ISOLATION AND ECOLOGY OF BACTERIAL POPULATIONS INVOLVED IN REDUCTIVE DECHLORINATION OF CHLORINATED SOLVENTS

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SUMMARY

Despite strict regulation, chlorinated organic contaminants such as tetrachloroethene (PCE) and trichloroethene (TCE) are still used as solvents and degreasing agents in many industrial applications. As a result of previous improper disposal practices, accidental spills, and continued use, PCE and TCE have become widely distributed in the environment. The discovery of bacteria that use chloroorganic compounds as electron acceptors has revolutionized bioremediation strategies at many sites contaminated with chlorinated ethenes. Generally, bioremediation is categorized by three applications: intrinsic bioremediation (natural attenuation), biostimulation, and bioaugmentation. All three approaches require sensitive methods to monitor the presence, distribution, and activity of the organisms of interest. Pure cultures are indispensable resources to develop an understanding the physiology of the dechlorinators and the biochemistry of the key enzymes.

In order to explore the distribution and function of dechlorinating populations, sediment-free, non-methanogenic, ethene-producing enrichment cultures were derived from various chlorinated ethene contaminated and non-contaminated site materials. The findings of this study demonstrate that *Dehalococcoides* species are intimately involved in complete reductive detoxification of chlorinated ethenes and are widely distributed in anoxic sediments and aquifers, including non-contaminated (pristine) environments. Careful examination of enrichment culture dechlorination kinetics, 16S rRNA gene based analyses, and reductive dehalogenase gene targeted PCR approaches revealed that complete reductive dechlorination is carried out by multiple dechlorinators. The findings of this study also suggest that the existence of a different *Dehalococcoides* population, other than strains 195, FL2 and BAV1, may be involved in the complete detoxification of chlorinated ethenes.

Two new dechlorinating species were isolated from contaminated and non-contaminated site materials. The first new isolate, designated strain SZ, was isolated from PCE-to-ethene

dechlorinating microcosms established with creek sediment. 16S rRNA gene sequence of the strain SZ indicates that the new isolate is affiliated with the genus *Geobacter* most closely related to *G. thiogenes*. Strain SZ is capable of stepwise dechlorination of PCE to *cis*-DCE, while the closest relatives were not able to dechlorinate PCE or TCE. Dechlorination of PCE or TCE by strain SZ was supported by acetate, hydrogen or pyruvate as electron donor. Chloroethene-dechlorinating populations have been shown to have distinct electron donor requirements. However, none of previously described chlorinated ethene degrading population can use both, acetate and hydrogen, as electron donors. PCE dechlorination by strain SZ uses both acetate and hydrogen as electron donors suggesting that the ability to versatile electron donor utilization may increase the efficiency of bioremediation approaches. Importantly, strain SZ reduced two environmental priority pollutants, PCE and U(VI) concomitantly and detected from both bio-stimulated chloroethene and uranium contaminated sites, strongly suggesting that strain SZ play a important roles in *in-situ* bioremediation of chloroethene and U(VI) contaminated sites.

The second, a new *Dehalococcoides* species designated strain GT, was isolated from contaminated site materials. Strain GT uses trichloroethene (TCE), *cis*-DCE, 1,1-dichloroethene (1,1-DCE), and the human carcinogen vinyl chloride (VC) as growth supporting electron acceptors producing products ethene and inorganic chloride. The new isolate shares common traits of *Dehalococcoides* such as ampicillin resistance, strict hydrogen-dependent metabolism, and a low hydrogen consumption threshold concentration. Culture-dependent and independent, 16S rRNA gene and reductive dehalogenase gene targeted PCR approaches suggested culture purity.

To investigate the reductive dechlorination of chlorinated ethenes in the presence of sulfate and to examine the effect of sulfide on dechlorination performance of several chloroethene dechlorinating pure and mixed cultures, dechlorination end points and rates were determined. Findings from this study demonstrate that complete reductive dechlorination can be achieved

under electron limiting conditions, but that sulfide toxicity affects chlorinated ethene degrading populations.

CHAPTER I

Introduction

Halogenated aliphatic compounds are common groundwater contaminants and are significant components of hazardous wastes and landfill leachates. Most hazardous halogenated aliphatic compounds released from industrial, commercial, and agricultural sources are brominated or chlorinated alkanes and alkenes that contain between one and three carbon atoms. Among the chlorinated alkene compounds, tetrachloroethene (PCE) and trichloroethene (TCE) are the most common contaminants at National Priority List (NPL) sites. PCE and TCE have superior solvent properties, exhibit high boiling points, are nearly inflammable, and are non-explosive. PCE and TCE are manufactured for use in metal degreasing, dry cleaning of fabrics, and cleaning agents. PCE is also used in the production of other chemicals, such as trichloroacetic acid and various fluorocarbons. Since the 1960s, about 85% of the 36,000 dry cleaners in the U.S. use PCE as their primary cleaning solvent. The common usage of chlorinated ethene makes them one of the most common groundwater pollutants. Typically, chlorinated ethene contamination results from improper waste disposal, spills and carelessness handling during industrial production, transportation, and solvent application. For example, from 1987 to 1993, one half of million kg of PCE was released to land and water (Environmental Protection Agency 's Toxic Chemical Release Inventory).

Due to the proven negative impact of chlorinated ethenes (PCE, TCE, DCEs, and VC) to human health and overall ecosystem function, regulatory agencies recognized the necessity of remediation and regulation of this class of chemicals. The Department of Health and Human Services (DHHS) has determined that PCE and TCE may reasonably be anticipated to be a carcinogen. The U.S. EPA strictly regulated Maximum Contaminants Level (MCL) to PCE and TCE are 5 ppb (parts per billion = 0.005 mg/L) in drinking water. Unfortunately, vinyl chloride

(VC) is a proven human carcinogen among the chlorinated ethenes (Federal Register, 1995) and MCL in drinking water is set at 2 ppb.

Various remedial treatment techniques have been applied to chlorinated ethenes contaminated sites (e.g. physical, chemical and biological treatments). The most common physical treatment technique, pump-and-treat, has been shown to remove PCE and TCE contaminants successfully from subsurface environments but typically fails to remove the contaminants in acceptable time frames. PCE and TCE form DNAPLs (dense nonaqueous phase liquid) because they have higher densities than water and low water solubilities. Even small amounts of residual PCE or TCE after physical treatment can serve as a constant source of PCE or TCE contamination. Chemical treatment techniques have also been shown to successfully remediate chlorinated solvent contaminated sites by using zero valent metals, such as zero valent iron. However, chemical treatments still have disadvantages including high-cost and inefficiency.

The discovery of chlororespiration opened a new field of biological remediation technologies for many contaminated sites. During chlororespiration, specialized microorganisms (dechlorinators) capture energy for growth by transferring electrons derived from an electron donor to a chlorinated compound (electron acceptor), substituting the chlorine substituent with a hydrogen atom (reductive dechlorination). The first evidence of chlororespiration was obtained when studying growth and dechlorination of 3-chlorobenzoate by *Desulfomonile tiedjei* strain DCB-1. The first bacterium that linked PCE dechlorinating to growth, *Dehalobacter restrictus* PER-K23, was described in 1993. Since then, remarkable progress has been made to understand the microbial reductive dechlorination of chlorinated ethenes. Both mixed and pure cultures that reductively dechlorinate PCE, TCE, DCEs, and VC with coupling to growth and energy conservation have been described in the literature. Over the last decade, at least 17 species have been isolated that perform partial or complete reductive dechlorination of chlorinated ethenes. Identification of pure strains capable of chlororespiration will give important information about

the diversity of organisms implicated in this process and also provide fundamental understanding of the diverse biochemical mechanisms involved.

Reductive dechlorination processes could be stimulated by adding suitable electron donors to indigenous dechlorinating populations (biostimulation) or by introducing dechlorinating populations (bioaugmentation). These applications are effective and environmentally acceptable approaches compared to current physical or chemical treatments. However, in order to make meaningful decisions in treatment selection, understanding the distribution and ecology of dechlorinating populations is essential.

The findings of this research are presented in five chapters, along with a relevant literature review in Chapter II. Chapter III presents the environmental distribution of chloroethene dechlorinating populations and quantification of *Dehalococcoides* derived reductive dehalogenase genes in ethene-producing enrichment cultures which were derived from various contaminated and non-contaminated site materials. Chapter IV describes a new acetate and hydrogen oxidizing, PCE- and U(VI)-reducing anaerobic bacterium, *Geobacter lovleyi* strain SZ and its environmental distribution. Chapter V describes the isolation and characterization of a new *Dehalococcoides* isolate, strain GT that detoxifies TCE, *cis*-DCE, 1,1-DCE, and human carcinogen VC as growth supporting electron acceptors to the benign end products ethene and inorganic chloride. The limitation of 16S rRNA gene based approaches to monitor *Dehalococcoides* is also discussed. The effect of sulfate and sulfide on the microbial reduction of chlorinated ethenes is examined in Chapter VI. Finally, Chapter VII presents the major conclusions of this study and discusses recommendations for future research endeavors.

CHAPTER II

Literature Review

2.1 Natural sources of halogenated compounds

More than 3,800 halogenated organic compounds are produced by living organisms or are formed during natural abiotic processes, such as volcanic activity, forest fires, and other geothermal processes (38-40). Although most of the biogenic halogenated organics are found in marine environments, many others are found in terrestrial plants, fungi, bacteria, insects, animals, and humans (7, 38, 40, 41, 50, 53, 58, 112). Interestingly, several halogenated compounds were identified as products of human white blood cell myeloperoxidase-induced halogenation acting on invading pathogens, as well as in various other disease processes. Myeloperoxidase from humans was shown to convert chlorophenols to chlorinated dioxins and dibenzofurans (113). A recent study of volcanoes has revealed an extraordinarily large numbers of organohalogens, including 100 organochlorines, 25 organobromines, 5 organofluorines, and 4 organoiodines, most of which are newly discovered compounds (56). Abrahamsson et al. (3) demonstrated the biogenic formation of tetrachloroethene (PCE) and trichloroethene (TCE) by algae. A recent study demonstrated the natural formation of vinyl chloride (VC) in soil, a compound which was previously believed to be of anthropogenic origin (57).

This natural production of halogenated organic compounds may have stimulated microorganisms to evolve degradation or detoxification pathways for halogenated compounds, making it feasible to use bioremediation of halogenated compounds in various contaminated sites.

2.2 Anthropogenic sources of halogenated compounds

Halogenated organic compounds are known to be one of the largest group of environmental chemicals and have great public health concerns. Large amounts of chlorinated organic compounds are produced and used in various industrial, agricultural and military

applications. As a consequence, chlorinated organic compounds have become the most common groundwater contaminants. Contaminants found in groundwater supplies are mainly volatile organic compounds. In particular, PCE and TCE are among the most abundant contaminants in subsurface environments. Due to their excellent solvent properties, PCE and TCE are widely used for dry cleaning, metal degreasing, textile finishing, dyeing and extraction processes. As a result, massive and continuous production and improper handling of chlorinated organic compounds have lead to serious contamination in the environment. The estimated annual industrial PCE production in 1994 was 246 million pounds (91). The production rates of TCE were an estimated 200 to 400 million pounds per year as of 1995 (110). All chlorinated ethenes are a threat to human health and VC is a proven carcinogen. VC contamination of groundwater results mainly from microbial reduction of PCE and TCE under anaerobic conditions (9). However, high dissolved concentrations of VC have been reported as the result of releases from PVC manufacturing operations (42). From 1987 to 1993, according to the EPA's Toxic Release Inventory, VC releases to water and land totaled over 38,000 pounds. Due to the negative health effect of chlorinated ethenes, United States Congress established the Superfund Program in 1980 to clean up many of chlorinated solvent contaminated sites (Superfund Sites). One recent EPA survey showed that about 50% of the National Priorities List (NPL) sites (http://www.epa.gov/superfund/sites/npl) were contaminated by chlorinated ethenes (e.g., PCE, TCE, DCEs, and VC). A total of 1,240 hazardous waste sites are listed on the NPL as of March 8, 2004.

2.3. Microbial oxidation of chlorinated ethenes under aerobic conditions

Oxidation is one important process in the biodegradation of both chlorinated aliphatic and aromatic compounds. Co-metabolic oxidation of chlorinated alkenes, short chain alkanes and some chloroaromatic compounds is catalyzed by monooxygenases and dioxygenases enzyme systems (24, 29, 32, 89). Dioxygenases incorporate both oxygen atoms from molecular oxygen

into the product while monooxygenases incorporate only one oxygen atom into the product and reduce the other to water. For each of these processes, the responsible microorganisms contain non-specific oxygenases that fortuitously oxidize chlorinated organic compounds to CO_2. This fortuitous transformation of chlorinated organic compounds by a microorganism that is unable to use chlorinated organic compounds as a source of energy is called cometabolism.

In a recent study, PCE degradation by *Pseudomonas stutzeri* strain OX1 under aerobic conditions was described. This study confirmed that a monooxygenase (toluene-*o*-xylene monooxygenase, ToMO) was involved in cometabolic degradation of PCE and TCE (90). Strain OX1 was isolated from activated sludge and grew on *o*-xylene, toluene, cresols, 2,3-dimethylphenol, and 3,4-dimethylphenols as sole carbon and energy sources. ToMO has a relaxed regiospecificity and a broad substrate range, and it oxidizes *o*-xylene, *m*-xylene, *p*-xylene, toluene, benzene, ethylbenzene, styrene, and naphthalene. This study assumes that oxidative PCE degradation is analogous to TCE, DCE, or VC oxidative degradation and involves a PCE-epoxide. Oxygenase-mediated TCE degradation has been studied in great detail and several pure cultures of methanotrophic bacteria have been obtained (63, 88, 103) but all isolated methanotrophic bacteria did not gain energy from TCE oxidation. Hence, cometabolic oxidation of chlorinated ethenes does not supply energy for microbial growth or metabolism. However, the requirements of primary substrates (e.g., toluene, phenol, methane, etc) and oxygen for the growth of bacteria cometabolizing chloroethenes hamper the application of this process in bioremediation are somewhat of a disadvantage.

A number of studies have demonstrated that microorganisms can metabolically degrade *cis*-DCE or VC under aerobic conditions. In contrast to co-metabolic PCE and TCE oxidative degradation, several recent investigations suggested that microorganism oxidize DCE or VC to CO_2 in the absence of a primary substrate in mixed and pure cultures. One pure culture, strain JS666, a member of the family *Comamonadaceae* in the β-*proteobacteria*, was isolated from a chloroethene contaminated groundwater processing pump-and-treat plant (22). Strain JS666 was

first aerobic bacterium that grew with *cis*-DCE as the sole source of carbon and energy. Several organisms have been isolated that grow on VC as a primary substrate. Organisms isolated to date that can grow on VC include *Mycobacterium aurum* strain L1 (1, 43, 44) and *Pseudomonas aeruginosa* strain MF1 (107). As the least chlorinated of the chloroethenes, VC has the greatest tendency to undergo oxidation. However, addition of oxygen to stimulate aerobic populations that can degrade chlorinated ethenes in an anaerobic aquifer system is expensive and in most cases impractical. Consequently, aerobic biodegradation of chloroethene contaminants in groundwater systems is limited to the edge of the contaminant plume, where dissolved oxygen has not been depleted by microbial respiration (10, 13).

2.4. Microbial oxidation of chlorinated ethenes under anaerobic conditions

Anaerobic *cis*-DCE and VC oxidation has been demonstrated under different electron accepting conditions including, Fe (III)-reducing, sulfate reducing, Mn (IV)-reducing and methanogenic conditions (10-12, 14, 45). Bradley and Chapelle (11) reported that DCE and VC can be mineralized under Fe (III)-reducing conditions, and that the bioavailability of Fe (III) is an important factor affecting the rates of anaerobic DCE and VC mineralization. The rate and magnitude of DCE oxidation did not differ significantly between Fe (III)-, sulfate-reducing and methanogenic conditions. The rate of VC oxidation was greater than the oxidation rate of DCE under each of the electron-accepting conditions. A subsequent investigation demonstrated that aquifer microorganisms can anaerobically oxidize DCE to CO_2 under Mn (IV)-reducing conditions (14). In general, the potential for biodegradation of highly reduced groundwater contaminants is greatest under aerobic conditions and least under methanogenic conditions. Hence, oxidative degradation of lower chlorinated ethenes (DCE or VC) can occur under anaerobic conditions (9). One recent study (45) showed that a Fe (III)-reducing enrichment culture was capable of anaerobic oxidation of *cis* DCE and VC, but the rate of *cis*-DCE degradation was 3.5 times higher than that of VC in the presence of glucose. These observations

indicate that oxidation of VC can occur under anaerobic conditions if a sufficiently strong oxidant is available to drive microbial degradation. Fe(III)-oxides are strong oxidants and are abundant in groundwater systems. An experiment conducted with addition of Fe (III)-oxides to anaerobic microcosms resulted in VC oxidation rates comparable to those observed under aerobic conditions. More importantly, microbial oxidation of VC under Fe (III)-reducing conditions provides a potential anaerobic alternative to the reductive dechlorination of VC to ethene. Given the potential environmental benefits of coupled anaerobic reductive and anaerobic oxidative biodegradation, the extent to which these processes co-occur in contaminated groundwater systems and identification of the responsible organisms merit further investigation.

2.5. Microbial reductive dechlorination of chlorinated ethenes under anaerobic conditions

Reductive dechlorination is the replacement of a chlorine substituent by hydrogen in the presence of a suitable electron donor and a catalyst. Highly chlorinated compounds such as PCE and TCE are generally resistant to oxygenolytic and hydrolytic dechlorination, but can be reductively dechlorinated. Due to the presence of the electronegative chlorine substituents, PCE and TCE are relatively oxidized and can act as electron acceptors. In contrast to cometabolic dechlorination, chlorinated ethene dechlorinating populations gain energy by coupling the oxidation of electron donors to the reduction of chlorinated compounds. This process is known as chlororespiration (also called dechlororespiration).

The first studies on the anaerobic degradation of chlorinated compounds concerned the reductive dechlorination of 3-chlorobenzoate to benzoate by *Desulfomonile tiedjei* strain DCB-1 (26, 95). It was first reported that the growth yield of strain DCB-1 increased stoichiometrically with the amount of 3-chlorobenzoate dechlorinated (25). In the early 1980s various reports described the microbial degradation of chloroalkanes in methanogenic cultures (8, 108, 109). The demonstrated prevalence of microbial reductive dechlorination dispelled the notion about the bio-recalcitrance of chloroethene contaminants in anaerobic environments. Considerable evidence

for reductive dechlorination of PCE, TCE, DCEs and VC has arisen from anaerobic microcosms, enrichment culture, and pure culture. However, dechlorination of chlorinated ethenes is often incomplete and frequently leads to the accumulation of *cis*-DCE and VC (79). VC accumulation is of most concern because VC is a human carcinogen. Hence, considerable efforts focused discovering microbes capable of complete reductive dechlorination of chlorinated ethenes to the benign product ethene. A few studies provided evidence that VC serves as a metabolic electron acceptor and that *Dehalococcoides* are involved in ethene formation (23, 28, 46, 82). Since many contaminated sites are anaerobic, stimulating reductive dechlorination is a promising strategy for bioremediation.

Reductive dechlorination processes can proceed metabolically or cometabolically. When no energy gained from reductive dechlorination, the process is cometabolic. Examples of cometabolism are the reductive dechlorination of PCE by sulfate reducers (21), methanogens (30, 54), or acetogens (101). Compared to chlororespiration, rates of cometabolic reductive dechlorination processes are low and contributions to the overall reductive dechlorination are probably negligible in natural environments. Hence, respiratory dechlorinators using chlorinated ethenes as electron acceptors in their energy metabolism, and hence, for growth, are likely to be major contributors to reductive dechlorination reactions in anaerobic environments. Even though isolates that dechlorinate PCE to TCE or *cis*-DCE were obtained from various sites (e.g., contaminated sites and pristine sites) and show remarkable differences in their substrate specificity, the level of phylogenetic diversity is surprisingly low compared to the physiological diversity. Most of the PCE-dechlorinating populations belong to different groups of the *Proteobacteria* and the low G+C content gram-positive bacteria including *Dehalobacter restrictus* strain PER-K23 (51), *D. restrictus* strain TEA (111), *Sulfurospirillum multivorans* (formerly *Dehalospirillum multivorans,* (74, 92), *S. halorespirans* strain PCE-M2 (74), *Desulfitobacterium hafniense* strain PCE-S (formerly *Desulfitobacterium frappieri* strain PCE-S, (80), *Desulfitobacterium hafniense* strain TCE1 (formerly *Desulfitobacterium frappieri* strain

TCE1, (35), *Desulfitobacterium* sp. strain PCE1 (36), *Desulfitobacterium* sp. strain Viet1 (65), *Desulfitobacterium metallireducens* (33), *Desulfitobacterium* sp. strain Y51 (98), *Desulfuromonas chloroethenica* strain TT4B (60, 61), *Desulfuromonas michiganensis* strains BB1 and BRS1 (97), *Clostridium bifermentans* strain DPH-1 (17), and *Enterobacter* sp. strain MS-1 (93).

A new, distinct group of dechlorinators, the *Dehalococcoides,* was discovered only recently. The first isolated *Dehalococcoides* species, *Dehalococcoides ethenogenes* strain 195, has been shown to completely degrade PCE to ethene (77). Strain 195 grows with PCE, TCE, *cis*-DCE, 1,1-DCE or 1,2-dichloroethane, but VC and *trans*-DCE do not support growth. VC is dechlorinated cometabolically to ethene at low rates by strain 195 (78). Another *Dehalococcoides* species, strain FL2, was isolated. Strain FL2 dechlorinates TCE, *cis*-DCE, and *trans*-DCE mainly to VC and ethene (48). Similar to strain 195, the final dechlorination step to ethene occurs at a very low rate (48). The first VC-dechlorinating *Dehalococcoides* species, strain BAV1, was isolated from a chlorinated ethene contaminated site. Strain BAV1 grew with VC as electron acceptor, and efficiently reduced VC to ethene. Besides VC, strain BAV1 dechlorinates *cis*-DCE, *trans*-DCE, 1,1-DCE, 1,2-dichloroethane, and vinyl bromide, and uses these compounds as growth supporting electron acceptors (47). Instead of utilizing chlorinated ethenes, *Dehalococcoides* sp. strain CBDB1 reductively dechlorinated chlorinated benzenes and polychlorinated dibenzodioxins (4, 16). These unusual bacteria are most closely affiliated with the Chloroflexi. *Dehalococcoides* species have an extremely narrow substrate range, and can only be grown with chloroorganic compounds as electron acceptors and hydrogen as an electron donor. All laboratory mixed cultures that dechlorinate beyond DCEs and produce ethene have been found to involve *Dehalococcoides* populations (28, 46, 49, 82). None of the microorganisms isolated so far, however, is capable of dechlorinating PCE or TCE to ethene with each dechlorination step supporting growth. Recently, two TCE-to-ethene dechlorinating *Dehalococcoides* species were identified, however, only present in the mixed cultures (28, 82).

The continuous discovery of novel dechlorinators suggests a broad distribution of reductive dechlorination in anaerobic environments. The characterization of new isolates is needed to gain more insights into the physiology, and ecology of dechlorinating capabilities and to fully exploit the reductive dechlorination process for bioremediation applications.

2.6. Reductive dehalogenases

To understand the biochemical mechanisms of chlororespiration, efforts have been made to identify and characterize the key enzymes. Dechlorinating bacteria are able to grow while they use chlorinated ethenes as terminal electron acceptors, i.e., the reduction of chlorinated compounds by specific enzymes is coupled to energy conservation via electron transport phosphorylation. Reductive dehalogenases (RDases) are the final reductases and the key catalysts in the respiratory chain of chlororespiring microorganisms.

PCE RDases were characterized from *Sulfurospirillum multivorans* (83-85), *Dehalobacter restrictus* (76), *Desulfitobacterium* sp. strain PCE-S (80, 81), *Desulfitobacterium* sp. strain PCE1 (15), *Clostridium bifermentans* strain DPH-1 (87), and *Desulfitobacterium* sp. strain Y51 (99). Common to all PCE RDases investigated so far are an iron-sulfur cluster and a corrinoid cofactor, both of which are involved in electron transfer to the chlorinated substrate. During the course of the catalytic reactions, the cobalt of the corrinoid is subjected to a change in its redox state.

Two membrane-bound RDases, PCE-RDase and TCE-RDase have been characterized from *Dehalococcoides ethenogenes* strain 195. The gene encoding TCE-RDase gene, *tceA,* involves in the complete dechlorination of TCE to ethene via *cis*-DCE and VC (75). A VC RDase gene was partially purified from *Dehalococcoides* sp. strain VS. The corresponding gene, *vcrA,* codes for a protein that reduces VC and all DCE isomers but not PCE or TCE (82). One recently discovered putative a VC RDase gene, designated *bvcA*, has been obtained from strain BAV1 and is likely involved in complete detoxification of VC (59).

It is important to note that almost all known RDases are purified from hydrogenotrophic dechlorinators. No RDase has ever been identified and characterized from acetate utilizing dechlorinating populations. Knowledge of hydrogen dependent reductive dechlorination alone could not provide a complete understanding of chlororespiration. Even though acetate is one of the most important substrates in anaerobic environments, less attention was given to the isolation and characterization of acetate-oxidizing dechlorinating populations. Hence, efforts should be made to gain insights into their physiology and ecology as well as to better understand their contributions to the reductive dechlorination processes.

2.7. Reductive dechlorination in the presence of other terminal electron acceptors

The distribution of terminal electron accepting processes under steady state conditions is often explained on a thermodynamic basis where microorganisms preferentially utilize the terminal electron acceptor yielding the most energy first (denitrification > Mn(IV) reduction > Fe(III) reduction > sulfate reduction > methanogenesis > acetogenesis). Anaerobic respiratory bacteria compete for suitable electron donors such as hydrogen and organic substrates such as acetate, and different terminal electron accepting processes have different electron donor threshold concentrations below which the terminal electron accepting process is no longer energetically favorable (66-69). The reduction of nitrate to gaseous N-compounds (e.g., denitrification) is energetically more favorable than reductive dechlorination. In contrast, acetogenic or methanogenic reduction of CO_2 to acetate or methane, respectively, is energetically less favorable than reductive dechlorination. Consequently, chloroethene dechlorinating populations should out-compete acetogenic and methanogenic populations, at least under electron donor-limiting conditions. Studies have described the influence of alternate electron acceptors (e.g., nitrate and sulfate) on reductive dechlorination (27, 31, 35, 102). Little, however, is known as to how chloroethene dechlorinating populations compete with sulfate-reducing bacteria for reducing equivalents. In addition, the effect of sulfide, the product of sulfate reduction, on

chloridogenic populations and their activity has not been explored. Other than nitrate and sulfate, the effect of Fe(III) reduction on dechlorination activity has been investigated for several PCE dechlorinating pure cultures. Interestingly, acetate-oxidizing, PCE-reducing dechlorinator (e.g., *Desulfuromonas michiganensis* strain BB1) reduces PCE and Fe(III) simultaneously. Understanding the effects of alternative electron accepting processes are important to predict and control dechlorination processes at field sites that contain other electron acceptors.

2.8. Metal and radionuclide contamination in environments

Metals and radionuclides are another group of environmental groundwater contaminants of concern. The total amount of natural uranium an Earth stays almost the same because it is an element, but radionuclides can move from place to place through natural processes or by human activities. Rain can wash uranium into lakes and rivers. Mining, milling, manufacturing, and other human activities also release uranium into the environment. Uranium-235 was used in nuclear weapons production and in nuclear reactors. Depleted uranium is used to make ammunition for military purposes, guidance devices and compasses, radiation shielding material, and X-ray targets (6, 106). For more than 50 years, the U.S. Department of Energy (DOE) has used uranium as a source of nuclear energy for both public and military purposes (105). It was estimated that more than 80% of DOE sites have radionuclide contamination (104). Obviously, metals and radionuclides cannot be biodegraded. However, microorganisms can interact with these contaminants and change their oxidation state through adding or removing electrons. The EPA has established an MCL of 30 µg/L for uranium in drinking water (http://www.epa.gov/safewater/mcl.html).

2.9. Microbial reduction of metal and radionuclide

Metals are commonly found in association with organic contaminants such as, chlorinated solvents and petroleum. Metal pollutants can be inadvertently released during the

manufacture of various industrial products, ranging from steel to computer components. As with radionuclides, metals cannot be degraded but they can be transformed through sorption, methylation, complexation, and change of valence states. At low concentrations, many metals are vital to life processes, often serving important functions in enzymes. However, above certain concentrations, metals can become toxic to microorganisms and to higher species. Heavy metal toxicity can result in damaged or reduced mental and central nervous function, lower energy levels, and damage to blood composition, lungs, kidneys, liver, and other vital organs. Microorganisms can alter the reactivity and mobility of metals, and thus facilitate the use of bioremediation as a form of treatment for metal contaminated environments.

A phylogenetically diverse group of bacteria and archaea is known to conserve energy to support growth by oxidizing hydrogen or organic compounds with the reduction of Fe(III). This group includes species from genera such as *Geobacter*, *Desulfuromonas, Pelobacter, Shewanella, Ferrimonas, Geovibrio, Geothrix* and others (reviewed in (70)). Among them, *Geobacter* species have been intensively studied because they completely oxidize organic compounds to carbon dioxide with Fe(III) serving as a terminal electron acceptor. In the same family, *Desulfuromonas michiganensis* strain BB1 has been reported to reduce simultaneously both PCE and Fe(III) as electron acceptors (97). Hence, *Geobacter* sp. and *Desulfuromonas* sp. are of obvious importance for developing strategies of bioremediation of organic contaminants as well as metals.

Initially, uranium reduction has been thought to be dominated by abiotic reactions such as reduction by sulfide or Fe(II) (34, 52, 55, 62). However, U(VI) reduction can also be catalyzed by Fe(III) reducers (5, 64, 72) and sulfate reducing microorganisms (18, 71, 73, 100). Members of the *Geobacter* genus are of importance as they have been found in a wide variety of anaerobic environments and molecular studies suggested that they are dominant members of Fe(III)-reducing microbial communities in subsurface environments (20, 96). Many dissimilatory metal reducers, especially *Geobacter* sp., can reduce U(VI) to insoluble U(IV) preventing the migration of uranium in groundwater by precipitation and immobilization in the form of UO_2 (uraninite)

(37). An early study established the growth-linked reduction of U(VI) to U(IV) by *G. metallireducens* strain GS-15 and *G. sulfurreducens* strain PCA (64, 72). Including strains GS-15 and PCA, several *Geobacter* species have been shown to have the ability to reduce U(VI) to U(IV). For example, whole-cell suspension of *Geobacter hydrogenophilus* stain H-2 and *Geobacter chapellei* strain 172 reduce U(VI), although it is not clear whether this type of metabolism can supply energy for growth (19). One potential strategy to immobilize uranium in the subsurface is to stimulate the activity of dissimilatory metal-reducing populations. For example, the addition of electron donor, acetate, to uranium contaminated subsurface sediments stimulated the growth of Fe (III) reducing populations, which reduced U(VI) to U(IV) effectively (5, 86, 94). Therefore, acetate addition to sediments contaminated with hexavalent uranium is likely to stimulate the growth and activity of known U(VI) reducing organisms, including *Geobacter* species.

Groundwater at uranium-contaminated sites often contains high concentrations of NO_3^- and SO_4^{2-} (1, 2). In the presence of NO_3^-, uranium reduction does not occur and removal of NO_3^- is a prerequisite for in situ uranium reduction (94). In addition to NO_3^- and SO_4^{2-}, U(VI) contaminated sites are also co-contaminated with organic solvents, such as PCE, TCE and PCBs (http://www.esd.ornl.gov/nabirfrc). However, little is known about the impact on uranium reduction by the presence of chlorinated electron acceptors.

2.10. References

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CHAPTER III

Environmental Distribution of Chloroethene-Dechlorinating Populations and Quantification of *Dehalococcoides*-Derived Reductive Dehalogenase Genes

Abstract

Sediment-free, non-methanogenic, ethene-producing enrichment cultures were derived from three chloroethene contaminated and two non-contaminated sites. 16S rRNA gene targeted PCR analysis revealed that *Dehalococcoides* populations existed in all ethene producing enrichment cultures indicating their involvement in complete reductive dechlorination of chlorinated ethenes to ethene. In addition to *Dehalococcoides* populations, tetrachloroethene (PCE) to 1,2-*cis*-dichloroethene (*cis*-DCE) dechlorinators were also detected in ethene producing enrichment cultures using 16S rRNA gene targeted PCR approaches. The presence of these partial dechlorinators as well as the dechlorination performance of the cultures in the absence of hydrogen and in the presence of ampicillin suggested that multiple dechlorinators coexisted and were involved in complete reductive dechlorination. Kinetic studies and qualitative/quantitative PCR approaches targeting the trichloroethene (TCE)-reductase (RDase) gene *tceA* and the vinyl chloride (VC)-RDase genes *vcrA* and *bvcA* showed that (i) *tceA, bvcA*, and *vcrA* were heterogeneously distributed in ethene producing cultures, (ii) *bvcA* and/or *vcrA* were present when complete reductive dechlorination of VC occurred, (iii) *vcrA* was detected more frequently than *bvcA* in ethene producing cultures, and (iv) unidentified RDase gene(s) existed in many ethene producing cultures.

3.1. Introduction

Chlorinated solvents like tetrachloroethene (PCE) and trichloroethene (TCE) are commonly used in dry cleaning operations, as degreasing solvents in many industrial applications, and as an ingredient in the manufacturing of other chemicals (11). As a consequence of extensive usage, improper disposal, and careless handling, chlorinated ethenes are among the most common contaminants observed in groundwater systems (1). Most chlorinated ethenes are suspected carcinogens among them, vinyl chloride (VC) is a known human carcinogen and generally considered to be a threat to human health (37). Although there are natural mechanisms for the formation of VC (18), VC contamination of groundwater results primarily from in complete microbial reductive dechlorination of poly-chlorinated ethenes at contaminated sites (12).

Because of adverse health effects and frequent occurrence of chlorinated ethenes in subsurface systems, the effects of microbial processes on the environmental fate of chloroethenes in groundwater have been intensively studied. The discovery of the microbial reductive dechlorination process revolutionized remediation strategies in many chloroethene contaminated environments. Laboratory and field evidences support that microorganisms can transform chlorinated ethenes to nontoxic products, ethene or ethane, under a variety of environmental conditions (2-8, 14, 15, 20, 25-27, 32). A relatively large number of organisms belonging to phylogentically diverse groups of bacterial genera are involved in partial reductive dechlorination of PCE to TCE and 1,2-*cis*-dichloroethene (*cis*-DCE) (33). The partial dechlorinators include *Dehalobacter*, *Desulfuromonas*, *Geobacter*, *Sulfurospirillum*, and *Desulfitobacterium* species (33, 35).

Reductive dechlorination past *cis*-DCE to VC and ethene has been exclusively linked to the members of *Dehalococcoides* cluster (7, 14, 15, 26, 27, 34). Because of the significance of *Dehalococcoides* in the reductive dechlorination process, recent efforts focused on the isolation of *Dehalococcoides* populations and the characterization of their functional genes (i.e., RDase

genes) involved in reductive dechlorination, especially the final reduction step of VC to ethene. The TCE-RDase gene, *tceA*, has been identified in the metabolic reductive dechlorination of TCE to VC in strains 195 and FL2 (15, 23) but not in strains BAV1, KB-1/VC-H_2, or VS. Recently, two putative VC-RDase genes, *bvcA* and *vcrA,* were identified from strains BAV1 and VS, respectively (19, 27). Despite the frequent detection of *Dehalococcoides* populations in many contaminated sites utilizing 16S rRNA gene-based approaches (16, 20, 25), little is known about the distribution of *Dehalococcoides*-derived RDase genes (19, 27), including *bvcA* and *vcrA*. The identification of *bvcA* and *vcrA* allows to study the environmental distribution of VC-respiring *Dehalococcoides* populations at contaminated and non-contaminated sites.

Hence, the objectives of this study were to (i) establish microcosms and develop enrichment cultures capable of complete reductive dechlorination of PCE to ethene from both contaminated and non-contaminated sites, (ii) evaluate the distribution and role of various chlorinated ethene dechlorinating populations in these cultures including partial dechlorinators, and (iii) analyze the distribution of the *Dehalococcoides* RDase genes (*tceA*, *bvcA*, and *vcrA*) with both qualitative and quantitative PCR approaches in ethene producing enrichment cultures derived from these sites.

3.2. Materials and Methods

3.2.1. Chemicals

PCE and TCE were purchased from Sigma-Aldrich Co. (St. Louis, MO). *cis*-DCE was obtained from Supelco Co. (Bellefonte, PA). Gaseous VC was obtained from Fluka Chemical Corp. (Ronkinkoma, NY), and ethene was purchased from Scott Specialty Gases (Durham, NC). All of the other chemicals used were reagent grade or better unless otherwise specified.

3.2.2. Sample collection

Samples were collected from chloroethene contaminated site aquifers and non-contaminated aquatic sediments. Sample sources are shown in Table 3.1 and 3.2. for

contaminated and non-contaminated sites, respectively. Individual samples were homogenized manually within an anaerobic glove box (Coy, Ann Arbor, MI) and kept at 4°C until use.

3.2.3. Microcosms and enrichment cultures

Microcosms were established inside an anaerobic glove box with a mixture of 95% N_2 and 5% H_2 [vol/vol] and were set up as either 20-ml vials containing ~2 g of sample and 8 ml of sterile, anoxic, reduced mineral salts medium (36) or 160-ml (nominal volume) serum bottles (Wheaton Co. Millville, NJ) containing ~20 g of aquifer or sediment material and 80 ml of medium. Microcosms were sealed with butyl-rubber stoppers (Geo-Microbial Technologies, Inc., Ochelata, OK) or Teflon-lined rubber stoppers (West Pharmaceutical Services, Lionville, PA) and aluminum crimp seals (Wheaton Co., Millville, NJ). Microcosms were amended with H_2 (40 µmol for 20 ml vial, 0.4 mmol for 160 ml bottle) plus 5mM acetate or 5mM lactate. Sterile H_2 was added via syringe. PCE-, TCE-, *cis*-DCE-, and VC-amended microcosms were established with contaminated aquifer materials, while only PCE-amended microcosms were established with non-contaminated sediment. Liquid chloroethenes were dissolved in hexadecane (100 µl total volume was added to 20 ml vials, 200 µl total volume was added to 160 ml serum bottles), resulting in final aqueous chlorinated ethene concentrations of 0.1 mM (see appendix for calculation). Gaseous VC was added using sterile plastic syringes as a single dose of 80 µl to 20 ml vials or 600 µl to 160 ml bottles resulting in a final aqueous concentration of 0.1 mM.

Sediment-free, non-methanogenic cultures (referred to as enrichment cultures) were developed by consecutive transferring 3% [vol/vol] of aqueous culture from the ethene producing microcosms into 160 ml bottles containing 100 ml of medium. More than 30 sequential transfers of liquid culture were performed for each enrichment culture. Methanogenesis was completely inhibited by amendment of 2 mM 2-bromoethanesulfonate during the second sequential transfer

Table 3.1. Characteristics of contaminated aquifer samples and microcosm test results

Sample designation	Sample location	Reported contaminants[a]	Date of collection	Dechlorination end products within microcosms enriched with[b]			
				PCE	TCE	*cis*-DCE	VC
Long Branch (MS-1, 12-14')[c]	Manufactured Gas Plant, Long Branch, NJ	PCE, TCE	11/2000	*cis*-DCE	*cis*-DCE	-	-
Hydrite 1 (OW 63, 20-22')	Hydrite Chemical Co., WI	PCE, TCE, *cis*-DCE	12/2000	*cis*-DCE	*cis*-DCE	-	-
Hydrite 5 (P 19, 40-42')	Hydrite Chemical Co., WI	*cis*-DCE, VC	12/2000	ETH	ETH	ETH	ETH
Hydrite 7 (P 19, 48-50')	Hydrite Chemical Co., WI	*cis*-DCE, VC	12/2000	ETH	ETH	ETH	ETH
Hydrite 8 (P 53, 74-76')	Hydrite Chemical Co., WI	*cis*-DCE, VC	12/2000	-	-	-	ETH
Hydrite 10 (P 53, 80-82')	Hydrite Chemical Co., WI	*cis*-DCE, VC	12/2000	-	ETH	ETH	ETH
FMC (M-2, 20-21')	FMC Corp., CA	TCE	3/2002	ETH	ETH	ETH	ETH
TRW Minerva (CSB-12, 10-10.5')	TRW, OH	TCE	5/2001	ETH	ETH	ETH	ETH

-; no dechlorination observed after 90 days of incubation

[a]; major chloroethene contaminants reported to be present at the site

[b]; all microcosms were amended with H_2 plus acetate or lactate as electron donor and carbon source, respectively. End products were determined after 90 days of incubation.

[c]; (actual designated site sampling well, depth below ground surface in feet)

Table 3.2. Characteristics of non-contaminated aquifer samples and microcosm test results

Sample designation	Sample location	Date of collection	Dechlorination end products within microcosms amended with PCE[a]
Kalamazoo River	MI, USA	7/2002	ETH
Su-Zi Creek	South Korea	6/2002	ETH
Anaerobic Digestor	South Korea	6/2002	*cis*-DCE
Gap Creek	South Korea	6/2002	*cis*-DCE
Yu-Seoung Creek	South Korea	6/2002	*cis*-DCE
Rice Paddy	Japan	7/2002	*cis*-DCE
Ute Reservoir	Germany	7/2002	*cis*-DCE
Key Largo	FL, USA	8/2002	*cis*-DCE

[a]; all microcosms were amended with H_2 plus acetate or lactate as electron donor and carbon source, respectively. End products were determined after 90 days of incubation.

(21). All dechlorinating enrichment cultures were amended with 5 mM acetate and had a H_2/CO_2 (80%/20% [vol/vol]) headspace and the same electron acceptor that was used in the original microcosm. All enrichment cultures were amended with electron acceptor concentrations ranging from 0.1 mM to 0.33 mM PCE, 0.32 mM TCE, 0.6 mM *cis*-DCE, and 0.55 mM VC. All cultures were incubated in an inverted position at room temperature without agitation in the dark.

3.2.4. Ampicillin treatment

All dechlorinating enrichment cultures were amended with ampicillin, a cell wall biosynthesis inhibitor that apparently does not affect *Dehalococcoides* species (14, 15, 26). A filter sterilized, aqueous anoxic stock solution (100 mg/ml) of ampicillin was added to a final concentration of 100 mg to 100 ml media before inoculation. Dechlorination activity of PCE was determined after 60 days of incubation for duplicate cultures compared with duplicate control cultures that did not receive ampicillin.

3.2.5. Qualitative PCR

Genomic DNA from chlorinated ethene degrading enrichment cultures was extracted by using the Qiagen Mini Kit (Qiagen, Valencia, CA). Group-specific 16S rRNA gene targeted primers were used to detect chloroethene-degrading populations from enrichment cultures. The presence of *Dehalococcoides*, *Desulfuromonas*, *Dehalobacter, Geobacter*, *Sulfurospirillum*, and *Desulfitobacterium* groups was determined by using 16S rRNA gene targeted specific primer pairs (Table 3.3.). For nested PCR, the initial amplification was performed with a pair of universal bacterial primers and then followed by a second round of PCR using specific primers (22). The presence of the TCE RDase gene (*tceA*) and the VC RDase genes (*bvcA* and *vcrA*) was also determined using primers specifically amplifying the RDase genes (see Table 3.3.).

All PCR amplification reactions were performed in a total volume of 20 µl. The reaction mixture contained 2 µl of 10 X reaction buffer (Applied Biosystems, Foster City, CA), 2 mM $MgCl_2$ (Applied Biosystems, Foster City, CA), 0.13 mg of bovine serum albumin/ml (Promega, Madison, WI), 200 µM concentrations of each deoxynucleoside triphosphate (Applied

Biosystems, Foster City, CA), 2.5 U of AmpliTaq polymerase (Gibco BRL, Gaithersburg, MD), 100 nM concentration of each primer, and 1 µl of template DNA (10-40 ng). Different concentrations of $MgCl_2$ were applied in PCR reactions targeting *Sulfurospirillum* (1.5 mM) and *Desulfitobacterium* (2.5 mM). Amplification was carried out in a 9700 GeneAmp PCR system (Applied Biosystems, Foster City, CA). Aliquots (3 to 5 µl) of PCR products were resolved in 1% (wt/vol) agarose gels in Tris-acetate-EDTA buffer (40 mM Tris, 1 mM EDTA, 20 mM acetic acid, pH 8.3) and stained in aqueous ethidium bromide solution (0.5 µg/ml) for 30 min. After destaining with water, the bands were visualized and visualized under UV light.

3.2.6. Quantitative Real-Time PCR (RTm PCR)

RTm PCR was applied to enumerate the total number of bacteria cells, the number of *Dehalococcoides* cells, and the number of *Dehalococcoides* derived RDase genes (*tceA, bvcA*, and *vcrA*) in selected PCE dechlorinating cultures. The following primers and probes were used (13, 29): for bacterial 16S rRNA gene, 5'- ATG GYT GTC GTC AGC T-3' (forward), 5'-ACG GGC GGT GTG TAC-3' (reverse) and 5'-FAM-CAA CGA GCG CAA CCC-TAMRA (probe); for *Dehalococcoides* 16S rRNA gene, 5'-CTG GAG CTA ATC CCC AAA GCT-3' (forward primer), 5'-CAA CTT CAT GCA GGC GGG-3' (reverse primer), and 5'-FAM-TCC TCA GTT CGG ATT GCA GGC TGA A-TAMRA (probe); for *tceA*, 5'-ATC CAG ATT ATG ACC CTG GTG AA-3' (forward primer), 5'-GCG GCA TAT ATT AGG GCA TCT T-3' (reverse primer), and 5'-FAM-TGG GCT ATG GCG ACC GCA GG-TAMRA (probe); for *bvcA*, 5'-AAA AGC ACT TGG CTA TCA AGG AC-3' (forward primer), 5'-CCA AAA GCA CCA CCA GGT C-3' (reverse primer), and 5'-FAM-TGG TGG CGA CGT GGC TAT GTG G-TAMRA (probe); for *vcrA*, 5'- CGG GCG GAT GCA CTA TTT T-3' (forward primer), 5'- GAA TAG TCC GTG CCC TTC CTC-3' (reverse primer), and 5'-FAM- CGC AGT AAC TCA ACC ATT TCC TGG TAG TGG-TAMRA (probe). Each MicroAmp optical tube had a 30 µl total reaction volume containing 1×TaqMan Universal PCR Master Mix (Applied Biosystems), forward primer (300 nM), reverse primer (300 nM), probe (300 nM), and DNA template. PCR cycle parameters were

as follows: 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 58°C (52°C for bacterial 16S rRNA gene targeting). PCR was performed in a spectrofluorimetric thermal cycler (ABI Prism 7700 Sequence Detection System, Applied Biosystems). A calibration curve (log DNA concentration versus a set cycle threshold value, C_T) was obtained using 10-fold serial dilutions of pure culture genomic DNA, or plasmid DNA carrying a cloned 16S rRNA gene, *bvcA*, *tceA*, or *vcrA* of *Dehalococcoides* sp. strain BAV1, strain FL2, and strain GT (see chapter V).

3.2.7. Analytical methods

Chloroethenes were quantified as described previously (36). Headspace samples (100 µl) were withdrawn with gastight 250 µl glass syringes (model, 1725, Hamilton Co., Reno, NV) and manually injected into a split injector operated at a split ratio of 2:1. Chlorinated ethene concentrations are reported as total mass per bottle (100 ml media containing 160 ml serum bottle).

Table 3.3. Sequences of 16S rRNA gene and *Dehalococcoides* derived RDase gene targeted primers and PCR conditions

	Forward primer (5'→3')	Reverse primer (5'→3')	Annealing temperature (°C)	Reference
Target species				
Dehalococcoides	GCGGTTTTCTAGGTTGTC	CACCTTGCTGATATGCGG	58	(22)
Desulfuromonas	AACCTTCGGGTCCTACTGTC	GCCGAACTGACCCCTATGTT	58	(22)
Dehalobacter	TGTATTGTCCGAGAGGCA	ACTCCCATATCTCTACGG	53	(30)
Geobacter	GAATATGCTCCTGATTC	ACCCTCTACTTTCATAG	53	(35)
Sulfurospirillum	GCTCTCGAAACTGGTTACCTA	GTATCGCGTCTCTTTGTCCTA	55	(9)
Desulfitobacterium	AATACCGN[a]ATAAGCTTATCCC	TAGCGATTCCGACTTCATGTTC	55	(10)
Universal Bacteria	AGAGTTTGATCCTGGCTCAG	ACGGTTACCTTGTTACGACTT	58	(22)
Targeted RDase gene				
tceA	ACGCCAAAGTGCGAAAAGC	TAATCTATTCCATCCTTTCTC	50	(23)
bvcA	TGCCTCAAGTACAGGTGG	ATTGTGGAGGACCTACCT	51	(19)
vcrA	TGCTGGTGGCGTTGGTGCTCT	TGCCCGTCAAAAGTGGTAAAG	55	(27)

[a]; N = A/C/T/G

The following thermocycling program was used for *Dehalococcoides, Desulfuromonas, Dehalobacter,* and *Geobacter* targeted, as well as the universal primers: 94 ºC for 2.2 min (1 cycle); 94 ºC for 30 sec, annealing temperature for 45 sec, and 72 ºC for 2.2 min (30 cycles); 72 ºC for 6 min (1 cycle).

The following thermocycling program was used for *Sulfurospirillum*: 95ºC for 2 min (1 cycle); 94 ºC for 30 sec, annealing temperature for 30 sec, and 72 ºC for 30 sec (30 cycles); 72 ºC for 7 min (1 cycle).

The following thermocycling program was used for *Desulfitobacterium*: 94ºC for 2.17 min (1 cycle); 94 ºC for 30 sec, annealing temperature for 30 sec, and 72 ºC for 1 min (30 cycles); 72 ºC for 7 min (1 cycle).

The following thermocycling program was used for *tceA* and *bvcA*: 94 ºC for 2.2 min (1 cycle); 94 ºC for 30 sec, annealing temperature for 45 sec, and 72 ºC for 2.2 min (30 cycles); 72 ºC for 6 min (1 cycle).

The following thermocycling program was used for *vcrA*: 92 ºC for 3.0 min (1 cycle); 94 ºC for 0.45 min, annealing temperature for 1 min, and 72 ºC for 2.2 min (30 cycles); 72 ºC for 6 min (1 cycle).

3.3. Results

3.3.1. Microcosms and enrichment cultures

Microcosms were established with contaminated aquifer materials and non-contaminated aquatic sediment samples. The end products of microbial reductive dechlorination were monitored after 90 days of microcosm incubation, and the results are summarized in Tables 3.1 and 3.2. Complete reductive dechlorination of chlorinated ethenes to ethene was observed in microcosms derived from both contaminated aquifer materials (Hydrite, FMC, and Minerva) and non-contaminated river and Creek sediments (Kalamozoo River and Su-Zi Creek). Although Hydrite 8 and 10 microcosms were developed from aquifer materials collected from different depths of the same sampling well (P 53, see Table 3.1.), different reductive dechlorination end products were obtained from these two sets of microcosms, suggesting a heterogeneous distribution of dechlorinating populations. Hydrite 10 microcosms reductively dechlorinated TCE, *cis*-DCE, and VC to ethene, but did not dechlorinate PCE. In the Hydrite 8 microcosms, VC was the only chloroethene to be reduced. Incomplete reductive dechlorination of PCE and TCE to *cis*-DCE was observed in Long Branch and Hydrite 1 microcosms. Table 3.2. describes end products of PCE dechlorination from non-contaminated sediment amended microcosms. Incomplete dechlorinating to *cis*-DCE was more commonly observed in microcosms developed from non-contaminated river sediment, while ethene was more commonly observed in microcosms developed from contaminated aquifer material.

Subsequent efforts focused on developing sediment-free enrichment cultures from PCE [Hydrite 7 (H7-PCE), Hydrite 5 (H5-PCE), Minerva (Min-PCE), FMC (FMC-PCE), Kalamazoo (Kal-PCE), and Su-Zi (S-PCE)], TCE [Hydrite 10 (H10-TCE)], and VC [Su-Zi (S-VC), Hydrite 10 (H10-VC), Hydrite 5 (H5-VC)] dechlorinating microcosms. All enrichment cultures continued to exhibit the same dechlorination activity as the original microcosms, even after more than 30 sequential transfers. For example, H7-PCE culture continued to dechlorinate PCE, TCE, *cis*-DCE and VC to ethene as depicted in Figure 3.1.

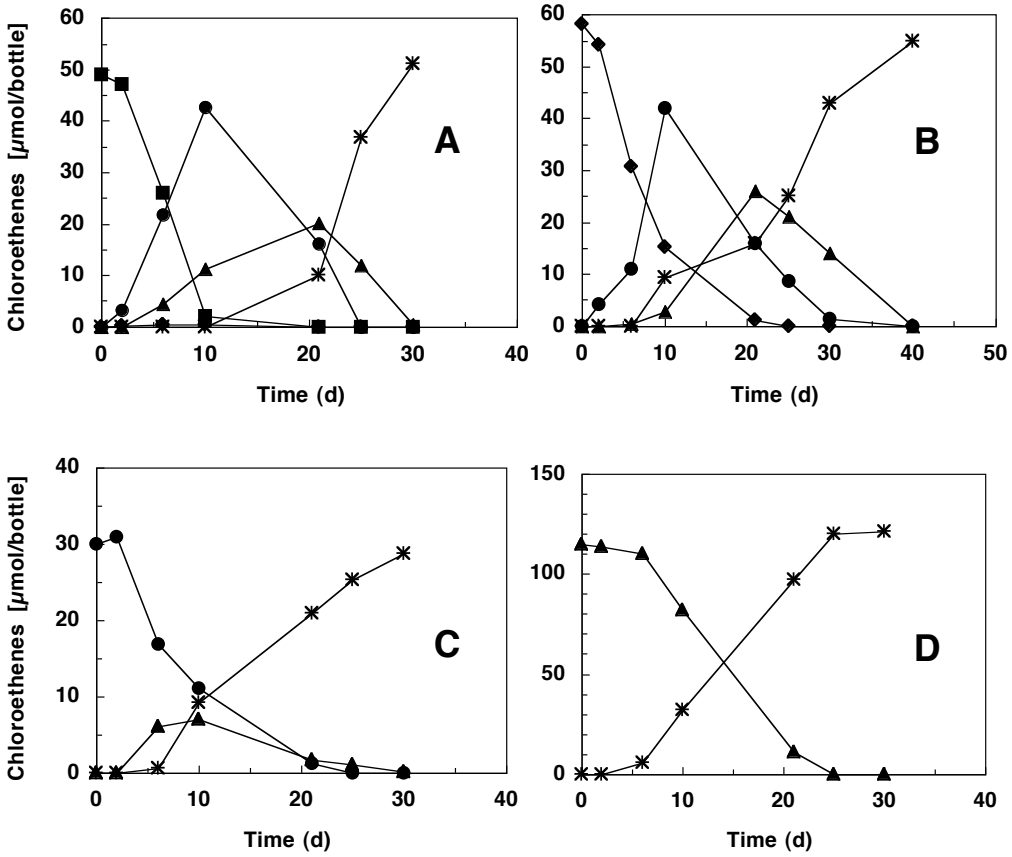

Figure 3.1. Reductive dechlorination of PCE (A), TCE (B), *cis*-DCE (C) and VC (D) to ethene by the H7-PCE culture amended with (5 mM) acetate and H_2. All data points were averaged from triplicate cultures. Symbols: ■; PCE, ◆; TCE, ●; *cis*-DCE, ▲; VC, ✳; ethene

3.3.2. Reductive dechlorination in the presence of ampicillin

The PCE dechlorinating enrichment cultures (H5-PCE, H7-PCE, Min-PCE, FMC-PCE, Kal-PCE, and S-PCE) did not possess ampicillin resistance and therefore no longer dechlorinated PCE, suggesting that the *Dehalococcoides* species present in these cultures did not contribute to PCE dechlorination (see Table 3.4.). However, the H10-TCE culture exhibited both complete reductive dechlorination and similar dechlorination kinetics in the presence of ampicillin as compared to control cultures suggesting that *Dehalococcoides* populations are involved in the complete reduction of TCE in this culture. Additionally, complete dechlorination of VC to ethene occurred in the presence of ampicillin in S-VC, H10-VC, and H5-VC (see Table 3.4.).

3.3.3. Reductive dechlorination of PCE in the absence of H_2

Since the known *Dehalococcoides* species are strictly hydrogenotrophic and are the only growth of organisms implicated in dechlorination past *cis*-DCE, all PCE dechlorinating cultures were amended with acetate (5 mM) as the sole electron donor and a N_2/CO_2 headspace to determine the role of acetotrophic dechlorinators in the complete reductive dechlorination of PCE. Dechlorination of PCE to *cis*-DCE was observed in H7-PCE and S-PCE cultures, while PCE was only dechlorinated to TCE in the Kal-PCE enrichment culture. No dechlorination of PCE was observed in H5-PCE, Min-PCE, or FMC-PCE in the absence of H_2 (see Table 3.4.).

3.3.4. Identification of chlorinated ethene degrading populations

Chlorinated ethene degrading populations were identified using specific 16S rRNA gene targeted PCR primer pairs tested on genomic DNA extracted from six PCE dechlorinating enrichment cultures, one TCE dechlorinating enrichment culture, and three VC dechlorinating cultures, as summarized in Table 3.4. *Dehalococcoides* species were detected from all ethene forming enrichment cultures with direct PCR. *Dehalococcoides* species, however, were not detected in Long Branch and H1 cultures exhibiting PCE-to-*cis*-DCE dechlorination (data not shown). *Dehalobacter* species were observed in H5-PCE and Min-PCE cultures. *Dehalobacter* was also detected in the H7-PCE culture during the early stages of enrichment (i.e., < 10 transfer)

Table 3.4. Distribution of chlorinated ethene dechlorinating populations, *Dehalococcoides* RDase gene, dechlorination end products in the presence of ampicillin, and the dechlorination end products in the absence of H_2 in ethene producing enrichment cultures

Enrichment cultures[a]	Dechlorination end product	Dechlorination end product in the presence of ampicillin	Dechlorination end product in the absence of H_2	16S rRNA gene targeted group						Chloroethene RDase gene targeted		
				Dhc	*Dsf*	*Dhb*	*Geo*	*Sulf*	*Dsb*	*tceA*	*bvcA*	*vcrA*
H7-PCE	**Ethene**	PCE	***cis*-DCE**	+	-	-	+	-	-	-	-	+
H5-PCE	**Ethene**	PCE	PCE	+	-	+	-	-	-	-	-	+
Min-PCE	**Ethene**	PCE	PCE	+	-	+	-	-	-	-	+	+
FMC-PCE	**Ethene**	PCE	PCE	+	-	-	-	-	-	+	-	+
Kal-PCE	**Ethene**	PCE	**TCE**	+	-	-	-	+	-	-	-	+
S-PCE	**Ethene**	PCE	***cis*-DCE**	+	-	-	+	-	-	-	-	+
H10-TCE	**Ethene**	**Ethene**	TCE	+	-	-	-	-	-	-	-	+
S-VC	**Ethene**	**Ethene**	N/D	+	-	-	-	-	-	-	-	+
H10-VC	**Ethene**	**Ethene**	N/D	+	-	-	-	-/+	-	-	+	-
H5-VC	**Ethene**	**Ethene**	N/D	+	-	-	-	-	-	-	-	+

[a]Enrichment culture abbreviation; H7: Hydrite 7, H5: Hydrite 5, Min: Minerva, Kal: Kalamazoo, S: Su-Zi, H10: Hydrite 10
N/D; not determined
All cultures were amended with H_2 plus 5 mM acetate.
Dhc; Dehalococcoides group
Dsf; Desulfuromonas group
Dhb; Dehalobacter group
Geo; Geobacter group
Sulf; Sulfurospirillum group
Dsb; Desulfitobacterium group
+; present (from direct PCR)
-; absent (16S rRNA gene targeted group: from direct and nested PCR; *Dhc* RDase gene targeted group: from direct PCR only)
-/+; absent in direct PCR but present in nested PCR

, but no visual band was obtained after further enrichment (data not shown). *Desulfuromonas* populations were detected in the FMC-PCE culture during early stages of enrichment (i.e., < 10 transfers), but *Desulfuromonas* was not detected in the FMC-PCE culture after further enrichment, or in of the any other ethene producing enrichment culture. Populations similar to *Geobacter* sp. strain SZ (35) were detected in H7-PCE and S-PCE cultures. *Sulfurospirillum* species were detected in the Kal-PCE culture via direct PCR and in the H10-VC culture during nested PCR. *Desulfitobacterium* species were not detected in any enrichment culture.

3.3.5. Detection of tceA, bvcA, and vcrA

Three known *Dehalococcoides* derived RDase genes (*tceA*, *bvcA*, and *vcrA*) were screened in ethene producing enrichment cultures, and the results are summarized in Table 3.4. *tce*A, which codes for an enzyme involved in TCE dechlorination in strains 195 and FL2 (15, 24), was only detected in the FMC-PCE culture. *bvcA* was detected in Min-PCE and H10-VC cultures. *vcrA* was detected in all cultures except H10-VC. Interestingly, both VC-RDase genes were detected in the Min-PCE culture suggesting that at least two different VC-respiring *Dehalococcoides* species co-exist in this culture. Additionally, *bvcA* was detected only in the H10-VC culture but not in the H10-TCE culture, even though these enrichment cultures were derived from sediment for the same aquifer sample.

3.3.6. Quantification of tceA, bvcA, and vcrA

The total numbers of bacterial and *Dehalococcoides* 16S rRNA gene copies, as well as chloroethene RDase genes (*tceA, bvcA*, and *vcrA*) were quantified from H7-PCE, S-PCE, and FMC-PCE. *Dehalococcoides* cells were quantified ranging from 9.04 x10^5 to 1.58 x 10^7 per ml in the three PCE dechlorinating cultures (see Table 3.5.). Compare with the total number of bacteria in all ethene producing PCE enrichment culture, *Dehalococcoides* cell number ranged from 1.3 to 7.7% (Figure 3.2.A.). *vcrA*, ranging from 4.04 × 10^5 to 1.23 × 10^7 copies per ml of culture, was

Table 3.5. Quantification of total bacterial and *Dehalococcoides* 16S rRNA gene copies, and chloroethene RDase genes in select PCE dechlorinating enrichment cultures

Enrichment cultures	Total quantified gene copies per ml culture				
	Bacterial 16S rRNA	*Dehalococcoides* 16S rRNA	*tceA*	*bvcA*	*vcrA*
H7-PCE	2.06×10^8 (9.76×10^7)	1.58×10^7 (1.01×10^6)	-	3.76 (2.32)	1.23×10^7 (2.52×10^5)
S-PCE	6.85×10^7 (2.62×10^7)	9.04×10^5 (1.03×10^5)	-	26.3 (7.8)	1.08×10^6 (1.10×10^5)
FMC-PCE	6.48×10^7 (2.85×10^7)	3.85×10^6 (3.55×10^5)	2.31×10^6 (3.25×10^5)	2.89 (0.8)	4.04×10^5 (2.79×10^4)

-; not quantifiable

All values are averaged from triplicate analysis. The numbers in parentheses represent standard deviation from triplicate analysis.

All cultures were amended with H_2/CO_2 (80%/20% [vol/vol]) headspace and 5 mM acetate.

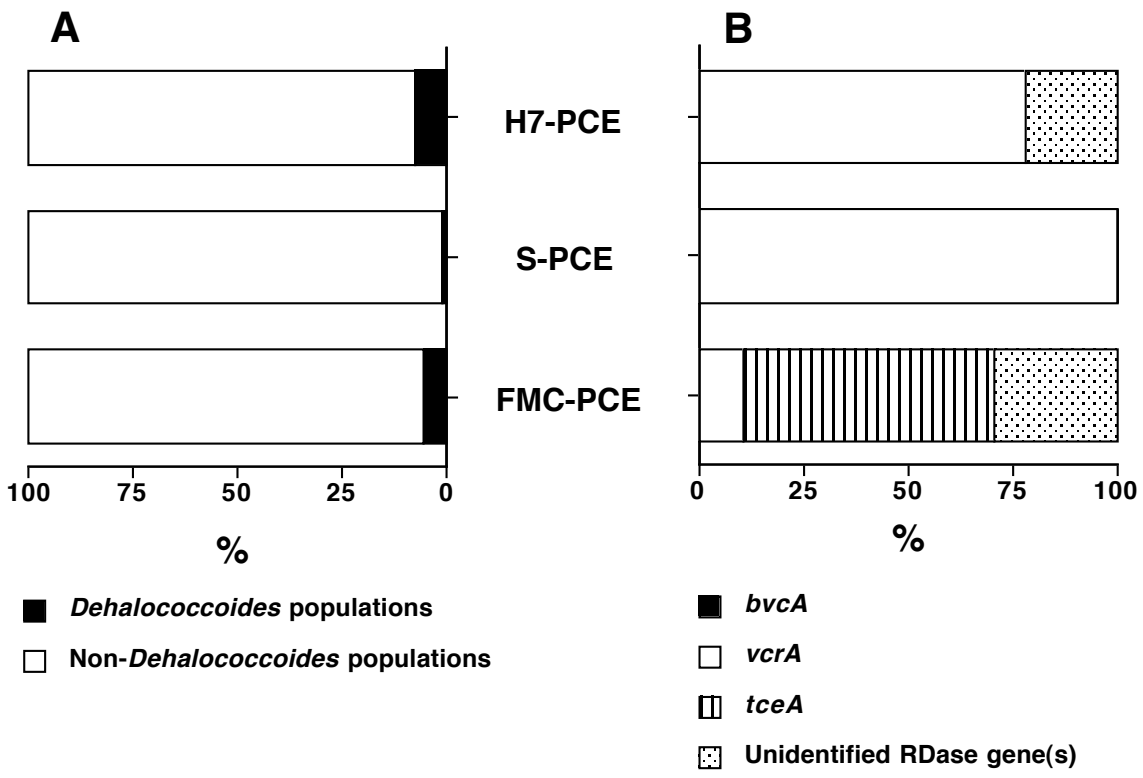

Figure 3.2. Proportion of *Dehalococoides* in PCE dechlorinating enrichment cultures (A). Proportion of the known and unidentified chloroethene RDase genes as a fraction of the total quantified *Dehalococcoides* population in PCE dechlorinating enrichment cultures (B). Proportion of *bvcA* is too small to depict.

detected in all three cultures. As shown with conventional PCR, *tceA* was only detected in the FMC-PCE culture but not in H7-PCE or S-PCE, suggesting that *tceA* was not required to promote complete detoxification of chlorinated ethenes. In contrast to conventional PCR (Table 3.4.), a detectable, albeit small, quantity of *bvcA* was detected in all three cultures, ranging from 3 to 26 copies per ml culture. All dechlorinating cultures possessed multiple RDase genes, however, *vcrA* was present in much higher quantities than *bvcA*, suggesting that populations possessing *vcrA* are more prevalent and involved to a greater extent in complete reductive dechlorination in these cultures. The proportion of *tceA*, *bvcA*, and *vcrA* as a fraction of the total number of *Dehalococcoides* species as determined from 16S rRNA gene analysis is shown in Figure 3.2.B. In H7-PCE, *Dehalococcoides* strains carrying *vcrA* or *bvcA* account for 77.9 ± 6.6% and <0.01% of the total number of *Dehalococcoides* species, respectively. Therefore, 22.2 ± 6.6% of the *Dehalococcoides* population does not contain *tceA*, *bvcA*, or *vcrA* but contains uncharacterized RDase gene(s). Similarly, in the FMC-PCE culture, 29.5 ± 15.7% of the *Dehalococcoides* population contains unidentified gene(s) since *tceA*, *bvcA*, and *vcrA* only account for 70.5 ± 15.7% of the total number of *Dehalococcoides* species. In contrast, *vcrA* and *bvcA* account for 119.5 ± 25.8% and <0.01% of the total number of *Dehalococcoides* species, respectively in S-PCE. Therefore, this culture consists almost exclusively a single *Dehalococcoides* species that contains the *vcrA* gene.

3.3.7. Development of Bio-Dechlor INOCULUM ™ for bioaugmentation

The same culture volume (1%, vol/vol) from two pure *Dehalococcoides* species (strain BAV1 and FL2) and three ethene producing, PCE reducing enrichment cultures, H7-PCE, H5-PCE, and FMC-PCE were combined in 100 ml of medium to develop a microbial consortium capable of dechlorinating chlorinated ethenes. Ethene, as a final dechlorination product, was formed from all chlorinated ethenes (i.e., PCE, TCE, *cis*-DCE, *trans*-DCE, 1,1-DCE and VC) by this culture with either lactate or hydrogen provided as an electron donor. A series of bioaugmentation studies was performed in microcosms, which did not exhibit complete reductive

dechlorination. Microcosms were established with aquifer materials from a TCE contaminated site (Plant 66, GA) and amended with hydrogen as an electron donor and TCE as an electron acceptor. No TCE dechlorination was observed over 50 days of incubation in room temperature. At day 50, 2% [vol/vol] of Bio-Dechlor culture was augmented. In bioaugmented cultures, TCE was dechlorinated and stoichiometric amounts of ethene were produced without a significant lag time (Figure 3.3). No dechlorination was observed in non-bioaugmented microcosms over a 100-day incubation.

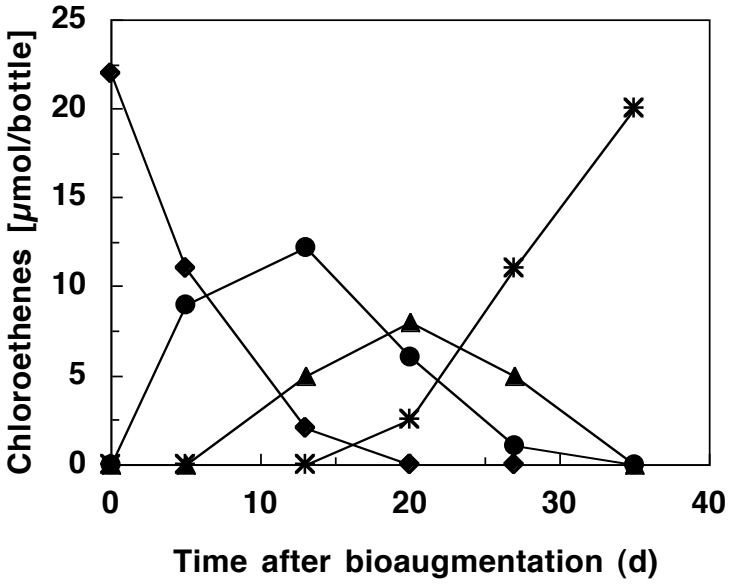

Figure 3.3. Reductive dechlorination of TCE in bioaugmented microcosms. Microcosms were established with TCE contaminated aquifer materials from the Plant 66 site in GA. Complete reductive dechlorination was achieved days 35 after augmentation. No dechlorination was observed in non-bioaugmented cultures over a 100 day of incubation (not shown). d, days.
Symbols: ◆; TCE, ●; *cis*-DCE, ▲; VC, ✳; ethene

3.4. Discussion

The combination of microcosm and enrichment culture studies with molecular-based analyses (16S rRNA gene and chloroethene RDase gene targeted) allowed for determination of the distribution of key dechlorinating organisms and assessing the potential for dechlorination activity at a particular chlorinated ethene contaminated site. This information aids in selecting the most promising remediation approach (e.g., biostimulation vs. bioaugmentation). The presence of *Dehalococcoides* species has been shown to be critical for the complete reductive dechlorination of chlorinated ethenes to ethene (7, 8, 16, 20, 25, 27), and the results presented here confirm this observation. Although these observations do not rule out the involvement of other undescribed and uncharacterized populations in ethene formation from chlorinated ethenes, *Dehalococcoides* populations were present in all ethene-producing enrichment cultures developed from both contaminated and non-contaminated sites. The environmental distribution of *Dehalococcoides* populations and the potential for dechlorinating activity, however, can be very heterogeneous, even at one site (e.g., Hydrite site, Table 3.1.). Hence, replicate samples from multiple locations within a plume should be analyzed to avoid false positive and false negative results.

Because of the high similarity (> 99%) of 16S rRNA gene sequences among *Dehalococcoides* species with different dechlorinating activities, analysis based on the 16S rRNA gene is insufficient to predict the potential for complete detoxification of chlorinated ethenes, especially for complete detoxification of VC (7, 13, 15). For example, Ritalahti and Löffler (28) describe two 1,2-dichloropropane (1,2-D) dechlorinating *Dehalococcoides* populations that share identical 16S rRNA genes with strain BAV1, but are incapable of dechlorinating DCEs or VC. Therefore, detection of *Dehalococcoides* populations based on the 16S rRNA gene at contaminated sites does not necessarily indicate that the desired reactions are occurring. Recently, genes involved in reductive dechlorination of VC to ethene were identified from *Dehalococcoides* sp. strains BAV1 and VS (19, 27), and molecular tools that allow specific

detection of these genes have been developed (29). Based on this information, and the previously identified gene responsible for TCE reduction (*tceA*) (15, 24), it is now possible to identify specific functional genes involved in chlorinated ethene dechlorination. The use of molecular techniques targeting these functional genes, coupled with 16S rRNA gene analysis, allows a better analysis of the potential for complete detoxification of chlorinated ethenes at contaminated sites.

Qualitative and quantitative PCR approaches to monitor *tceA*, *vcrA*, and *bvcA* from ethene-producing, *Dehalococcoides*-containing enrichment cultures showed that these functional genes were heterogeneously distributed in all PCE-, TCE-, and VC- reducing enrichment cultures. Multiple RDase genes existed in many ethene-producing cultures, but all ethene-producing enrichment cultures contained *bvcA* or *vcrA* or both in the case of Min-PCE. *vcrA* was detected more frequently and in higher quantities than *bvcA* in the enrichment cultures described here, suggesting that populations possessing *vcrA* are more prevalent and involved to a greater extent in complete reductive dechlorination of VC.

Quantitative analysis of *Dehalococcoides* 16S rRNA genes and RDase genes (*tceA, bvcA,* and *vcrA*) gave a more detailed structure of the *Dehalococcoides* community within the tested enrichment cultures. The results presented here imply that *Dehalococcoides* populations with previously unidentified and uncharacterized RDase genes are present in some of the enrichment cultures described. Although this analysis assumed that all *Dehalococcoides* have one 16S rRNA gene operon per genome, the same genome size (31), and only one VC RDase gene per genome, characterizing enrichment cultures in this manner may help guide further research with cultures that contain potentially new isolates with novel functions and characteristics. Analyzing enrichment cultures this way could also be an interesting way to track changes within the *Dehalococcoides* population based on enrichment conditions (e.g., electron acceptor provided) or during field application (e.g., bioaugmentation at contaminated sites).

For example, this technique could have been used to analyze the enrichment of two different VC-dechlorinating *Dehalococcoides* organisms in the H10-TCE and H10-VC enrichment cultures. Utilization of different electron acceptors (i.e., TCE or VC) in microcosms and subsequent enrichment cultures established from sediment from a single contaminated sediment core sample (Hydrite 10, see Table 3.1.), allowed for the enrichment of populations that contained primarily *bvcA* (H10-VC) or *vcrA* (H10-TCE). These findings imply that enriching with a different electron acceptor could selectively enrich a specific *Dehalococcoides* population over time, which could be monitored using the techniques employed in this work. This is similar to previous findings where enrichment with two different electron acceptors (TCE and 1,2-D) selectively enriched two different *Dehalococcoides* populations with different substrate specificities (15, 28).

In addition to *Dehalococcoides* species, partially dechlorinating populations that dechlorinate PCE and TCE to *cis*-DCE were also detected in five from six PCE dechlorinating enrichment cultures. Although a relatively large number of partial dechlorinators have been isolated and characterized, the presence of these dechlorinators and their functional role in the complete reductive dechlorination of PCE in cultures containing multiple dechlorinating species has not been extensively examined. The results presented here show that partial dechlorinators coexisted with *Dehalococcoides* and played an important role in the complete reductive dechlorination of PCE. All six PCE dechlorinating enrichment cultures did not dechlorinate PCE in the presence of ampicillin, indicating that populations other than *Dehalococcoides* species (e.g., *Dehalobacter*, *Geobacter*, and *Sulfurospirillum*) are involved in the initial PCE dechlorination step. Presumably, PCE is dechlorinated to either TCE or *cis*-DCE by partial dechlorinators and then *Dehalococcoides* populations use TCE or *cis*-DCE as an electron acceptor leading to complete dechlorination. Partial dechlorinators, however, were not detected in the FMC-PCE culture, suggesting that additional dechlorinating population(s) other than the ones analyzed for may be involved in the reductive dechlorination process. It is not clear why

Dehalobacter and *Desulfuromonas* populations were initially present in H7-PCE and FMC-PCE, respectively, but not detected after further enrichment (>30 transfers). One possible explanation is that initially multiple dechlorinating populations coexist, but one partial dechlorinator out-competes another for the same electron acceptor/donor. For example, a SZ like strain of *Geobacter* (a PCE-to-*cis*-DCE dechlorinator) species may have become the dominant partial dechlorinator in the H7-PCE (see Table 3.4.) culture due to their ability to use both hydrogen and acetate as electron donors to support reductive dechlorination (35), whereas *Dehalobacter* species are strictly hydrogenotrophic (17). This hypothesis is also supported by the fact that H7-PCE and S-PCE contained strain SZ like *Geobacter* species (see Table 3.4.) and was the only cultures that dechlorinated PCE to *cis*-DCE in the absence of hydrogen in H7-PCE and S-PCE enrichment cultures.

In summary, the findings of this study demonstrate that *Dehalococcoides* populations are intimately involved in complete detoxification of chlorinated ethene compounds and are widely distributed in anoxic environments, including non-contaminated environments. Previous efforts to determine the distribution of *Dehalococcoides* at contaminated sites have mainly relied on PCR approaches targeting the *Dehalococcoides* 16S rRNA gene. However, RDase gene based PCR approaches used here provided meaningful information regarding VC reducing *Dehalococcoides* populations and will be a useful tool for monitoring VC reducing populations in microcosm and enrichment culture studies as well as sites undergoing biostimulation and bioaugmentation. Careful examination of enrichment culture kinetics and gene-based molecular PCR approaches revealed that complete reductive dechlorination processes are carried out by multiple dechlorinating organisms and imply that undescribed *Dehalococcoides* population(s) may be involved in the complete detoxification of chlorinated ethenes. Further research is warranted to obtain new *Dehalococcoides* isolates and to identify RDases gene(s) in order to develop a comprehensive understanding of the dechlorinating populations involved in chloroethene reductive dechlorination in contaminated subsurface environments.

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CHAPTER IV

Characterization, Description and Specific Detection of *Geobacter lovleyi* Strain SZ sp. nov., a Novel Uranium-Reducing and Tetrachloroethene (PCE)-Dechlorinating Bacterium

Abstract

A bacterial isolate, designated strain SZ, was obtained from non-contaminated creek sediment microcosms based on its ability to derive energy from acetate oxidation coupled to tetrachloroethene (PCE)-to-*cis*-1,2-dichloroethene (*cis*-DCE) dechlorination. Hydrogen and pyruvate served as alternate electron donors, and the range of electron acceptors included (reduced products given in brackets) PCE and trichloroethene (TCE) [*cis*-DCE], nitrate [ammonium], fumarate [succinate], Fe(III) [Fe(II)], malate [succinate], Mn(IV) [Mn(II)], U(VI) [U(IV)], and elemental sulfur [sulfide]. PCE and soluble Fe(III) (as ferric citrate) were reduced at rates of 56.5 and 164 nmol min^{-1} mg of $protein^{-1}$, respectively, with acetate as the electron donor. Inhibition studies with chloramphenicol suggested that dechlorination and reduction of Fe(III) (as ferric citrate) were inducible activities. Competing electron acceptors such as U(VI) and nitrate did not inhibit PCE dechlorination and were consumed concomitantly. With PCE, Fe(III) (as ferric citrate), or nitrate as electron acceptors, H_2 was consumed to threshold concentrations of 0.078 ± 0.032, 0.156 ± 0.07 and 0.5 ± 0.062 nM, respectively, and acetate was consumed to 3.0 ± 2.08 nM, 1.2 ± 0.53 and 3.6 ± 0.25 nM, respectively. Apparently, electron acceptor-specific acetate consumption threshold concentrations exist, suggesting that, similar to the hydrogen threshold model, the measurement of acetate threshold concentrations offers an additional diagnostic tool to delineate terminal electron-accepting processes in anaerobic subsurface environments. 16S rRNA gene sequence analysis affiliated strain SZ with the genus *Geobacter*, with *Geobacter* (formerly *Trichlorobacter*) *thiogenes* as the closest relative (98.4% sequence identity). Furthermore, phylogenetic analysis of 16S rRNA gene sequences recovered from PCE-dechlorinating consortia and chloroethene-contaminated subsurface environments suggested that *Geobacter lovleyi* belongs to a distinct dechlorinating clade within the *Geobacter* group. A

specific, 16S rRNA gene-targeted primer set detected strain SZ in two PCE-dechlorinating consortia, groundwater samples from a chloroethene-contaminated site and bio-stimulated uranium contaminated FRC sites. These findings suggest that *Geobacter* spp. not only play relevant roles in bio-reduction of metals and radionuclides but also contribute to reductive dechlorination of priority pollutants (i.e., chlorinated ethenes). Substrate versatility, consumption of electron donors to low threshold concentrations, and simultaneous reduction of electron acceptors suggest that strain SZ-type organisms have desirable characteristics for bioremediation applications. Phylogenetic and phenotypic characteristics classify strain SZ as the type strain of the new species, *Geobacter lovleyi* sp. nov.

4.1. Introduction

Environmental pollutants such as chlorinated organic solvents, toxic metals and radionuclides (e.g., uranium) are strictly regulated due to the negative effects on ecosystem function and human health. Tetrachloroethene (PCE) and trichloroethene (TCE) are frequently used as solvents and degreasing agents. As a result of extensive usage, improper disposal and accidental spills, PCE and TCE became widely distributed, pervasive groundwater contaminants. Uranium was released in significant amounts from nuclear weapon complexes managed by the U. S. Department of Energy (DOE) during the cold war arms race (45). It was estimated that more than 80% of DOE sites have radionuclide contamination and at least 27% have volatile organic hydrocarbons as co-contaminants (44).

Considerable knowledge accrued on the fate of specific contaminants and the bacteria involved in the transformation and degradation pathways, which have lead to successful bioremediation field studies. For instance, acetate additions to stimulate dissmilatory metal-reducing organisms promoted reduction of soluble U(VI) to insoluble U(IV), and contributed to plume containment (2, 14, 20). Biostimulation and bioaugmentation approaches were successfully implemented at chloroethene contaminated sites (12, 26, 36). The majority of sites, however, contain multiple contaminants, and mixed waste scenarios are a major challenge successful remedies must address. Many U(VI)-impacted DOE sites report elevated concentrations of nitrate, sulfate, chlorinated solvents (e.g., PCE, TCE), and other chloroorganic pollutants, including polychlorinated biphenyls (http://public.ornl.gov/nabirfrc/frcdoc-figures.cfm). Unfortunately, mixed waste scenarios received very little attention, and the effects of co-contaminants on desired biotransformation processes are unclear. Obviously, populations that consume toxic electron acceptors (i.e., oxidized radionuclides, chloroorganic compounds) under a range of redox conditions (e.g., in the presence of alternate, energetically favorable oxidants) are desirable at sites impacted with nitrate, uranium and chlorinated ethenes.

In this study, we report the isolation, characterization and specific detection of a novel *Geobacter* species, strain SZ, capable of coupling the oxidation of acetate and H_2 to the reduction of a variety of electron acceptors, including PCE, TCE, nitrate, and uranium.

4.2. Materials and Methods

4.2.1. Isolation procedure and growth conditions

Su-Zi Creek sediment was collected in June 2002 near Seoul, South Korea. The sampling site is located residential the suburbs with no reported contamination. Microcosms were established inside a glove box (Coy Laboratory Products Inc., Grass Lake, MI) filled with a mixture of 95% N_2 and 5% H_2 [vol/vol]. Approximately 2 g of sediment was transferred to 20-ml (nominal capacity) glass vials and 9 ml of sterile, anoxic, reduced bicarbonate buffered (30 mM) medium amended with 10 mM acetate was added (43). All the vials received 0.5 µl neat PCE as electron acceptor and were sealed with Teflon-lined rubber stoppers. Sediment-free, non-methanogenic, dechlorinating cultures were obtained after sequential transfers (1% [vol/vol]) to fresh medium. PCE-dechlorinating enrichment cultures were transferred to 20-ml vials containing bicarbonate-buffered and HEPES-buffered (10 mM) medium amended with 10 mM acetate and 1 µl neat PCE. Dilution-to-extinction series were established in liquid and semisolid medium (0.5 % [wt/vol] SeaPlaque® agarose, Cambrex Bio Science Rockland Inc., Rockland, ME). PCE (2 µl dissolved in 100 µl hexadecane) was added by syringe after the agarose had solidified. The vials were incubated upside down and PCE was only available to the bacteria via the headspace.

4.2.2. 16S rRNA gene sequencing and phylogenetic analysis

Genomic DNA was obtained from PCE/acetate, Fe(III)/acetate, and nitrate/acetate-grown cultures. Individual genomic DNA was extracted with a QIAamp DNA mini kit (QIAGEN, Valencia, CA). Related 16S rRNA gene sequences from cultured organisms and environmental clones were identified by BLAST analysis and obtained from GenBank. Distance matrices and

phylogenetic trees were generated with related species and environmental clones based on 16S rRNA gene sequences following sequence alignment using the MegAlign program of the Lasergene software package (DNA Star Inc., Madison, MI). Bootstrap values were calculated with the MEGA software program for 1,000 replicates (24). The nearly complete 16S rRNA gene sequence (1,478 bp) of strain SZ has been deposited in GenBank under accession number AY914177.

4.2.3. Restriction Fragment Length Polymorphism (RFLP) analysis of PCR amplified 16S rRNA genes

Genomic DNA was extracted from acetate-fed cultures containing Fe(III)-citrate, nitrate, PCE or fumarate as an electron acceptor. 16S rRNA genes were PCR amplified using a universal bacterial primer pair (8F and 1525R), and 50 ng of genomic DNA as template (27). PCR and restriction fragment length polymorphism (RFLP) analysis using *HhaI*, *MspI*, and *RsaI* were performed as described previously (27). Controls included genomic DNA from *G. thiogenes*, *G. sulfurreducens*, and *D. michiganensis* strain BB1.

4.2.4. PCR-based detection of strain SZ

The nearly complete 16S rRNA gene sequence of strain SZ was aligned with sequences from close relatives listed in Table 4.1, and the Oligo Design and Analysis Tools (www.idtdna.com) were used to select the forward primer Geo196F [5' GAA TAT GCT CCT GAT TC 3'] and the reverse primer Geo999R [5' ACC CTC TAC TTT CAT AG 3']. BLAST analysis suggested specificity of the primer pair to the target sequence. Direct and nested PCR were performed as described previously (27). Briefly, all amplification reactions were performed in a total volume of 20 µl with 100 nM concentration of each primer, and 10-20 ng of template DNA. Annealing temperatures ranging from 36 to 57ºC were tested using an Eppendorf Mastercycler (Eppendorf, Germany). To verify primer specificity, genomic DNA of close relatives and selected chloroethene dechlorinating species, including, *G. thiogenes*, *G. sulfurreducens*, *G. metallireducens*, *D. michiganensis* strain BB1 and strain BRS1, *Sulfurospirillum multivorans*,

Dehalobacter restrictus, *Dehalococcoides* species (strain FL2, strain BAV1, and strain 195), and *Desulfitobacterium* sp. strain Viet1 was subjected to PCR amplification with Geo196F and Geo999R.

Five PCE-to-ethene dechlorinating consortia derived from geographically distinct chloroethene-impacted sites (e. g., the Hydrite culture, Wisconsin; the Minerva culture, Ohio; the FMC culture, California; the KB-1 culture, Ontario; and the Bachman culture, Michigan) were tested. In addition, DNA extracted from groundwater samples collected from two wells (MW7 and MW33) at a chloroethene-contaminated site in Georgia and eight different community DNA from samples collected from the FRC site [one from a denitrifying Fluidized Bed Reactor, three wells from the non-biostimulated areas (FW016, FW106, and TPB16) and four from wells located in biostimulated areas (FW026, FW029, FW101-2, and FW102-3)] were screened.

G. sulfurreducens and *G. metallireducens* were kindly provided by Kelly Nevin, University of Massachusetts, Amherst, and Benjamin Griffin, Michigan State University, East Lansing, provided a culture of *G. thiogenes* strain K1 (8). Genomic DNA of *Dehalococcoides ethenogenes* strain 195 was generously provided by Steve Zinder, Cornell University, Ithaca, NY. Melanie Duhamel, University of Toronto, Ontario, Canada, kindly provided genomic DNA of culture KB-1 (11). *Sulfurospirillum multivorans* and *Dehalobacter restrictus* were obtained from DSMZ. Strains BB1, BRS1, FL2, BAV1 and Viet1 were isolated in our laboratory.

4.2.5. Substrate utilization and growth conditions

Electron acceptor utilization was tested in bicarbonate-buffered medium amended with 5 mM acetate. The following compounds were added to duplicate 160-ml serum bottles containing 100 ml of medium (aqueous concentrations are given in parentheses): PCE (0.17-0.33 mM), TCE (0.32-0.63 mM), *cis*-DCE (0.28 mM), *trans*-1,2,-dichloroethene (0.21 mM), 1,1,-dichloroethene (0.19 mM), vinyl chloride (0.1 mM), carbontetrachloride (0.1 mM), 1-chloroethane (0.1 mM), 1,1-dichloroethane (0.1 mM), 1,2-dichloroethane (0.1 mM), 1,1,1-trichloroethane (0.1 mM), 1,1,2-trichloroethane (0.1 mM), 1,1,2,2-tetrachloroethane (0.1 mM), trichloroacetate (0.5-2 mM),

trifluoroacetate (1 mM), U(VI) (0.1-2 mM), Fe(III)-citrate (5-10 mM), NO_3^- (5-10 mM), NO_2^- (1-10 mM), fumarate (5-10 mM), malate (5 mM), SO_4^{2-} (5-10 mM), SO_3^- (0.5-5 mM), poorly crystalline Fe(III) oxide (5-10 mM), MnO_2 (1-2 mM) and oxygen (5-10% [vol/vol]). Liquid chloroethenes were added undiluted or from autoclaved hexadecane stock solutions 1 day prior to inoculation (43). All other halogenated solvents were added undiluted using a gas-tight Hamilton syringe (1800 series, Hamilton, Reno, NV). Gaseous compounds were added by plastic syringe. Non-volatile compounds were added from aqueous, anoxic, neutralized, sterilized stock solutions by syringe. Oxygen was added as sterilized air (breathing quality, Airgas, Inc. Randor, PA) to medium that did not receive reductants (*L*-cysteine and $Na_2S\times9H_2O$) and resazurin. Elemental sulfur was added as an aqueous, repeatedly pasteurized (at 90°C), powdered S^0 suspension. Soluble ferric iron was added from an aqueous, neutralized, autoclaved ferric citrate stock solution (0.5 M). Poorly crystalline Fe(III) oxide and MnO_2 were synthesized and added as described by Lovley and Phillips (32, 33). A U(VI) stock solution was prepared by dissolving 0.635 g uranyl acetate in 50 ml of a 30 mM bicarbonate solution (16). All cultures received a 1% (vol/vol) inoculum from an acetate/PCE-grown culture that had consumed all PCE.

The ability of the new isolate to use alternate electron donors was evaluated in duplicate bottles amended with 0.33 mM PCE or 5 mM Fe(III)-citrate. The following substrates were tested at concentrations given in parentheses: H_2 (50,000 ppmv, 123 μmol), pyruvate (5 mM), yeast extract (0.01-0.1%, wt/vol), formate (10 mM), propionate (10 mM), lactate (10 mM), citrate (5 mM), succinate (5 mM), butyrate (10 mM), benzoate (10 mM), glucose (10 mM), methanol (0.08-0.4 mM), toluene (0.5 mM), benzene (0.5 mM) and ethanol (0.1-0.3 mM). Sodium salts were added from anoxic, sterile stock solutions before inoculation. Sterile H_2 gas was added by syringe, and undiluted alcohols, toluene and benzene were added with a Hamilton syringe. Electron donor oxidation was judged by its consumption and the reduction of PCE or Fe(III) compared to control cultures that received no electron donor. All cultures received a 1% (vol/vol) inoculum from an acetate/PCE-grown culture that had consumed all acetate.

Possible carbon sources were examined in duplicate cultures amended with PCE or Fe(III)-citrate and H_2 (50,000 ppmv or 123 µmol). Formate, propionate, lactate, citrate, succinate, butyrate, glucose (5 mM each), and yeast extract (0.1% wt/vol) were tested. Reduction of PCE and Fe(III) was monitored and compared with duplicate control cultures that did not contain H_2. The bottles received a 1% (vol/vol) inoculum from an acetate/PCE-grown culture that had consumed all acetate.

4.2.6. Growth linked uranium reduction by strain SZ

Triplicate bottles (160 ml serum bottle containing 100 ml of medium) were amended with 100 µM uranium as an electron acceptor, H_2 (1.96 mmol) as an electron donor, and 5 mM lactate as a carbon source. Quadruple control cultures were amended electron donor and carbon source but no uranium. Experiments were initiated by transferring 2% PCE/acetate grown strain SZ inoculum to culture vessels. Triplicate abiotic control cultures received the same concentration of electron acceptor, electron donor, and carbon source but a 2% filter-sterilized inoculum. Once uranium concentration approached zero, additional 100 µM uranium was spiked to uranium-reducing cultures and monitored again. Genomic DNA was obtained from live cultures except abiotic control cultures. To detach all sorbed particles from the glass surface, brief, 2-3 second sonication in a water bath was applied. Individual genomic DNA was obtained with an InstaGene™ Matrix (Bio-Rad Laboratories, Hercules, CA). Briefly, cell pellets from 1 ml culture sample were incubated at 56°C for 20 min with 200 µl of InstaGene™ Matrix solution followed by an 8 min incubation in boiling water. Genomic DNA was obtained from supernatant by centrifugation at 11,000 rpm, 2 min at room temperature.

Total 16S rRNA gene copy numbers were determined through quantitative Real-Time PCR (RTm PCR). *Geobacteraceae* 16S rRNA-gene targeted primer, Geo564F [5'-AAG CGT TGT TCG GAW TTA T-3'] and Geo840R [5'-GGC ACT GCA GGG GTC AAT A-3'], was used for RTm PCR as described (7). Reaction for RTm PCR were performed in a total volume of 30 µl with 300 nM concentration of each primer, 15 µl QuantiTect SYBR Green PCR master mix

(Qiagen), 10.2 µl RNase-free water (Qiagen), and 3 µl template of 10-fold diluted DNA. The PCR conditions were as follows; 2 min at 50°C and 15 min 95°C, followed by 40 cycles of 30 sec at 94 °C, 30 sec 57°C, and 30 sec 72°C. RTm PCR carried out in an ABI Prism 7000 Sequence Detection system (Applied Biosystems). A calibration curve was generated by using 10-fold serial dilutions of genomic DNA from fumarate/acetate grown *G. sulfurreducens* cells. Total 16S rRNA gene copies were estimated under the assumption of an average molecular weight of 660 for a base pair in double-strained DNA, two 16S rRNA gene operons per *Geobacter* genome, and genome size of 3.81 Mbp (38).

4.2.7. PCE dechlorination in phylogenetically related species

G. thiogenes, *G. sulfurreducens*, and *G. metallireducens* were tested for their ability to dechlorinate PCE. Duplicate cultures were amended with 0.1 mM PCE as electron acceptor and 5 mM acetate as electron donor. Experiments were initiated by adding 5% [vol/vol] inoculum from Fe(III)-citrate/acetate grown *G. sulfurreducens*, *G. metallireducens* or fumarate/acetate grown *G. thiogenes* cultures. Reductive dechlorination of PCE was monitored over a 3-month incubation period. All cultures were incubated statically at room temperature in the dark.

4.2.8. Determination of electron donor consumption threshold concentrations

Triplicate bottles were amended with excess electron acceptor (0.33 mM PCE, 5 mM Fe(III)-citrate or 5 mM nitrate), H_2 as the sole electron donor (8,333 ppmv, 20.4 µmol), and 5 mM lactate as a carbon source. One set of triplicate control cultures was amended with each electron acceptor, lactate as carbon source, and had N_2/CO_2, but no hydrogen, in the headspace. The inoculum (1% vol/vol) was transferred from dechlorinating cultures that had consumed all acetate. Concentrations of chlorinated compounds, Fe(III), Fe(II), nitrate, ammonium and H_2 were monitored overtime. After a constant H_2 threshold concentration was reached, all cultures were respiked with 20.4 µmol of H_2, and H_2 consumption was monitored again.

The acetate consumption threshold concentration for strain SZ was determined using the same three terminal electron acceptors, PCE, Fe(III)-citrate, and nitrate. Strain SZ cultures were

amended with 0.1 mM acetate and 0.33 mM PCE, 7.5 mM of Fe(III)-citrate, or 2 mM nitrate. The cultures were incubated at room temperature until reduction of the respective electron acceptor ceased due to electron donor limitation. For the dechlorinating cultures, ^{14}C-acetate (59 μCi/mmol, Sigma-Aldrich, MO) was added to triplicate cultures at concentrations of 447 nM and 894 nM. Triplicate cultures amended with Fe(III) or nitrate received 894 nM ^{14}C-acetate as electron donor. After no further acetate consumption occurred in dechlorinating cultures, an additional 894 nM ^{14}C-acetate was added and its consumption monitored. All cultures were incubated at room temperature in the dark without agitation.

4.2.9. Rate and yield measurements

Reduction rates were determined in triplicate 100-ml cultures containing 0.33 mM PCE, 3 mM Fe(III)-citrate or 3.5 mM NO_3^- and 5 mM acetate that had received a 3% (vol/vol) inoculum from acetate, PCE, Fe(III)-citrate or nitrate pre-grown cultures, respectively. After complete reduction of the electron acceptor provided, reduced products (*cis*-DCE, ferrous iron, ammonium) and acetate concentrations were determined. The protein increase (difference between initial and final protein concentrations) was estimated as described below.

4.2.10. Influence of different electron acceptors on PCE dechlorination

To test the influence of alternate electron acceptors on PCE reductive dechlorination, duplicate cultures amended with PCE + Fe(III)-citrate, PCE + U(VI), and PCE + nitrate with acetate (10 mM) as the electron donor were established. PCE, Fe(III)-citrate, U(VI), and nitrate were added in concentrations of 0.33, 5, 0.3, and 5 mM, respectively. The experiments were initiated by adding 3% [vol/vol] inocula of a strain SZ culture grown with PCE and acetate that had consumed all PCE. Control cultures were amended with the same electron acceptors but received the same amount of filter-sterilized inocula. Consumption of electron acceptors and production of *cis*-DCE, Fe(II) and ammonium were monitored over time. In addition, the effects of sulfur oxyanions (sulfate, up to 10 mM; and sulfite, up to 5 mM) on reductive dechlorination were examined.

4.2.11. Induction studies

To determine whether PCE and soluble Fe(III) reduction activities were constitutive, experiments with the protein biosynthesis inhibitor chloramphenicol were conducted (39). Chloramphenicol was added from an ethanol stock solution (30 mM) to a final concentration of 300 μM to cultures grown with 10 mM fumarate and 20 mM acetate that had completely consumed fumarate. PCE (0.33 mM) or Fe(III)-citrate (5 mM) were added to duplicate cultures with or without chloramphenicol, and reduction was monitored. Control cultures received the same volume of ethanol without the antibiotic to assess the effects of the solvent.

4.2.12. Microscopy

Light micrographs were obtained with a BX-40 Olympus microscope after staining the cells and flagella with silver nitrate as described (48). Cells were collected by centrifugation (1,288 × g for 20 min at room temperature) from cultures grown with soluble (nitrate, PCE, fumarate) and insoluble (poorly crystalline Fe(III) oxide, elemental sulfur, MnO_2) electron acceptors. Scanning electron microscopy (SEM) micrographs were obtained from fumarate-grown cultures as described previously (17).

4.2.13. Analytical techniques

Chloroethenes, chloroethanes and volatile fatty acids were quantified as described previously (18). Dehalogenation of trichloroacetate and trifluoroacetate was determined by chloride and fluoride ion release. Oxygen concentration in the headspace was measured with a Hewlett-Packard model 5890 GC equipped with a thermal conductivity detector and a Chrompack Molsieve 5 Å fused silica column (10 m × 0.53 μm). For analysis of ^{14}C-acetate, 1 ml of culture suspension was made basic by adding 10 μl of 1M NaOH, and pushed through a Millipore 0.2 μm cellulose membrane filter. Radio-labeled acetate was quantified with a HP 1050 HPLC equipped with a C-61OH Carbohydrate Supelcogel column (Supelco, Bellefonte, PA) and a 500 TR series flow scintillation analyzer (Packard Instrument, Meriden, CT). The solvent system was 0.1% H_3PO_4 at a flow rate of 0.5 ml/min. Inorganic anions and cations were

analyzed with an ion chromatograph equipped with a CD 20 conductivity detector and two AS11-HC 2 mm columns for anions, and a CS12A column for cations (Dionex Sunnyvale, CA). Formation of N_2O, a potential denitrification product, was monitored on a HP 6890 gas chromatograph equipped with a HP-1 column (30 m length, 0.32 mm diameter, 0.25 µm film thickness) and ^{63}Ni-electron capture detector. Sulfide was determined colorimetrically as described previously (6). Fe(II) production was measured using the ferrozine method (42). The decrease of Mn(IV) and U(VI) was monitored spectrophotometrically as described (4, 22). Growth was monitored by measuring electron acceptor consumption or protein increase. The Coomassie Plus Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, Il) was used in accordance with the manufacturer recommendations to estimate the protein content. Cell pellets were harvested from 3 to 6 ml of cultures fluid by centrifugation (15,777 × g for 30 min at room temperature). Whole-cell lysis was achieved by adding 100 µl of 1M NaOH and heating to 90ºC for 10 min in a closed, 1.5-ml plastic tube. The suspension of lysed cells was gently mixed with an equal volume of Commassie Plus Reagent solution, and incubated for 10 min at room temperature. Protein was quantified spectrophotometrically by comparing the sample absorbances at 595 nm with protein standards (bovine serum albumin) of known concentration treated in the same way as the samples.

4.3. Results

4.3.1. Enrichment and isolation

Microcosms established with Sulfi creek sediment amended with acetate and H_2 completely dechlorinated PCE to ethene after 6 weeks of incubation. A sediment-free, non-methanogenic, *cis*-DCE-producing culture was obtained after 20 sequential transfers (1% [vol/vol]) to completely synthetic, defined, bicarbonate-buffered medium with acetate as electron donor and PCE as electron acceptor. Isolation of a dechlorinating organism, designated strain SZ, was achieved through three consecutive dilution-to-extinction series in semisolid medium. Small (diameter < 0.2 mm), ellipsoid shaped, white colonies formed after 2 months with PCE provided as electron acceptor in 10^{-5} dilution tubes. Larger (diameter 2-3 mm), ellipsoid shaped, reddish colonies developed down to the 10^{-6} diluted tubes within 2 weeks with fumarate as electron acceptor. Isolated colonies grown with PCE and fumarate were transferred to liquid medium, and PCE-to-*cis*-DCE-dechlorination, Fe(III)-to-Fe(II), and nitrate-to-ammonium reduction activity were recovered within 1 week of incubation.

4.3.2. Culture purity and phylogeny

Microscopic uniformity, recovery of PCE, Fe(III), and nitrate reduction activities from single colonies, identical 16S rRNA gene sequences obtained with genomic DNA extracted from PCE-, Fe(III)-, and nitrate-grown cultures, and indistinguishable RFLP patterns that matched those predicted from *in silico* digests suggested culture purity. RFLP patterns of *MspI*- and *RsaI*-digested amplicons generated from *G. thiogenes, G. metallireducens*, and *G. sulfurreducens* genomic DNA showed unique patterns different from those of strain SZ. *HhaI*-digested amplicons corroborated culture purity but failed to distinguish strain SZ from *G. thiogenes, G. metallireducens*, and *G. sulfurreducens*. Analysis of the 16S rRNA gene sequence indicated an affiliation with the genus *Geobacter* displayed in the phylogenetic tree shown in Figure 4.1. The 16S rRNA molecule of strain SZ shares the secondary structure characteristics of the 16S rRNA molecule for members of the *Geobacter* cluster (29). The closest relative is the TCA-

dechlorinating species *G. thiogenes*. Both organisms have distinct physiological characteristics (Table 4.1.) suggesting that they belong to different species.

4.3.3. Morphology of strain SZ

Cells were rod-shaped, 1-1.4 μm long by 0.4 μm wide. Cell morphology was constant under different growth conditions and during different growth phases. Motility was observed under all growth conditions including in cultures grown with insoluble electron acceptors (i.e., poorly crystalline Fe(III) oxide and MnO_2). Figures 4.2.A and 4.2.B illustrate silver nitrate-stained flagella of cells grown with PCE and nitrate. SEM micrographs revealed flagellated, rough-surfaced, rod shaped cells in the exponential growth phase (Figure 4.2.C). Spores were never observed.

4.3.4. Substrate utilization

Strain SZ coupled acetate oxidation to the reduction of PCE (Figure 4.3), TCE, Fe(III)-citrate (Figure 4.4), poorly crystalline Fe(III) oxide, nitrate, fumarate, malate, elemental sulfur, U(VI), and Mn(IV). *cis*-DCE was the only product detected from PCE and TCE dechlorination. Succinate accumulated in cultures that received fumarate and malate, and sulfide was produced in cultures amended with sulfur flower. Nitrate was reduced to ammonium, and the intermediate formation of nitrite was not observed. No N_2O formation occurred during growth with nitrate. Strain SZ did not reduce TCA, TFA, *cis*-DCE, *trans*-DCE, 1,1-DCE, VC, 1-chloroethane, 1,1-dichloroethane, 1,2-dichloroethane, 1,1,1-trichloroethane, 1,1,2-trichloroethane, 1,2-dichloropropane, nitrite, sulfate, thiosulfate, or sulfite with acetate as electron donor under the conditions tested. In addition to acetate, pyruvate and H_2 served as alternate electron donors. The following compounds were not utilized as electron donors for PCE and Fe(III) reduction: yeast extract, formate, propionate, lactate, citrate, succinate, butyrate, benzoate, glucose, toluene, benzene, methanol, and ethanol. Sustained PCE and Fe(III) reduction with H_2 as electron donor required the presence of acetate or lactate. H_2 oxidation and electron acceptor reduction ceased in cultures lacking acetate (or lactate), and could not be stimulated through the addition of formate,

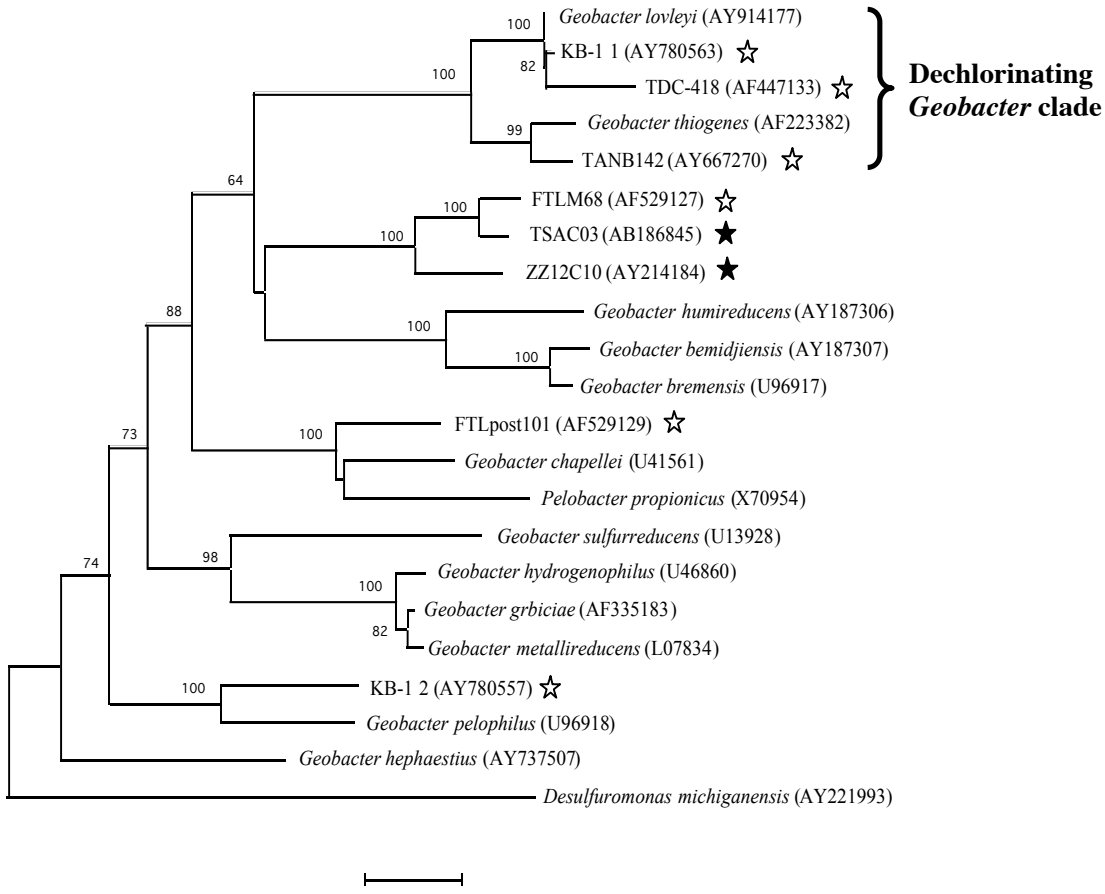

Figure 4.1. Phylogenetic tree of strain SZ and related species and environmental clones based on 16S rRNA gene sequences. The bootstrap values at the nodes are based on 1,000 replicates, and only values >50% are shown. The tree was generated for 1,290 bp aligned positions using Clustal W (MegAlign). Stars indicate environmental clone sequences; open stars indicate that these sequences were retrieved from dechlorinating enrichments cultures or chloroethene-contaminated sites. The scale bar represents 1 bp substitution per 100 nucleotides.

Table 4.1. Phenotypic differences between *Geobacter thiogenes* and *Geobacter lovleyi*

Organism	Electron donors	Electron acceptors	Reductive dechlorination activity	Sulfur-sulfide redox cycle	Formation of sulfur granules	Acetate consumption threshold for chlororespiration	Motility	Source
Geobacter lovleyi	Acetate, H_2 Pyruvate	PCE, TCE, Fe(III) [a], Mn(IV), U(VI), nitrate, fumarate, malate, sulfur	Inducible	Not detected	No	3 nM	Flagellar motility	Non-contaminated freshwater sediment
Geobacter thiogenes [b]	Acetate	TCA, fumarate, sulfur Fe(III) [c]	Constitutive	Yes	Yes	0.1 mM	Non-motile	Contaminated soil

[a]; Reduction occurred with soluble ferric iron and poorly crystalline ferric oxides
[b]; Data from references [De Wever, 2000 #45;Lovley, 2004 #23;Nevin, 2004 #12]
[c]; Reduction of soluble ferric iron was reported as "inconsistent" (8)
[d]; Apparently, a sulfur-sulfide redox cycle functions as an electron shuttle between acetate and the chlorinated electron acceptor (TCA)

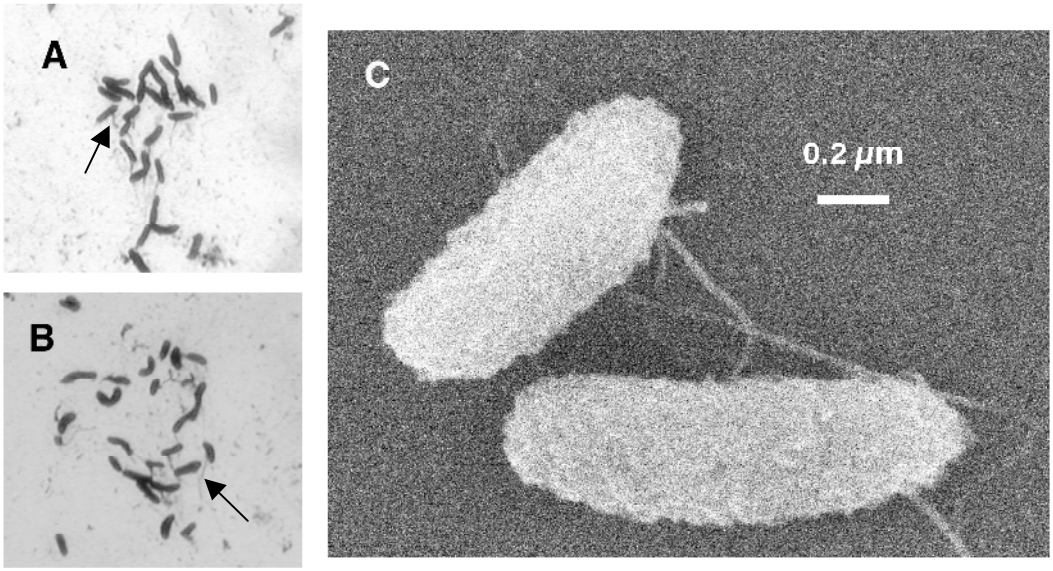

Figure 4.2. Micrographs of *Geobacter lovleyi* strain SZ. Light microscopic pictures of silver nitrate-stained cells grown with PCE and acetate (A), and nitrate and acetate (B). The arrows indicate silver-stained flagella. Scanning electron micrograph of cells grown with fumarate and acetate (C).

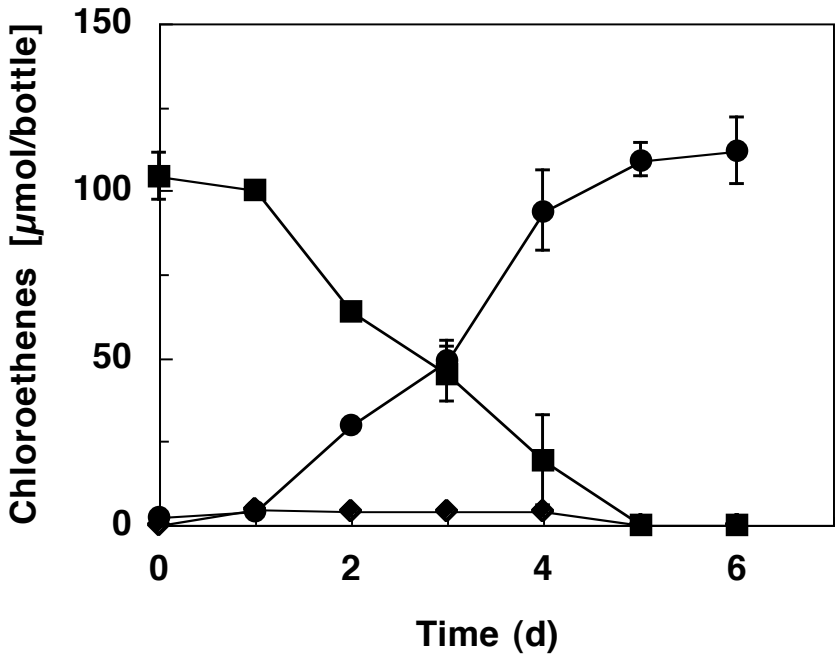

Figure 4.3. Dechlorination of PCE to *cis*-DCE with the intermediate formation of small amounts of TCE by strain SZ with acetate as electron donor. Data were averaged from triplicates, and error bars depict standard deviation. Symbols: ■; PCE, ◆; TCE, ●; *cis*-DCE

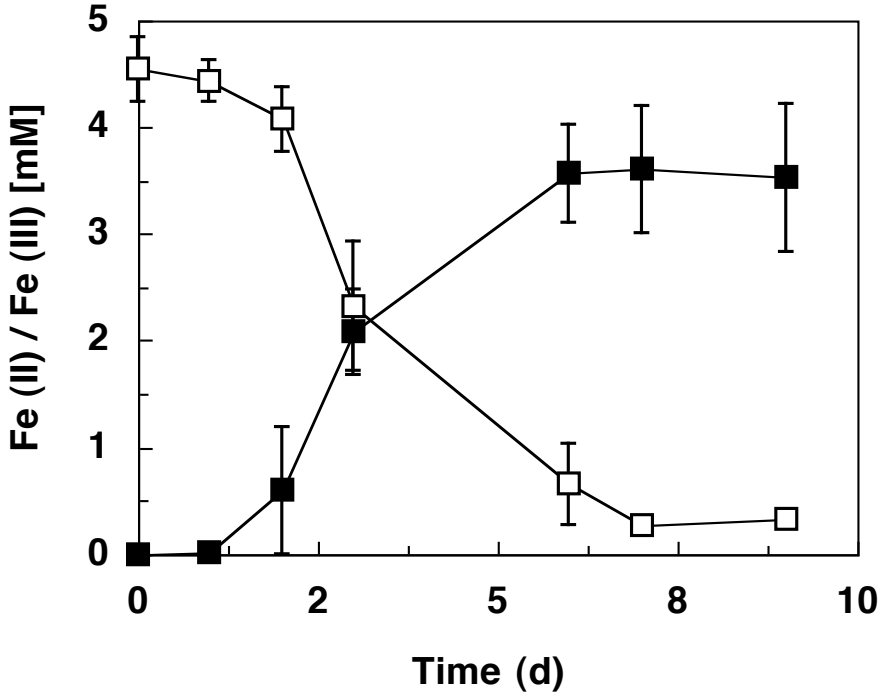

Figure 4.4. Reduction of soluble ferric iron by strain SZ with acetate as electron donor. Data from triplicates were averaged, and error bars depict standard deviation. Symbols: ■; Fe(II), □; Fe(III)

propionate, citrate, succinate, butyrate, glucose, or yeast extract, suggesting that these organic compounds cannot fulfill the carbon source requirements of strain SZ.

Under the conditions applied, strain SZ dechlorinated PCE at a maximum rate (average ± standard deviations) of 56.5 ± 0.84 nmol min^{-1} mg of $protein^{-1}$ with acetate as electron donor. Soluble Fe(III) was reduced at a maximum rate of 164 ± 43 nmol min^{-1} mg of $protein^{-1}$. Closely related species including *G. thiogenes, G. metallireducens*, and *G. sulfurreducens* were unable to dechlorinate PCE.

4.3.5. Growth linked uranium reduction by strain SZ

As depicted in Figure 4.5, strain SZ coupled H_2 oxidation to the reduction of uranium. At day 7, 100 µM uranium was completely consumed and additional 100 µM uranium feeds were completely consumed at increasing rates. No uranium reduction occurred in abiotic control cultures over the incubation time. In order to demonstrate growth-linked uranium reduction by strain SZ, the total 16S rRNA gene copies from uranium reducing cultures and control cultures were quantified. Genomic DNA was extracted from uranium reducing cultures when a total of 300 µM uranium was consumed and from control cultures that did not received uranium as an electron acceptor. Uranium reducing cultures that had reduced a total of 300 µM of uranium contained (1.19 ± 0.28) × 10^7 16S rRNA gene copies ml^- (average ± standard deviations). Very similar 16S rRNA gene copy numbers were quantified from 2% inoculum (6.10 ± 0.13) × 10^6 and from control cultures that did not received uranium (5.96 ± 1.65) × 10^6. These results suggest that uranium reduction by strain SZ linked to growth and no growth occurred in the absence of uranium as a sole electron acceptor.

4.3.6. Electron donor consumption threshold concentrations

In the presence of excess Fe(III)-citrate, and nitrate, strain SZ consumed H_2 to concentrations (average ± standard deviations) of 0.078 ± 0.032, 0.156 ± 0.07, and 0.5 ± 0.062 nM, respectively. No *cis*-DCE, Fe(II), or ammonium formation was observed in control cultures

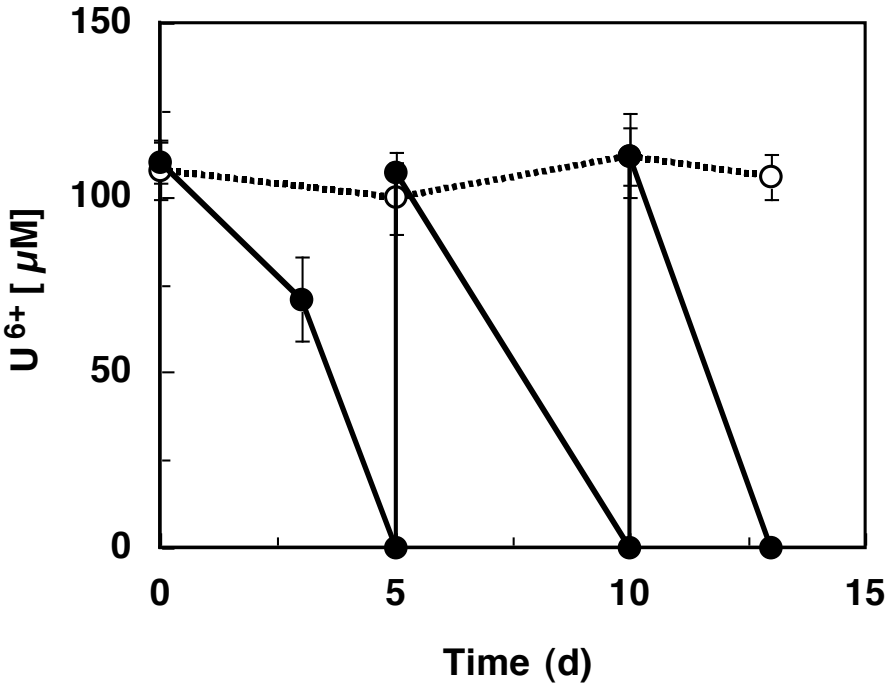

Figure 4.5. Reduction of U(VI) by strain SZ with H_2 as electron donor and lactate as a carbon source. Control cultures received the same electron acceptor, electron donor, and carbon source but a 2 % filter-sterilized inoculum.
Data were averaged from triplicates, and error bars depict the standard deviation.
Symbols: ●, U(VI) depletion; ○, U(VI) depletion in control cultures

that did not receive H_2. Consistent residual acetate concentrations (average ± standard deviations) of 3.0 ± 2.08, 1.2 ± 0.53, and 3.6 ± 0.25 nM were observed when PCE, Fe(III)-citrate, and nitrate, respectively, were present in excess.

4.3.7. Induction studies

Experiments with the protein biosynthesis inhibitor chloramphenicol suggested that PCE and Fe(III) reduction activities are inducible in strain SZ. Fumarate-grown cells failed to reduce PCE or Fe(III) in the presence of chloramphenicol (Figure 4.6). In contrast, PCE or Fe(III) reduction in cultures without the antibiotic started after a lag time of less than 1 day. Similar lag times and reduction rates were observed in cultures receiving ethanol without the antibiotic, suggesting that the alcohol exhibited no unspecific toxic effects.

4.3.8. Dechlorination in presence of alternate electron acceptors

As depicted in Figure 4.7, the presence of U(VI) did not inhibit PCE dechlorination, and both electron acceptors were reduced simultaneously. At day 6, both PCE and U(VI) were completely consumed. Neither PCE nor U(VI) reduction were observed in control cultures. Similarly, strain SZ reduced Fe(III) or nitrate concomitantly with PCE (data not shown). Sulfate (10 mM) and sulfite (5 mM) did not affect PCE dechlorination whereas the presence of oxygen completely inhibited growth of strain SZ.

4.3.9. PCR-based detection of strain SZ

At an annealing temperature of 53ºC, PCR with 16S rRNA gene-targeted primers Geo196F and Geo999R reproducibly yielded the correct sized amplicons with genomic DNA of strain SZ as template. Sequencing confirmed amplicon identify with the 16S rRNA gene fragment of strain SZ. Neither direct nor nested PCR performed with purified genomic DNA of *G. thiogenes, G. metallireducens,* and *G. sulfurreducens* yielded any amplification products. Similarly, none of the chlorinated ethene-dechlorinating pure cultures yielded amplification products (data not shown). Apparently, the designed primer pair distinguishes strain SZ from

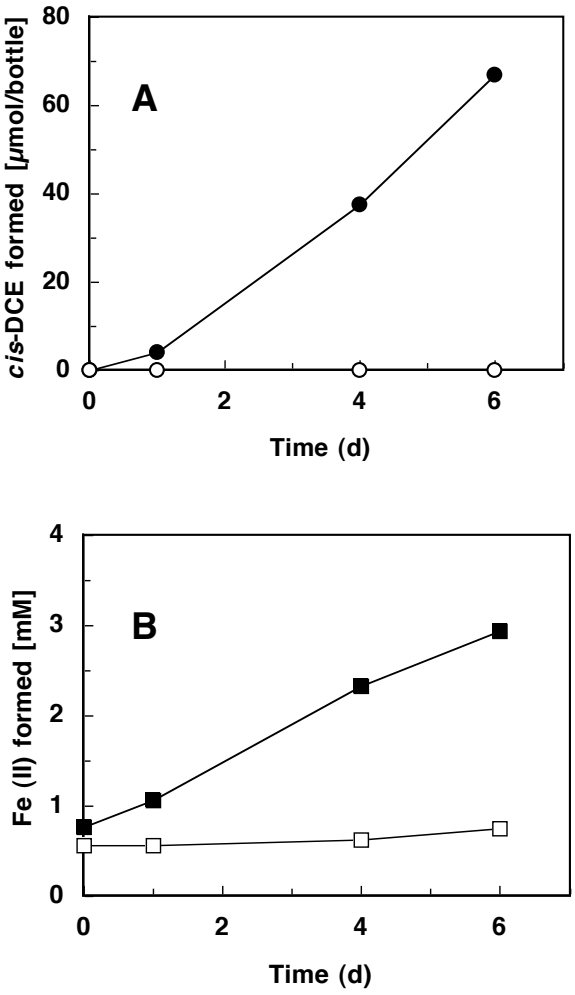

Figure 4.6. Effect of chloramphenicol on the reduction of (A) PCE and (B) Fe(III)-citrate. All cultures had completely consumed 10 mM fumarate before the experiments were initiated.
Symbols: ●, *cis*-DCE formation in the absence of chloramphenicol; ○, *cis*-DCE formation in the presence of chloramphenicol; ■, Fe(II) formation in the absence of chloramphenicol; □, Fe(II) formation in the presence of chloramphenicol. Data were averaged from duplicate cultures.

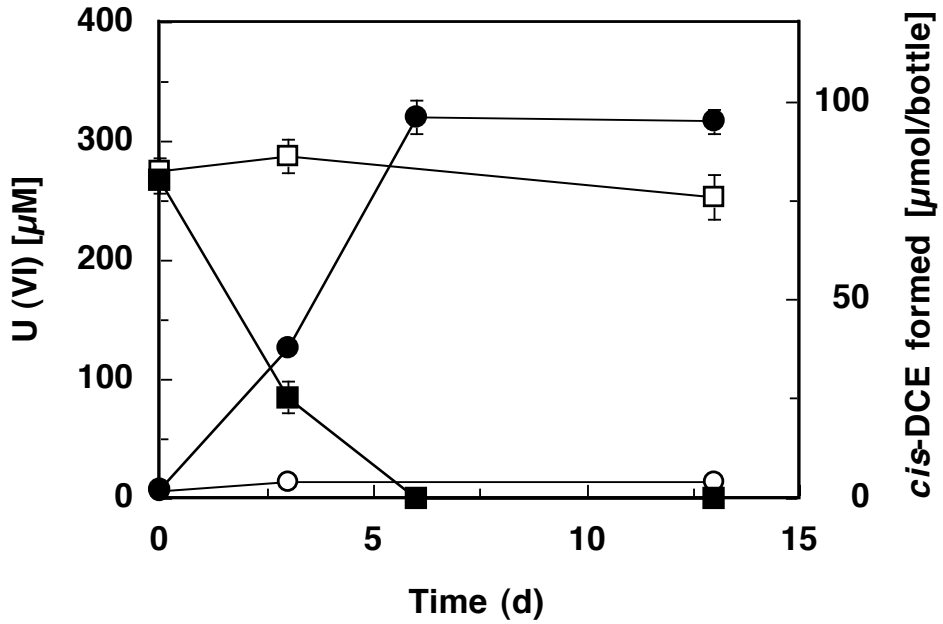

Figure 4.7. Concomitant reduction of PCE and U(VI) by strain SZ with acetate as electron donor. Control cultures received a filter-sterilized inoculum.
Symbols: ●, *cis*-DCE formation; ○, *cis*-DCE formation in control cultures; ■, U(VI) depletion; □, U(VI) depletion in control cultures. Data are averaged from duplicate cultures, and error bars depict standard deviation.

closely related *Geobacter* spp. and known chloroethene-dechlorinating bacteria. Direct PCR indicated the presence of strain SZ-like organisms in the PCE-to-ethene dechlorinating Hydrite and KB-1 consortia. A signal was also obtained with DNA extracted from the groundwater samples collected from a chloroethene-impacted site in Georgia, but only when the nested PCR approach was used (Figure 4.8.A). Positive signals were obtained in DNA extracted from groundwater samples collected from bio-stimulated FW026, FW101-2, and FW102-3 wells through direct PCR approaches with the strain SZ-type 16S rRNA gene targeted primer. In the nested PCR, positive signal was obtained from FW029 samples in addition to FW026, FW101-2, and FW102-3 samples. However, none of the samples with genomic DNA from non-stimulated FW016, FW106, and TPB16 wells yielded amplification products through direct and nested PCR (Figure 4.8.B). Sequencing of the amplicons confirmed their identity with the partial 16S rRNA gene sequence of strain SZ.

4.4. Discussion

The *Geobacter* group attracts considerable attention because its members are widespread in anoxic environments, and play key roles in environmentally relevant processes (30). *Geobacter* species affect iron geochemistry in anoxic subsurface environments, reduce toxic metals and radionuclides, and oxidize a variety of organic hydrocarbons (e.g., toluene, phenol) (1, 30). Although *Geobacter* species display versatility with regard to electron donor and electron acceptor utilization, metabolic reductive dechlorination (chlororespiration) was only described in one species to date: *G. thiogenes* coupled growth to the reductive dechlorination of TCA to DCA (8). The new isolate, strain SZ, is the first *Geobacter* species that uses PCE and TCE as metabolic electron acceptors. Growth with PCE as electron acceptor has been demonstrated previously for two strains in the *Desulfuromonas* cluster (23, 43), and the discovery of strain SZ suggests that metabolic reductive dechlorination is more widely distributed among the *Geobacteraceae.*

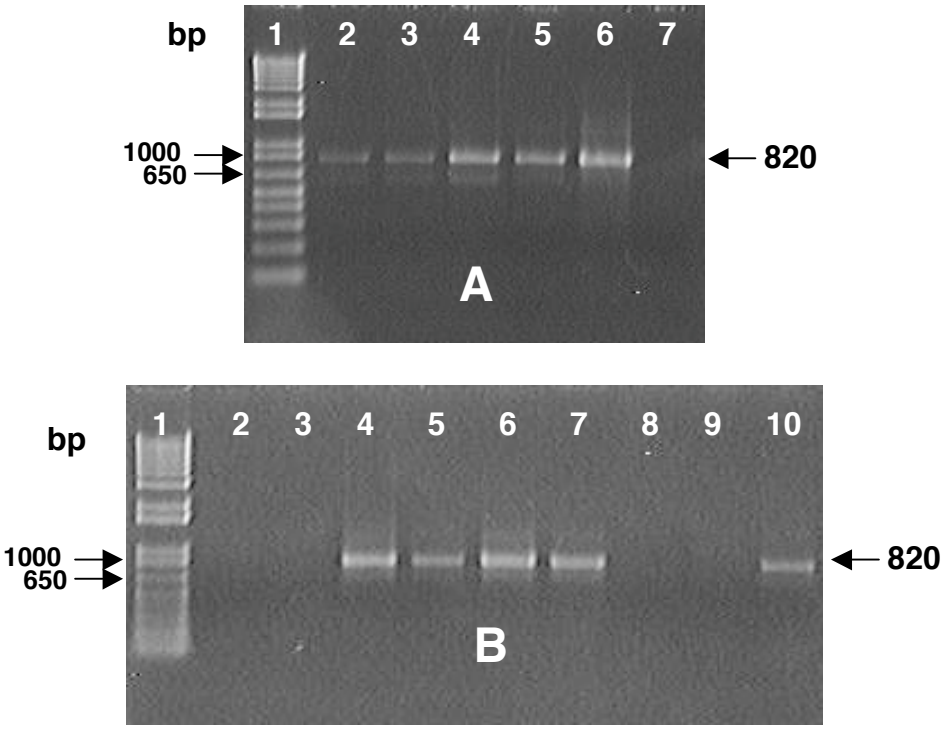

Figure 4.8. (A) Detection of strain SZ with the 16S rRNA gene targeted primers (Geo196F and Geo999R) in PCE dechlorinating enrichment cultures and groundwater samples. Lane1, DNA size marker; lane 2, the Hydrite culture; lane 3, the KB-1 culture; lane 4 and 5, groundwater samples collected from two wells (MW7 and MW33, respectively.) at a chloroethene contaminated site in Georgia, lane 6, strain SZ; lane 7, H_2O.
(B) Detection of strain SZ in biostimulated uranium contaminated FRC site samples via nested PCR. Lane1, DNA size marker; lane 2, stimulated FBR; lane 3, non-stimulated FW016 site; lane 4, bio-stimulated FW026; lane 5, bio-stimulated FW029; lane 6, bio-stimulated FW101-2; lane 7, bio-stimulated FW102-3; lane 8, non-stimulated FW106; lane 9, non-stimulated TPB16; lane 10, strain SZ.

Microbial reduction of soluble U(VI) to insoluble U(IV) is a promising strategy for containment of uranium plumes. Laboratory and field studies demonstrated that adding acetate can promote activity of native *Geobacter* species and result in U(VI) reduction and immobilization (2, 14, 20). PCE and nitrate are frequently encountered co-contaminants at uranium-contaminated sites (e.g., S-3 Pond site located near the Y-12 Plant in Bear Creek Valley, Oak Ridge, U.S. Department of Energy Natural and Accelerated Bioremediation Research Field Research Center [FRC]). The changes in Gibbs free energy under standard conditions ($\Delta G°'$) associated with PCE-to-*cis*-DCE dechlorination, nitrate reduction to ammonium, and uranium reduction are -72.5, -61.9 and -37.0 kJ per mol of electrons transferred (free energy of formation values from references (10, 25, 47)), respectively. Electron acceptors are typically oxidized sequentially, with the energetically more favorable electron acceptor consumed first. Hence, one would predict that PCE dechlorination precedes ammonification and U(VI) reduction. Although standard conditions rarely apply to real world conditions, thermodynamic calculations, assuming environmentally relevant concentrations of reactants and products and low pH values (e.g., FRC site groundwater), predict the same sequence of TEAPs. Such patterns of sequential reduction of electron acceptors have been observed at field sites and confirmed in pure culture studies (3, 15, 46). Although different TEAPs appeared to be inducible by the respective electron acceptor, strain SZ reduced nitrate, Fe(III)-citrate, or U(VI) and PCE simultaneously, suggesting no tight control over consumption of reducing equivalents in less favorable TEAPs. It is currently unclear if energy is captured from all simultaneously operating reductive pathways, and the significance of this electron acceptor utilization pattern is unclear. From an ecological point of view, however, this type of behavior might give strain SZ an edge over competing populations in oligotrophic, heterogeneous subsurface environments with periodically changing influx/availability of substrates.

Strain SZ exhibited flagellar motility when grown with insoluble (i.e., amorphous ferric iron, MnO_2) and soluble electron acceptors. This contrasts observations made with *G. thiogenes*,

which never displayed motility, and *G. metallireducens*. Childers et al. (5) suggested that flagella synthesis in *G. metallireducens* is regulated by the available electron acceptor, and only occurs when insoluble substrates are used. Apparently, the synthesis of the flagellar apparatus underlies different regulatory mechanisms in members of the *Geobacter* group, and further research is warranted to elucidate motility controls, and how motility affects contaminant transformation.

Acetate and H_2 are key intermediates in the anaerobic degradation of organic matter, and fluxes of both H_2 and acetate control microbial redox processes in subsurface environments. A variety of materials have been suggested and used for delivery of reducing equivalents to support a desired terminal electron accepting process, such as reductive dechlorination (13). Biostimulation ultimately increases the flux of H_2 and acetate (18) but competition for reducing equivalents, in particular from H_2, often limits its success. Obviously, organisms with greater electron donor versatility, in particular those that utilize acetate and H_2, are more desirable in bioremediation applications aimed at stimulating reductive processes. To our knowledge, strain SZ is the first chlorinated ethene-degrading organism described to utilize both, H_2 and acetate, as electron donors. This physiological feature offers a distinct advantage in subsurface environments where competition for reducing equivalents is fierce. Further, strain SZ consumes H_2 and acetate to low concentrations. It has been shown previously that chloroethene-respiring populations exhibit low H_2 threshold concentrations, and maintain H_2 concentrations below those needed to sustain other terminal electron accepting process such as methanogenesis, acetogenesis, and sulfate reduction (28, 37, 41, 50). Indeed, the H_2 consumption threshold of 0.078 nM measured in PCE-fed cultures of strain SZ is in good agreement with reported threshold values for chlororespiration (28). Analogously, acetate threshold concentrations depend on the thermodynamics of the TEAP. Although obviously relevant, information on acetate consumption threshold concentrations is scarce. Lovley and Phillips (31) showed that acetate threshold concentrations in Fe(III)-reducing sediments were lower than in sulfate-reducing and methanogenic sediments. Under mesophilic conditions, the acetate threshold concentration for

aceticlastic methane formation ranged from 69 to 1180 µM (49). *Desulfobacter postgatei*, an acetate-oxidizing sulfate reducer, consumed acetate to concentrations below 1 µM (21). Recently, He and Sanford (19) determined acetate threshold concentrations in *Anaeromyxobacter dehalogenans* strain 2CP-C cultures for two different TEAPs. Strain 2CP-C consumed acetate to concentrations of 69 nM with 2-chlorophenol as electron acceptor (chlororespiration), and an even lower value (< 1 nM) when grown with soluble ferric iron. The observed acetate threshold value of 3.0 nM in strain SZ cultures grown with PCE is at least three orders of magnitude lower than those reported for methanogens and sulfidogens. According to the threshold model, acetate-oxidizing dechlorinators should out-compete acetotrophic methanogens and sulfidogens for acetate. Hence, analogously to the H_2 consumption threshold model, acetate consumption threshold concentrations could serve as a diagnostic tool for the presence and activity of acetotrophic dechlorinators, and to delineate TEAPs in subsurface environments.

The 16S rRNA gene-targeted PCR approach distinguished strain SZ from closely related *Geobacter* species, including its closest relative *Geobacter thiogenes*. Hence, the primer set Geo196F/Geo999R will be useful to test enrichment cultures and environmental samples for the presence of strain SZ-type organisms. A specific, 16S rRNA gene-targeted primer set detected strain SZ in two PCE-dechlorinating consortia and groundwater samples from a bio-stimulated chloroethene-contaminated site as well as bio-stimulated uranium contaminated FRC sites strongly suggest that strain SZ not only plays relevant roles in bio-reduction of uranium but also contributes to reductive dechlorination of priority pollutants (i.e., chlorinated ethenes). In deed, physiological characteristics of strain SZ, for instance, the ability to reduce multiple FRC contaminants (uranium, chloroethenes, and nitrate), versatile electron donor utilization (acetate and hydrogen), consumption of electron donors to low threshold concentrations, support environmental niche of strain SZ in especially, nutrient limited FRC site. Interestingly, 16S rRNA gene sequences with high similarity to that of strain SZ were detected in a TCE-contaminated deep, fractured basalt (34), and in two PCE-to-ethene-dechlorinating mixed

cultures, including consortium KB-1 (9, 11) as presented in Table 4.2. The KB-1 consortium was maintained for many years with PCE as electron acceptor suggesting that the *Geobacter* species present are capable of capturing energy from chloroethene reductive dechlorination. Recent findings suggest that lateral transfer events have contributed to the dissemination of reductive dehalogenase genes (35, 40). Although we failed to identify other *Geobacter* species that dechlorinated PCE, further studies are needed to explore whether the ability to dechlorinate is more widely distributed amongst the *Geobacter* cluster, or restricted to a single clade, currently comprising two isolates, *G. thiogenes* strain K1 and *G. lovleyi* strain SZ.

4.5. Description of *Geobacter lovleyi* sp. nov.

Geobacter lovleyi (love.li'.i. N.L. gen. masc. n. *lovleyi* of lovley), named to recognize the contributions of Derek R. Lovley to our understanding of microbial metal and radionuclide reduction. *Geobacter lovleyi* is a rod shaped, motile, Gram-negative, anaerobic bacterium with cell dimensions of 1-1.4 × 0.4 μm. Electron donors include acetate, pyruvate and H_2. PCE, TCE, nitrate, soluble and insoluble forms of ferric ion, manganic ion, sulfur, fumarate, malate, and U(VI) are used as electron acceptors. PCE is reduced to *cis*-DCE as the final product by an inducible enzyme system. Strain SZ was isolated from non-contaminated freshwater sediment collected from Su-Zi Creek, South Korea. Phylogenetic and phenotypic characteristics place strain SZ in the *Geobacter* cluster within the family *Geobacteraceae* in the δ-subclass of the *Proteobacteria*, and warrant classifying strain SZ as the type strain of the new species, *Geobacter lovleyi* sp. nov. Strain SZ has been deposited at DSMZ and ATCC.

Table 4.2. Pairwise % similarity values for *Geobacter lovleyi* and related species and environmental clone sequences

		% Similarity [a]									
Species	No.	1	2	3	4	5	6	7	8	9	10
G. lovleyi	1	100									
G. sp. clone KB-1 1[b]	2	99.9	100								
G. thiogenes	3	98.4	98.3	100							
G. sp. clone TANB142[c]	4	98.2	98.1	99.1	100						
G. sp. clone TDC-418[d]	5	98.3	98.2	96.7	96.5	100					
G. chapellei	6	93.8	93.7	93.5	93.7	91.7	100				
G. metallireducens	7	93.2	93.2	92.3	92.3	91.2	93.0	100			
G. hydrogenophilus	8	92.4	92.3	91.4	91.5	90.4	92.7	98.6	100		
G. sulfurreducens	9	92.5	92.5	92.5	92.6	90.5	92.1	95.4	94.7	100	
D. michiganensis	10	88.6	88.6	88.5	88.5	86.6	89.1	91.1	90.5	90.4	100

[a]; % similarity values were calculated for 1,290 bp aligned positions using the MegAlign program
[b]; Environmental clone sequence, detected in the PCE-to-ethene-dechlorinating consortium KB-1 (AY780563)
[c]; Environmental clone sequence, detected in TCE-contaminated, deep, fractured basalt (AY667270)
[d]; Environmental clone sequence, detected in a PCE-to-ethene-dechlorinating mixed culture (AF447133)

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CHAPTER V

Quantitative PCR Confirms Purity of Strain GT, a Novel Trichloroethene-to-Ethene Respiring *Dehalococcoides* Isolate

Abstract

An apparently pure culture of a novel *Dehalococcoides* strain capable of trichloroethene (TCE)-to-ethene reductive dechlorination was obtained from chlorinated ethene contaminated aquifer material. Culture-dependent analyses and a variety of qualitative techniques targeting the 16S rRNA gene performed on genomic DNA from TCE, *cis*-1,2-dichloroethene (*cis*-DCE), 1,1-dichloroethene (1,1-DCE) and vinyl chloride (VC) grown cultures suggested culture purity. A careful quantitative analysis of the 16S rRNA gene and reductive dehalogenase genes (i.e., *vcrA*, *tceA*, and *bvcA*) implicated in chloroethene dechlorination in *Dehalococcoides*, however, revealed that culture GT consisted of two distinct *Dehalococcoides* populations. These findings suggest that commonly used qualitative 16S rRNA gene-based procedures are insufficient to verify purity of *Dehalococcoides* cultures. Subsequent transfers along with real-time PCR monitoring yielded a pure culture, designated strain GT. Phylogenetic analysis revealed that strain GT is affiliated with the Pinellas group of the *Dehalococcoides* cluster and shares 100% 16S rRNA gene sequence identity with two other *Dehalococcoides* isolates, strain FL2 and strain CBDB1. Strain GT, however, is distinct and respires the priority pollutants TCE, *cis*-DCE, 1,1-DCE and VC, thereby producing the innocuous compounds ethene and inorganic chloride. The *vcrA* gene implicated in DCE-to-ethene dechlorination in *Dehalococcoides* sp. strain VS was detected in strain GT but the *tceA* and *bvcA* genes were absent. Strain GT required hydrogen as an electron donor, dechlorinated TCE, *cis*-DCE, 1,1-DCE, and VC to ethene at rates up to 40, 41, 62 and 127 μmol L^{-1} d^{-1}, respectively.

Frequent detection of *vcrA* containing strain GT-like *Dehalococcoides* populations from many microbial consortia such as, KB-1, Bio-Dechlor, and SDC-9 (SHAW dechlorinating culture-9), suggest that strain GT type of *Dehalococcoides* may play an important role in complete

detoxification of chloroethenes. Importantly, strain GT respires vinyl chloride and efficiently produces ethene, making strain GT desirable for bioremediation applications.

5.1. Introduction

Chlorinated ethenes are pervasive groundwater contaminants as results of extensive usage, improper disposal, accident spill, and incomplete microbial dechlorination of highly chlorinated compounds such as tetrachloroethene (PCE) or trichloroethene (TCE).

A breakthrough in the anaerobic treatment of chloroethene-contaminated sites was the discovery of bacteria that use chloroorganic compounds as electron acceptors to drive their energy metabolism. This metabolic reductive dechlorination process, also known as (de)chlororespiration, is the focus of current bioremediation approaches to contain or remediate chloroethene plumes. Numerous bacterial isolates that reductively dechlorinate chloroethenes have been described (25), however, no single organism has the ability to couple energy generation with each reductive dechlorination step leading from PCE to ethene. The majority of isolates dechlorinate PCE to *cis*-DCE, and the ability to respire PCE to *cis*-DCE is distributed amongst phylogenetic groups and includes members of the *Desulfuromonas*, *Dehalobacter*, *Sulfurospirillum*, *Enterobacter*, and *Desulfitobacterium* (15). Bacteria with the ability of incomplete PCE dechlorination to *cis*-DCE are often present at contaminated sites, or transformation to *cis*-DCE can be achieved by biostimulation (10, 14). In contrast, reductive dechlorination past DCE has been exclusively linked to members of the *Dehalococcoides*, a deeply branching group within the Chloroflexi (green non-sulfur bacteria). Driving the reductive dechlorination process to completion (i.e., formation of ethene) is critical to achieve detoxification, and hence, the *Dehalococcoides* group received considerable attention from the bioremediation community (6, 14, 19). *Dehalococcoides ethenogenes* strain 195 was the first *Dehalococcoides* isolate described to dechlorinate PCE to ethene (21), however, careful investigations demonstrated that strain 195 failed to grow with VC, and that the final dechlorination step from VC to ethene was cometabolic and required the presence of a polychlorinated ethene (20). *Dehalococcoides* sp. strain BAV1 was the first isolate capable of coupling growth to VC reductive dechlorination, which was a relevant observation suggesting

that efficient chloroethene dechlorination without a VC stall is feasible (7, 8). Strain BAV1 respired all DCE isomers as electron acceptors but dechlorinated PCE and TCE only in the presence of a growth-supporting DCE isomer or VC (8). Another *Dehalococcoides* isolate, strain FL2, was shown to reduce TCE to ethene though the PCE-to-TCE and VC-to-ethene steps were cometabolic and required the presence of a growth-supporting electron acceptor (i.e., TCE, *cis*-DCE, *trans*-DCE) (9). A major hurdle for in-depth studies of this interesting bacterial group lays in the fastidious growth and the time-consuming and tedious isolation procedures (5, 22).

The known *Dehalococcoides* strains implicated in chloroethene reductive dechlorination share highly similar 16S rRNA genes (1, 5, 8, 9, 22). In fact, strain BAV1, a VC-respirer, strain FL2, an organism that cometabolizes VC, and strain CBDB1, an isolate that cannot grow with chloroethenes, share 16S rRNA gene sequences with greater than 99.9% identity. Hence, gene targets that provide higher resolution than the 16S rRNA gene are being thought for site assessment and bioremediation monitoring. Three such gene targets have been identified: *tceA* encoding a TCE reductive dehalogenase (RDase) in strain 195 and strain FL2, *vcrA*, a VC RDase in strain VS, and *bvcA*, a VC RDase in strain BAV1 (13, 18, 22).

Here we describe the first *Dehalococcoides* isolate that uses TCE, *cis*-DCE, 1,1-DCE, and VC as metabolic electron acceptors. Culture purity was demonstrated through the combined application of qualitative and quantitative 16S rRNA gene- and RDase gene targeted approaches, and we show that commonly used 16S rRNA gene based techniques are insufficient to verify *Dehalococcoides* culture purity.

5.2. Materials and Methods

5.2.1. Chemicals

PCE and TCE were purchased from Sigma-Aldrich Co. (St. Louis, MO). All other liquid chlorinated organic compounds were obtained from Supelco Co. (Bellefonte, PA). Gaseous VC was obtained from Fluka Chemical Corp. (Ronkonkoma, NY), and ethene was purchased from

Scott Specialty Gases (Durham, NC). Fluorinated ethene compounds were purchased from SynQuest Laboratories, Inc. (Alachua, FL). All of the other chemicals used were reagent grade or better unless otherwise specified. DNA extraction kits were purchased from QIAGEN (Valencia, CA) and Bio-Rad (Bio-Rad, Hercules, CA). Taq DNA polymerase and PCR buffer were from Applied Biosystems (Foster City, CA), bovine serum albumin and restriction endonucleases were from Promega (Promega Biosciences, Inc., San Luis Obispo, CA). The oligonucleotide primers for PCR were purchased from Integrated DNA Technologies (Coralville, IA).

5.2.2. Source of dechlorinating culture and isolation

A sediment free, non-methanogenic, non-acetogenic enrichment culture was derived from TCE-to-ethene-dechlorinating microcosms established with aquifer material from the chloroethene-contaminated site (Hydrite Chemical Co. Cottage Grove, WI). Dechlorinating culture was maintained and transferred in 160 ml glass serum bottle containing 100 ml mineral salt medium (27) amended with 5 mM acetate plus H_2/CO_2 [80%/20%] headspace and 0.32 mM TCE (5 µl TCE dissolved in 200 µl hexadecane) as electron acceptor more than three years. Routinely, L-cysteine (0.2 mM), $Na_2S•9H_2O$ (0.2 mM) and DL-dithiothreitol (DTT, 0.5 mM) were used as reductants (hereafter DTT- medium) for all experiments. Five times consecutive transfers received 1mg/ml of ampicillin and five times consecutive extinction-to-dilution series in 24 ml vials amended with 0.5 µl of neat TCE were performed. Following this treatment, 6 times additional transfers [0.5% vol/vol] to VC (0.55 mM) amended DTT-medium followed by 3 times transfers [0.5% vol/vol] to TCE (0.32 mM) DTT-medium were performed in 160 ml serum bottle.

5.2.3. Determination of substrate utilization

Alternated electron acceptors (aqueous concentrations are given in parenthesis), including PCE (0.33 mM), *cis*-DCE (0.32 mM), 1,2-*trans*-dichloroethene (*trans*-DCE, 0.21 mM), 1,1-dichloroethene (1,1-DCE, 0.19 mM), VC (0.19-0.55 mM), monochloroethane (0.1 mM), 1,1-dichloroethane (0.1 mM), 1,2-dichloroethane (0.1 mM), 1,1,1-trichloroethane (0.1 mM), 1,1,2-

trichloroethane (0.1 mM), carbon tetrachloride (0.1 mM), 1,2-dichloropropane (0.1 mM), vinyl bromide (0.1 mM), 1,1-dichloro-2,2-difluoroethene (0.1 mM), 1,2-dichloro-1,2-difluoroethene (0.1 mM), 2-chloro-1,1-difluoroethene (0.1 mM), 1,1-difluoroethene (0.1 mM), chlorotrifluoroethene (0.1 mM), trichlorofluoroethene (0.1 mM), sulfate (0.1-5 mM), fumarate (1-5 mM), nitrate (0.1-5 mM), and ferric citrate (5 mM) were tested in DTT medium with hydrogen as electron donor and acetate as carbon source. The inoculum (3% vol/vol) was transferred from TCE dechlorinating cultures that had consumed all TCE. Besides DTT medium, growth of strain GT was determined in reduced anaerobic complex media, such as full or half strength tryptic soy broth, received 3% inoculum transferred from either actively TCE or VC dechlorinating cultures. Autoclaved liquid chloroethenes, chloroethanes, and fluorinated ethenes were added neat or dissolved in hexadecane (27). Gaseous halogenated compounds were added by sterile syringe. Non-halogenated compounds were added from aqueous, anoxic, neutralized, sterilized stock solutions by syringe. All compounds were added before inoculations.

Alternated electron donors, glucose (2 mM), lactate (5 mM), pyruvate (5 mM), formate (5 mM), and yeast extract (0.01 wt/vol) were examined in 100 ml DTT-medium with TCE as an electron acceptor. The inoculum (3% vol/vol) was transferred from TCE dechlorinating cultures that had consumed all hydrogen. All possible electron donors were added from aqueous, anoxic, neutralized, sterilized stock solutions by syringe before inoculation.

Hydrogen consumption threshold concentrations were determined from in the excess of VC, and limit of hydrogen as described elsewhere (17).

All cultures were sealed with black butyl rubber stopper incubated upside down at room temperature without shaking in the dark, unless indicated otherwise.

5.2.4. 16S rRNA gene analysis and presence of reductive dehalogenase genes

Total genomic DNA was extracted from actively dechlorinating cultures using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA) or Insta Gene Matrix (Bio-Rad, Hercules, CA). Nearly complete 16S rRNA gene sequences were obtained from actively TCE, *cis*-DCE, 1,1-DCE, or VC

dechlorinating culture genomic DNA that yielded amplified 16S rRNA gene products with the universal bacterial primer pair (8F and 1541R) (16). PCR amplified 16S rRNA gene products from TCE or VC dechlorinating culture genomic DNA were digested for 3 h with enzymes *HhaI, MspI,* and *RsaI* at 37 ºC and terminated reaction by incubating 65 ºC for 10 min. Fragments were resolved by electrophoresis for 1 hr on 2.5% agarose gel (Invitrogen, Carlsbad, CA). 16S rRNA genes of VC dechlorinating culture genomic DNA were amplified with a hexachloro-fluorescein (HEX)-labeled primer, 8F-hex (5'-AGA GTT TGA TCC TGG CTC AG-3') and unlabeled primer 1541R. Fluorescent labeled terminal fragments obtained by digesting PCR products with *HhaI, MspI,* and *RsaI* and analyzed at High Throughput Sequencing and Genotyping Unit, University of Illinois, Urbana. PCR-DGGE analyses were performed by Microbial Insight (Rockford, TN) using universal bacterial primers, 27F and 519R (23) and *Dehalococcoides* specific primer, 1F-GC and 259R (5) as described. All 16S rRNA gene-based analyses were conducted with actively dechlorinating culture genomic DNA. The partial 16S rRNA gene sequence (1,299 bp) of strain GT was submitted to Genbank (AY914178).

The presence of *Dehalococcoides* derived chloroethene dehalogenases genes, TCE reductive dehalogenase, *tceA* and the VC reductive dehalogenases, *bvcA* and *vcrA* were tested as described (13, 18, 22). For the nested PCR (16), initial amplification was performed with a pair of degenerate PCR primers, RRF2 and B1R (13). The second PCR was performed with *bvcA*- or *tceA*-specific primers using the amplified products from first PCR as template. vcrAf and vcrAr primers (22) were used for amplification of parts of *vcrA* and for sequencing of amplicons from TCE and VC dechlorinating GT cultures.

Controls included genomic DNA from the following pure cultures *Dehalococcoides* strains FL2 and BAV1 (8, 9), *Desulfuromonas michiganensis* strain BB1 (27), *Dehalobacter restrictus* (11) and *Geobacter lovleyi* strain SZ (26) and ethene producing *Dehalococcoides* containing microbial consortia, KB-1 (5), Bio-Dechlor and SDC-9 (SHAW Dechlorinating Culture-9).

5.2.5. Quantitative Real-Time PCR (RTm PCR)

Total numbers of bacterial-, *Dehalococcoides*- 16S rRNA gene, *tceA*, *bvcA*, and *vcrA* were quantified using RTm PCR approaches (7, 24). The following primers and probes were used: for bacterial 16S rRNA gene, 5'- ATG GYT GTC GTC AGC T-3' (forward), 5'-ACG GGC GGT GTG TAC-3' (reverse) and 5'-FAM-CAA CGA GCG CAA CCC-TAMRA (probe); for *Dehalococcoides* 16S rRNA gene, 5'-CTG GAG CTA ATC CCC AAA GCT-3' (forward primer), 5'-CAA CTT CAT GCA GGC GGG-3' (reverse primer), and 5'-FAM-TCC TCA GTT CGG ATT GCA GGC TGA A-TAMRA (probe); for *tceA*, 5'-ATC CAG ATT ATG ACC CTG GTG AA-3' (forward primer), 5'-GCG GCA TAT ATT AGG GCA TCT T-3' (reverse primer), and 5'-FAM-TGG GCT ATG GCG ACC GCA GG-TAMRA (probe); for *bvcA*, 5'-AAA AGC ACT TGG CTA TCA AGG AC-3' (forward primer), 5'-CCA AAA GCA CCA CCA GGT C-3' (reverse primer), and 5'-FAM-TGG TGG CGA CGT GGC TAT GTG G-TAMRA (probe); for *vcrA*, 5'- CGG GCG GAT GCA CTA TTT T-3' (forward primer), 5'- GAA TAG TCC GTG CCC TTC CTC-3' (reverse primer), and 5'-FAM- CGC AGT AAC TCA ACC ATT TCC TGG TAG TGG-TAMRA (probe). Each MicroAmp optical tube had a 30 µl total reaction volume containing 1×TaqMan Universal PCR Master Mix (Applied Biosystems), forward primer (300 nM), reverse primer (300 nM), probe (300 nM), and DNA template. PCR cycle parameters were as follows: 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 58°C (52°C for bacterial 16S rRNA gene targeting). PCR was performed in a spectrofluorimetric thermal cycler (ABI Prism 7700 Sequence Detection System, Applied Biosystems). A calibration curve (log DNA concentration versus a set cycle threshold value, C_T) was obtained using 10-fold serial dilutions of pure culture genomic DNA, or plasmid DNA carrying a cloned 16S rRNA gene, *bvcA*, *tceA*, or *vcrA* of *Dehalococcoides* sp. strain BAV1, strain FL2, and strain GT.

5.2.6. Analytical techniques

Chloroethenes, chloroethanes and fluorinated ethenes were quantified with a Hewlett-Packard model 6890 gas chromatography equipped with a HP-624 column (60 m length, 0.32 mm

diameter, 1.8 µm film thickness) and a flame ionization detector (FID). Headspace samples (100 µl) were withdrawn with gastight 250 µl glass syringes (model, 1725, Hamilton Co., Reno, NV) and manually injected into a split injector operated at a split ratio of 2:1. To maintain the constant pressure in culture bottles, 100 µl of sterile N_2 was injected prior to withdrawing. Chloroethene concentrations are reported as total mass per 160 ml serum bottle unless otherwise indicated. Volatile fatty acids and hydrogen concentrations were quantified as described before (17, 27).

5.3. Results

5.3.1. Isolation of Dehalococcoides sp. strain GT

A TCE-to-ethene dechlorinating enrichment culture was obtained from TCE-fed, ethene producing microcosm using sequential transfers to medium amended with acetate, hydrogen and TCE. Following repeated transfers in the presence of ampicillin, microscopic analysis revealed a homogeneous culture consisting of small cells (<1 μm in diameter) with a disc-shaped morphology characteristic for *Dehalococcoides*. No visible growth occurred in reduced anaerobic complex media such as tryptic soy broth over a six month incubation. Amplicons generated with universal bacterial primer (8F and 1541R) using genomic DNA obtained from TCE and VC dechlorinating cultures as template yielded identical restriction patterns with all restrictions enzymes tested (Figure 5.1). T-RFLP analysis confirmed the RFLP results and yielded single peaks of 198, 443, and 513 base pairs in length from *HhaI, RsaI,* and *MspI* digested amplicons (Figure 5.2). DGGE analysis with universal primers 27F/519R yielded a single band expected for a pure culture. Further, DGGE analysis with the *Dehalococcoides*-specific primers 1F-GC/259R yielded identical bands that were indistinguishable from the band generated from *Dehalococcoides* sp. strain FL2 genomic DNA (Figure 5.3). *Dehalococcoides* sp. strain BAV1 DNA, which was included in the analysis with the *Dehalococcoides*-specific primers, yielded a band with different migration properties. The results shown in Figure 5.3 suggest that the sequence contributed by the sequence new *Dehalococcoides* isolate shared an identical 16S rRNA gene sequence with strain FL2 but differed from that of strain BAV1. Sequence alignments demonstrated that the sequence obtained from the novel TCE-to-ethene-dechlorinating isolate shared and identical 16S rRNA gene sequence with that of strain FL2 but differed one base pair at position 136 from that of strain BAV1. Culture-based approaches, microscopic analysis, and 16S rRNA gene-bases analyses all suggested culture purity. To further characterize the culture and corroborate culture purity, quantitative RTm PCR analysis using *Bacteria*- and *Dehalococcoides*-targeted primers and RDase gene-targeted primers.

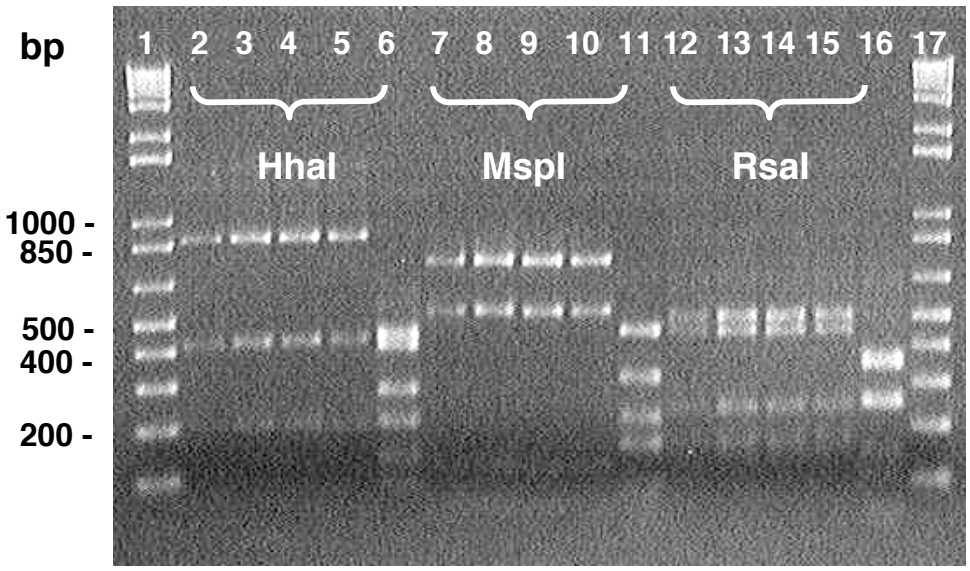

Figure 5.1. Restriction patterns in Restriction Fragment Length Polymorphism (RFLP) of 16S rRNA gene generated with universal bacterial primers.
Lane 1 and 17, ladder; lane 2,7,12, TCE-grown GT culture; lane 3,8,13, VC-grown GT culture; lane 4,9,14, VC-grown strain BAV1; lane 5,10,15, TCE-grown strain FL2; lane 6,11,16, PCE grown *Geobacter* sp. strain SZ.

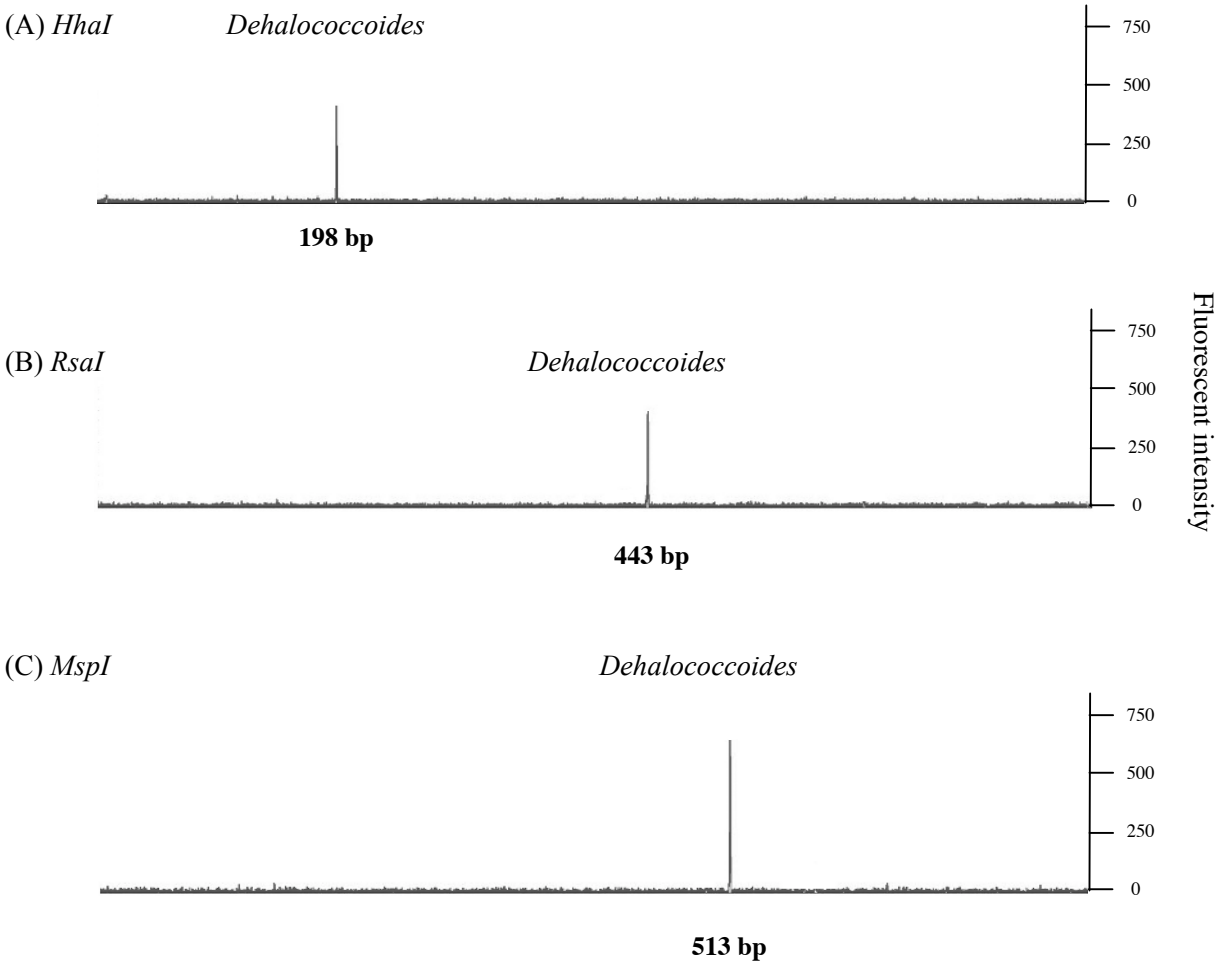

Figure 5.2. Terminal Restriction Fragment Length Polymorphism (T-RFLP) profiles after digesting the 16S rRNA gene amplicons obtained with genomic DNA from strain GT grown with VC. *HhaI*, *RsaI*, and *MspI* digests reveal peaks expected for the Pinellas group of *Dehalococcoides* spp.

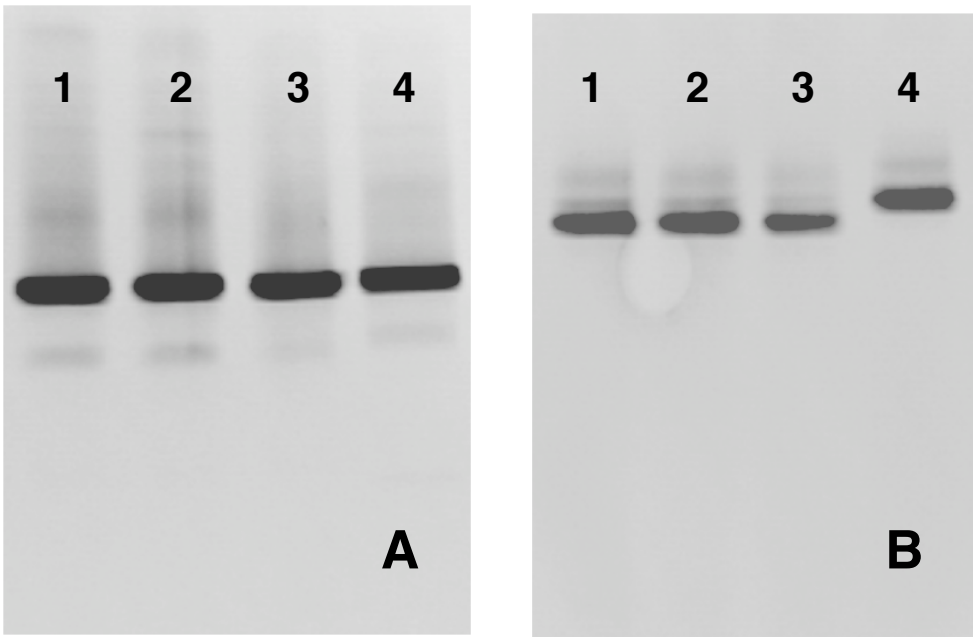

Figure 5.3. Denaturing Gradient Gel Electrophoresis (DGGE) with a universal bacterial primer PCR (A) and a *Dehalococcoides* specific primer PCR (B). Amplified PCR fragments were electrophoresed on 8-10% acrylamide gel with 30-65% (for universal bacterial primer amplified PCR product), and 45-65% (for *Dehalococcoides* specific primer amplified PCR product) denaturant. Lane1, TCE-grown strain GT; lane 2, VC-grown strain GT; lane 3, strain FL2; lane 4, strain BAV1.

The total bacterial cell numbers (2.26 x10^7 to 1.18 x10^8 16S rRNA gene copies per ml) almost equal the total *Dehalococcoides* cell numbers (3.46 x10^7 to 1.26 x10^8 16S rRNA gene copies per ml), suggesting that all cells in this culture were *Dehalococcoides*. An almost equal number of *vcrA* gene copies were enumerated indicating the *Dehalococcoides* cells in the culture carry this gene. Surprisingly, the *bvcA* and *tceA* genes were also quantifiable in this culture, though at much lower numbers ranging from 4.9×10^2 to 6.1×10^2 gene copies per ml of culture (Figure 5.4 column A). These findings suggested that the culture consisted solely of *Dehalococcoides* but also indicate the presence of multiple *Dehalococcoides* strains. Apparently, these different strains harbored identical 16S rRNA gene sequence and could not be resolved by 16S rRNA gene-based approaches. The strategy to further purify the TCE-to-ethene-dechlorinating culture involved transfers with VC as electron acceptor in an attempt to eliminate the strain carrying *tceA*. Figure 5.4 column B shows the RTm PCR results of a culture following six subsequent transfers with VC. *tceA* was no longer detectable though *bvcA* was still quantifiable. Hence, the culture was fed TCE again, and transfers occurred immediately after the onset of TCE dechlorination. Following three consecutive transfers, RTm PCR analysis failed to detect *bvcA* and *tceA*, and the total cell numbers inferred from the quantification of bacterial 16S rRNA genes, *Dehalococcoides* 16S rRNA genes, and the *vcrA* gene suggested that a pure culture consisting of a single *Dehalococcoides* organism was obtained (Figure 5.4 column C). PCR with RDase gene-targeted primer pair RRF2 and B1R yielded amplicons of the expected size (1,500 to 1,700 bp), however, nested PCR with *tceA*- and *bvcA*-specific primers did not yield a visible band, whereas *vcrA* was detected in direct PCR with a *vcrA*-targeted primer pair. The isolate was designated *Dehalococcoides* sp. strain GT (for Georgia Tech), and grew with TCE, *cis*-DCE, 1,1-DCE, and VC as electron acceptors and hydrogen as an electron donor.

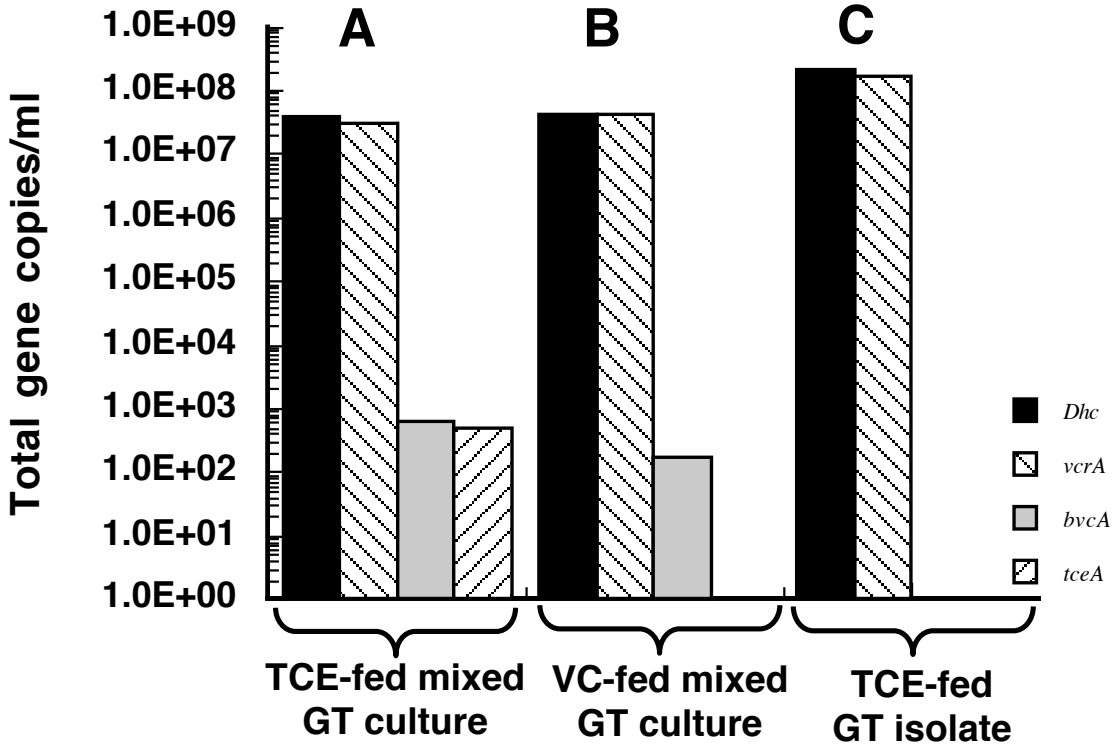

Figure 5.4. Real-Time PCR (RTm PCR) analysis of *Dehalococcoides* 16S rRNA gene copy numbers and distribution of chloroethene reductive dehalogenase genes in GT cultures. Ethene was the major dechlorination products (>90%) at the time of DNA extraction in all dechlorinating cultures. Analysis of RTm PCR in TCE-fed (column A) and VC-fed (column B) GT cultures were conducted April 2004 and October 2004 at least five separate RTm PCR analyses, respectively.

5.3.2. Physiological characteristics of strain GT

Figure 5.5 shows the dechlorination of TCE (A), *cis*-DCE (B), and VC (C) to ethene with hydrogen as the electron donor and acetate as the carbon source. Dechlorination started after a lag time of 2 weeks, and differences in lag times between the different electron acceptors were not apparent. Similar lag times of 2 weeks were observed in both TCE-and VC-fed cultures when incubated at 22 and 30 °C, however, only negligible dechlorination occurred at 35°C over a 3 month incubation period (data not shown). Only small amounts of *cis*-DCE (<19 μM) and VC (<22 μM) were transiently formed in TCE-amended cultures, whereas a substantial buildup of VC (up to 50% of the initial amount of *cis*-DCE added) occurred in *cis*-DCE-fed cultures. TCE, *cis*-DCE, 1,1-DCE, and VC were dechlorinated to ethene at rates of up to 40, 41, 62, and 127 μmol/L/d, respectively. No dechlorination occurred in cultures lacking hydrogen or acetate, suggesting that strain GT is strictly hydrogenotrophic and requires acetate as a carbon source. TCE, *cis*-DCE, 1,1-DCE, and VC were the only growth-supporting electron acceptors, and could not be replaced with PCE, *trans*-DCE, monochloroethane, 1,1-dichloroethane, 1,2-dichloroethane, 1,1,1-trichloroethane, 1,1,2-trichloroethane, carbon tetrachloride, 1,2-dichloropropane, vinyl bromide, 1,1-dichloro-2,2-difluoroethene, 1,2-dichloro-1,2-difluoroethene, 2-chloro-1,1-difluoroethene, 1,1-difluoroethene, chlorotrifluoroethene, trichlorofluoroethene, sulfate, fumarate, nitrate, and ferric citrate. Strain GT failed to dechlorinate PCE, even when PCE was added to actively TCE-, *cis*-DCE-, or VC dechlorinating cultures. The addition of ampicillin to the culture medium did not prohibit the dechlorination of TCE, *cis*-DCE, 1,1-DCE or VC to ethene with hydrogen as an electron donor and acetate as a carbon source. No growth occurred in half or full-strength tryptic soy broth medium over a 6 month incubation period.

Strain GT consumed hydrogen concentrations to 0.98 ± 0.17 ppmv (0.76 ± 0.13 nM) when provided with an excess of VC. No dechlorination was observed in cultures grown under the same conditions without hydrogen. Replacing the electron donor, hydrogen, with glucose,

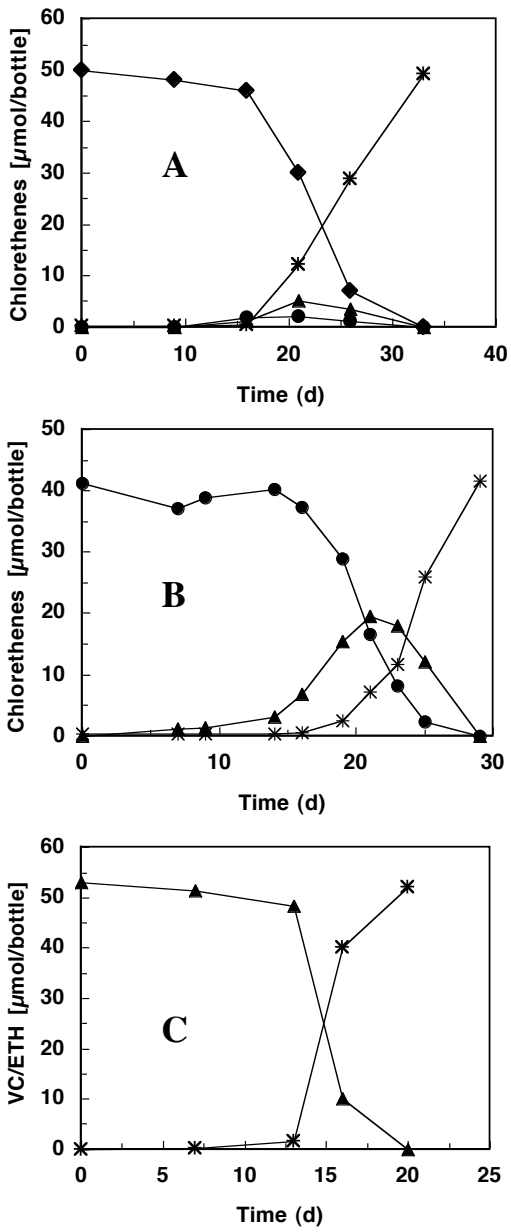

Figure 5.5. Reductive dechlorination of TCE (A), *cis*-DCE (B), and VC (C) to ethene by strain GT. Hydrogen was provided as electron donor and acetate as carbon source. All data were averaged from triplicate cultures. Symbols: ◆; TCE, ●; *cis*-DCE, ▲; VC, ✳; ethene.

formate, lactate, pyruvate, or yeast extract did not lead to dechlorination of TCE, *cis*-DCE, 1,1-DCE or VC.

5.3.3. Detection of vcrA in strain GT

The *vcrA* gene implicated in DCE and VC dechlorination in strain VS (22) was consistently detected in cultures of isolate GT. Amplification with *vcrA*-specific primers with genomic DNA extracted from TCE, *cis*-DCE, and VC grown GT cultures yielded amplicons of the expected size and sequencing confirmed identity to the strain VS *vcrA* gene over 379 base pairs stretch analyzed. Quantified total *vcrA* and *Dehalococcoides* 16S rRNA gene copies from VC dechlorinating cultures ranged from 1.59 to 2.6 × 10^8 and 1.13 to 2.28 × 10^8 per ml sample, respectively.

5.3.4. Growth-linked VC dechlorination

16S rRNA gene and *vcrA* gene targeted quantitative RTm PCR verified growth of strain GT with VC as electron acceptor. The dechlorination of VC to ethene was coupled to an increase in both 16S rRNA gene copies (data not shown) and *vcrA* gene copies (Figure 5.6). In cultures not provided with a VC as an electron acceptor, the cell numbers increased insignificantly from 4.29 ± 1.2 × 10^5 (i.e., cells introduced with the inoculum) to 5.38 ± 2.1 × 10^5. From the linear proportion of the curve (i.e., during the exponential growth phase), a doubling time of 2.98 days was estimated.

5.3.5. Environmental detection of vcrA containing Dehalococcoides species

The *vcrA* gene identified in strain GT and strain VS, was detected in three commercially available, *Dehalococcoides* containing ethene producing bio-augmentation cultures, KB-1, Bio-Dechlor, and SDC-9. *vcrA* was consistently detected in all three tested cultures regardless of electron acceptor utilized (Figure 5.7).

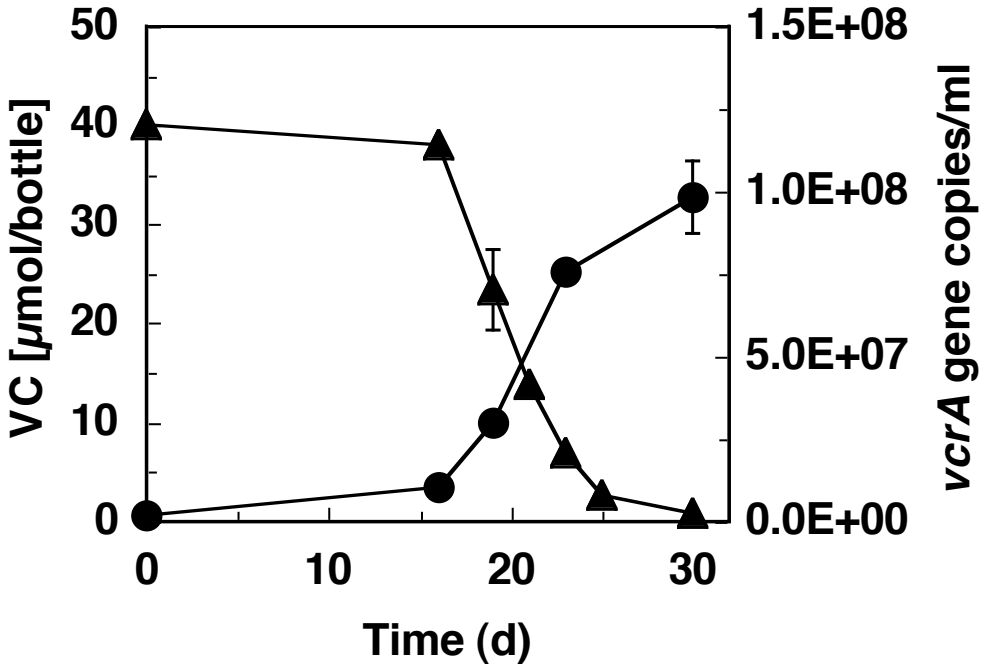

Figure 5.6. An increase in *vcrA* gene copies as determined by RTm PCR during the reductive dechlorination of VC to ethene by culture GT. Data points were averaged from triplicate cultures and error bars represent the standard deviation. Symbols: ●; *vcrA* and ▲; VC

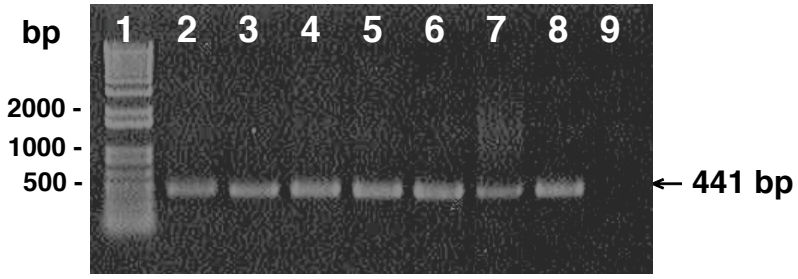

Figure 5.7. Detection of the *vcrA* gene in chloroethene dechlorinating, ethene producing enrichment cultures. Lane 1, size marker; lane 2, 3 and 4, genomic DNA from PCE, TCE, and VC-dechlorinating KB-1 culture, respectively; lane 5 and 6, genomic DNA from PCE and TCE-dechlorinating Bio-Dechlor Inoculum, respectively; lane 7, genomic DNA from TCE-dechlorinating SHAW culture; lane 8, genomic DNA from TCE-dechlorinating strain GT; lane 9, genomic DNA from TCE-dechlorinating strain FL2.

5.4. Discussion

A new *Dehalococcoides* species, strain GT, was isolated from chloroethene-impacted aquifer material. Similar to other *Dehalococcoides* isolates, strain GT had a highly restricted metabolism, and required hydrogen as an electron donor, and a chloroorganic compound (i.e., TCE, *cis*-DCE, 1,1-DCE or VC) as electron acceptor. Strain GT is affiliated with the Pinellas group of the *Dehalococcoides* cluster and exhibits physiological differences with regard to electron acceptor utilization as summarized in Table 5.1. Importantly, strain GT is the first Pinellas group isolate carrying the complete TCE-to-ethene pathway indicating that a single organism is capable of detoxification of the common environmental pollutant TCE. Hence, the notion that efficient TCE dechlorination requires more than one dechlorinating population should be revised. Dechlorination activities similar to that of strain GT have been described for a *Dehalococcoides* organism detected in consortium KB-1 (5) and culture VS (3, 22) suggesting that this physiology might be more widely distributed among the *Dehalococcoides*.

The presence of *vcrA* in the strain GT suggests that identical genes are shared between members of the Victoria and Pinellas groups. Similarly, the *tceA* gene originally detected in *Dehalococcoides ethenogenes* strain 195 (placed in the Cornell group), was also present in strain FL2, a member of the Pinellas group (9, 18). On the other hand, isolates that share the 16S rRNA gene signature sequences of the Pinellas group respire different chlorinated substrates. For instance, strains GT and BAV1 respire chlorinated ethenes whereas strain CBDB1 does not (2, 8). A recent study by Hölscher et al. (12) demonstrated that highly similar reductive dehalogenase genes are shared among *Dehalococcoides* strains, and that unique RDase genes exist that distinguish different *Dehalococcoides* strains. The division of the *Dehalococcoides* into the Victoria, Pinellas and Cornell groups was originally suggested by Hendrickson et al. and based on

Table 5.1. Utilization profiles of chlorinated ethenes as an electron acceptor in defined *Dehalococcoides* cultures

Cultures	Electron acceptors	Group[a]	Reference
Strain 195	PCE, TCE, *cis*-DCE, 1,1-DCE	C	(21)
Strain BAV1	*cis*-DCE, *trans*-DCE, 1,1-DCE, VC	P	(8)
Strain FL2	TCE, *cis*-DCE, *trans*-DCE	P	(9)
Strain KB-1/VC[b]	TCE, *cis*-DCE, VC	P	(5)
Strain VS[b]	TCE, *cis*-DCE, 1,1-DCE, VC	V	(4, 22)
Strain GT	TCE, *cis*-DCE, 1,1-DCE, VC	P	This study

[a] Group designations are according to Hendrickson et al. (10).
C, Cornell; V, Victoria; P, Pinellas (16S rRNA gene sequence sub-group).
[b] Present in mixed culture

16S rRNA gene sequence differences (10). With the new physiological information, however, it becomes apparent that this grouping does not reflect the physiological properties of its members.

The difficulty in culturing and isolating *Dehalococcoides* populations has hindered a comprehensive understanding of their distinctive characteristics. In this study, culture purity of strain GT was examined throughout the 16S rRNA-gene and RDases based approaches. All examined 16S rRNA gene based analyses including, sequencing, RFLP. T-RFLP, and DGGE with culture genomic DNA as well as microscopic uniformity strongly suggests pure culture of strain GT, however, only RTm-PCR for RDases analysis demonstrated that mixed of multiple *Dehalococcoides* species, mostly strain GT small portion of strain FL2-type and strain BAV1.

A relevant finding from this study is that 16S rRNA gene-based analyses are not sufficient to prove the purity of a *Dehalococcoides* culture. In our efforts to isolate strain GT, we derived a culture that contained a single Pinellas 16S rRNA gene sequence. Obviously, all 16S rRNA gene-based assays would detect a single sequence and suggest culture purity. Unfortunately, *Dehalococcoides* are fastidious growers, and obtaining isolated colonies is very challenging. The quantitative assessment of RDase genes demonstrated that we initially derived a culture that contained at least three distinct *Dehalococcoides* strains, which could not be distinguished by 16S rRNA gene analyses. Hence, a careful quantitative assessment of 16S rRNA gene copy number and RDase gene targets is recommended to verify purity of *Dehalococcoides* cultures. The RDase gene-targeted RTm PCR approach is an excellent tool to complement 16S rRNA gene-based approaches to verify purity of *Dehalococcoides* cultures.

Interestingly, it has been reported that *bvcA* was also present in cultures of KB-1 and Bio-Dechlor (13), suggesting that competition between the two different VC-dechlorinating *Dehalococcoides* populations for the same electron acceptor and the same reducing equivalents should be considered for providing relevant information on bioremediation technologies.

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CHAPTER VI

Effect of Sulfur Oxyanions and Sulfide on the Bacterial Reduction of Chlorinated Ethenes

Abstract

A study was developed to clarify the effect of microbial reductive dechlorination of chloroethene in the presence of sulfate reduction. Both dechlorinating cultures and sulfate reducing cultures were derived from the same chloroethene impacted sites materials (the Hydrite and the FMC site). Different dechlorination endpoints and dechlorination rates were observed in the presence of sulfate and viable sulfate reducing populations and compared to in the absence of dechlorinating populations. Sulfide, a product of sulfate reduction, was found to inhibit reductive dechlorination when in excess of the electron donor. However, in the Hydrite site enrichment culture, complete reductive dechlorination could be achieved only when the culture maintained hydrogen concentrations below 16,600 ppmv, without stimulating sulfate reduction processes. All tested chlorinated-ethene dechlorinating pure cultures, including *Desulfuromonas michiganensis* strain BB1, *Sulfurospirillum multivorans*, *Desulfitobacterium* sp. strain Viet1, *Dehalococcoides* sp. strain FL2 and strain BAV1, showed that 2 mM sulfide has an inhibitory effect (except *Sulfuromonas multivorans*). These findings demonstrate that complete reductive dechlorination can be achieved in electron limiting conditions, but that sulfide can be toxic for chlorinated ethene degrading populations. Therefore, a careful examination of the indigenous dechlorinating populations and sulfate reducers is warranted when considering bioremediation of chlorinated ethene in the presence of high sulfate concentrations.

6.1. Introduction

Bacterial populations that use chloro-organic compounds as electron acceptors are known as chloridogenic or (de)chlororespiring bacteria. Chloridogenic populations capture the energy released in reductive dechlorination reactions and thereby reduce the chloro-organic compounds. Such populations efficiently catalyze reductive dechlorination reactions and are promising candidates for bioremediation efforts at sites contaminated with chlorinated solvents. Reductive dechlorination is a thermodynamically favorable process, with standard Gibbs free energy changes in the range of -150 kJ per chloride released with hydrogen as electron donor. The reduction of nitrate to gaseous N-compounds (e.g., denitrification) is energetically more favorable than reductive dechlorination, and thus reductive dechlorination does not occur in the presence of nitrate. In contrast, acetogenic or methanogenic reduction of CO_2 to acetate or methane, respectively, is energetically less favorable than reductive dechlorination. Consequently, chloridogenic populations outcompete acetogenic and methanogenic populations, at least under electron donor-limiting conditions. While several studies confirm that nitrate reduction inhibits reductive dechlorination (1, 9, 27), other studies show that activities of acetogenic and methanogenic are negligible in actively dechlorinating cultures under electron donor limiting conditions (5, 7, 25, 28). These observations are also reflected in hydrogen consumption threshold concentrations, which decrease as the terminal electron accepting process (TEAP) becomes energetically more favorable (16).

The inhibitory effect of sulfate and other sulfur oxyanions on the degradation of chlorophenols, polychlorinated biphenyls (PCBs), chlorinated benzenes and chloroanilines has been investigated (2, 8, 19, 22-24). Sulfate reduction is less favorable than chlororespiration but little is known about the competition for reducing equivalents between chlorinated ethene degrading populations and sulfate-reducing bacteria. In addition, the effect of sulfide, a major product of sulfate reduction, on chlorinated ethene degrading populations and their activity has not been explored. Many chlorinated solvent contaminated sites have relatively high sulfate

concentrations (0.1 mM to 10 mM). The interaction between two terminal electron accepting processes, reductive dechlorination and sulfate reduction, must be considered on a site-by-site basis to provide meaningful decisions about the type of remediation strategies employed.

The objectives of the present study were to investigate the reductive dechlorination of chlorinated ethene in the presence of sulfate reduction and to examine the effect of sulfide on dechlorination performance of several chloroethene dechlorinating pure cultures and mixed cultures.

6.2. Materials and Methods

6.2.1. Chemicals

The highest purity chemicals were purchased from Aldrich (Milwaukee, WI), Sigma Chemical Co. (St. Louis, MO) and J.T Baker (Phillipsburg, NJ).

6.2.2. Organisms, medium and growth conditions

Non-methanogenic, sediment-free, ethene-producing mixed cultures were derived from two chloroethene-contaminated sites, the Hydrite Chemical Co from Wisconsin and the FMC Co. from California (26). Routinely, PCE-dechlorinating enrichment cultures were maintained to 160-ml vials containing 100 ml reduced, anaerobic, synthetic, bicarbonate-buffered medium (26) amended with PCE as electron acceptor. Hexadecane (200 µl) containing 5 to 10 µl of PCE was added to 100 ml cultures to obtain aqueous PCE concentrations ranging from 0.17 to 0.33 mM (or 49 to 98 µmoles/bottle). Hydrogen (H_2/CO_2 headspace or 1ml hydrogen) served as the electron donor for the Hydrite site enrichment culture, whereas 5 mM lactate served as the electron donor for the FMC site enrichment culture.

Sulfate reducing cultures were also derived from the same contaminated sites by enriching with sulfate instead of chlorinated ethene compounds as an electron acceptor. Two individual sulfate reducing cultures were maintained to bi-carbonated medium amended with 5-

10 mM sulfate and H_2 (for Hydrite culture) or lactate (FMC culture) was provided as electron donor.

Methanogens were completely inhibited in both ethene producing enrichment cultures by the presence of chloroethenes and after three sequential 2-bromoethanesulfonate treatments.

Competition co-culture experiments were initiated by adding the same volume [3% vol/vol] two derived populations (dechlorinating and sulfate reducing populations) to 100 ml culture media with 0.17 mM PCE, 8 mM of sulfate and electron donor. Liquid chloroethenes were added undiluted or from autoclaved hexadecane stock solutions 1 day prior to inoculation (26). Sulfate and lactate were added from anoxic, filter-sterilized stock solutions. All cultures were incubated in the dark at room temperature with the butyl rubber stopper down without shaking.

The following pure cultures were used in this study: *Desulfuromonas michiganensis* strain BB1 (26), *Sulfurospirillum multivorans* (18), *Desulfitobacterium* sp. strain Viet1 (15), *Dehalococcoides* sp. strain FL2 (12), and *Dehalococcoides* sp. strain BAV1 (10).

To determine microbial reductive dechlorination endpoint in the presence of different sulfide concentrations to chlorinated ethene dechlorinating pure cultures (described above), cultures were amended with electron acceptor, electron donor (Table 6.2, first two column) and different concentrations of sulfide. Sterile, anoxic, sulfide stock solution (0.5 M) was added as needed. To prevent transfer of oxidized forms of sulfide, pre-washed $Na_2S{\times}9H_2O$ was used to prepare a concentrated sulfide stock (0.5M) solution.

6.2.3. Detection of chloroethene dechlorinating populations

Genomic DNA was extracted using the Qiagen Mini Kit (Qiagen, Valencia, CA). To determine the presence or absence of chloroethene dechlorinating populations (i.e., the *Dehalococcoides Desulfuromonas*, and *Dehalobacter* group), 16S rRNA gene primers targeting known chloroethene dechlorinators were tested on genomic DNA extracted from the two ethene-producing enrichment cultures. The quality of the extracted genomic DNA was resolved in 1%

agarose gel in Tris-aceate-EDTA buffer and stained in aqueous ethidium bromide solution (0.5 µg/ml) for 30 min.

6.2.4. Growth conditions in defined co-culture experiments

The co-culture experiments were conducted in 160 ml serum bottles containing 100 ml bicarbonate-buffered medium amended with 5 mM of acetate and a H_2/CO_2 headspace.

The experiments were initiated by adding 1% of PCE/acetate grown strain BB1, 5 % of VC/ H_2 grown strain BAV1 and 5 µl PCE in 200µl hexadecane. After PCE depletion and the completion of ethene formation, an additional 5 µl of neat PCE was added. Different concentrations of sulfide (0.2 mM, 1 mM and 2 mM) from a concentrated sulfide stock (0.5 M) were added.

6.2.5. Analytical methods

Chloroethenes were quantified as described before (11). Chloroethene concentrations are reported as total mass per 160 ml serum bottle. For measurement of volatile fatty acids (VFA), aqueous samples were withdrawn by syringe, filtered with a 0.2 µm Nylon Membrane filter, and kept at –20°C until analysis. For analysis of VFA, 475 µl of sample was added to 25 µl of 1M H_2SO_4 in an autosampler vial and analyzed using a Waters HPLC. Sulfate analysis was conducted by using DX-100 Ion Chromatography equipped with a conductivity detector and a Dionex IonPac® AS14A column. Dionex standard sodium carbonated eluent was used at a flow rate of 1.0 ml/min. Dissolved sulfide concentrations were measured from aqueous samples using a modified Cline assay (3). Hydrogen concentration was measured by direct injection of 3 ml headspace samples into a RGA3 Reduction Gas Analyzer equipped with 1 ml sampling loop and a reduction gas detector. Hydrogen concentrations are expressed in ppmv (1 ppmv = ca. 0.1 Pa = ca. 10^{-6} atm) and quantified as described before (16).

6.3. Results

6.3.1. Microbial reductive dechlorination in the presence of sulfate

In the absence of sulfate, complete PCE reductive dechlorination was observed with both Hydrite (Figure 6.1.A) and FMC site enrichment cultures (Figure 6.1.B). Hydrogen was shown to be the only electron donor that supported complete reductive dechlorination for Hydrite site enrichment culture (after >30 times transfer), whereas lactate was required as the electron donor in FMC site enrichment culture. Sulfate reducers capable of reducing sulfate to sulfide were derived from both the Hydrite site enrichment culture and FMC site enrichment culture, and these cultures utilize the same electron donors as needed for complete reductive dechlorination (data not shown). In the presence of excess electron donor and sulfate, incomplete reductive dechlorination products and dechlorination rates were observed when both sulfate reducing populations and dechlorinating populations were present. As illustrated in Figure 6.2.A, incomplete conversion of PCE to *cis*-DCE instead of ethene was observed for the Hydrite site enrichment cultures over 120 days incubation. Sulfate reduction was observed after conversion of PCE to *cis*-DCE. Sulfate reduction did not continue to completion, even in the presence of excess electron donor. At day 100, hydrogen concentrations were maintained above 300,000 ppmv. When both sulfate reducers and dechlorinating populations derived from the FMC site enrichment culture were present, VC accumulated instead of ethene, and PCE was still present at the end of experiments (Figure 6.2.B). Sulfate reduction was more apparent after VC formation ceased. Sulfide accumulated in the Hydrite and FMC site enrichment cultures to a concentration of 3.0 mM and 2.6 mM, respectively. Under electron donor limiting conditions, however, further reductive dechlorination was achieved in the presence of sulfate reducers for the Hydrite site enrichment cultures. As shown in Figure 6.3, rapid PCE to *cis*-DCE conversion was observed under electron donor limiting conditions and in the presence of sulfate reducers and 8 mM sulfate; however, sulfate reduction did not occur. Because of the lack of an electron donor, reductive dechlorination was not extensive. The hydrogen concentration in this culture was

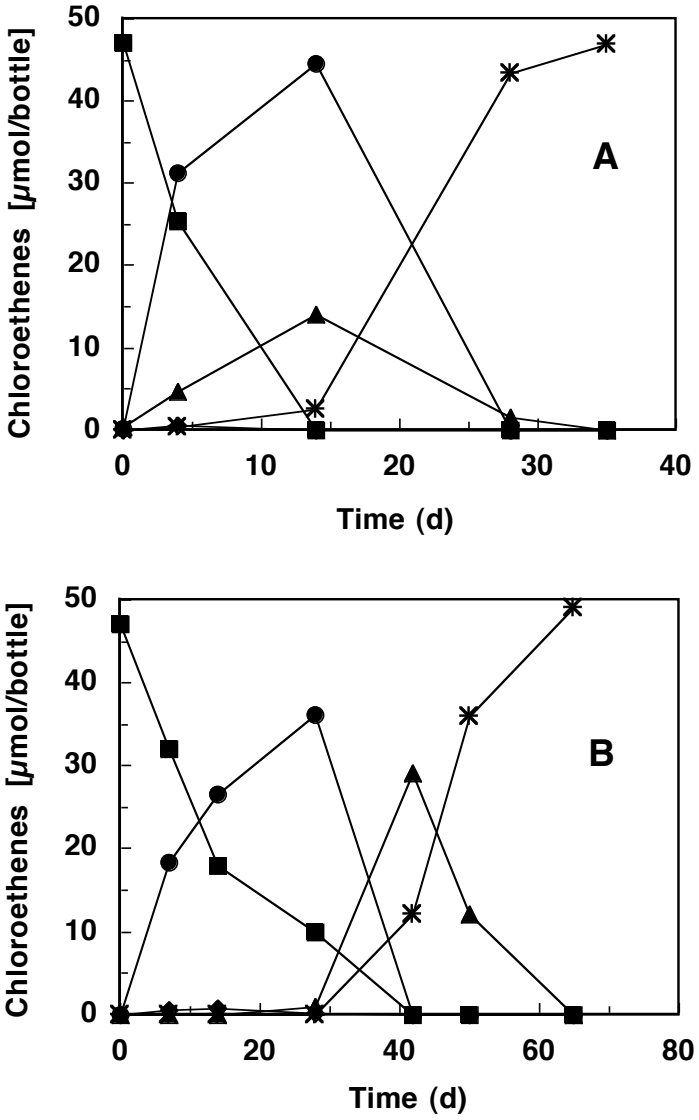

Figure 6.1. Microbial reductive dechlorination of PCE in the absence of sulfate. (A) Hydrite site enrichment culture with hydrogen as the electron donor and (B) FMC site enrichment culture with lactate as the electron donor. Data were averaged from duplicate cultures. Symbols: ■; PCE, ◆; TCE, ●; *cis*-DCE, ▲; VC, ✳; ethene.

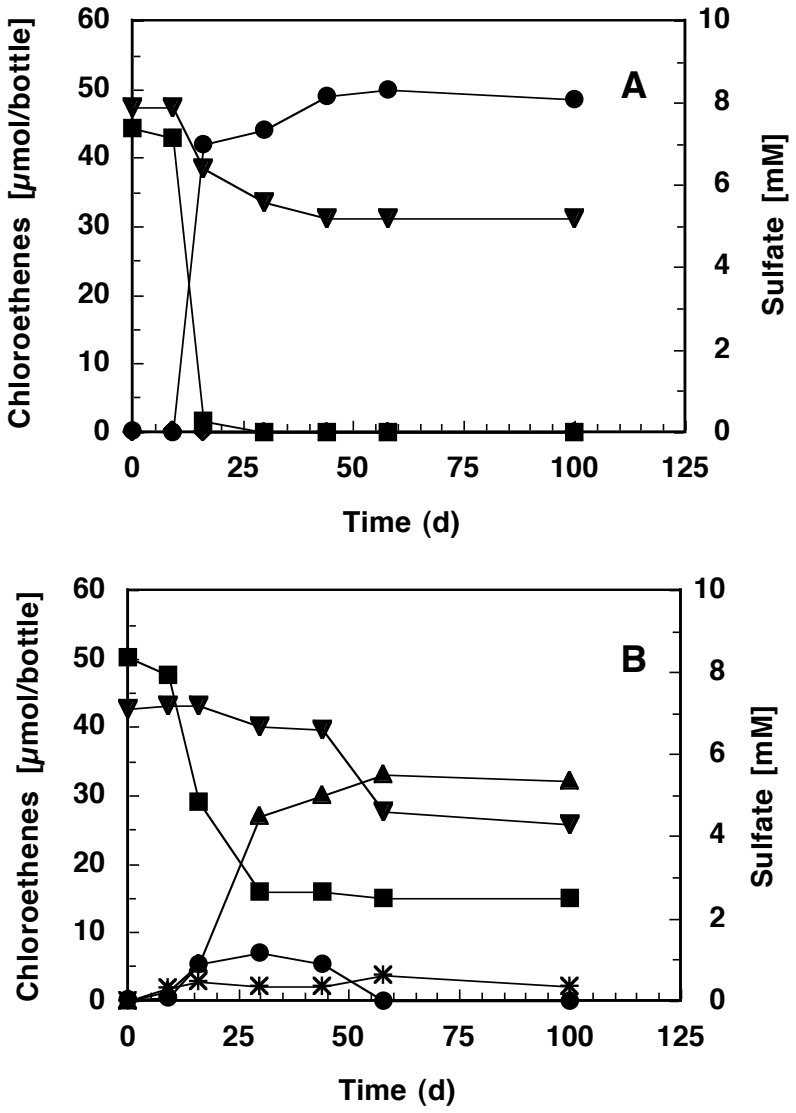

Figure 6.2. Microbial reduction of PCE and sulfate in the presence of excess electron donor; (A) Hydrite site enrichment cultures, (B) FMC site enrichment cultures. Incomplete reductive dechlorination occurred, *cis*-DCE accumulation in the Hydrite, and VC accumulation in the FMC. Culture bottles received the same volume of inoculum derived from dechlorinating culture and sulfate reducing culture (3% each vol/vol). Data were averaged from duplicated cultures. Symbols: ■; PCE, ◆; TCE, ●; *cis*-DCE, ▲; VC, ✳; ethene, ▼; sulfate.

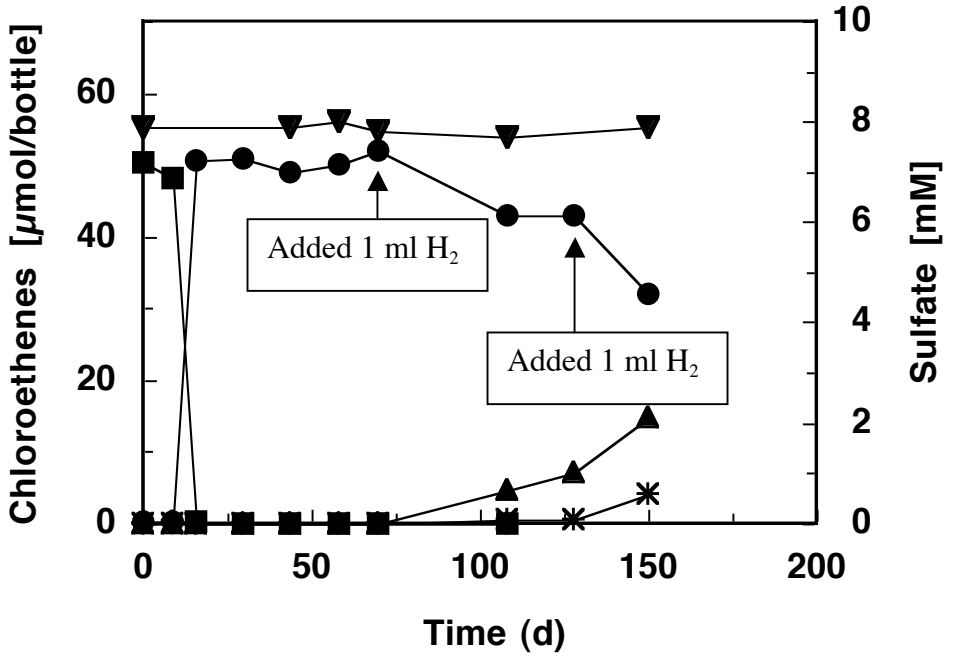

Figure 6.3. Microbial reductive dechlorination of PCE in the Hydrite site enrichment cultures under electron donor limiting conditions. The culture was amended with 5 mM acetate and 1 ml of hydrogen. Hydrogen concentration was maintained below 2 ppmv when dechlorination stopped. At days 75 and 125, cultures received an additional 1 ml of hydrogen. Further dechlorination was achieved without stimulating sulfate reduction.
Symbols: ■; PCE, ◆; TCE, ●; *cis*-DCE, ▲; VC, ✳; ethene, ▼; sulfate.

below 1.2 ppmv 70 days after the initiation of the experiments. An additional 1 ml of hydrogen (ca. = 16,600 ppmv) was added at both day 75 and day 125, resulting in further dechlorination of *cis*-DCE to ethene, without the formation of sulfide.

6.3.2. Identification of the chloroethene-dechlorinating populations

In order to identify the chloroethene-degrading population(s), PCR reactions using 16S rRNA gene primers targeting known chloroethene dechlorinators were tested on DNA extracted from both enrichments cultures. As shown in Table 6.1, *Dehalococcoides* sp. were detected in both ethene forming enrichment cultures, whereas two different *cis*-DCE forming populations, *Dehalobacter* sp. and *Desulfuromonas* sp., were detected in the Hydrite and FMC site enrichment culture, respectively. Therefore, it is assumed that at least one *Dehalococcoides* sp. is involved in ethene formation, and a heterogeneous distribution of dechlorinating populations exists in the derived enrichment cultures.

6.3.3. Effect of microbial reductive dechlorination in the presence of sulfide

Both ethene producing enrichment cultures were tested with different sulfide concentrations to clarify inhibitory effects of sulfide on the ability of these cultures to perform complete reductive dechlorination. At 2mM of sulfide, *cis*-DCE accumulated in the Hydrite site enrichment culture with slow conversion to VC (Figure 6.4.A). No further dechlorination was observed at the conclusion of the experiment. Ethene formation did not occur at this sulfide concentration (Figure 6.4.A), but complete conversion of PCE to ethene occurred at 0.2 mM of sulfide (Figure 6.1.A). Complete inhibition occurred at sulfide concentrations of 5.0 mM for the Hydrite site enrichment culture (Figure 6.4.B) and 2.0 mM for the FMC site enrichment culture (data not shown).

Taking into account the identified dechlorinating populations at each site, careful inspection shows that sulfide inhibition may be population specific. At a sulfide concentration of 2 mM, it is apparent that the *Dehalobacter* group in the Hydrite site enrichment culture was not inhibited since PCE was converted to *cis*-DCE; however, the lack of further dechlorination

Table 6.1. Detection of chloroethene dechlorinating populations using specific 16S rRNA gene targeted primers

Enrichment cultures	Dechlorination end product	PCR primers targeting group		
		Dehalococcoides	*Dehalobacter*	*Desulfuromonas*
Hydrite	Ethene	+	+	-
FMC	Ethene	+	-	+

- : not detected
+ : detected

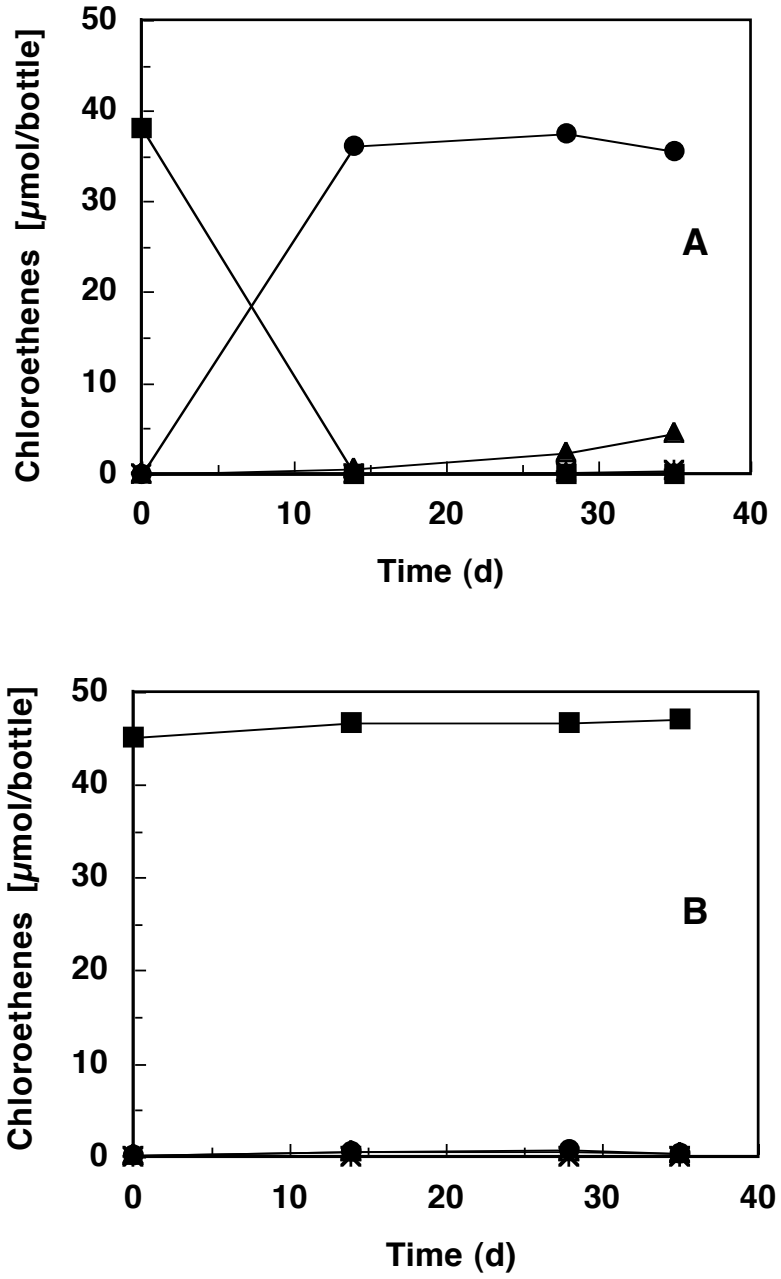

Figure 6.4. Microbial reductive dechlorination of PCE by Hydrite enrichment culture in the presence of (A) 2 mM of sulfide and (B) 5 mM of sulfide. At the end of experiments, ethene production was not observed. Data were averaged from duplicate cultures. Symbols: ■; PCE, ◆; TCE, ●; *cis*-DCE, ▲; VC, ✳; ethene.

indicates that the *Dehalococcoides* group was inhibited at this concentration. Likewise, FMC site enrichment culture was composed of two dominant dechlorinating populations, the *Desulfuromonas* group and the *Dehalococcoides* group. The *Desulfuromonas* group in the FMC site enrichment culture appears to be inhibited at 2mM of sulfide as evidenced by the lack of PCE reduction (data not shown). These findings suggest that the toxicity effect of sulfide is unique to specific sites and dechlorinating populations.

6.3.4. Effect of sulfur oxyanions and sulfide on chloroethene degrading pure cultures

By varying the concentrations of sulfate and sulfide in the media, the influence of these sulfur containing compounds upon the dechlorination performance of five pure chlorinated ethene degrading cultures was determined. The pure cultures tested represent a diverse group with a broad range of activities (dechlorination rates and endpoints) and included *Desulfuromonas michiganensis* strain BB1, *Sulfurosprillum multivorans*, *Desulfitobacterium* sp. strain Viet1, *Dehalococcoides* sp. strain FL2 and *Dehalococcoides* sp. strain BAV1. The presence of sulfate (10 mM) had no effect on the dechlorination performance of each pure culture compared to cultures grown in the absence of sulfate. However, concentrations of sulfide as low as 1.0 mM inhibited reductive dechlorination by *Desulfitobacterium* sp. strain Viet1. All cultures were inhibited at 2 mM sulfide, except *Sulfurosprillum multivorans,* which continued to dechlorinate in the presence of 5 mM sulfide (Table 6.2). Sulfide, a sulfate reduction product, clearly showed inhibitory effects to the activity of several dechlorination populations.

6.3.5. Influence of sulfide to defined coculture experiments

In order to explain the observed results from the FMC site enrichment culture, two pure chloroethene dechlorinating strains were combined in a co-culture to show a sulfide toxicity effect. Different sulfide concentrations (0.2 mM, 1 mM, and 2mM) were tested in the defined co-culture experiment, which was composed of two pure cultures: *Desulfuromonas michiganensis* strain BB1 and *Dehalococcoides* sp. strain BAV1. The co-culture was amended with 5 mM of acetate, a H_2/CO_2 (80:20 vol/vol) headspace, and 5 µl PCE in 200 µl hexadecane.

Desulfuromonas michiganensis strain BB1 couples acetate oxidation to PCE reduction, converting PCE to *cis*-DCE (26); *Dehalococcoides* sp. strain BAV1 reductively dechlorinates *cis*-DCE to ethene with hydrogen as the electron donor (10). Therefore, PCE was expected to be completely converted to ethene in the co-cultures. Figure 6.5 shows that PCE was degraded in a stepwise fashion to *cis*-DCE and VC before being converted to stoichiometric amounts of ethene within 27 days of inoculation. At day 27, each set of duplicate co-culture was amended with 5 µl of neat PCE and a different amount of sulfide, resulting in sulfide concentrations of 0.2 mM (no amendment), 1 mM, and 2 mM, respectively. PCE was completely reduced to ethene in the co-cultures, which had no additional amendments of sulfide or 1mM sulfide (data for 0.2 mM is shown in Figure 6.5; no data are shown for 1 mM sulfide). At day 43, all PCE was completely reduced to ethene in the coculture, which was amended with no additional sulfide. This shows that sulfide had no inhibitory effect upon *Desulfuromonas michiganensis* strain BB1 or *Dehalococcoides* sp. strain BAV1 at these concentrations. *Desulfuromonas michiganensis* strain BB1, however, was inhibited in the presence of 2 mM sulfide, resulting in incomplete conversion of PCE to *cis*-DCE. No further ethene formation was observed.

Table 6.2. Reductive dechlorination end products in pure chloroethene dechlorinating cultures in the presence of sulfate or sulfide

Cultures	Electron acceptor/donor	Dechlorination end products					
		Sulfate (mM)	Sulfide (mM)				
		10	0.2[a]	1.0	2.0	5.0	10.0
Desulfuromonas michiganenesis strain BB1	PCE/acetate	*cis*-DCE	*cis*-DCE	*cis*-DCE	PCE/TCE/ *cis*-DCE	PCE	PCE
Sulfurospirillum multivorans	PCE/H_2	*cis*-DCE	*cis*-DCE	*cis*-DCE	*cis*-DCE	PCE/TCE/ *cis*-DCE	PCE
Desulfitobacterium sp. strain Viet1	PCE/pyruvate	TCE	TCE	PCE	PCE	PCE	PCE
Dehalococcoides sp. strain FL2	TCE/H_2	VC	VC	VC	TCE	TCE	TCE
Dehalococcoides sp. strain BAV1	VC/H_2	ETH	ETH	ETH	VC	VC	VC

All data were from triplicate culture

[a]: Defined bicarbonated buffer media containing 0.2 mM of sulfide)

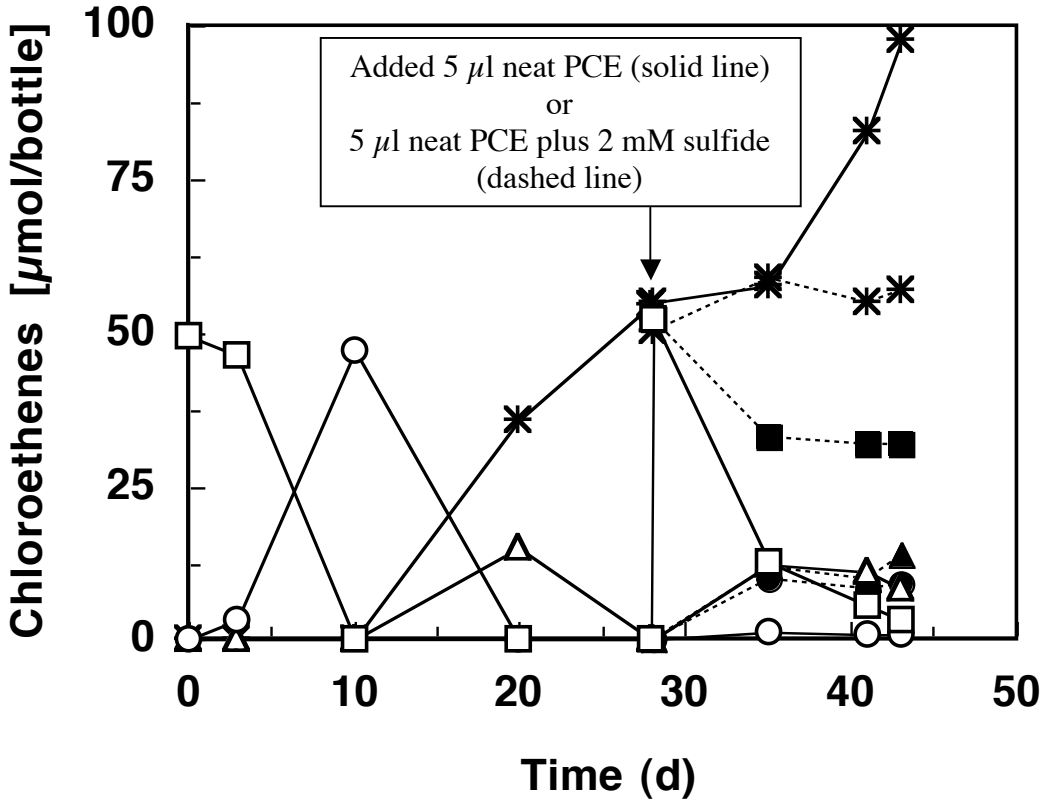

Figure 6.5. Complete reductive dechlorination of PCE in co-cultures of *Desulfuromonas michiganensis* strain BB1 and *Dehalococcoides* sp. strain BAV1. All co-cultures were supplied with 5 mM acetate, a H_2/CO_2 headspace and 5 µl of PCE in 200 µl hexadecane. At day 27, duplicate cultures received an additional 5 µl of neat PCE (solid line, open symbols), while the other duplicate cultures received 5 µl neat PCE plus 2 mM of sulfide (dashed line, closed symbols). Data were averaged from duplicate cultures.
Symbols: squares; PCE, diamonds; TCE, circles; *cis*-DCE, triangles; VC, asterisks; ethene.

6.4. Discussion

Currently, the addition of hydrogen, or substrates that can be fermented to hydrogen, to chloroethene-impacted sites is considered critical for successful remediation. This is because only one known species, *Dehalococcoides* sp., can completely detoxify chloroethenes to ethene, and this species strictly requires hydrogen as an electron donor. Lactate is often used to stimulate microbial activity at contaminated sites. Lactate is fermented by soil microbes, producing propionate, acetate, and hydrogen as major products. In consequence, chloroethene dechlorinating populations should compete with sulfate reducers for the same reducing equivalent (e.g., hydrogen or acetate). An excess of hydrogen, however, may be used by sulfate reducers and allow them to thrive even though *Dehalococcoides* sp. can dechlorinate at much lower hydrogen levels. In the presence of sulfate and viable sulfate reducing populations, sulfate will be transformed to sulfide, which, according to the data presented, shows inhibitory effects to dechlorinating populations. Thus, complete reductive dechlorination was not guaranteed in the presence of sulfide, a sulfate reduction product. Therefore, the addition of excess hydrogen to stimulate reductive dechlorination and site cleanup may lead to inhibition of microbial reductive dechlorination.

This study demonstrates that sulfate reduction and the microbial reductive dechlorination process may compete for the same reducing equivalents. In consequence, incomplete reductive dechlorination products were observed due to sulfide accumulation.

In addition, careful examination of the sulfate reduction product, sulfide, showed a toxicity effect to chlorinated ethene degrading populations both in mixed enrichment cultures and pure cultures. Several studies with freshwater sediments and mixed culture systems have compared competition for the same electron donor (i.e., hydrogen) between dechlorinating process and other hydrogen dependent processes, such as acetogenesis and methanogenesis (17, 28).

It has been speculated that sulfur oxyanions inhibit reductive dechlorination due to competition between sulfate reduction and the chlororespiration process for the same reducing equivalents. Under sulfate reducing conditions, reductive dechlorination of chloro-organic compounds (e.g., chlorophenols, chlorobenzoates, chloroanilines, PCBs etc.) is severely inhibited or is not detected in many cases (2, 8, 19, 22-24).

Ethene-producing enrichment cultures and sulfate reducing cultures were obtained from two chlorinated solvent contaminated sites, Hydrite, and FMC site. These two cultures, dechlorinating cultures and sulfate reduction cultures, utilize the same electron donor for complete reductive dechlorination of PCE or sulfate reduction. These results imply that dechlorinating populations and sulfate reducers could coexist in many contaminated sites. Townsend et al. (27) showed that sulfate reduction and reductive dechlorination of 3-chlorobenzoate can occur simultaneously in aquifer slurries.

In the enrichment cultures described here, two different terminal electron accepting processes compete for the same electron donor: hydrogen or lactate, for the Hydrite and FMC sites, respectively. The results of the previously described experiments demonstrate that the presence of excess electron donor can stimulate both reductive dechlorination and sulfate reduction. Interestingly, neither process continued to completion, even under the presence of enough electron donor to stimulate reductive dechlorination or sulfate reduction. Our results, however, showed that sulfate reduction did not continue to completion in the presence of excess electron donor, implying that indigenous sulfate reducing population are also inhibited by sulfide. Klemps et al. (13) showed that above 5 mM sulfide concentration has a toxicity effect to sulfate reducing bacteria.

On the other hand, complete reductive dechlorination proceeded in the presence of sulfate under electron donor limiting conditions, whereas the sulfate reduction process was not stimulated. Previous results (16) have demonstrated that dehalogenating bacteria can be excellent competitors for hydrogen. Dehalogenating bacteria maintained hydrogen concentrations below

the threshold hydrogen concentrations needed by other organisms utilizing the hydrogenotrophic terminal electron acceptor processes (e.g., methonogenesis, acetogenesis, etc.). Mazur and Jones (21) reported that a significantly lower hydrogen concentration was observed in the sediment slurries amended with PCE, as compared to slurries without PCE, under sulfate reducing conditions. The results presented here show that under electron donor limiting conditions, dechlorinating bacteria can achieve complete reductive dechlorination while maintaining the hydrogen concentrations below the threshold hydrogen concentrations needed for sulfate reduction to occur.

Surprisingly, microbial reductive dechlorination of chlorinated ethenes was inhibited by a sulfate reduction product, sulfide, rather than sulfate. The experiment testing different sulfide concentrations showed that each site has different dechlorination end points in the presence of concentrations of sulfide, suggesting sulfide toxicity was specific for different dechlorinating populations. The same amount of sulfide (2mM) was added to both enrichment cultures, which resulted in *cis*-DCE accumulation in the Hydrite site enrichment culture (Figure 6.4) and in no dechlorination in the FMC site enrichment culture (data not shown). The Hydrite and FMC site enrichment cultures contained multiple dechlorinating populations exhibiting different dechlorination activities and different levels of sulfide toxicity. More precisely, these results imply that *Dehalobacter* sp., which was detected in the Hydrite site enrichment culture enrichment culture, was less susceptible to sulfide toxicity than *Desulfuromonas* sp., which was detected in the FMC site enrichment culture. These results partially explain why many chlorinated ethene contaminated sites have accumulated incomplete reductive dechlorination products such as *cis*-DCE or VC, even in the presence of *Dehalococcoides* spp.

Most interestingly, certain concentrations of sulfide inhibited all five pure chlorinated ethene degrading cultures, while the presence of sulfate did not inhibit microbial reductive dechlorination (Table 6.2). Given the fact that growing *Dehalococcoides* sp. can be inhibited by the presence of 2 mM sulfide, complete microbial reductive dechlorination is not guaranteed

solely because *Dehalococcoides* sp. is present. Furthermore, in defined coculture experiments, *Desulfuromonas michiganensis* strain BB1 and *Dehalococcoides* sp. strain BAV1 were inhibited in the presence of 2 mM of sulfide, but not at 1 mM sulfide. Krumholz (14) speculated that residual sulfide from polysulfide has toxicity to *Desulfuromonas chloroethenica* strain TT4B. This is very useful information to consider when attempting to ensure complete reductive dechlorination at any site where sulfate and sulfate reducers are present in sufficient quantities to possibly inhibit dechlorination.

Sulfate has been reported to suppress or enhance reductive dechlorination in enrichment cultures and some pure cultures. Townsend and Suflita (27) demonstrated that sulfate inhibits dehalogenation in *Desulfomonile tiedjei* under growth conditions. They also suggested that inhibition may be due to sulfur oxyanions serving as the preferred electron acceptors and repressing the expression of reductive dehalogenases in bacteria that contain both sulfur oxyanions and chloroaromatic respiratory activities. None of tested above chlorinated ethene degrading pure cultures used sulfate as their alternative electron acceptor, therefore, competition respiratory activities were ruled out. PCE dechlorination by *Desulfitobacterium frappieri* strain TCE1 was not negatively influenced in the presence of relatively high sulfate concentrations (6). The other sulfur oxyanions, sulfite and thiosulfate have been shown to inhibit reductive dechlorination processes. These inhibition mechanisms are not clear, but Deweerd et al. (4) speculated that sulfite and thiosulfate have different chemical reactivity compared with chemically inert sulfate to dechloriantion involved proteins. Reductive dechlorination activity of strain BB1 was completely inhibited in the presence of 1 mM of sulfite (26). Two reductive dehalogenases from *Dehalococcoides ethenogenes* strain 195 showed that complete inhibition was observed in the presence of 2 mM of sulfite (20).

Our results, using indigenous dechlorinating population and sulfate reducers, indicate that complete reductive dechlorination can be achieved in the electron limiting conditions, but that sulfide has a toxicity effect on dechlorinating populations. A careful evaluation of the indigenous

dechlorinating populations and sulfate reducers is warranted when considering treatment technologies to achieve complete reductive dechlorination in the presence of high sulfate concentrations at specific chlorinated ethene contaminated sites.

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CHAPTER VII

Conclusions and Recommendations

Dehalococcoides-containing, ethene-forming enrichment cultures were derived from aquifer materials from various chlorinated ethene contaminated sites and non-contaminated sites. These ethene forming enrichment cultures are a significant source of information allowing for a further understanding of microbial reductive dechlorination processes. Additionally, these cultures are meaningful sources to obtain dechlorinating isolates. Findings of this research not only advance our understanding of the unique physiology of *Geobacter lovleyi* strain SZ, and *Dehalococcoides* sp. strain H10, but also expand their potential for environmental significances and their applications in bio-remediation.

Combined kinetic studies and 16S rRNA gene and chloroethene RDases. *tceA*, *vcrA*, and *bvcA*, targeted quantitative and qualitative PCR approaches in ethene producing enrichment cultures provided following information; 1) all ethene forming enrichment cultures contain at least one *Dehalococcoides* species; 2) in addition to *Dehalococcoides* populations, tetrachloroethene (PCE) to 1,2-*cis*-dichloroethene (*cis*-DCE) dechlorinators were also detected in many ethene producing enrichment cultures and coexisted with *Dehalococcoides* species; 3) different *Dehalococcoides* species are present in many ethene producing enrichment cultures, including species yet to be isolated (e.g., strains 195, FL2, BAV1, and CBDB1); 4) heterogeneous distribution of VC RDases, *bvcA* and *vcrA*, is common; 4) *bvcA* or *vcrA* gene containing enrichment cultures effectively reduce VC and formed ethene; 5) *vcrA* was detected more frequently than *bvcA* from most ethene producing cultures, and 6) unidentified RDases existed in many ethene producing cultures.

A new isolate, designated strain SZ, was obtained from a PCE-to-ethene enrichment culture derived from non-contaminated aquatic sediment amended microcosms. The 16S rRNA gene sequence of strain SZ indicated that the new isolate was affiliated with the genus *Geobacter*

most closely related to *G. thiogenes*. Strain SZ is capable of stepwise dechlorination of PCE to *cis*-1,2-dichloroethene (*cis*-DCE), while the closest relatives were not able to dechlorinate PCE or TCE. Reductive dechlorination of PCE and TCE was supported by acetate, hydrogen and pyruvate as electron donor. Chloroethene-dechlorinating populations have been shown to have distinct electron donor requirements. For example, *Dehalobacter* and *Dehalococcoides* populations are strictly hydrogen dependent. *Desulfuromonas* populations, such as, *Desulfuromonas chloroethenica* strain TT4B and strain BB1, use acetate rather than hydrogen for reductive dechlorination of PCE. However, no dechlorinating populations, which can couple the oxidation of both acetate and hydrogen to the reduction of PCE has been demonstrated. PCE dechlorination by strain SZ uses both acetate and hydrogen as electron donors suggesting that the ability to versatile electron donor utilization may increase the efficiency of bioremediation approaches. Surprisingly, strain SZ reduced two environmental priority pollutants, PCE and U(VI) concomitantly, suggesting that strain SZ holds promise for *in-situ* bioremediation of chloroethene and U(VI) contaminated sites. In deed, specific detection of strain SZ-type of organism in currently on-going bio-stimulated chloroethene contaminated sites and uranium contaminated FRC sites strongly imply that strain SZ is playing an important roles in biotransformation of chloroethenes and uranium.

Isolating an acetate-oxidizing, PCE-dechlorinating dechlorinator, strain SZ, may lead to a better understanding of the characteristics and environmental significances of acetate-oxidizing dechlorinating populations. However, all known reductive RDases are purified from hydrogen dependent dechlorinators simply because of limited numbers of acetate oxidizing dechlorinators. Further studies are needed to identify and characterize functional genes that are involved in a process of interest such as reductive dechlorination and uranium reduction.

A complete TCE, *cis*-DCE, and human carcinogen VC degrading new *Dehalococcoides* sp. strain H10 was isolated. Because of high similarity of 16S rRNA gene sequences among the *Dehalococcoides* species, 16S rRNA gene based purity analyses such as, sequencing, RFLP, T-

RFLP, and DGGE are insufficient to verify the culture purity. In this study, *Dehalococcoides* related chloroethene RDases, *tceA*, *bvcA* and *vcrA* targeted quantitative real time PCR approaches intuitively demonstrates culture purity of strain H10. Ampicillin resistant growth, a strict requirement for hydrogen as electron donor, and hydrogen threshold concentrations supported the involvement of *Dehalococcoides*. More directly, 16S rRNA-gene targeted quantitative RTm-PCR approaches showed that an increase in *Dehalococcoides* 16S rRNA gene copies was dependent on the presence of TCE, *cis*-DCE, and VC as growth supporting electron acceptors. Presence of *vcrA* and the absence of *tceA* and *bvcA* genes and different electron acceptor utilization patterns in strain H10 could differentiate with pure *Dehalococcoides* strains 195, FL2, and BAV1. Frequent detection of *vcrA* containing strain H10-like *Dehalococcoides* populations from bio-augmentation microbial consortia such as, KB-1, Bio-Dechlor, and SDC-9 (SHAW dechlorinating culture-9) and many ethene forming enrichment cultures, suggest that strain H10 type of *Dehalococcoides* may play an important role in complete detoxification of chloroethenes. Hence, further research, for instance, competition experiments between two different VC degrading *Dehalococcoides* species, strain H10 and BAV1, for the same electron acceptor and reducing equivalents in lab and in-situ conditions, should be considered for providing relevant information for bioremediation approaches.

Relatively little is known about how the presence of sulfate reducing process affects the reductive dechlorination process, although such information is relevant for engineered bioremediation approaches. This research examined effect of reductive dechlorination under sulfate reducing conditions and also the influence of the product of sulfate reduction, sulfide. The following conclusions were drawn from these experiments: 1) different, mostly incomplete, reductive dechlorination endpoints and rates were observed in the presence sulfate reducing populations as compared to those cultures grown in the absence of sulfate reducing populations, 2) neither process continued to completion, in the presence of excess electron donor, 3) complete reductive dechlorination can be achieved under the electron limiting conditions, hence, simply the

increase of electron donor and the presence of *Dehalococcoides* populations did not guaranteed complete reductive dechlorination in presence of viable sulfate reduction, 4) the sulfate showed no inhibition effect on all test dechlorinating mixed and pure cultures, 5) the sulfate reduction product, sulfide, showed an inhibitory effect to all tested dechlorinating enrichment and pure cultures, 6) sulfide toxicity is specific for different dechlorinating populations. Hence, a careful examination of the indigenous dechlorinating populations and sulfate reducers is warranted when considering bioremediation of chlorinated ethene in the presence of high sulfate concentrations.

Overall, this research provides a better understanding of the environmental microbiology and ecology involved in reductive dechlorination of chlorinated ethenes, and may have important implications for bioremediation. This information is critical in order to provide meaningful decision of engineered bioremediation, biostimulation and bioaugmentation at any given contaminated sites because contamination of soil and groundwater with chlorinated ethenes is a threat to human health and overall ecosystems. Therefore, the knowledge of the distribution, function, and specific requirements of dechlorinators is essential to amenable bioremediation technologies to detoxify many contaminated sites.

APPENDIX A

16S rRNA gene sequence of *Geobacter lovleyi* strain SZ

(GenBank accession number AY914177)

CTCAGAACGAACGCTGGCGGCGTGCCTAACACATGCAAGTCGAACGGAGTGAAGGAGCTTGCTCT
TTCATTTAGTGGCGCACGGGTGAGTAACGCGTAGATAATCTGCCTTGGACTCTGGGATAACATCT
CGAAAGGGGTGCTAATACCGGATAAGCCCAGATGGCGTAAGTCATTGCGGGAAAAGGGGGCCTCT
GAATATGCTCCTGATTCAAGATGAGTCTGCGTACCATTAGCTAGTTGGTAGGGTAAGAGCCTACC
AAGGCGACGATGGTTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAG
ACTCCTACGGGAGGCAGCAGTGGGGAATTTTGCGCAATGGGGGAAACCCTGACGCAGCAACGCCG
CGTGAGTGATGAAGGCTTTCGGGTCGTAAAGCTCTGTCTAGAGGGAAGAAATGATAGTCGGTTAA
TACCCGGTTTTCTTGACGGTACCTCTGAAGGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGT
AATACGGAGGGTGCAAGCGTTGTTCGGATTTATTGGGCGTAAAGCGCGTGTAGGCGGTTTGTTAA
GTCTGATGTGAAAGCCCTGGGCTCAACCTGGGAAGTGCATTGGAAACTGGCAGACTTGAATACGG
GAGAGGGTAGTGGAATTCCTAGTGTAGGAGTGAAATCCGTAGATATTAGGAGGAACACCGGTGGC
GAAGGCGGCTACCTGGACCGATATTGACGCTGAGACGCGAAAGCGTGGGGAGCAAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAGGTGTTGCGGGTATTGACCCCTGCAGTG
CCGCAGCTAACGCATTAAGTACTCCGCCTGGGAAGTACGGTCGCAAGACTAAAACTCAAAGGAAT
TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGCAGAACCTTACC
TGGTCTTGACATCTACGGAACCTCTATGAAAGTAGAGGGTGCCTTTCGGGGAGCCGTAAGACAGG
TGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CCTATCCTCAGTTGCCATCATTAAGTTGGGCACTCTGTGGAGACTGCCGGTGTCAAACCGGAGGA
AGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGACCAGGGCTACACACGTGCTACAATGGCC
GGTACAAAGAGTTGCGATGCCGCGAGGTGGAGCTAATCTCATAAAGCCGGTCTCAGTTCGGATTG
GAGTCTGCAACTCGACTCCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGA
ATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGGGAGTCGATTGGTCCCGAAGTACG
TGAGCTAACCCTTTTGGGAGGCAGCGTCCTAAGGAATGGTCGGTGACT

APPENDIX B

16S rRNA gene sequence of *Dehalococcoides* sp. strain GT

(GenBank accession number AY914178)

TTATGCATGCAAGTCGAACGGTCTTAAGCAATTAAAGATAGTGGCGAACGGGTGAGTAACGCGTA
AGTAACCTACCTCTAAGTGGGGGATAGCTTCGGGAAACTGAAGGTAATACCGCATGTGGTGGGCC
GACATATGTTGGTTCACTAAAGCCGTAAGGCGCTTGGTGAGGGGCTTGCGTCCGATTAGCTAGTT
GGTGGGGTAATGGCCTACCAAGGCTTCGATCGGTAGCTGGTCTGAGAGGATGATCAGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGCAAGGAATCTTGGGCAATGGGCGAAA
GCCTGACCCAGCAACGCCGCGTGAGGGATGAAGGCTTTCGGGTTGTAAACCTCTTTTCATAGGGA
AGAATAATGACGGTACCTGTGGAATAAGCTTCGGCTAACTACGTGCCAGCAGCCGCGGTAATACG
TAGGAAGCAAGCGTTATCCGGATTTATTGGGCGTAAAGTGAGCGTAGGTGGTCTTTCAAGTTGGA
TGTGAAATTTCCCGGCTTAACCGGGACGAGTCATTCAATACTGTTGGACTAGAGTACAGCAGGAG
AAAACGGAATTCCCGGTGTAGTGGTAAAATGCGTAGATATCGGGAGGAACACCAGAGGCGAAGGC
GGTTTTCTAGGTTGTCACTGACACTGAGGCTCGAAAGCGTGGGGAGCGAACAGAATTAGATACTC
TGGTAGTCCACGCCTTAAACTATGGACACTAGGTATAGGGAGTATCGACCCTCTCTGTGCCGAAG
CTAACGCTTTAAGTGTCCCGCCTGGGGAGTACGGTCGCAAGGCTAAAACTCAAAGGAATTGACGG
GGGCCCGCACAAGCAGCGGAGCGTGTGGTTTAATTCGATGCTACACGAAGAACCTTACCAAGATT
TGACATGCATGTAGTAGTGAACTGAAAGGGGAACGACCTGTTAAGTCAGGAACTTGCACAGGTGC
TGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTTTGGTTAAGTCCTGCAACGAGCGCAACCCTT
GTTGCTAGTTAAATTTTCTAGCGAGACTGCCCCGCGAAACGGGGAGGAAGGTGGGGATGACGTCA
AGTCAGCATGGCCTTTATATCTTGGGCTACACACACGCTACAATGGACAGAACAATAGGTTGCAA
CAGTGCGAACTGGAGCTAATCCCCAAAGCTGTCCTCAGTTCGGATTGCAGGCTGAAACCCCGCCT
GCATGAAGTTGGAGTTGCTAGTAACCGCATATCAGCATGGTGCGGTGAATACGTTCTCGGGCCT

APPENDIX C

Ferrozine method for determination of Fe(II) and Total Fe

Solutions

1. 0.5 M HCl solution
2. Ferrozine buffer: 0.2g ferrozine, 12.0g HEPES buffer in 1L water
3. Hydroxylamine ferrozine buffer: 1g hydroxylamine in 100 ml Ferrozine buffer

Assay (in 1.5 ml Eppendorf tube)

HCl extractable Fe(II)

1. 1:10 dilution of the sample (the dilution may vary, but must be 1:10 or more dilute) is extracted in 0.5 M HCl (100 μl 0.2μm-filtered sample to 900 μl HCl solution).
2. Incubate 30 minutes
3. 1:10 dilution of HCl extracted samples into Ferrozine buffer (100 μl sample to 900 μl Ferrozine buffer)
4. Incubate 15 minutes
5. Measure absorbance at 562 nm

Total Fe

Use the same protocol as above, but used the Hydroxylamine buffer instead of the Ferrozine buffer

Fe(III)

Substract total HCl extractable Fe(II) from quantified total Fe

Standard preparation

1. Dissolve Ferrous ammonium sulfate to N_2 purged, acidified water (100 µl HCl per 100 ml water) get a final 100 mM of Fe(II)
2. Dilute Fe(III) standard solution to get desired concentration inside glove box
3. Follow the same protocol as above.

Note

All measurements must be done quickly.
Linear range up to 10 mM Fe(II) and Total Fe
Store Ferrozine and hydroxylamine buffer in dark bottle at 4 °C

References

Stookey, L. L. 1970. Ferrozine- a new Spectrophotometric reagent for iron. Anan. Chem. **42**:779-781

APPENDIX D

Spectrophotometric determination of U(VI)

Solutions

1. Complexing solution:
   Dissolve 25 g of (1,2-cyclohexyenedinitrilo) tetraacetic acid (CyDTA),
   Dissolve 5 g of sodium fluoride,
   65 g of sulphosalicylic acid in 800 ml water
   Neutralize to pH 7.85 with 40 % sodium hydroxide
   Dilute to 1L
2. Buffer solution:
   Dissolve 149 g of triethanolamine in 800 ml water
   Neutralize to pH 7.85 with perchloric acid
   Stand overnight and readjust pH 7.85
   Dilute to 1 L
3. Ethanol
4. Bromo-PADAP solution:
   Dissolve 0.05 g 2-(5-Bromo-2-Pyridylazo)-5-Diethylaminophenol in 100 ml ethanol

Assay (in 2.0 ml Eppendorf tube)

1. Dispense 100 μl filtered sample in tube
2. Add 150 μl complexing solution
3. Add 150 μl buffer solution
4. Add 800 μl ethanol
5. Add 150 μl Bromo-PADAP solution
6. Add 650 μl DI water
7. Incubate 40 minutes at room temperature
8. Measure absorbance at 578 nm

Standard preparation

Prepare 30 mM uranium stock solution
Dissolve 0.653 g uranyl acetate in 50 ml of a 30 mM bicarbonate solution
Dilute uranium stock solution to get desired concentration

Note

Linear range up to 300 µM U(VI)

Reference

Johnson, D. A., and T. M. Florence. 1971. Spectrophotometric determination of uranium(VI) with 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol. Anal. Chim. Acta. **53:**73-79.

APPENDIX E

DNA isolation from bacterial cell

Solution

InstaGene™ Matrix (Bio-Rad, Hercules, CA)

Assay (in sterilized 1.5 ml Eppendorf tube)

1. Make cell pellet
2. Add 200 μl InstaGene Matrix to the pellet and incubate at 56 °C for 20 minutes
3. Vortex at maximum speed for 10 seconds
4. Place the tube in a 100 °C boiling waterbath (Hot Pot) for 8 minutes
5. Vortex at maximum speed for 10 seconds
6. Centrifuge at 11,000 rpm for 2 minutes
7. Use supernatant for PCR

Note

1. Store the prepared samples at -20 °C
2. Repeat step 5 and 6 for reusing

APPENDIX F

Dilution and extinction series (media and low melting agarose)

Procedure

1. Prepare anaerobic media (about 200 mL)
2. Prepare clean 20 ml vials (10 for liquid media dilution, 10 for agarose dilution)
3. Add 0.5 % Sea Plaque Low Melting Agarose (LMA) to 10 vials (0.05 g in 10 ml total culture volume)
4. Dispense liquid media to vials
   Flush headspace of both prepared media bottle and vials while dispensing 9 ml media to each vial
5. Dispense liquid media to LMA containing vials
   Add a couple of drops of media to make agar wet before flushing
   After inserting N_2-line, add remaining liquid media for a total of 9 ml
   Flush for ~30 seconds
   Repeat for each of the 10 vials containing LMA
6. Crimp all vials
7. Autoclave
8. Let liquid media cool down.
   Solid media should be held in a 45°C water bath before inoculation.
9. Inoculation
   This is performed in the clean hood. Begin by making an ice bath.
   All agar-containing vials should remain in the water bath.
10. Add vitamins and electron acceptor/donor to liquid dilution media
    Perform serial dilutions from original culture by transferring 1ml
    You will have 10^{-1}, 10^{-2}, 10^{-3}, …10^{-10}
    Change needle and syringe every 3 transfers
11. Remove 1-3 agarose containing vials from water bath and add vitamins and electron acceptor/donor.
    (if you want to use chlorinated compound as an electron acceptor, add after procedure 18)
12. Replace vials in water bath, repeat this step for all agarose containing vials
13. Retrieve one vial of LMA from bath
14. Inoculate 1 ml from the most (10^{-10}) to the east (10^{-1}) diluted liquid culture.
    Inject very gently and roll gently to mix inoculum throughout the agarose-containing culture.
    Use new needle and syringe every 3 transfers.
    Each solid culture will be 10^{-1} the dilution of the liquid cultures giving you 10^{-11}, 10^{-10}, 10^{-9}…10^{-2}
15. Submerge agar culture in ice bath right side up.
16. After solidification (~10 minutes) incubate upside down.

APPENDIX G

Quantification of *Geobacter* 16S rRNA gene copies

Primers

Geobacteraceae 16S rRNA-gene targeted primer pair
Geo564 Forward 5'-AAG CGT TGT TCG GAW TTA T
Geo840 Reverse 5'-GGC ACT GCA GGG GTC AAT A

Real time (RTm)-PCR reaction master mix

Component	Stock solution	Final Concentration	µl per 30 µl reaction mixture
Water	-	-	10.2
SYBR Green	2X	1X	15
Forward primer	10 µM	300 nM	0.9
Reverse primer	10 µM	300 nM	0.9
DNA	-	-	3

PCR cycle (ABI Prism 7000 Sequence Detection System, Applied Biosystems)

1 cycle of 2 min at 50 °C and 15 min at 95 °C
40 cycles of 30 sec at 94 °C and 30 sec at 57 °C
1 cycle of 30 sec at 72 °C

Calibration curve

The total *Geobacter* 16S rRNA gene copy numbers were calculated following equation with the assumption of an average double strand DNA molecular weight of 660, two 16S rRNA gene operons per a *Geobacter* genome, and a genome size of 3.5 Mbp.

$$\text{16S rRNA gene copies in DNA per reaction mix} = \frac{[(\text{DNA ng}/\mu\text{l}) \times (6.02\times10^{23})]}{[(3.5 \times 10^{6}) \times 2 \times (660) \times 10^{9}]}$$

Reference

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

Methé, B. A., K. E. Nelson, J. A. Eisen, I. T. Paulsen, W. Nelson, J. F. Heidelberg, D. Wu, M. Wu, N. Ward, M. J. Beanan, R. J. Doson, R. Madupu, L. M. Brinkac, S. C. Daugherty, R. T. DeBoy, A. S. Durkin, M. Gwinn, J. F. Kolonay, S. A. Sullivan, D. H. Haft, J. Selengut, T. M. Davidsen, N. Zafar, O. White, B. Tran, C. Romero, H. A. Forberger, J. Weidman, H. Khouri, T. V. Feldblyum, T. R. Utterback, S. E. V. Aken, D. R. Lovley, and C. M. Fraser. 2003. Genome of *Geobacter sulfurreducens*: metal reduction in subsurface environments. Science **302:**1967-1969.

APPENDIX H

Preparation of MnO_2 stock solution (0.125 M)

Procedure

1. Prepare 30 mM $MnCl_2$ and 20 mM $KMnO_4$ solutions
2. Slowly adding a 250 ml solution of $MnCl_2$ (30 mM) to a 250 ml basic solution of $KMnO_4$ (20 mM)
3. Stir continually with a magnetic stir bar
4. Allow to settle down to bottom of beaker
5. To remove dissolved chloride, washed with filtered E-pure water, centrifuge the suspension at 5000 rpm for 15 min at least 3 times.
6. Suspend MnO_2 total 100 ml filtered, E-pure water to sterile 160 ml bottle and microwave until it boils for a few second and closed with autoclaved stopper
7. Make anoxic MnO_2 solution using hungate

Reference

Lovley, D. R., and E. J. P. Phillips. 1988. Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. Appl. Environ. Microbiol. **54:**1472-1480.

APPENDIX I

Calculation of aqueous chloroethene concentration (C_e)

$$C_e = \frac{Q}{[(V_w/1000) + (K_{om} \times f_{om} \times M_{sed})/1000 + (K_{ow} \times V_{org})/1000 + (H_c \times V_v/1000)]}$$

Where

C_e = aqueous concentration (mM)
Q = total mass of chloroethene (mg)
V_w = volume of aqueous (ml)
K_{om} = organic matter-water partition coefficient (L/kg)
f_{om} = fraction of organic matter
M_{sed} = total mass of sediment (g)
K_{ow} = octanol-water partition constant (2)
V_{org} = total volume of organic (hexadecane) phase (ml)
H_c = dimensionless Henry's law constant (1)
V_v = total head space volume (ml)

For example, 3 µl PCE dissolved in 200 µl hexadecane to 160 ml bottles containing 100 ml of medium resulting in 0.1 mM aqueous concentration

3µl PCE = 4.869 µg PCE

$$C_e = \frac{4.869\mu g}{[100ml/1000 + (758 \times 0.2\ ml)/1000 + (0.72 \times 60\ ml)/1000]}$$

= 0.1 mM

References

Gossett, J. M. 1987. Measurement of Henrys law constants for C_1 and C_2 chlorinated hydrocarbons. Environ. Sci. Technol. **21:**202-208.

Pankow, J. F., and J. A. Cherry. 1996. Dense chlorinated solvents and other DNAPLs in groundwater. Waterloo Press, Portland, OR.

APPENDIX J

Preparation of poorly crystalline Fe(III): FeOOH

Procedure

1. Dissolve $FeCl_3 \times 6H_2O$ in filtered E-pure water to provide 0.4 M final concentration
2. Stir continually while slowly adjusting the pH to 7.0 dropwise with 10 N NaOH
3. (It is extremely important not to let the pH to 7.0 even momentarily during the neutralization step because this will result in a Fe (III) oxide that is less available for microbial reduction)
4. Continue to stir 30 min once pH 7.0 is reached and recheck pH to be sure it has stabilized at pH 7.0
5. To remove dissolved chloride, washed with filtered E-pure water, centrifuge the suspension at 5000 rpm for 15 min at least 3 times. Discard supernatant, resuspend Fe (III) oxide (tapped with spanner). Make the same final volume that you use to make 0.4 M initially (e.g, dissolve 10.8128 g of $FeCl_3 \times 6H_2O$ in 100 ml, after centrifuge make sure you make all suspension in 100 ml volume)
6. Then fill ~50 ml FeOOH solution to sterile 160 ml bottle and microwave until it boils for a few second and closed with autoclaved stopper
7. Make anoxic FeOOH solution using hungate

Reference

Lovley, D. R., and E. J. P. Phillips. 1986. Organic matter mineralization with reduction of ferric iron in anaerobic sediments. Appl. Environ. Microbiol. **51:**683-689.

APPENDIX K

Chloroethene standards

Chloroethens Standard Preparation

1. Gather the following materials:
   - 10 ml vials
   - Teflon (gray) septum
   - Methanol.
   - 10 ml pipette and pipette bulb
   - Autopipette and tips
   - Chlorinated ethenes
2. Weigh vial with septum. Record weight.
3. In cold room, add 10 ml of methanol. Cap and weigh again.
4. Add appropriate amount of chlorinated ethene (between 25 – 500 ml) in cold room. Cap and weigh again. (The appropriate amount can be determined from the concentration range needed for the calibration curve. The max amount of standard (methanol solution) that can be added is 0.5 ml. Determine approximate concentration needed in the standard so that when diluted into calibration standards it will yield an appropriate concentration.)
5. Repeat step 4 for the other chlorinated ethenes desired in standard, weighing each time.

Calibration Curve

1. A calibration curve for 160 ml vials with 100 ml of media, make a salt solution corresponding to media.
2. Dispense salt solution into vials (the number of vials equals the number of points in the calibration).
3. In hood, add 0.5 ml of standard to one vial and seal with Teflon septums. Repeat for various dilutions. Include one without addition of standard.
4. Allow to equilibrate for 2 days.
5. Measure with GC

APPENDIX L

Spectrophotometric determination of hydrogen sulfide

Solution

1. 2% (w/v) zinc acetate solution
   dissolve 2 g zinc acetate in 80 ml DI water, added 0.2 ml acetic acid, make up to 100 ml with DI water
2. 0.4% (w/v) N,N-dimethyl-p-phenyenediamine sulfate (DPPDS) solution
   dissolve 0.4 g DPPDS in 20 ml DI water, add 20 ml H_2SO_4, make up to 100 ml with DI water
3. 0.5% (w/v) $FeNH_4(SO_4)_2$
   dissolve 0.5 g $FeNH_4(SO_4)_2$ in 20 ml DI water, add 20 ml H_2SO_4, make up to 100 ml with DI water
4. Mix solution 2 and 3 in a ratio 1:1

Assay

1. Add 100 µl solution 1
2. Add 100 µl diluted sample (dilute with anoxic DI water)
3. Add 100 µl solution 2 and mix
4. Immediately add 100 µl solution 3 and mix
5. Incubate at room temperature for 30 min
6. Measure absorbance at 670 nm

Note

Linear range up to 30 µM

Reference

Cline, J. D. 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanogr. **14:**454-458.

APPENDIX M

Spectrophotometric determination of Mn(IV)

Procedure

1. 2 mM Benzidine hydrochloride reagent in 10% acetic acid
   dissolve 0.5144 g/L into 10% glacial acetic acid
2. Mn(IV) standard solutions from 0-100 µM
3. Dilute with DI water to within the standard range
4. Add 100 µl diluted sample (or standard solution) to 900 µl DI water
5. Add 500 µl benzidine hydrochloride reagent, mix, and immediately filter (0.22 μm) into a plastic cuvette
6. Monitor absorbance at 424 nm. It will rapidly increase as reaction proceeds, then slowly fall.
7. Record the highest absorbance value for each sample

Reference

Burnes, B. S., M. J. Mulberry, and T. J. DiChristina. 1998. Design and application of two rapid screening techniques for isolation of Mn(IV) reduction-deficient mutants of *Shewanella putrefaciens*. Appl. Environ. Microbiol. **64:**2716-2720.