Genetic Techniques for the Verification and Monitoring of Dihaloethane Biodegradation in New Mexico Aquifers

BY

Rebecca A. Reiss
Principal Investigator
Biology Department
New Mexico Institute of Mining and Technology

and

Peter Guerra
Research Associate
Environmental Engineering
New Mexico Institute of Mining and Technology

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ABSTRACT

The dihaloethanes 1,2-dibromoethane (EDB) and 1,2-dichloroethane (EDC) are used in industrial applications. Both are carcinogenic and cytotoxic. The primary source of dihaloethane contamination is associated with petroleum refining industries and fuel dispensing systems. In New Mexico, approximately 175 locations have dihaloethane-contaminated soil and groundwater. The objective was to determine the potential application of molecular biological tools to monitor biodegradation potential of contaminated aquifers.

Sites for preliminary experiments were in Ribera and Socorro, New Mexico. Methods for isolation of microbes from aquifer samples included centrifugation and micro-filtration. Both were adequate, but micro-filtration on-site allowed the collection of larger sample volumes and eliminated the need to transport water to the lab. Once isolated and concentrated, the samples were divided for DNA and protein isolation. Polymerase chain reaction (PCR) was used to amplify the16SrRNA gene from the DNA. The PCR product was cloned and sequenced. Bacterial species were determined by sequence comparison to GenBank. Attempts to amplify the gene for dehaloalkane dehalogenase (*dhlA*) from the DNA proved inconclusive. However, enzyme activity was detected in protein extracts from contaminated aquifers. The ability to quantify enzyme activity directly from groundwater provides a rapid method for estimation of biodegradation potential.

Keywords: Dihaloethanes, dehalogenase, biodegradation, polymerase chain reaction (PCR), enzyme activity.

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JUSTIFICATION

Dihaloethane contamination of groundwater

Ethylene dibromide (1,2-dibromoethane or EDB) and ethylene dichloride (1,2-dichloroethane, 1,2 DCA, or EDC) are halogenated aliphatic hydrocarbons, a category of xenobiotic compounds. Halogenated hydrocarbons cover a broad range of compounds containing one or more halogen atoms (fluorine, chlorine, bromine, iodine, and/or astatine). EDB and EDC are heavily used for industrial, petrochemical, food-industry and agricultural applications. Both compounds were used as lead scavengers in leaded fuels. According to the EPA's Toxic Release Inventory (TRI) database (www.epa.gov/enviro/html/tris/ez.html), approximately 2,670 lbs. of EDB and 433,000 lbs. of EDC were released onto land and into water between 1987 and 1993 in America. In New Mexico more than 175 locations contain EDB or EDC contaminated soil and/or groundwater, the primary source of drinking water. The principal source of EDB and EDC contamination in New Mexico is associated with release at petroleum refining industries and fuel dispensing systems. Leaky Underground Storage Tanks (LUSTs) are a major contributor of dihaloethane contamination.

Effects of dihalogens on human health

Both EDB and EDC are probable carcinogens (29, 30). EDB also has been found to be carcinogenic to fish (23). In addition to being carcinogenic, EDB causes neural tube damage in rat embryo culture (5), and has been implicated in liver and kidney damage,

and reproductive lesions such as reduced sperm health (8, 68). Genotoxicity as measured by sister chromatid exchange was significant at one part per million (ppm) (7).

The molecular nature of dihaloethane carcinogenesis is beginning to be understood. Inside the nucleus, EDB is conjugated to glutathione by glutathione S-transferase. This complex can bind to DNA, forming DNA-adducts. During DNA replication, the strand containing the DNA-adduct may be misread, resulting in base substitutions (9, 37, 38). The resulting mutations can cause cancer. EDB can also be metabolized by cytochrome P450, but this pathway is not as well characterized (90).

Dihaloethane degradation

The Maximum Contaminant Levels (MCLs) established under the National Primary Drinking Water Regulations are 0.05 µg/L and 5µg/L for EDB and EDC, respectively. Persistence of EDB and EDC contamination can vary greatly between soil types. Laboratory studies indicate that half-life values range from 1 day to 60 weeks in surface soils (72). Evaporation and photochemical reaction were noted as the processes responsible for removal of the majority of dihalogens from surface soils. However, *in-situ* testing detected EDB in shallow surface soil 19 years after its last known application (76). The long persistence was attributed to entrapment in intraparticle micropores of the soil and low rate biodegradation. Additionally, low octanol-water partitioning coefficient values and detection in many aquifers indicate that EDB and EDC will leach in soil. Once dissolved in groundwater, volatilization is limited; therefore, the first route for

removal is through microbial degradation, although a small fraction may be hydrolyzed by geochemical and biological-byproduct reactions involving hydrogen sulfide (2).

Researchers from Dow Chemical demonstrated EDC biodegradation under aerobic, sulfate reducing, and methanogenic conditions in microcosms prepared with EDC contaminated aquifer material and groundwater (72). The ability of aquifer microorganisms to degrade EDC has bearing on the risk factors associated with human exposure, and potential application in the remediation of groundwater. Results from an investigation performed at a Gulf Coast site indicated that EDC biodegraded through a series of steps that included the byproduct 2-chloroethanol (47). Half-lives for EDC biodegradation determined using the Gulf Coast site aquifer samples ranged from 2 months to 4.2 years. These data were used to support natural attenuation monitoring and estimate the risk of exposure when considering potential human exposure pathways.

A technical report published in 1987 discussed the natural biodegradation rate of EDB in sediments collected in Windsor Locks and Simsbury, Connecticut (58). The objective of this study was to determine the importance of microbial degradation of EDB in groundwater located beneath farmland. Ethylene dibromide was used as a soil fumigant in agriculture between approximately 1950 and 1975. Degradation experiments were carried out at environmentally significant concentrations (<5 µg/L). Results were quite favorable; first-order half-lives of EDB degradation under aerobic and anoxic conditions ranged between 35 days and 350 days. At one of the sites, rates were faster in samples collected from within the EDB plume, suggesting that the microbial consortia had

adapted to EDB as a substrate. However, the report concluded with a reoccurring theme: the EDB degradation in the subsurface is not consistent with the rates determined in the laboratory.

EDC degrading microorganisms that were enriched and isolated under ideal conditions were used to inoculate a full-scale groundwater remediation system (78). The primary treatment for incoming groundwater pumped from the subsurface consisted of a rotating biological contactor (RBC) inoculated with laboratory-cultivated microbes that degrade EDC. RBC technology has been extensively applied in wastewater treatment. Further treatment and polishing was accomplished through a dual media filtration/adsorption (sand followed by activated carbon) system. Results from four years of operation indicated that more than 90% of the EDC present in the influent was biodegraded, and not just adsorbed.

Microbial utilization of halogenated hydrocarbons as a substrate requires the removal of the halogens, leaving behind an easily degradable carbon skeleton. Carbon-halogen bonds can be cleaved through enzymatic processes. The enzymes referred to as dehalogenases are responsible for breaking carbon-halogen bonds and are specific to the type of compound they degrade. Haloalkane dehalogenase catalyzes the removal of a halogen group from halogenated aliphatic hydrocarbons. This initial reaction is the rate-limiting step in the biodegradation of EDB and EDC (67). Both compounds can enter the metabolic pathway of microbes that contain the gene for haloalkane dehalogenase (*dhlA*). The *dhlA* gene is on a 200 kilobase plasmid, pXAU1, isolated from *Xanothbacter*

autrophicus strain GJ10 (80). The *dhlA* gene has been cloned and sequenced from *X. autotropicus* (33), the kinetics of the enzyme have been studied (67), and the structure of the protein has been established by x-ray crystallography (88). Site-directed mutagenesis has been used to determine the critical amino acids (35, 42, 63). Thus, the catalytic activity of the *dhlA* gene product is well characterized.

Another haloalkane dehalogenase capable of degrading EDB but not EDC was discovered in *Rhodococcus rhodochrous* (44). The enzyme is coded for by the plasmid gene *dhaA*, which was cloned and sequenced (44, 60, 62). The *dhlA* and *dhaA* gene products exhibit some structural similarities, but contain limited homology at the nucleic acid level. The *dhaA* gene has been isolated and sequenced from both gram-positive and gram-negative bacteria, an indication that it can be passed between species, a process known as horizontal transfer (61, 85).

These studies rely on the ability to culture the responsible species. Molecular techniques are providing new tools to study the biodegradation of xenobiotics independent of the ability to culture the species.

Molecular genetic techniques

Molecular techniques are a new tool for the investigation of microbial diversity. The amplification by polymerase chain reaction (PCR) of the 16S ribosomal gene (16S rDNA) is the predominant method for molecular characterization of complex microbial consortia (69). To estimate diversity, the PCR product can be analyzed by terminal

fragment length polymorphism (T-RFLP) (31). The sequencing of PCR products allows the identification of species that cannot be cultured (13). With genetic information, phylogenetic methods are used to identify species and to build trees to determine the evolutionary relationships between microbial species.

The combination of traditional environmental microbiological and molecular genetic techniques is expanding our understanding of the diversity of species involved in biodegradation. For guidance on which species to expect in contaminated aquifers, it is necessary to turn to the literature. Reviews of dehalogenation in bacteria reveal that at least five different chemical strategies are used by a plethora of species (18). A 16S ribosomal RNA phylogenetic analysis of anaerobic bacteria capable of reductive dehalogenation indicate that most of the species are proteobacteria and low G+C gram-positive anaerobes (27). Other forms of dehalogenation are catalyzed by aerobic bacteria. Figure 1 is a phylogenetic tree that includes species capable of dehalogenation for which 16S rRNA gene sequences are available. Table 1 is a listing of the species used to build the tree. From this data, it is clear that the ability to dehalogenate aliphatic hydrocarbons is widespread in nature. This information is useful to identify groups that may be present in contaminated aquifers. However, the ability of bacteria to transfer useful DNA between species complicates the evolution of intrinsic bioremediation. In addition, difficulties with the isolation of all species responsible severely limit the understanding of bioremediation.

Table 1: Microbes with dehalogenase activity

Species/Strain	GenBank Accession Numbers	Compounds dehalogenated	References
Desulfitobacterium sp. Viet-1	AF357919	Tetrachloroethene	(73)
Desulfomonile teidjei str. DCB-1	M26635	3-chlorobenzoate	(12, (83)
Mycobacterium sp. GP1	AJ012626	1,2-dibromoethane	(62)
Rhodococcus erythropolis M15-3	AJ250925	Haloalkanes	(61)
Rhodococcus globerulus	U89713	Substituted biphenols	(71)
Methylobacterium sp. A4	AF361189	Dichloromethane	(34)
Methylobacterium dicloromethanicum	AF227128	Dichloromethane	(15)
Methylopila helvetica DM9	AF227126	Dichloromethane	(15)
Ancylobacter aquaticus	M62790	Dichloroethane	(77, 78)
Xanthobacter autotrophicus	U62888	Dichloroethane	(86)
Brevundimonas vesicularis	AJ007801	Lindane	(82)
Sphingomonas paucimobilis	AF039168	Lindane	(82), (54)
Hyphomicrobium sp. SAC-1	AF279790	Dichloromethane	(41)
Hyphomicrobium sp. SAN-1	AF279791	Dichloromethane	(41)
Methylophophilus leisingerii	AF250333	Dicholormethane	(14)
Achromobacter xylosoxidans	AF232712	Dichlorophenoxyacetic ac	(70)
Burkholderia sp. LB400	U86373	PCBs	(71)
Burkholderia sp. EN-B9	AF074712	PCBs	(71)
Comamonas acidovorans MC1	AF149849	Dichloropropionate	(70)
Comamonas testosterioni MBIC3840	AB007996	TCE	(57)
Pseudomonas aeruginosa	AF237678	PCBs	(81), (28)
Pseudomonas putida	D84020	PCBs	(81), (28)
Pseudomonas cichorii	AB021398	1,3-dichloropropene	(87)
Stenotrophomonas maltophilia	AF017749	2,2-dichoropropionate	(70)
Dehalospirillum multivorans str.K	X82931	Tetrachloroethene	(55, 56)
Dehalococcoides ethenogenes	AF004928	Tetrachloroethene	(53), (17)
Bacterium CBDB1	AF230641	Trichloroethane	(17)

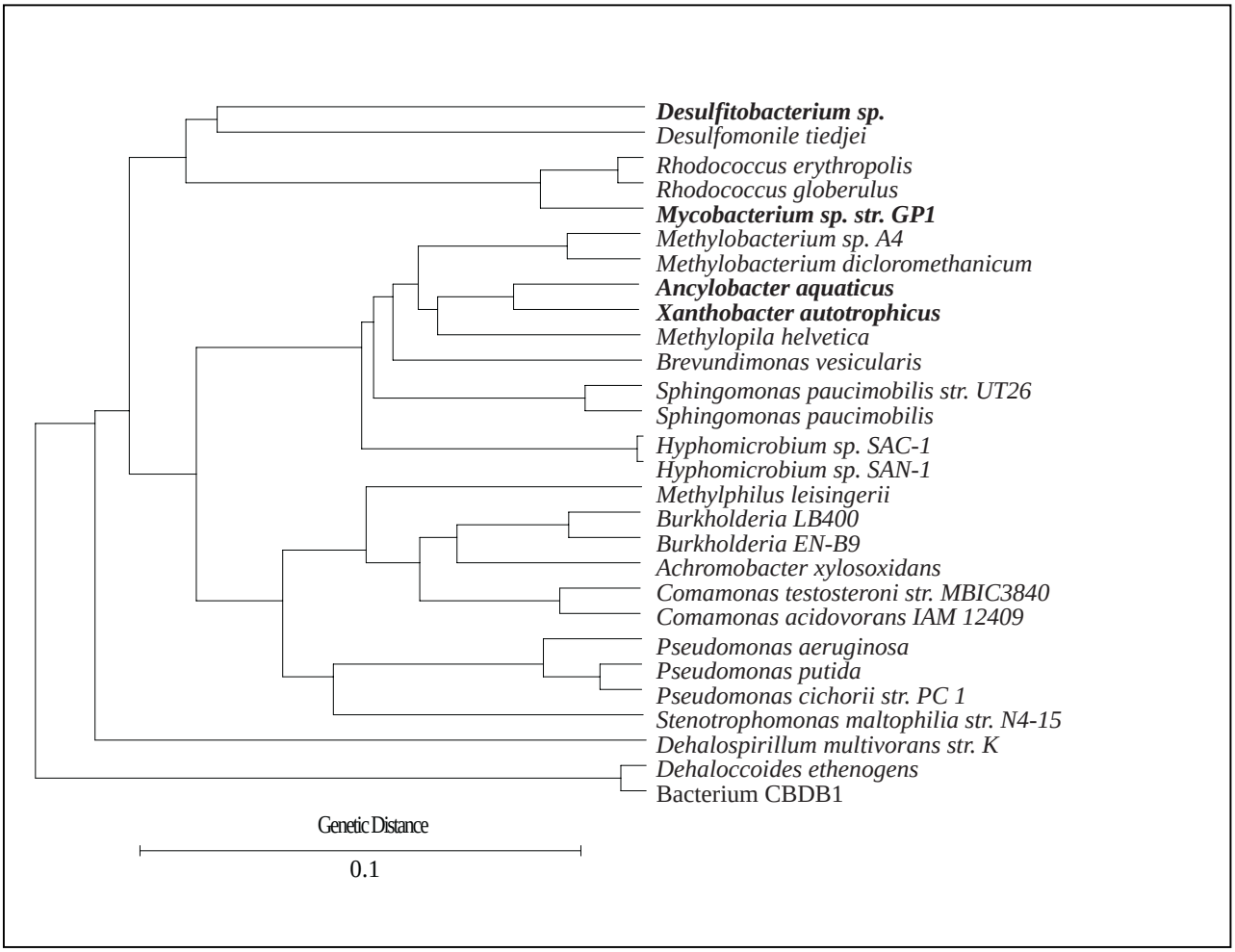

Figure 1: Phylogenetic tree of species with dehalogenase activity
Unrooted Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) tree of species known to degrade halogenated hydrocarbons (from Table 1). The species shown in bold are known to degrade EDB and EDC. The tree is based upon 1020 out of 1324 possible positions within the 16S rDNA gene. The scale indicates the genetic distance; 0.1 corresponds to 10 changes per 100 bases.

Objectives

The first objective of the original proposal was to determine the distribution of the dehalogenase gene (*dhlA*) in New Mexico aquifers. Although preliminary results suggested that the *dhlA* gene could be detected by PCR, a reliable assay was not developed. However, dehalogenase activity was detected from crude protein extract. Protein extraction followed by direct enzyme assay was not proposed since it has not

been reported in the literature. Enzyme detection is commonly carried out on batch reactor samples, but not directly on protein isolated directly from groundwater. This method has several advantages over the DNA method. First, it is not prone to contamination since there is no amplification. Second, it is a measure of overall activity and provides a means to measure biodegradation potential. Third, the protein(s) responsible for the dehalogenase activity from each well can be isolated using standard protein purification techniques. Once purified, the sequence of amino acids can be used to infer the nucleic acid sequence and primers specific for that well can be developed.

The second objective was to identify the microbes that may harbor dehalogenase activity. Comparison of microbial consortia in contaminated and uncontaminated wells provides circumstantial evidence for the microbes that may harbor the activity. Clones from 16S rDNA libraries were sequenced. This is a labor-intensive and time-consuming procedure, but it yields important information that can be used to develop an environmental microarray capable of detecting rapidly the species present in a well sample.

METHODS

Collection sites

Groundwater was collected from monitoring wells in Ribera and Socorro, New Mexico. Both sites have groundwater that is contaminated with various levels of EDB and/or EDC and wells that have no detectable contamination (20-22). Table 2 lists the wells sampled during the course of this project.

Table 2: Groundwater sampling sites

Location[1,2]	Designation	EDC Levels (µg/L or ppb)	EDB Levels (µg/L or ppb)
Ribera, NM	Monitoring Well 2 (MW2)	200	14
Ribera, NM	Monitoring Well 4 (MW4)	8.7	0.05
Ribera, NM	Monitoring Well 6 (MW6)	13.0	0.04
Ribera, NM	Monitoring Well 8 (MW8)	ND[3]	ND
Ribera, NM	On-Site Water Supply (OSS)	ND	ND
Socorro, NM	Monitoring Well 12 (MW12)	9.0	0.04
Socorro, NM	Monitoring Well 20 (MW21)	0.3	ND
Socorro, NM	Monitoring Well 21 (MW 20)	0.6	ND

1. Ribera contamination analysis sample – May 31, 2001 (22)
2. Socorro contamination analysis sample – Sept. 27, 1995 (21)
3. ND – Not Dectected

Water collection and sample concentration

Two methods of water collection and sample concentration were used. The original method was to bail manually from the wells using sterile-teflon bailers. Samples were poured into sterile biological oxygen bottles and kept on ice for transport back to the lab. The microbes were concentrated by centrifugation at 10,000 x *g* for 90 minutes at 4°C. The pellets from a total of 1.5 L of groundwater were resuspended in 50 mL of Tris-sulfate buffer (10 mM Tris pH 7.2, 1 mM EDTA, 1 mM β-mercaptoethanol). The samples were centrifuged again at 10,000 x *g* for 30 minutes. Samples from which total protein was to be isolated were resuspended in approximately 5 mL of Tris-sulfate buffer kept on ice or at 4°C°. Isolation of proteins was done as soon as possible (often the same day). Material from which DNA was to be extracted was stored at –20°C for at least 24 hours.

The second groundwater sampling method was developed to increase the volume of water from which microbes can be isolated. This is especially important for the protein analysis. A Hammerhead two-inch pump (Cat. # H23SEB, QED Environmental, Ann Harbor, MI) was lowered into the well and water was pumped through a Gelman 0.2 micron filter capsule (Cat. # 12117, Pall Gelman, Ann Harbor, MI.) Up to 40 liters were filtered on site. The microbes and sediment were removed from the filters by agitation on a Berrell model 75 (Pittsburgh, PA) wrist action shaker for at least 48 hours and back-flushing filters at least three times with 50 mL of Tris-sulfate buffer. The material flushed from the filters was centrifuged at 10,000 x *g* for 90 minutes at 4 °C. The pellets

were resuspended in 5 mL Tris-sulfate for protein isolation. Pellets for DNA extraction were frozen.

Problems were encountered with filters clogging from sediment in some wells. To investigate the relationship of the sediment mass in the wells to microorganism concentration, one liter of water was collected after purging the well but prior to filtration. Another liter was collected after filtration. These samples were filtered through pre-weighed 90 mm Gelman A/E glass-fiber filters. After drying, the filters were weighed and the amount of sediment calculated was compared to the DNA and protein concentration isolated from each well.

Positive control preparation

Xanthobacter autotrophicus strain GJ10 (ATCC Cat. No. 43050) was used as a positive control because it harbors the *dhlA* gene. *X. autotrophicus* GJ10 was grown aerobically in nutrient broth (36) for at least 24 hours at 30°C. Citrate was used as the carbon source for growth and in some cases, *X. autotrophicus* cultures were supplemented with up to 1 mM EDC to insure the expression of the *dhlA* gene and increase enzyme production. Liquid cultures were centrifuged at 10,000 x *g* for 90 minutes at 4°C and treated the same as the environmental samples.

Protein extraction

The cell suspensions were sonicated at 100 watts continuously for 45 seconds three times to disrupt membranes. Extracts were kept on ice during sonication to limit heating, which is detrimental to the enzyme activity. Cellular debris and other solids were removed from solution by ultra-centrifugation at 45,000 x *g* for 30 minutes. The resulting supernatants were the crude protein extracts. In some experiments, protease inhibitor cocktail (Sigma Cat. # P8465, St. Louis, MO) was added to prevent enzymatic breakdown of the proteins. The protein concentration in each extract was determined using protein assay dye reagent concentrate (Bio-Rad Cat # 500-0006, CA). A standard curve was generated for a Beckman DU-600 spectrophotometer using bovine serum albumin (BSA).

Proteins were visualized by denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gels consisted of a 12.5% acrylamide separating gel and a 6% stacking gel. Samples were heated to 90°C in sample loading dye. Proteins were stained with Coomassie Brillant blue R-25. Pre-stained broad range molecular weight markers (Bio-Rad Cat. # 161-0318, Hercules, CA) were run on all gels. Results were digitized on a Kodak EDAS 120 photodocumentation system (Rochester, NY).

Enzyme assays

The assay for dehalogenase activity was based on previously described procedures (36). This assay method relies on quantifying the amount of chloride released from EDC when a protein extract is added. Chloride concentration was determined based on color change

measured as absorbance at 460 nm following the addition 0.25 M ferric ammonium sulfate ($(NH_4)Fe(SO_4)_2 \bullet 12H_2O$) dissolved in 9 M nitric acid and a saturated solution of mercuric thiocyanate ($Hg(SCN)_2$) in methanol. The displacement of the thiocyanate ion from mercuric thiocyanate by chloride in the presence of ferric iron produces a yellow ferric thiocyanate complex. The color of this complex is stable and proportional to the chloride ion concentration (3). In addition, plasticware was avoided during preparation and execution of the assay and calibration curve due to incompatibilities with the substrate (EDC) and other reactants.

The calibration curve for chloride measurements was prepared using seven different sodium chloride solutions: zero, 0.14 mM (5 mg/L), 0.28 mM (10 mg/L), 0.56 mM (20 mg/L), 1.13 mM (40 mg/L), 2.82 mM (100 mg/L), and 7.05 mM (250 mg/L). Each concentration was prepared in triplicate to obtain a more accurate calibration curve. Samples were treated as described above and the absorbance at 460 nm was measured. Absorbance was plotted against chloride concentration to generate the calibration curve.

The Method Detection Limit (MDL) was calculated pursuant to the EPA's approach (4). The same methods and proportions used to prepare and analyze each assay sample was used to determine the MDL. A 10 mg/L sodium chloride solution in Tris-sulfate buffer was used as the standard. Eight aliquots were analyzed and the results were used to determine the standard deviation. The MDL was calculated as the product of the standard deviation and the Student's t value for a 99% confidence level.

Enzyme activities were calculated on protein extracts, *X. autotrophicus* samples, as well as groundwater samples collected from Socorro (MW12) and Ribera (MW2, MW4 and MW8). These assays consisted of adding EDC to a final concentration of 5 mM to the protein extract and measuring the change in chloride concentration over time. The activity of the enzymes present in the crude extract is directly proportional to the rate of chloride production, since the first rate-limiting step in metabolic breakdown of the substrate is removal of the halogen.

The protein extract from the *X. autotrophicus* grown in the laboratory was used as the positive control and this extract was diluted 20:1 using the 10-mM Tris-sulfate buffer. The volume of groundwater protein extracts was adjusted to 9.0 mL with Tris-sulfate buffer. Each assay consisted of nine parts of extract and one part 50 mM EDC dissolved in ultra pure water. Assays were conducted at 30°C in a temperature controlled warm room. Screw capped glass tubes were used for the assays to limit volatilization. A negative control consisting of Tris-sulfate buffer and EDC was included during each experiment.

Time for each assay began when the EDC stock solution was added to the crude protein extract. Aliquots were removed at 15-minute time intervals. To each aliquot 0.2 volume of 0.25 M ferric ammonium sulfate and 0.2 volume of saturated mercuric thiocyanate solutions were added in order. Since the addition of the ferric ammonium sulfate rapidly lowers the pH of the sample, the enzyme-substrate reaction is immediately quenched.

Color was allowed to develop for at least ten minutes prior to measurement. The absorbance at 460 nm was measured on a Beckman DU-600 spectrophotometer.

Each assay was set up and run simultaneously three times, so that an average chlorine concentration could be determined for each time interval. The assays were carried out at a pH of 7.2 ±0.1 with the exception of two assays performed on samples from Ribera (MW2 and MW4). These additional assays were performed at a pH of 5.9 ±0.1, which was achieved by using pH 5.9 Tris-sulfate buffer during preparation of the protein extracts and assay reactions.

The activity of each extract was calculated from the slope of the line produced when the chloride concentration was plotted against time. Specific activity was calculated as the chloride release rate divided by the protein concentration of the extract, as determined from the Bio-Rad protein assay (see protein extraction). The normalizes the activities so all extracts can be compared. Since the role of the enzyme is removal of chloride, it is traditional to express the unit activity as micromoles of chloride released per minute; therefore, the specific activity is expressed as a unit of activity per mass of protein (U/g).

The Michaelis-Menton rate constant (K_m) and the maximum velocity (V_{max}) were estimated by measuring the rate of chloride released at six different substrate (EDC) concentrations; 6 mM, 19 mM, 31 mM, 50 mM, 63 mM and 88 mM. Each reaction consisted of 1 mL of protein extract and one of the above substrate concentrations. Samples from each of the six reactions were collected and treated with the reactants

following 40 minutes of incubation at 30°C. A negative control in which 10 mM Tris-sulfate buffer was used in place of protein extract was prepared and was treated identically.

Rate constant enzyme assays were performed using protein extract obtained from two monitoring wells located in Socorro (MW12 and MW21) and two monitoring wells from Ribera (MW2 and MW4). Each assay was set up in duplicate so that an average chloride concentration could be determined for each substrate concentration.

K_m and V_{max} were estimated by plotting the reciprocal of the rate of reaction (chloride release per minute) against the reciprocal of the substrate (EDC) concentration and fitting a straight line through the data. The y-intercept of this line is equal to the reciprocal of V_{max}, the x-intercept is equal to the negative reciprocal of K_m, and the slope of the plot is the ratio of K_m to V_{max}. The units of V_{max} and K_m are mM chloride per minute and mM EDC, respectively. In addition, since the reaction between dehalogenase and one molecule of EDC yields one molecule of chloride and one molecule of chloroethanol, the units of V_{max} are directly interchangeable with mM EDC used per min. The enzymatic metabolism of chloroethanol involves the release of the other chloride halogen. In *X. autotropicus*, the 2-chloroethanol dehydrogenase activity has a pH optima of 9.0 and does not affect chloride production in crude extracts without additional stimulation (32). Therefore, additional chloride halogen release from the chloroethanol byproduct is not expected and will not affect the assay results.

DNA extraction

DNA was extracted using a G-nome DNA isolation kit ® (Qbiogene Cat.# 2010-200, Carlsbad, CA). The frozen pellets were thawed and were immediately resuspended in 1.85 mL of cell suspension solution. The manufacturer's protocol was followed.

Quality control PCR (qcPCR) was used to test for *Taq* polymerase inhibitors in the DNA preps (66). Most samples contained inhibitors, so further purification was necessary. Geneclean III (Qbiogene Cat. # 1102-200) is a silica resin to which DNA is bound in high-salt conditions. The resin is washed and the DNA is eluted from the resin in low-salt conditions. In most cases, this eliminated inhibitors. Occasionally, samples would still contain inhibitors of PCR. These samples were further processed through Microcon® YM-100 filters (Millpore Corporation, Bedford, MA) following manufacturer's instructions. DNA was quantified by optical density reading at 260 nm on a Beckman DU-600 spectrophotometer.

16S rDNA PCR, cloning and sequencing

A portion of the 16S rRNA gene (rDNA) was amplified from the DNA using the following primers: rRNA341F 5'- CCTACGGGAGGCAGCAG -3' and rRNA 926R 5'-CCGTCAATTCCTTTRAGTTT-3' (51). These primers amplify a 585 basepair section of *E. coli* 16S rRNA gene and are within a region conserved in eubacteria. The reaction mix included 10 ng of template DNA in 5 µL of GeneReleaser (BioVentures, Murfeesboro, TN) that was heated to 80°C for 5 minutes. After heating, 2.5 µL of Opti-prime Buffer 7 (Stratagene Cat. # 200429, La Jolla, CA), 0.5 picomoles of each primer,

and 1 unit of Ampitaq Gold® DNA Polymerase (Applied Biosystems Cat. # 4311806, Foster City, CA) was added for a total volume of 25 μL. Cycling conditions were as follows: 95°C for 5 minutes, 5.0 sec per degree ramp to 50°C, 72°C 1 min (1 cycle), 96°C for 30 sec, 55°C for 1 min, 72°C 1 min (5 cycles), 96°C for 30 sec, 60°C 1 min, 72 °C for 1 min (28 cycles), 72°C, 10 minutes (1 cycle). PCR products were fractionated on a 1.2% agarose gel and stained with ethidium bromide. Bands were visualized under short-wave UV light and digitized on a Kodak EDAS 120 documentation system.

Adequate product was obtained from two wells in Ribera, MW2 and OSS, to make a rDNA library. The PCR product was purified and cloned using the pPCRscript-AMP cloning kit (Stratagene Cat.# 211188, La Jolla, CA). The bacterial library was plated on plates containing Lubria broth (LB), ampicillin (AMP), 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-Gal), and isopropyl-β–D-thiogalactopyranoside (IPTG) following the manufacturer's protocol. White colonies were selected and tested for the presence of an insert by PCR with the T7 and T3 primers that flank the insert. Plasmid DNA was purified from 2 mL cultures of bacteria with inserts using the StrataPrep® plasmid miniprep kit (Stratagene Cat. # 400761, La Jolla, CA). DNA was quantified by optical density reading at 260 nm on a Beckman DU-600 Spectrophotometer.

Individual clones were digested with the restriction enzyme *Hin*fI and the patterns compared. Those clones chosen for sequencing had different restriction patterns. For each sequencing reaction, 500 ng of DNA were mixed with 4.0 μL of ABI Big Dye Terminator Version 3 (ABI Cat. # 4390242), and 3.2 picomoles of primer in a total

volume of 10 µL. The cycling parameters were as follows: 96°C for 30 sec, 1.2 sec per degree ramp to 50°C then 15 sec, 1.2 sec per degree ramp to 60°C, then 4 min (30 cycles). Products were run on an Applied Biosystems Prism-310 DNA analyzer. Each clone was sequenced at least twice in both directions. Sequences were aligned with Sequence Navigator (Applied Biosystems, Foster City, CA). Consensus sequences were submitted to Basic Local Alignment Sequence Tool Analysis (BLAST) on GenBank (http://www.ncbi.nlm.nih.gov) for identification (1). Sequences were also submitted to CHIMERA-CHECK program in the Ribosomal Database Project (52) (http://rdp.cme.msu.edu).

dhlA PCR

The conditions to amplify the *dhlA* gene were established using DNA from *Xanthobacter autotrophicus* strain GJ10 as a positive control. A nested PCR strategy was designed in which two consecutive rounds of PCR are performed. Primers were designed using the *dhlA* gene (GenBank accession #M26950) sequence template (Figure 2). To increase sensitivity, the primers for the second round (dhla1 and dhla2) were labeled with the fluorescent dye FAM. Detection by capillary electrophoresis on the ABI Prism 310 Genetic Analyzer was performed using 3.0% GeneScan® polymer (ABI, Foster City, CA) under non-denaturing conditions. Each injection contained GeneScan® 2500 TAMRA labeled size markers (ABI Cat# 410545, Foster City, CA).

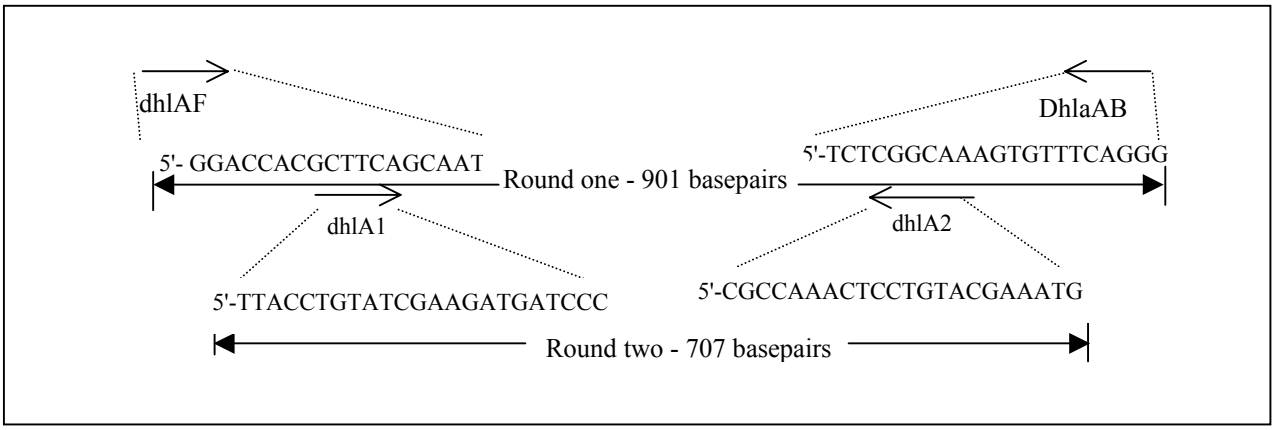

Figure 2: Nested PCR strategy
The primers dhlAF and dhlAB amplify a 901 basepair fragment of the dhlA gene. The product of this first round reaction is used as a template for second round primers and dhlA2, which produces a 707 basepair fragment.

RESULTS

Collection methods

Although both manual bailing and on-site filtration produced results for DNA and protein extractions, filtration has several advantages. First, microbes from much larger water samples are obtained without transporting large amounts of water back to the lab. Second, the microbes are concentrated and can be washed on-site, which saves time. The major disadvantage is the filters can clog quickly, depending on the type of sediments in the well. Since some of the microbes may be associated with sediment, it is important to include the sediment in the extractions. Table 3 provides results from a collection made in June of 2001 in Ribera. The yield from MW6 (2.1 µg /L) was sufficient to visualize the proteins on a SDS-PAGE gel (Figure 3). Lane S3 contains 6 µg of protein, which corresponds to about 2.8 Liters of groundwater.

Table 3: On-site filtration results

Well[1]	Liters filtered [2]	Pre filtration sediment (grams)	Post filtration sediment (grams)	Protein Yield µg/L	DNA Yield µg/L	Concentration factor[3]
MW2	24	2.01	0.23	0.22	0.5	2667
MW6	40	0.42	0.25	4.17	2.1	4444
MW12	2	3.62	3.49	0.43	1.5	222
MW16	6	0.15	0.81	0.43	1.5	666

1. 20 L were purged from each well before collection. Water was collected until the filter was clogged.
2. Water was filtered through a 0.2 micron filter capsule.
3. Total liters filtered/ final sample volume after ultracentrifugation.

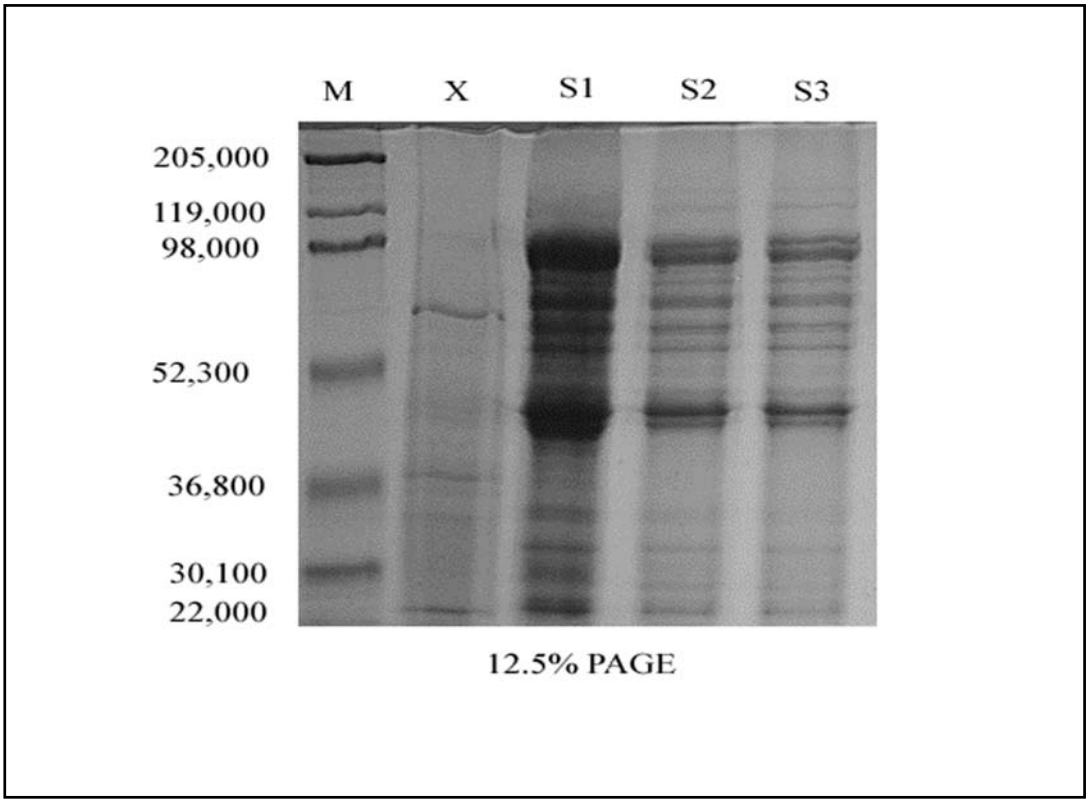

Figure 3: SDS-PAGE gel of proteins found in groundwater
The water sample was collected from Ribera, New Mexico from Monitoring Well 6 (MW6). Protein was isolated from a filter capsule that filtered approximately 40 liters of water. Lane M represents the molecular weight marker. The sizes of the marker are noted along the left side of the gel. The next lane, X, is protein extracted from *X. autotrophicus*. Samples S1, S2, and S3 represent 24 µg, 12 µg and 6 µg, respectively. This represents the amount isolated from to 11.2, 5.6, and 2.8 liters of groundwater.

Enzyme activity

The results for enzyme activity were obtained from wells that were manually bailed and are shown in Table 4. Figure 4 are the Lineweaver-Burk double-reciprocal plots of the data for each well. The K_m for haloalkane dehalogenase produced by *X. autotropicus* was not derived in this study, this value is reported to be greater than 400 mM (67).

Table 4: Enzyme assay results

Sample (Location)	EDC Concentration mMolar	Specific Activity (U/g)[1]	K_m mM EDC	V_{max} mM/min
X. autotropicus 1	0	0.60±0.28	NA[3]	NA
X. autotropicus 2	>989.6	1.06±0.12	NA	NA
MW2 (Ribera)	$3.7x10^{-3}$	$2.5x10^{-2}$±$1.6x10^{-3}$	97	0.13
MW4 (Ribera)	$1.2x10^{-4}$	$8.3x10^{-2}$±$2.3x10^{-2}$	127	0.19
MW8 (Ribera)	BDL[2]	BDL	NA	NA
MW12 (Socorro)	$2.2x10^{-3}$	$1.9x10^{-2}$±$1.5x10^{-3}$	161	0.11
MW21 (Socorro)	$1.1x10^{-5}$	NA	138	0.08

1. Specific Activity - One unit (U) of enzyme activity equals one mole chloride released per minute per gram of protein.
2. BDL - Below detectable limits
3. NA – Not Assayed

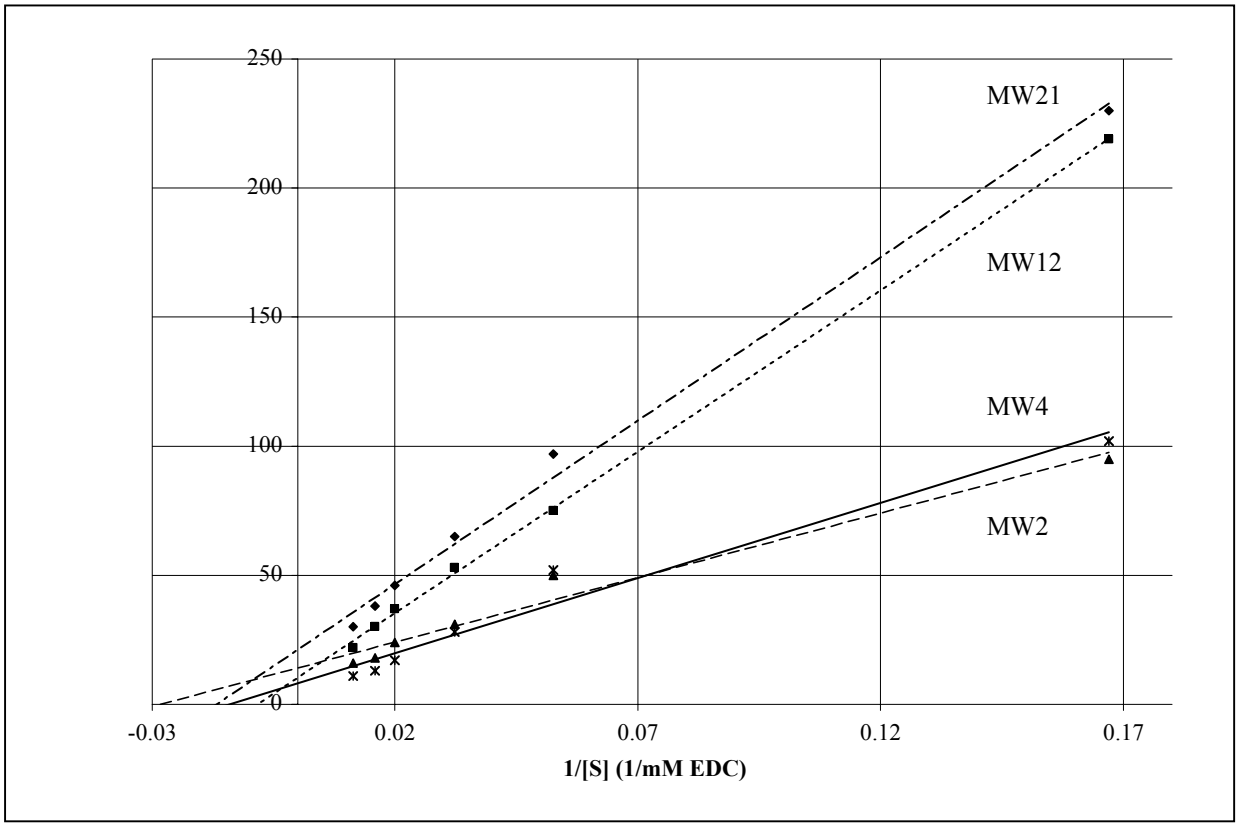

Figure 4: Lineweaver-Burk plots
The plots of the data for each well is shown. The x-intercept is $-1/K_m$, the y-intercept is $1/V_{max}$, the slope equals the ration of K_m to V_{max}.

16S rDNA sequencing

Adequate 16S rDNA PCR product was obtained from two wells in Ribera (MW2 and OSS) to produce 16S libraries for these wells. Currently, 88 clones from the MW2 library and 82 from the OSS library have been isolated. Of these, 67 and 53 from MW2 and OSS, respectively, have been tested for inserts. A total of 70 plasmids have been purified. Clones were selected for sequencing based on categorization by *HinfI* digests. To date, five clones have been sequenced. The results of the BLAST analysis on these clones are shown in Table 5.

Table 5: DNA sequencing results

Well – Clone #	GenBank Accession Number	Possible Classification	References
MW2-03	AY122595	Green-nonsulfur	(12, 89)
MW2-07	AY122596	*Desulfotomaculum*	(48, 77)
MW2-13	AY122597	δ- Proteobacteria	(11, 49, 64, 65)
MW2-28	AY122598	*Clostridium*	(10, 24, 43)
MW2-67	AY122600	*Spirochaeta*	(12)
OSS-11	AY122601	*Sphingomonas*	(6, 16, 79)
OSS-15	AY122602	*Methylobacterium*	(26, 59)
OSS-33	AY122603	*Desulfotomaculum*	(19, 50, 75)
OSS-34	AY122604	*Ochrobactrum*	(46, 74)
OSS-41	AY122605	*Hydrogenophaga*	(45)
OSS-45	AY122606	*Methylobacterium*	(26)

Two of the microbes found in the contaminated groundwater (MW2-03 and MW2-67) are most similar to sequences found in other chlorinated-solvent contaminated environments. Clone MW2-03 has 95% identity to an uncultured green-nonsulfur bacterium identified in an anaerobic digester (12) and 93% identity to a clone identified in a trichlorobenzene degrading consortium (89). Clone MW2-67 is 100% homologous to a Spirochaete found in the same studies (12, 89). Clone MW2-07 has 95% identity to an uncultured eubacterium in an aquifer contaminated with chlorinated solvents, including EDC (13). This clone also shares significant homology (95%) with an uncultured *Desulfotomaculum*

found in rice paddies (77) and a strain isolated from a phenol-degrading culture (94%) (48). Clone MW2-13 is 99% homologous to an uncultured bacterium found in groundwater containing chlorobenzene (GenBank Accession #AY050586, Alfreider, unpublished). Weaker homology (90%) was found to a clone found near uranium mines (64) and a species discovered in coastal marine environments in South Carolina (11). Eighty-nine percent homology was detected with an uncultured bacterium from Arctic Ocean sediments (65) and a clone isolated from a depth of 158 m in Suruga Bay, Japan (49). Clone MW2-28 is 92% homologous to *Clostridium*, which is a diverse, endospore forming anaerobic bacteria that has been found in forest soils and rice paddies (10, 24, 43).

Six clones from the on-site supply library have been sequenced. OSS-11 exhibits 97-98% homology to strains of marine (6, 16) and freshwater (79) *Sphingomonas*. Clones OSS-15 and OSS-45 are closely related to *Methylobacterium*. OSS-15 exhibits 100% homology to species found in accretion ice (GenBank accession # AF395034), soybean root nodules (GenBank accession # AF293375), potable water (26), Scotch Pine buds (59), and coastal marine waters (11). OSS-45 is 95% homologous to *Methylobacterium rhodinium* (26). Clone OSS-33 shares 88% homology to *Desulfotomaculum*, an endospore-forming sulfate-reducing bacterium (19, 50, 75). This genera is also represented in the contaminated well (MW2-07). OSS-34 is 99% similar to *Ochrobactrum,* a widespread α-proteobacteria associated with roots (46) and halobenzoate-degrading consortia (74). The clone OSS-41 is 98% homologous to *Hydrogenophaga*, a β-proteobacteria found in reactors in wastewater treatment (45).

Dehalogenase gene amplification

Although preliminary experiments with dhlAF and dhlaB primers suggested that it might be possible to amplify the *dhlA* gene from DNA extracted from groundwater, the process was found to be unreliable. Sequence analysis on PCR products from the groundwater samples gave the same sequence as the positive control, *X. autotropicus*. From this data, contamination from the positive control can not be ruled out. Precautions are always taken to prevent such contamination from occurring, such as the use of aerosol-resistant tips on pipetors. Negative controls are always run along with experiments to detect contamination. However, to insure that such cross-contamination could not occur, the conditions required by the FBI for human forensic samples were adopted (84). PCRs from groundwater were set up in a sterile hood on a different floor. The field in the hood was exposed to UV light for at least 10 minutes prior to the experimental procedure. The PCR product was never taken into the lab were the reactions were set up.

Under these conditions, the *dhlA* gene was not detected in any groundwater samples. Although the conditions for capillary electrophoresis of nested PCR products were worked out using *X. autotropicus*, a signal was never observed in groundwater. PCR with alternative primers (87) was also unsuccessful.

DISCUSSION

Collection methods

Manual bailing of wells provides adequate material for a few experiments. However, in order to obtain adequate protein to visualize on a SDS-PAGE gel (Figure 3), it was necessary to filter 40 L of water. Although there is a trend that suggests that high sediment concentration reduces the volume of water that can be filtered, this was not true for MW6. The filter clogged after only six liters, but the amount of sediment is the lowest (Table 3). This suggests that other factors, such as particle size and porosity characteristics, play a role in the success of this method. Experiments to investigate this are necessary to refine the on-site filtration method.

In all cases, the sediment was processed as part of the sample. It was assumed that significant proportions of the microbes were bound to the sediments. The methods developed during this project provide a framework to test this assumption. Filtration of groundwater through a series of filters with increasingly smaller pores followed by DNA and protein assays will provide valuable information on the characteristics of microbes in groundwater.

Cell lysis

Sonication is a standard method for lysing cells for protein preparation. For DNA isolation, freeze-thaw followed by SDS treatment is one of several standard methods used. The efficiency of lysis was not measured in this study, but is an important consideration for future studies. To optimize the lysis for protein isolation, the

relationship between the length and intensity of sonication on the activity recovered is necessary. Other DNA extraction methods include agitation with glass beads and treatment with guanidine thiocyanate. Comparison of the DNA yield using different procedures will indicate the most complete extraction method.

Protein Analysis

The ability to visualize the proteins in groundwater (Figure 3) combined with an enzyme assay make it possible to purify proteins directly from groundwater without culturing individual species. The key to this process is the concentration of groundwater microbes prior to protein extraction. Samples that were hand bailed allowed a concentration factor of about 338- fold. On-site filtration increases the concentration factor to above 4000-fold (Table 3). The concentration factor can be further increased by microfiltration of the crude extracts. New techniques in proteomics require extremely small amounts of protein for amino acid sequencing. The ability to study the structure of proteins responsible for the similar activity in widely separated aquifers will provide some answers to fundamental questions regarding microbial evolution. For example, has this activity evolved separately in widely separated aquifers, or is this due to horizontal gene transfer? If horizontal gene transfer is involved, how does this occur over long distances? If the proteins are different, then convergent evolution is responsible. Comparison of multiple contaminated sites will advance our understanding of the process of microbial evolution. Not only will such information be important to bioremediation, but will also be important in the study of medical microbiological issues, such as the evolution of antibiotic resistance.

Enzyme activity is detectable in protein extracts from groundwater samples collected from wells contaminated with EDC. Activity was below detectable limits the one well (MW8) with no contamination. However, no linear relationship between contaminant concentration and enzyme activity was detected in this study. There are many factors that influence microbial growth besides concentration of a single carbon source. Additional regions must be investigated before significant conclusions can be made.

The experiments at pH 5.9 provide additional evidence that this is a hydrolytic enzymatic process and not an abiotic process. The relationship between pH and dehalogenase is grounded in the hydrolytic nature of the enzyme. Upon release of haloalcohol byproduct (chloro- or bromoethanol), the cleaved halogen (chloride or bromide) and hydrogen left behind during hydrolysis remains bound to the active cavity. Reactivation of the enzyme will not occur until the trapped chlorine is stripped from the active cavity and a new water molecule is positioned for hydrolysis. Release of the chloride and hydrogen from the active cavity is influenced by diffusion of hydrogen within the vicinity of the enzyme. Therefore, enzyme activity is adversely affected at higher hydrogen ion concentrations (i.e. at pH 5.9 instead of pH7.2).

Comparison between the rate constants indicates that there is variability in the enzyme kinetics of the groundwater samples tested. The average value for K_m was 131 mM with a standard deviation of 26 mM. The average V_{max} is 0.13 mM/min with a standard deviation of 0.05 mM/min. This variation may be due to characteristics unique to each well that affects the expression of dehalogenase gene(s). The Socorro and Ribera sites

are separated by 224 km, so the difference could reflect different enzymes at work. Additionally, enzyme rate constants and activity estimated from crude extracts produced from different regions of a contamination plume will yield insight into the spatial variability in biodegradation across a particular site. There are numerous hydogeochemical and climatic factors that influence microbial growth, which may effect enzyme kinetics. Sampling error could be a factor as well.

Since the rate constants K_m and V_{max} estimated from groundwater protein extract are based upon first-order kinetics, they provide a means to estimate the first-order biodegradation rate constant. Since these rate constants were estimated using extract concentrated from fresh, uncultured groundwater samples, they can be used to estimate the kinetics of biodegradation expected *in-situ*.

The rate of EDC degradation can be expressed as follows:

$$rate = -v = \frac{V_{\max}[S]}{K_m + [S]} \qquad \text{Equation 1}$$

where: [S] = concentration of substrate (EDC)

Because this reaction is liquid phase, the stoichiometric balance can be expressed as follows:

$$-\frac{dC_{EDC}}{dt} = -v \qquad \text{Equation 2}$$

where: C_{EDC}= concentration of EDC in solution (mM)

t = time (minutes)

Substituting Equation 1 into Equation 2, solving for time, and integrating yields:

$$t = \int_{C_{EDC}}^{C_{EDC\ (o)}} \frac{dC_{EDC}}{-v} = \int_{C_{EDC}}^{C_{EDC\ (o)}} \frac{K_m + C_{EDC}}{V_{\max} C_{EDC}} dC_{EDC} \qquad \text{Equation 3(a)}$$

$$t = \frac{K_m}{V_{\max}} \ln \frac{C_{EDC\ (o)}}{C_{EDC}} + \frac{C_{EDC\ (o)} - C_{EDC}}{V_{\max}} \qquad \text{Equation 3(b)}$$

where: $C_{EDC(o)}$ = initial concentration of EDC in solution (mM)

Equation 3(b) can be expressed in terms of conversion (X) as follows:

$$C_{EDC} = C_{EDC(0)}(1 - X) \qquad \text{Equation 4}$$

$$t = \frac{K_m}{V_{\max}} \ln \frac{1}{1 - X} + \frac{C_{EDC(o)} X}{V_{\max}} \qquad \text{Equation 5}$$

Equation 5 represents the time expected for the desired conversion (i.e. 99.99%) based on the enzyme concentration in the extract. The estimate for conversion time that is expected for the concentration of enzyme *in-situ* is the product of the time estimate from Equation 5 and the concentration factor from groundwater sample to extract. Since 1.25 liters of groundwater sample was concentrated to 3.7 mL of protein extract, the factor is 338.

Using Equation 5, the K_m and V_{max} values estimated from each groundwater sample, and the extract-to-groundwater concentration factor; the EDC-biodegradation curve (time vs.

concentration) was plotted from a fully saturated concentration of 88 mM EDC to a final concentration of 0.0088 mM or 99.99% conversion (Figure 5). Since K_m and V_{max} are based on first-order models, biodegradation by first-order decay can be expressed as follows:

$$C_{EDC} = C_{EDC(o)}e^{-kt} \qquad \text{Equation 6}$$

where: k = first-order biodegradation rate constant ($year^{-1}$)

t - time (year).

Results from these comparisons yielded an estimate for first-order biodegradation rate constants, which were estimated between 1.94 and 2.21 $years^{-1}$ at the Ribera site and 0.86 and 1.02 $years^{-1}$ at the Socorro site. This analysis is important since first-order biodegradation is used in many fate and transport models, which are applied to ascertain the risk of human an ecological exposure to contaminants in groundwater (40). Time estimates for 50% and 99.99% conversion and first-order biodegradation rate constants for each well tested are summarized in Table 6.

These estimates are based upon laboratory conditions, which are unlikely to reflect the environment within the cell. For example, the K_m of the extracts are much greater than the concentrations in the groundwater, which brings up the argument whether this reaction will proceed at such low concentrations. If the microbes actively transport EDC,

it is likely that the concentration within a cell is high enough for the reaction to proceed at a biologically significant rate.

Table 6: First-order biodegradation rates

Groundwater Sample	50% Conversion (Days)	99.99% Conversion (Years)	k $years^{-1}$
MW2 (Ribera)	123	4.6	1.94
MW4 (Ribera)	107	4.0	2.21
MW12 (Socorro)	236	8.7	1.02
MW21(Socorro)	267	9.8	0.86

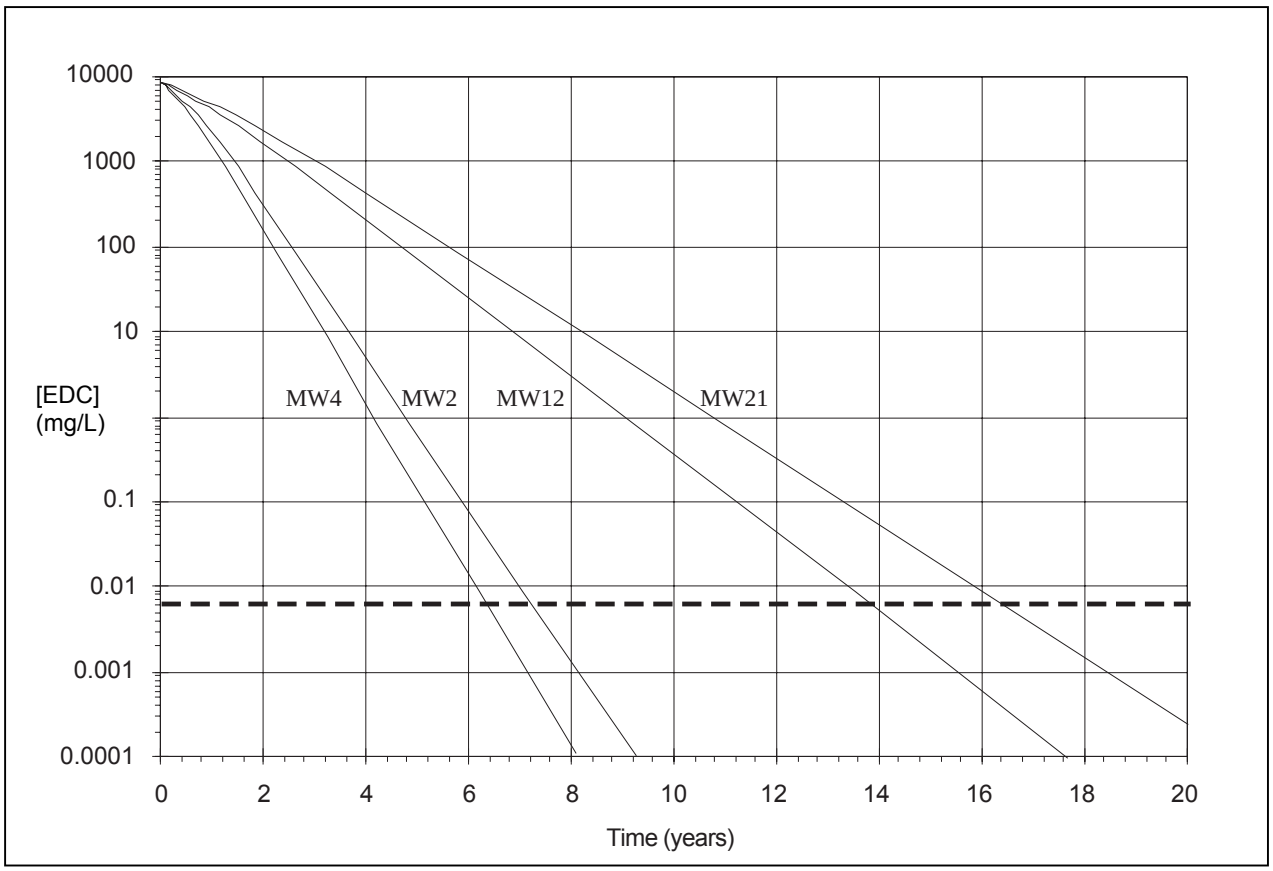

Figure 5: Estimated *in-situ* biodegradation rates
The expected decrease of EDC concentration is shown for monitoring wells 2 and4 (Ribera, NM), and wells 12 and 21 (Socorro, NM). The dotted line is the maximum allowable contaminant level for EDC established by the EPA in 1996.

These preliminary experiments indicate that direct enzyme assays from uncultured groundwater samples have the potential to become a powerful method in the environmental scientists' toolbox. First, it is a direct measure of the biodegradation potential of organisms in the contaminated environment. Second, it can be performed quickly on a small sample of water, eliminating changes that occur during culturing. These characteristics make direct assay a powerful tool to monitor intrinsic biodegradation or enhanced bioremediation processes. For example, spatial and

temporal differences observed in biodegradation rates might provide site-specific data that can be used to enhance remediation.

As is common in research, more questions are raised than are answered. Future experiments include the range of rates within a single well to determine the variation within a well. Testing additional contaminated wells is necessary to determine any relationship between contaminant level and biodegradation rates. Any factor, such as chemical and hydrogeological characteristics of the region, can influence microbial growth, which will affect enzymatic rates. Although environmental data, such as temperature, pH, substrate concentration are available the aquifers, there is a paucity of information on the micro-environment within the microbes in which these reactions take place.

16S rDNA sequencing

The bacteria identified thus far in the contaminated aquifer are related to species common in other solvent-contaminated environments. Most of the matches are from uncultured bacteria and are most likely representatives of green-nonsulfur bacteria, δ-*Proteobacteria*, *Desulfotomaculum*, *Clostridium*, and *Spirochaeta*. Two of these species, *Desulfotomaculum* and *Spirochaeta* were also detected in chlorinated solvent contaminated environments, supporting the hypothesis that a consortium of bacteria is an important characteristic of environments undergoing intrinsic bioremediation (13).

Although none of the bacteria identified in this study are known to harbor dehalogenase activity (Table 1), several are present in consortia that degrade halogenated compounds

(48, 77). It is possible that all bacteria were not amplified or sequenced. More likely, this finding emphasizes the limited understanding of the molecular basis of biodegradation and the need for continued research.

The bacteria identified in the on-site potable water supply are members of the genera *Sphingomonas*, *Methylobacterium, Ochrobactrum, Hydrogenophaga, and Desulfotomaculum. Sphingomonas* and *Methylobacterium* have been cultured from other oligotrophic environments and their presence in potable groundwater is not unexpected (26,59). *Ochrobactrum* are associated with a halobenzoate-degrading consortium (74) and *Hydrogenophaga* was found in waste-water treatment plants (45). The sulfate-reducer *Desulfotomaculum* is the only genera common to both wells in this study.

Additional cloning and sequencing from the MW2 and OSS rDNA libraries is proceeding. Once a more complete sample is obtained, a complete phylogenetic analysis will be performed using PAUP (Phylogenetic Analysis Using Parismony), allowing comparisons to analyses performed for similar environments (13). In addition, this information has been supplied to Argonne National Labs in Illinois to design an environmental micro-array capable of rapidly detecting species in groundwater samples.

Dehalogenase gene amplification

The technique of PCR with primers for dehalogenase genes was unreliable. The low number of targets within any single sample is one problem, but the specificity of the

primers also plays a role. A series of degenerate primers for two classes of dehalogenase have been described (25). Primers for group I α-halocarboxylic acid dehalogenase (deh) genes are: dehI $_{for1}$(5'-ACG YTN SGS GTG CCN TGG GT-3') and dehI$_{rev1}$ (5'AWC ARR TAY TTY GGA TTR CCR TA). The primer dehI $_{for1}$ can be used with a different reverse primer, dehI$_{rev2}$ (5'SGC MAK SRC NYK GWA RTC ACT-3') to amplify a smaller region. Group 2 genes can be amplified using dehII$_{forI}$ (5'-TGG CGV CAR MRD CAR CTB GAR TA-3' and dehII$_{revI}$ (5'-TCS MAD SBR TTB GAS GAN ACR AA-3'). These primers can detect dehalogenase from pure cultures (25). It remains to be seen if these primers can be used to amplify successfully the dehalogenase gene from groundwater DNA.

From a practical standpoint, protein analyses hold much greater potential for estimation of first-order biodegradation rates. Proteomics may solve the problem of gene detection by PCR. Once proteins are isolated and purified, the protein sequence can be determined from very small samples. The nucleic acid sequence can be inferred from the protein sequence, and more specific primers can be designed.

PRINCIPAL FINDINGS

Potential of direct enzyme assays

Although protein analysis was not initially proposed, this research demonstrates that it has the greatest potential for monitoring intrinsic bioremediation of dihaloethanes. Protein isolation is rapid, easy, and is less subject to false-positive results than PCR. The assay for chloride production can be used to directly estimate biodegradation rates in groundwater, which can be incorporated into attenuation models that include non-biotic factors. The methods developed for collection and concentration of groundwater microbes provide adequate yield to perform additional protein purification protocols without culturing. Purification of the enzyme(s) responsible for dehalogenase activity will facilitate the comparison of genes responsible for biodegradation between different aquifers. Once this type of data is available, it will then be possible to distinguish between the convergent evolution or horizontal transmission of genes for degradation.

Sequence comparison

DNA sequence comparison is a powerful technique, but it is very labor-intensive. The information gained in this project can be used to develop an environmental micro-array that can quickly identify species. Combined with enzyme activity data it will be possible to evaluate consortium from different aquifers for the ability to degrade xenobiotics. These tools will advance the knowledge of natural bioremediation and suggest methods to enhance the process.

SUMMARY

Attempts to detect specific genes for dihaloethane biodegradation using polymerase chain reaction (PCR) in New Mexico aquifers proved unreliable. However, it was discovered that the enzyme activity was detectable in crude protein extracts made from groundwater samples. Direct enzyme assays for monitoring biodegradation potential has three major advantages over PCR. First, it is a direct measure of the biodegradation activity, not just the potential. Just because a gene is present, it does not mean it is expressed. Second, it provides an accurate estimate even if more than one gene is involved. Third, it is not prone to contamination because there is no amplification of product.

First-order biodegradation rate constants were calculated from rate constants determined from direct enzyme assays on crude protein extracts. This is a distinct improvement over current batch-reactor methods because it is a rapid method that does not require culturing so there is no loss of species from the consortium. These rate constants can be incorporated into existing models for natural attenuation.

The application of direct enzyme assays to monitoring of biodegradation does not require information regarding the microbial consortium of an aquifer. However, combining enzyme data with information regarding species diversity will advance the understanding of biodegradation. By comparing the enzyme data to the microbial consortium of numerous wells, a better understanding of the bacterial species responsible for biodegradation will result.

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