Assessment of the Distribution of Toxic Release Inventory Facilities in Metropolitan Charleston: An Environmental Justice Case Study.” *American Journal of Public Health*. doi:10.2105/AJPH.2012.300700.

Chapter 6 Appendix: Figures

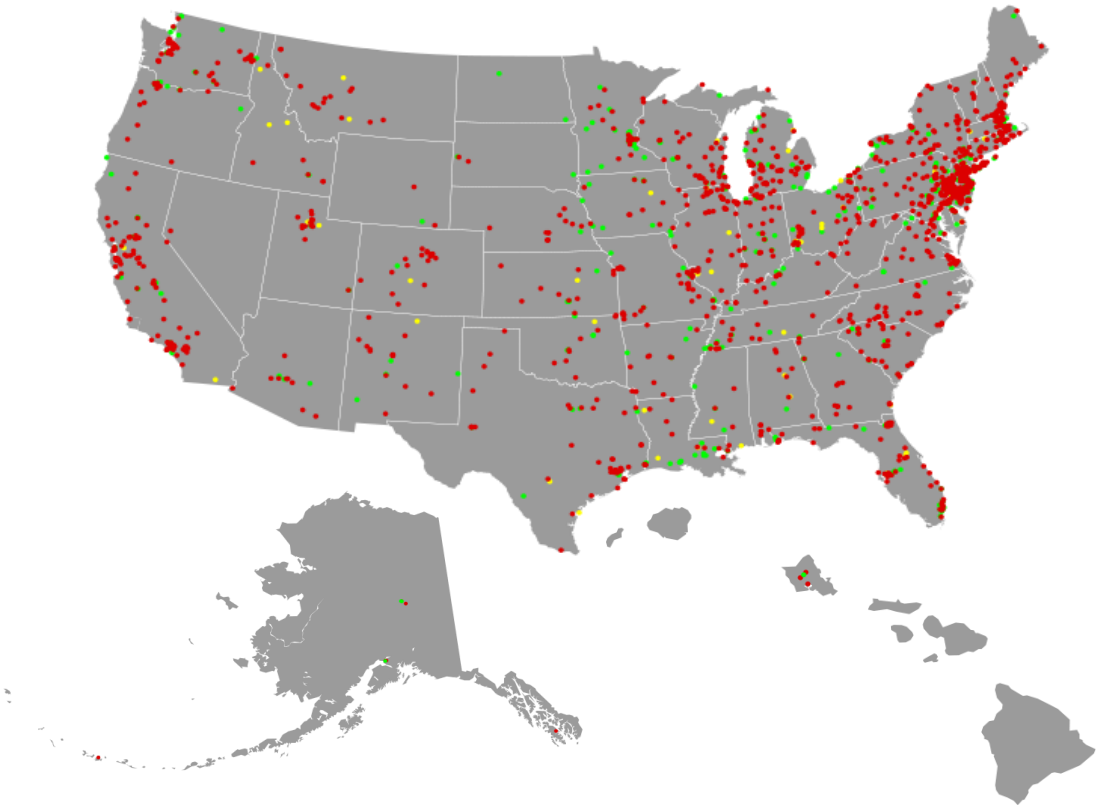


Figure 6.1. Map of Superfund sites in the United States as March 31, 2010. Red dots indicates indicate final sites in the National Priority List, yellow are proposed sits, and green are deleted sites (O. O. of S. R. and T. I. US EPA, n.d.; S. US EPA, n.d.-d).

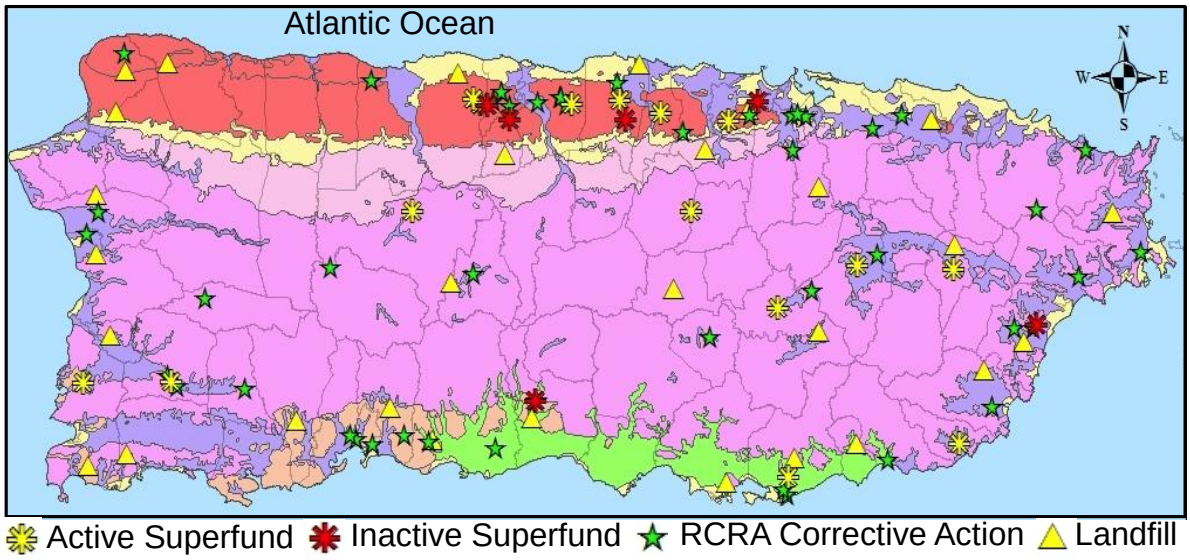


Figure 6.2. Impacted areas in Puerto Rico.

Hydrogeology and contaminated sites are indicated; the north coast limestone aquifer is depicted in orange and light pink color. Resource Conservation and Recovery Act (RCRA) facilities are also included; these are sites where releases of hazardous waste into soil, ground water, surface water, sediments, and air have occurred; requiring the investigation and cleanup, or remediation. Forty-five percent of all Superfund sites are located in the northern karst region of the island, which includes one of the largest and most productive sources of groundwater. Evidence suggests that the higher preterm birth rates in Puerto Rico cannot be explained by changes in obstetric practices and that exposure to hazardous chemicals contributes to preterm birth (Padilla et al., 2011).

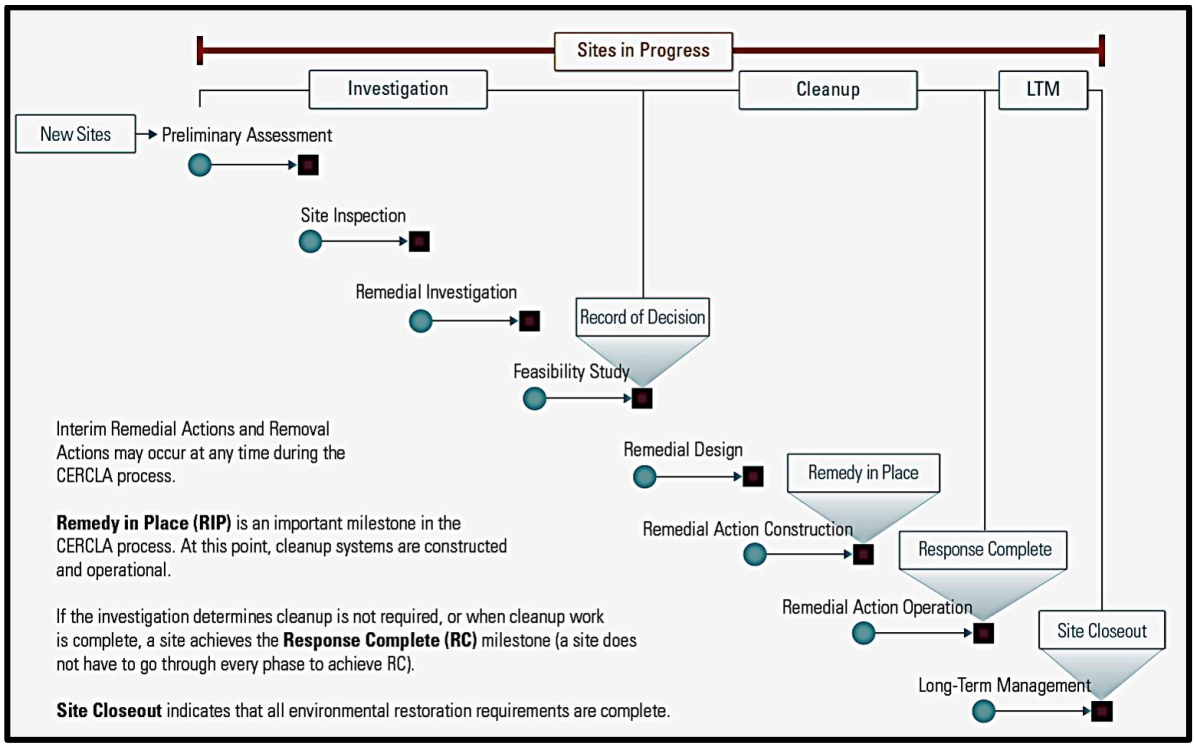


Figure 6.3. DoD CERCLA environmental restoration phases and milestones. Figure from the National Academies Report (National Research Council, 2013).

Chapter 6 Appendix: Tables

Table 6.1. Number of Federal and general sites for each status and milestone as of January 24, 2014:

Status	Non-Federal (General)	Federal	Total
Proposed Sites	49	4	53
Final Sites	1162	157	1319
Deleted Sites	358	17	375
Milestone	**Non-Federal (General)**	**Federal**	**Total**
Partial Deletions	42	18	60*
Construction Completions	1085	72	1157

Chapter 7
Conclusion and recommendations

Summary of research findings and future research needs

Bioaugmentation is now a widely accepted strategy for the remediation of chlorinated solvents. Microbial consortia containing *Dehalococcoides* are the basis for successful bioremediation efforts at sites (i.e., drinking water aquifers) contaminated with chlorinated ethenes. *Dehalococcoides* species have a highly restricted energy metabolism and require halogenated compounds to make a living. All *Dhc* strains share highly similar 16S rRNA genes (>98 nt % identity) but may exhibit different dechlorination activities. For example, strain FL2 and strain 195 cannot grow with VC while strains BAV1, GT and VS use VC as an electron acceptor and efficiently detoxify VC to ethene. Hence, gene targets providing higher resolution than the 16S rRNA gene are desirable for site assessment and bioremediation monitoring to link cause-and-effect relationships between microbial activity and contaminant detoxification.

The research efforts encompassed herein elucidated the RDase involved in the 1,2-D dichloroelimination. Results showed that 1, 2-D-respiring Chloroflexi organisms (*Dhc* and *Dhgm*) appear to be major contributors to this activity at the sites investigated. The sensitive *dcpA*-targeted PCR assays developed in this dissertation provide a more comprehensive molecular biological tool approach that can now be applied to tackle 1,2- D-contaminated sites. The assays resulted in an invention disclosure filed to UTRF (University of Tennessee Research Foundation) and a patent application to USTPO http://www.google.com/patents/US20140072965.

Although the 1,2-D RDase in the dehalospiring Chloroflexi *Dhc* and *Dhgm* has been identified the equivalent RDase in *Desulfitobacterium dichloroeliminans* and *Dehalobacter* sp. remains to be elucidated. To achieve this and based on the lessons learned on *Dhc* transcription, expression; the use of DNA/RNA molecular tools along with already-optimized BN-PAGE methodology, a multiple lines of evidence approach, similar to the one employed to identify *dcpA* should be used to assign function to the 1,2- D RDase in *Dhb* and *Desulfitobacterium.*

Dhgm is also known to grow on 1,1,2-TCA and 1,2-DCA and the RDase involved in these additional dichloroelimination reactions are inconclusive. Of particular interest are the enzymes involved in 1,2,3-TC dechlorination, by *Dhgm* spp., the only group characterized to use this contaminant as electron acceptor. TCP is a persistent groundwater pollutant, and although a federal MCL has not been established several states already have state-regulatory levels. Furthermore the EPA has added TCP in their "contaminate candidate list" which are unregulated chemicals that are anticipated to require future regulation. Therefore, as concern for 1,2,3-TCP grows as an emerging contaminant, the elucidation of the dechlorination mechanism by *Dhgm* is an area of interest and future research.

The sequencing of the *Dhc* RC and KS genomes provided clues about possible additional substrate utilization otherwise unknown. So far strain *Dhc* RC and KS are maintained in 1,2-D as its sole electron acceptor, since they fail to grow on other chlorinated solvents (TCE, VC). Interestingly the findings presented indicated multiple non-identical RDase genes are harbored in *Dhc* RC and KS genomes. Aside from *dcpA*, only one other RDase gene present has assigned function, *pcbA*; a gene recently implicated in PCB and PCE dechlorination (Wang et al., 2014). PCB is highly toxic and recalcitrant and since only a few strains have been reported to posses the ability to dehalogenase PCB congeners, efforts to grow RC and KS on PCB should be attempted. Concomitant dechlorination trials with PCE should also be performed, to investigate co-contaminate inhibition or dual transformation of PCE/PCB.

The discovery of *dcpA* in *Dhc* strain RC and KS lead to the discovery of the 1,2-D RDase also in in *Dhgm* strain BL-DC-9, indicating HGT between these groups of organohalide respiring bacteria. While a mechanism has been proposed for the VC-to-ethene RDase (*vcrA*) by site-specific integration via a *ssrA* module, this dissertation effort elucidated the sharing of a genomic island encompassing the *dcp* operon in *Dhc* RC and KS and *Dhgm* and a mechanism by phage insertion and transposition are proposed, among others. Since bioremediation monitoring relies on biomarker detection and quantification, understating of RDase genes mobility in the environment is relevant for scientists and practitioners alike.

The future is Green

A wave of initiatives for a more environmentally conscious society has emerged and sustainable remedial practices are now, more than ever part of the conversation. As stated by the American Society for Testing and Materials (ASTM) "Green remediation shouldn't be an oxymoron. When it comes to handling contaminated sites, from a small to a huge location the goal is to protect human health and the environment." Historically, site restoration remedies have been implemented without consideration of green or sustainable concepts in order to restore sites; but things are changing. A framework has been in the works for sustainable remediation. Since the 80s a series of executive orders regarding sustainability, environmental protection and energy efficiency have been drafted, and in the last decade the EPA's Green Remediation Strategy was launched, also the Sustainable and Remediation Forum (SURF) composed or private sector leaders and practitioners was created. In recent years, Guidance documents have been drafted by stakeholders and last year the ASTM, which is globally recognized as a leader in the development and delivery of international voluntary consensus standards, developed the first "Green and Sustainable clean up standards", but still the implementation of sustainable practices in voluntary. On February 2014, the Horinko Group published a white paper entitled "the Rise and Future of Green and Sustainable Remediation" led by Ms. Marianne Horinko, past EPA administrator, alluding to the up-and coming interest of this topic. Once upon a time, the drafting of environmental regulations and policies were dependable, on analytical chemistry advances: as technology and instruments increased their sensitivity to detect contaminants in the environment, specific statues for maximum contaminant levels were drafted or amended. A similar scenario is in the works nowadays, not with by state-of-the-art technology primed in a more mindful decision making process with uniting efforts across disciplines and experts, which is crucial to the enhancement and restoration of impacted sites.

The future is bright

We are living in exciting times. In 2013, Chen et al., isolated an aerobic *Comamonas* strain (designated 7D-2) capable of growing by reductive bromination of bromoxybil, a compound

used in herbicides (Chen, 2013). Further characterization of the dehalogenating mechanism uncovered a new class of reductive dehalogenase found in aerobes designated as BhhA. Several *bhhA* homologues have been identified in a variety of aerobic genera (e.g. *Roseobacter, Silicibacer, Sulfitobacter*) indicating a wide distribution of these enzymes and suggesting important contributions to the orgahalogen cycle in nature (Chen, 2013).

Alongside, Bommer *et. al*. achieved the first crystal structure of the Pce RDase, resulting in the first reductive dehalogenase crystalized (Bommer, M. Kunze C, Fesseler J, Schubert T, Diekert G, 2014). Furthermore, during the final stages of this research the VcrA RDase of strain became the first Dhc RDase heterologously expressed in *E. coli* and reconstituted to its active form (Parthasarathy A, Stich TA, Lohner ST, Lesnefsky A, Britt RD, 2015). These advances had shed light into the characteristics and biochemistry of these particular enzymes and are paving the path for the next generation of discoveries. In the near future RDase genes could be genetically modified resulting in protein with modified motifs or substituents to add or increase the spectrum of halogenated compounds that that they can dechlorinate. The implications of such RDases engineering efforts could be enormous.

Nevertheless, early efforts used to characterized the Pce RDase in dehalogenating bacteria and classical approaches that included reverse genetics, and transcriptional approaches were successful in assigning function to six *Dhc* RDases (e.g. *vcrA*, *pcbA*, *bvcA*, *dceA*, *cbrA*, *dcpA*). These RDases were identified, without the need of a genetic system, heterologous expression or complete enzyme purification. Moreover, the present study assigned function to the 1,2-D to propene RDase in *Dhc* strain RC and KS, and also in *Dhgm* strain BL-DC-9 (Padilla-Crespo et al., 2014) with similar integrated techniques. Therefore, biomarker discovery and RDase characterization will remain an emerging filed of research with vast opportunities since dozens of putative RDase genes with unknown functions are harbored in *Dhc* genomes while hundreds of putative RDase genes homologues linger in the databases awaiting for their functional characterization. Combined with 16S rRNA gene-targeted approaches, these RDase gene targets will assist in site assessment and bioremediation monitoring.

The future is green, and the future is bright.

References

Bommer, M. Kunze C, Fesseler J, Schubert T, Diekert G, D. H. (2014). Structural basis for organohalide respiration. *Science*, *346*(6208), 455–458.

Chen, K. et al. (2013). The molecular characterization of the enzymes involved in herbicide degradation of a brominated aromatic. *Molecular Microbiology*, *89*(6), 1121–1139.

Padilla-Crespo, E., Yan, J., Swift, C., Wagner, D. D., Chourey, K., Hettich, R. L., Löffler, F. E. (2014). Identification and environmental distribution of dcpA, which encodes the reductive dehalogenase catalyzing the dichloroelimination of 1,2-dichloropropane to propene in organohalide-respiring Chloroflexi. *Applied and Environmental Microbiology*, *80*(3), 808–818.

Parthasarathy A, Stich TA, Lohner ST, Lesnefsky A, Britt RD, S. A. (2015). Biochemical and EPS-spectroscopic investigation into heterologously expressed vinyl chloride reductive dehalogenase (VcrA) from *Dehalococcoides mccartyi* strain VS. *J Am Chem Soc.*, *137*(18), 3525–3532.

Wang, S., Chng, K. R., Wilm, A., Zhao, S., Yang, K.-L., Nagarajan, N., & He, J. (2014). Genomic characterization of three unique *Dehalococcoides* that respire on persistent polychlorinated biphenyls. *Proceedings of the National Academy of Sciences of the United States of America*, (111), 12103–12108.

Vita

Elizabeth was born to parents Isabel M. Crespo-Arbelo and Francisco Padilla-Marty. She was raised in Mayagüez Puerto Rico, and was valedictorian at both her Middle School and High School Graduations. She earned a double degree in Industrial Biotechnology and Industrial Microbiology from the University of Puerto Rico-Mayagüez, where she graduated Magna Cum Laude. As an undergraduate student, she performed research at top research centers: Harvard Medical School, the U.S. Department of Energy, Lawrence Berkeley National Laboratory, the University of Wisconsin-Madison and the Georgia Institute of Technology. The recipient of a competitive NSF Gradate Research Fellowship, Elizabeth is the primary author and coauthor of more than 30 conference proceedings and peer-reviewed publications. In 2013 she received the University of Tennessee Cokkinias Outstanding Graduate Student Award. Furthermore, during her graduate studies Ms. Padilla received several awards including Best Poster at the 7th International Symposium for Subsurface Microbiology (Shizouka, Japan) and the Environmental Science Student Award at "Sustainability Live", United Kingdom's largest environmental symposium. She was also the winner of the Battelles's 2013 Student Paper Competition Winner at the Seventh International Conference on Remediation of Contaminated Sediments (Dallas, TX). Her efforts have been recognized by organizations such as the Federation of European Microbiological Societies, the Goizueta Foundation, the American Society of Microbiology, the Hispanic Business, NASA, among others. In the summer of 2012 she had the opportunity to work at the National Foundation Offices, where she performed a comprehensive analysis on the funding awarded to research on microbial communities. While At NSF she advocated not only for the microbes "and their funding rights", but also for opportunities for woman and minorities in the sciences. She was selected as 2013-2014 STEM (Science, Technology, Engineering and Math) Graduate Fellow for the Congressional Hispanic Caucus Institute and worked in the Committee of Science and Space Technology at the U.S. House of Representatives. Elizabeth is committed to education and outreach; she has served as a mentor in the Minority Striving and Pursuing Higher Degrees of Success in Earth System Science Professional Development Program (MS PHD'S) and the NASA Student Ambassadors Program. Ms. Padilla has volunteered in outreach activities from the Consortium for Ocean Leadership, SACNAS (Society

for Advancement of Chicano/Latino and Native Americans in Science), and ASLO-MP (American Society of Limnology and Oceanography Multicultural Program). Her ultimate goal is to be in a position where she teach, do research, as well as advocate for funding of basic/applied research and broadening participation initiatives. Elizabeth is passionate about environmental microbiology, wanting to share her knowledge with the community in order to give smart solutions to enhance bioremediation and detoxification of contaminated sites and inspire students, especially woman and minorities, into pursuing careers in science. Among her dearest moments in life are having the opportunity of being the only westerner selected to participate of a highly competitive summer course in Singapore, where she was taught, on-by-one, by researchers she had always admired on paper; including Kenneth Nealson (who first isolated *Shewanella oneidensis*), and having had the opportunity to organize and lead a Policy Summit in Capitol Hill on a topic of her passion "Green and Sustainable Remediation". At the Summit, Congressman's Lujan gave the opening remarks, and Elizabeth was introduced by Dr. Marcy Gallo, Director of the Subcommittee on Environment of the Staff Science Committee at the U.S. House of Representatives. Lastly, in a humid and warm night in VA, as a midsummer night's dream, she danced on stage with her long time heroes the singer-songwriters "The Gipsy Kings". Later that evening she had the opportunity to meet the masters and during dinner they sang her acapella "La Gitana Mora" (the Arabian Gispsy), Elizabeth's favorite song.