MOLECULAR ANALYSIS OF DIVERSITY, GENE EXPRESSION AND ACTIVITY OF MINERAL-ASSOCIATED BACTERIA

by

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ABSTRACT

This dissertation investigated the diversity and hydrogenase activity and gene expression of mineral-associated microorganisms. Surface-associated microbes have been shown to dominate diversity and activity in the environment, however molecular analysis of sediment-associated communities is hindered by both inaccessibility to the subsurface and co-extraction of inhibitory compounds. In order to analyze microbial communities in which the environmental conditions previously had precluded the use of traditional sediment extraction techniques, biofilm coupons (metal, mesh cylinders containing surrogate substrata) were used to recover microorganisms able to attach and compete in a biofilm. The community recovered from the contaminated site using hematite as a surrogate substratum was dominated by microbes most closely related to *Alcaligenes* sp. (metal-tolerant), *Frateuria* sp. (acidophilic), and *Methylobacterium radiotolerans* (radionuclide-tolerant) which together reflect the acidic, metal-, and radionuclide-contaminated environment. Hematite, as compared to other substrata, was shown to recover communities most closely representative of sediment communities inhabiting the saturated zone.

Surface-associated cells have been shown to express greater activity than suspended populations and mineral-associated sulfate-reducing bacteria (SRB) mediate the formation of different secondary minerals as compared to suspended cells. In order to investigate the affect of surface-association on enzyme activity, hydrogenase enzyme activity was compared in hematite-associated and suspended populations of the SRB *Desulfovibrio desulfuricans* Essex 6. Hydrogenase activity of surface-associated populations was higher than that displayed by suspended cells. Hydrogenase likely affects the pH and pE of the conditions immediately surrounding the cell. The greater rate of activity may be one factor which contributes to the formation of a mineral phase not observed in the presence of suspended populations of this bacterium. In order to determine the portion of cells expressing hydrogenase in the surface-associated populations, in situ reverse-transcription PCR was applied to the hematite-associated cells and all cells were expressing the [NiFe] hydrogenase gene. This thesis demonstrates that environmental conditions of contaminated subsurface environments select for microorganisms able to tolerate or utilize the contaminants. Also, the hydrogenase activity of surface-associated populations is not representative of the suspended cells thus indicating the importance of studying attached populations where enzyme activity likely influences the conditions at the mineral-microbe interface.

CHAPTER 1

SUBSURFACE MICROBIOLOGY

Iron and Sulfur Cycles

Biological and chemical processes control the biogeochemical cycling of several elements integral to life. Microorganisms have developed metabolic pathways to utilize different oxidation states of a variety of elements including iron, nitrogen, and sulfur. Assimilatory pathways dictate the incorporation of elements into biomass whereas dissimilatory pathways are used for energy generation (19). Biogeochemical cycling of sulfur is an important mode of energy generation in subsurface microorganisms and effects of these metabolic pathways are important to subsurface geochemistry and bioremediation.

Sulfur commonly exists in the environment in three oxidation states (sulfide, S^{2-}; elemental sulfur, S^0; sulfate, S^{6+}). The predominant reservoirs in the subsurface are elemental sulfur deposits, fossil fuel complexes, and pyrite (FeS_2) or gypsum ($CaSO_4 \cdot 2\ H_2O$). Sulfur cycling is carried out by a variety of microorganisms able to utilize any of the three oxidation states for energy generation (Table 1.1). Sulfur oxidation in soil communities is typically carried out by microbes including *Thiobacillus denitrificans* and *Achromatium* spp. which use reduced sulfur species as inorganic electron donors (11).

Dissimilatory sulfate reduction is carried out by sulfate-reducing bacteria (SRB) such as *Desulfovibrio* spp. and *Desulfotomaculum* spp. Many SRB can generate energy not only by reducing sulfate to sulfide, but also by reducing nitrate or by fermentation

pathways (substrate level phosphorylation). Sulfate reduction is an environmentally significant process in which the end-product, sulfide, can react with a variety of metals influencing their mobility in the subsurface. For instance, sulfide is able to precipitate divalent metals (represented as M^{2+}) such as Fe(II), Hg(II), and Pb(II) as insoluble metal-sulfides (FeS, HgS, PbS) (Eqn. 1, (4)). Sulfide has also been shown to mediate the reductive dissolution of Fe(III) oxides such as hematite (α-Fe_2O_3) (Eqn. 2, (34)). These reactions are important in the mineral transformations and subsurface transport of heavy metals.

$$M^{2+} + S^{2-} \rightarrow MS \quad (1)$$

$$8Fe_2O_3 + 8H_2S + 32H^+ \rightarrow 16Fe^{2+}_{(aq)} + S_8^0 + 24H_2O \quad (2)$$

Table 1.1. Key reactions of the sulfur cycle.

Key Reactions and the Sulfur Cycle	
Process	**Representative Organisms**
Sulfide/sulfur oxidation (aerobic) ($H_2S \rightarrow S^0 \rightarrow SO_4^{2-}$)	*Thiobacillus* spp. *Beggiatoa* spp.
Sulfate reduction (anaerobic) ($SO_4^{2-} \rightarrow H_2S$)	*Desulfovibrio* spp. *Desulfobacter* spp.
Sulfur reduction (anaerobic) ($S^0 \rightarrow H_2S$)	*Desulfuromonas* spp. *Desulfovibrio* spp.

Adapted from Madigan, 2003 (10)

Microbial Diversity

Pristine environments are inhabited by microorganisms adapted to the ambient oligotrophic conditions. Iron, sulfur, nitrogen, and carbon cycling likely dominate the metabolic physiologies of the inhabiting populations. *Arthrobacter* spp. and a variety of

pseudomonads comprise up to 40% of the culturable microorganisms isolated from the soil (21). The interesting feature of the communities inhabiting pristine sites, however, is not necessarily the diversity but the differences between the populations inhabiting unperturbed sites as compared to those occupying anthropogenically contaminated environments. These comparisons provide a basis for inferences regarding the effects of contaminants on indigenous populations and organisms able to tolerate or utilize the contaminants.

Subsurface metal and radionuclide contamination are the result of the era of nuclear weapons development which has left over 3,000 sites and 6.4 billion cubic meters of soil, groundwater, and environmental media moderately to highly contaminated with organic and inorganic substances (29, 30). The microbial diversity at these sites indicates that the pressure imposed by the contaminants selects for indigenous populations not only able to tolerate but thrive under the harsh conditions. For example, acidophilic bacteria can be isolated from acid mine drainage (3, 7) and metal-tolerant microbes from metal-contaminated media (23). Not only are these environments inhabited by microbes tolerant of the conditions but many of the populations are able to utilize or detoxify the contaminants. Examples include the isolation of a tetrachloroethylene (PCE)-dechlorinating organism from a PCE-contaminated site (35), the recovery of a U(VI)-reducing bacterium isolated from a uranium-contaminated aquifer(33) , and the characterization of a chromium-reducing pseudomonad recovered from an environment contaminated with chromium (22). The ability of the indigenous populations to utilize, immobilize, and/or degrade the contaminants demonstrates the possibility of remediating

these areas by taking advantage of populations with key physiologies related to metal-reduction or dechlorination.

Bioremediation of metals and radionuclides

Bioremediation is defined by the National Research Council as the treatment of contaminated areas by exploitation of natural biodegradative processes (28). *In situ* bioremediation is an inexpensive treatment of organic and inorganic contaminants as compared to other contemporary methods including chemical treatment, passive reactive barriers (underground treatment walls), and pump-and-treat techniques (30). There are two main pathways for *in situ* microbial bioremediation: the addition of exogenous microorganisms or the use of indigenous communities. Bioaugmentation is the injection of natural or genetically-modified organisms expressing key physiologies for remediation. In order for a successful bioaugmentation treatment, the microorganisms added to the environment must first be able to reach the contaminant plume and subsequently develop a niche to compete with the existing populations adapted to the ambient conditions (20). These two hurdles can reduce the efficiency and applicability of bioaugmentation, however the technique may be optimal where indigenous populations have not yet adapted to the contaminants (such as a chemical spill) or where the conditions are too harsh for the natural populations (39).

Biostimulation and natural attenuation are often more successful and economically feasible approaches since the indigenous populations carry out the remediation events (20). The addition of nutrients (nitrogen, phosphorous), electron

donors (acetate, glucose), or complex sugars/proteins (molasses, whey) to contaminant plumes has been shown to stimulate the microbial activity resulting in contaminant degradation and metal immobilization. Biostimulation was successfully applied to uranium-contaminated sites in Rifle, Colorado and Oak Ridge, Tennessee. The injection of acetate (Rifle) or glucose and ethanol (Oak Ridge) resulted in significant reduction of soluble U(VI) in the groundwater (2, 27) and an increase in the dissimilatory metal-reducing bacteria (DMRB) and sulfate-reducing bacteria (SRB) at the Colorado site (2). DMRB and SRB have previously been shown to catalyze the immobilization of uranium, therefore enrichment of these populations at the Colorado site suggests they were integral to remediation of the U(VI).

In order to successfully employ biostimulation, it is important that the key physiologies and populations required for the activity of interest are present at the site and optimal conditions supporting their growth can be obtained (20). 16S ribosomal DNA (i.e. 16S rRNA gene) has been used as a phylogenetic marker for characterizing microbial diversity. Comparison of the 16S rRNA gene to those of characterized microorganisms can be used to suggest the likely physiologies of the populations based on the physiology of closely related organisms. However, to determine if the inhabiting microorganisms are capable of carrying out the process of interest, functional gene or enzyme analysis should be employed.

Functional gene analysis and enzymatic assays have been used to characterize the specific activities of microbial populations inhabiting a variety of sites. SRB have been detected at several contaminated areas and have been shown to immobilize metals both

chemically by the production of sulfide and enzymatically by electron transport proteins (13, 14, 17, 18). Functional gene analysis can be used to detect the presence of microorganisms capable of dissimilatory sulfate reduction using a gene target specific for this activity. Dissimilatory sulfite reductase (dsr), which is unique to SRB, is constitutively expressed (25) and catalyzes the last step of dissimilatory sulfate reduction in which bisulfite is reduced to sulfide (19). The dsr gene has been used to characterize SRB diversity in several environments (6, 24, 32) and is a valuable tool for defining the potential of a community to carry out dissimilatory sulfate reduction.

Hydrogenase is a key enzyme for energy and electron flow in SRB and the most extensively studied enzyme within the genus *Desulfovibrio.* Hydrogenase directly catalyzes the production and/or consumption of hydrogen according to the reaction $H_2 \leftrightarrow 2H^+ + 2e^-$ therefore likely influencing the redox chemistry surrounding the cell. This reaction is crucial to SRB metabolism based on the production of electrons for driving the reduction of sulfite to sulfide. Three different hydrogenases have been characterized within the genus *Desulfovibrio*: the [Fe], [NiFe], and [NiFeSe] hydrogenases, of which the [NiFe] hydrogenase is the only hydrogenase expressed in all species (38). Hydrogenase enzyme assays use viologen dyes as artificial electron donors to characterize the overall hydrogenase activity of SRB. In addition, analysis of the sequence of [NiFe] hydrogenase genes recovered from subsurface communities offers insight to the phylogeny of the population based on sequence similarity comparisons to hydrogenases of characterized SRB. Thus, hydrogenases are useful in assessing SRB activity and phylogeny.

Studies of indigenous subsurface microbial communities have revealed differences in activity of surface-associated versus free-living populations (1, 8, 9, 31, 37). In experiments conducted on extant microbial communities inhabiting mesotrophic lakes, groundwater and sediments attached populations demonstrated higher rates of activity as compared to free-living populations based on measurements of nutrient uptake (10, 36), production of extracellular enzymes (12), or ability to degrade hydrocarbons (10). Surface-associated microbes are capable of directly mediating changes in the redox state and solubility of several metals including those complexed as insoluble metal oxides (15, 16, 26). These mineral-microbe interactions influence subsurface geochemistry by mediating dissolution of the mineral surface and/or precipitation and deposition of secondary metal oxides (5). The majority of studies investigating microbial activity, including enzyme activities such as hydrogenase have only examined suspended populations, thus disregarding the populations likely to be displaying the greatest rates of activity and mediate changes in the geochemistry of the site.

In summary, indigenous microbial communities can be stimulated to remediate contaminated subsurface sites. It is important to understand the microbial diversity and metabolic capabilities of the community to carry out key processes such as metal-reduction or sulfate reduction. Analysis of 16S rDNA can suggest the likely physiology of the microorganisms present; however, functional gene analysis or enzyme assays provide more substantial evidence regarding the ability of a community to carry out the process of interest. It is important to understand the subsurface diversity either by investigation of 16S rRNA genes or analysis of functional genes involved in the specific

process. Since the majority of the diversity and activity have been found to be in microorganisms associated with surfaces, it is also important to understand how surface-associated growth affects the overall activity of the populations.

The Scope of the Thesis

The goal of this thesis was to investigate and compare the microbial diversity of a contaminated aquifer and to determine the effect of mineral-associated growth on the activity of hydrogenase enzymes expressed by the SRB *D. desulfuricans* Essex 6. The first objective of the thesis was to characterize and compare the indigenous microbial communities at an acidic, metal-, and radionuclide-contaminated site at a Field Research Center within the Oak Ridge Reservation in Tennessee. The use of traditional sediment extraction techniques was precluded by the contaminants; therefore biofilm coupons (metal, mesh cylinders filled with surrogate substrata) were used to sample populations able to adhere to the substrate and compete in a biofilm. Hematite was used as a surrogate substratum to recover and compare populations inhabiting a pristine and a contaminated region of the aquifer (Chapter 2). The second objective of the thesis was to compare the microbial communities that had developed on different surrogate substrata at the pristine site (Chapter 2). The third objective of the thesis was to investigate the effect of hematite-associated growth on hydrogenase activity in *Desulfovibrio desulfuricans* Essex 6 when cultured under sulfate-reducing and pyruvate -fermenting conditions (Chapter 3). The fourth objective of this thesis was to investigate hydrogenase gene expression of mineral-associated and suspended populations of *D. desulfuricans* Essex 6

(Chapter 3). This dissertation demonstrates the importance of evaluating the particle-associated populations in order to better understand the overall microbial ecology.

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CHAPTER 2

COMPOSITION AND DIVERSITY OF MICROBIAL COMMUNITIES RECOVERED FROM SURROGATE MINERALS INCUBATED IN AN ACIDIC URANIUM-CONTAMINATED AQUIFER

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The terminal restriction fragment length analysis was carried out by Dr. David Cummings at the INL, ID. Phylogenetic analyses were performed by Lynn Petzke at the INL. These data are included in this thesis for completeness of the microbial diversity analysis.

Abstract

Our understanding of subsurface microbiology is hindered by the inaccessibility of this environment, particularly when the hydrogeologic medium is contaminated with toxic substances. In this study, surrogate geological media contained in a porous receptacle were incubated in a well within the saturated zone of a pristine region of an aquifer to capture populations from the extant communities. After an eight-week incubation, the media were recovered and the microbial community that developed on each medium was compared to the community recovered from groundwater and native sediments from the same region of the aquifer using 16S rDNA-based T-RFLP. The groundwater and sediment communities were highly distinct from one another, and the

communities that developed on the various media were more similar to groundwater communities than to sediment communities. 16S rDNA clone libraries of communities that developed on particles of a specular hematite medium incubated in the same well as the media used for T-RFLP analysis were compared with those obtained from an acidic, uranium-contaminated region of the same aquifer. The hematite-associated community formed in the pristine area was highly diverse at the species level, with 25 distinct phylotypes identified, the majority of which (73%) were affiliated with the β-Proteobacteria. Similarly, the hematite-associated community formed in the contaminated area was populated in large part by β-Proteobacteria (62%); however, only 13 distinct phylotypes were apparent. The three numerically dominant clones from the hematite-associated community from the contaminated site were affiliated with metal- and radionuclide-tolerant or acidophilic taxa, consistent with the environmental conditions. Only two populations were common to both sites.

Introduction

Effective management and reclamation of contaminated subsurface geological media requires an understanding of the composition of extant microbial communities. To date, characterization of indigenous subsurface microbial populations has mainly focused on microbes collected from sediment or rock samples (3, 6, 10, 24, 42) or groundwater (5, 7, 29). Subsurface sediment-based diversity studies are often impractical because sediment cores are both expensive and difficult to acquire. While groundwater is more readily obtained than sediments, contaminated groundwater poses the added problem of

secondary waste generation. Due to these limitations, studies requiring numerous samples (*e.g.*, temporal studies) are unrealistic with current methods. It has been suggested that those microorganisms most likely to be involved in contaminant transformation are often associated with the surfaces of the geological matrix through which the groundwater percolates (16, 19, 21, 23, 30). While water sampling techniques allow for temporal and large-scale spatial sampling of transient or planktonic communities, they may not capture key populations partitioned on the solid phases.

Recently, investigators have used surrogate solid media contained in a porous receptacle, referred to hereafter as a biofilm coupon, to sample microbial communities in the subsurface with a propensity to associate with surfaces (43). The retrievable coupon can contain particles such as hematite or quartz with surface properties representative of the natural geological matrix. These surfaces serve as sites for colonization by planktonic microbial populations, and promote close physical and temporally stable cooperative associations between members of the community. Biofilm coupons thus offer a pragmatic means of recovering populations of microorganisms that form communities optimized to function under a particular set of environmental conditions.

One objective of this study was to compare subsurface microbial communities that developed on media in biofilm coupons to communities associated with the sediment and groundwater phases. Intact-biofilm polymerase chain reaction (IB-PCR), a modified PCR method that circumvents the need to separate DNA from other sample components prior to amplification of community 16S rRNA genes, is described for use in situations where recovery of DNA from samples containing certain mineral phases is problematic.

A second objective of the study was to characterize and compare the indigenous microbial communities that developed on media (specular hematite particles) in biofilm coupons incubated at two saturated subsurface sites at a U. S. Department of Energy (DOE) Field Research Center (FRC) near Oak Ridge, Tennessee. At one site, the subsurface is contaminated with radionuclides, metals, organics, and nitric acid, and is currently under investigation for potential biological remediation. A nearby pristine region of the aquifer possesses similar hydrogeological characteristics and provides for a suitable comparison with the contaminated site. The results suggest that phylogenetically distinct microbial communities inhabit the two FRC sites, pristine and contaminated. Furthermore, microbial diversity at the contaminated site was diminished relative to the pristine community, and was dominated by organisms apparently adapted to the harsh conditions.

Materials and Methods

Site description

The U. S. DOE's Office of Science recently designated an area of the Oak Ridge Reservation (Oak Ridge, Tennessee) as a Field Research Center (FRC) in order to bring together scientists from multiple disciplines with the common goal of acquiring fundamental knowledge in support of *in situ* bioremediation. The FRC lies within the boundaries of the Y-12 National Security Complex near the Oak Ridge National Laboratory. The contaminant plume occupying the underlying aquifer is the result of leakage from two primary sources: the Boneyard/Burnyard and the S-3 Ponds. The

Boneyard/Burnyard consisted of unlined trenches in which a variety of wastes including magnesium chips, solvents, and laboratory chemicals were burned from 1943 until 1968. The unlined S-3 Ponds received U-bearing liquids mixed with nitric acid from 1951 until 1983 when they were denitrified and capped. Due to the co-disposal of nitric acid with the wastes, nitrate concentrations typically near 8,000 mg L^{-1} have been measured in the groundwater and the pH varies between 3.0 and 6.5 (Table 2.1). The contaminant plume occupies two distinct geological formations, Maynardville limestone and Nolichucky shale, and is enriched in organics (*e.g.*, chloroaliphatics and aromatics), metals (*e.g.*, Ni, Al, and As), and radionuclides (*e.g.*, ^{238}U and ^{99}Tc). ^{238}U has been detected at concentrations as high as 43 pCi g^{-1} soil and 13,600 pCi L^{-1} groundwater. By contrast, radionuclides and organic contaminants are below detection in the Background Area, and metals are at concentrations typical of the region (20, 49). For further information on this field site we direct the reader to the DOE FRC web page (http://www.esd.ornl.gov/nabirfrc/index.html).

Table 2.1. Groundwater characteristics of Background Area wells and Area 3 well FW026

				Dissolved Components (mg L^{-1})			
Well	**Area**	**pH**	**ORP[b]**	**Total Fe[c]**	**Nitrate[d]**	**Uranium[e]**	**TOC[f]**
FW300	**Background**	**6.13**	**NA**	**0.05**	**1.2**	**0.00**	**25.96**
FW303	**Background**	**7.52**	**+ 437**	**NA**	**NA**	**NA**	**NA**
FW026	**Area 3**	**3.43**	**+ 354**	**17.4**	**8262**	**42.74**	**68.7**

[a] Most data have not yet been compiled for well FW303, therefore data from well FW300, approximately 10.5 m southeast of well FW303, are provided as a proxy.
[b] Oxidation-reduction potential (mV), calibrated to the standard hydrogen electrode.
[c] Total dissolved Fe was determined by ICP.
[d] Dissolved nitrate was determined by IC.
[e] Dissolved uranium was determined by ICP-MS.
[f] Total organic carbon was determined by combustion.

Sampling mineral surface-associated microbial communities with biofilm coupons

To entrain and capture microbial populations from the subsurface, biofilm coupons composed of capped stainless steel mesh cylinders (25.4 cm x 1.27 cm) (46) were filled with various minerals, hereafter referred to as coupon-associated media, and lowered by fishing line into the saturated zone of pristine (Background Area well FW303) and contaminated (Area 3 well FW026) regions of the aquifer where they were incubated for approximately eight weeks. Prior to deployment, minerals and coupons were sterilized by combustion (550^{o}C, 6 h). Minerals included micaceous, specular hematite (1-3 mm diameter) from Minas Gerais, Brazil (kindly provided by K. Rosso, Pacific Northwest National Laboratory), illite shale (Ward's Natural Science, cat no. 46 V 0315), uncontaminated saprolite from the FRC subsurface (a gift from P. Jardine, Oak Ridge National Laboratory), and course quartz sand. The different coupon-associated media were separated within the coupon apparatus by plugs of glass wool. Following

retrieval, the coupons were shipped to the INEEL at 4°C by overnight courier where they were frozen at -80°C until 16S rDNA analysis, as described below. For geochemical characteristics of the Background Area and Area 3, see Table 2.1.

Retrieval of subsurface groundwater and sediments

Triplicate 4-L aliquots of groundwater were pumped from a depth of 5.5 m from well FW303, and planktonic and colloid-associated cells were captured on 0.22-μm filters. Sediments were collected within 1 m of well FW303 from depths of 4.9 m (unsaturated), 5.1 m (variably saturated), and 5.4 m (saturated) using a hollowstem auger fitted with a 5.08 cm-diameter split-spoon sampler. Filters and sediments were frozen immediately and shipped to the INEEL on dry ice within 72 h. Additionally, core material from the original FW303 (background) and FW026 (contaminated) boreholes was acquired from FRC archives and used for 16S rDNA clone library construction (see below); these were shipped and stored at 4°C for approx. three weeks prior to analysis.

DNA extraction from colonized coupon-associated media, sediments, and groundwater filters

Community genomic DNA was obtained from hematite (triplicate 1.5-g samples), quartz sand (9.2 g), illite shale (6.9 g), saprolite (3.8 g), glass wool separating saprolite and illite (1.3 g), glass wool separating hematite and sand (0.76 g), sediments (10 g each), and groundwater filters using bead-beating kits from either MoBio (UltraClean Soil DNA Kit or UltraClean Mega Prep Soil DNA Kit, depending on sample size) or Bio101 (FastDNA SPIN Kit for Soil). Sediments were allowed to thaw at 4°C overnight before DNA extraction. Bead-beating was performed in a water bath at 65°C, shaking at 400

rpm (30 min.); all other steps were carried out as recommended by the manufacturers. DNA from the Mega Prep kit was eluted in 8 mL water and concentrated to 100 μL by precipitation in NaCl and ethanol. DNA from the smaller kits was eluted in 50 μL water and not concentrated further.

Polymerase chain reaction (PCR) of 16S rRNA genes

16S rRNA genes were PCR-amplified from each community genome extracted with the bead-beating kits. Each 50-μL reaction contained the following (final conc.): 1X PCR buffer (Invitrogen), 2 mM $MgCl_2$ (Invitrogen), 200 μM of each deoxynucleotide triphosphate (Invitrogen), 0.5 μM of each forward and reverse primer (Invitrogen), 0.4 μg μL^{-1} molecular-grade bovine serum albumin (New England BioLabs), and 0.25 U Taq DNA Polymerase (Invitrogen). For clone libraries, conserved regions of the 16S rRNA gene were targeted with eubacterial forward primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and universal reverse primer 1492R (5'-GGTTACCTTGTTACGACTT-3'). For T-RFLP analysis, fluorescently labeled 16S rDNA PCR products were generated using primer 8F-FAM (eubacterial primer 8F modified with phosphoramidite fluorochrome 5-carboxyfluorescein, Invitrogen) in conjunction with 907R (5'-CCGTCAATTCMTTTRAGTTT-3'). Five μL of genomic DNA was used as template for the PCR reactions. The following cycling conditions were applied: 4 min. at 94°C followed by 30 cycles of denaturation at 94°C (1 min.), annealing for 1 min. across a broad range of temperatures, primer extension at 72°C (1.5 min.), and a final extension step at 72°C (4 min.). The Mastercycler Gradient thermocycler

(Eppendorf) was used to generate a gradient of annealing temperatures from 40-60^{o}C. Distinct PCR reactions, the quality of which was verified by 1.5% agarose gel electrophoresis, were incubated at annealing temperatures of 40.1, 42.7, 46.7, 49.9, 50.0, 52.7, 56.8, and 60.0^{o}C, and the products were combined prior to cloning or T-RFLP.

Intact-biofilm polymerase chain reaction

The DNA extracts from hematite media amplified poorly; therefore a whole-cell PCR approach was developed to overcome any limitations imposed by the extraction method. Community 16S rRNA genes were directly amplified from hematite media using intact-biofilm PCR (IB-PCR) in which colonized particles were added directly to PCR tubes containing water and buffer and heated at 99^{o}C for 15 min. to lyse the attached cells. The temperature was subsequently lowered to 80^{o}C, and the remaining PCR reaction components were added before initiating the same cycling regime as described above.

Terminal restriction fragment length polymorphism (T-RFLP) analysis

T-RFLP was used to compare the microbial communities from the pristine well colonizing the various coupon-associated media with one another and with the communities associated with the groundwater and sediments. Twenty-µL from each replicate PCR reaction (multiple annealing temperatures) were combined and purified with the Wizard PCR Preps DNA Purification kit (Promega). Approx. 200 ng of amplicons from each community DNA were digested in triplicate with 10 U of the restriction endonuclease *Msp*I (New England BioLabs) at 37^{o}C, 3 h. Restriction

fragments were purified with 3 μL sodium acetate (3 M, pH 5.2) and 66 μL ethanol (70%), air-dried, and re-suspended in 10 μL water. Samples were denatured by heating to 95^{o}C for 3 min. followed by submersion in an ice bath. The denatured DNA (2 μL), along with the internal standard Rox 1000 (Applied Biosystems), was loaded onto a model 377 DNA sequencer (Applied Biosystems) employing a Cambrex Long Ranger XL 5% polyacrylamide denaturing gel and electrophoresed at 51^{o}C, 3000 V, 4.5 h. The resulting data were analyzed using Genescan v 2.1 (Applied Biosystems). Peaks with less than 50 fluorescence units were discarded, as were those that were not present in at least two of the three replicate profiles.

Statistical analyses of T-RFLP data

Replicate T-RFLP profiles were aligned manually and a consensus profile was generated for each community that consisted of mean fragment lengths and fluorescence intensities. The consensus profiles were aligned manually for comparison. Jaccard similarity coefficients, which make use of only presence/absence data, and Euclidean distances, which represent weighted continuous data (both peak position and peak height), were calculated using the paleoecology statistics freeware package PAST (18). Cluster analyses of the T-RFLP patterns were performed applying the unweighted pair-group average (UPGMA) algorithm to the distance measures in either PAUP (version 4.0b10; Sinauer Associates, Inc.) or PAST.

Construction and screening of 16S rDNA clone libraries

Clone libraries of amplified 16S rRNA genes from the populations colonizing hematite media were constructed from the IB-PCR reactions. Amplification reactions from the eight different annealing temperatures were combined and purified with the Wizard PCR Preps DNA Purification kit (Promega). Amplicons were ligated overnight at 4°C into the pGEM-T Easy vector (Promega) and transformed into competent *E. coli* JM109 cells. Ligation and transformation were carried out as recommended by the manufacturer. Transformed cells were plated onto S-Gal agar (Sigma) with 100 μg mL^{-1} ampicillin (sodium salt) and incubated at 37ºC for 16 h. Approximately 100 white colonies from each library were chosen at random and used as templates for whole-cell PCR. The whole-cell PCR mix was the same as that described above for IB-PCR with the following exceptions: cells from a single transformed *E. coli* colony were used as the DNA source in place of hematite media, thermocycling employed a single annealing temperature of 50ºC, and primers M13F (5'-GTAAAACGACGGCCAG-3') and M13R (5'-CAGGAAACAGCTATGAC-3'), flanking the insertion site on the vector, were used to re-amplify the insert. Re-amplified inserts were digested with the restriction endonucleases *Msp*I and *Hin*P1I (New England BioLabs) in NEB2 buffer (37°C, 5 h) for restriction fragment length polymorphism (RFLP) analysis, and resolved in 3% agarose (NuSieve 3:1 agarose). Clones were conservatively clustered into RFLP types from which a single representative was sequenced in full for a minimum of 2X coverage.

Sequencing and phylogenetic analysis

Purified plasmids (QIAprep Spin Miniprep Kit, Qiagen) or M13 PCR products were sequenced using primers M13F, M13R, 515F (5'-GTGCCAGCMGCCGCGGTAA-3', where M = A or G), 519R (5'-ATTACCGCGGCTGCTGG- 3'), 1100F (5'-CAACGAGCGCAACCCT-3'), and 1100R (5'-AGGGTTGCGCTCGTTG-3'). Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an ABI 3700 Automated DNA Sequencer (Applied Biosystems).

Electropherograms were edited using the Chromas freeware (version 1.45; School of Health Science, Griffith University, Gold Coast Campus, Southport, Queensland, Australia) and sequences were assembled with the BioEdit Sequence Alignment Editor freeware (version 5.0.9; http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (17). Sequences were aligned with ClustalW and grouped together based on sequence similarity using Sequence Grouper (Andrew Shewmaker, INEEL). Because of known errors introduced during PCR and cloning (50), sequences with ≥ 97% sequence similarity to one another were considered indistinguishable and treated as a single phylotype.

All assembled sequences were examined for chimeric artifacts using CHIMERA_CHECK from the Ribosomal Database Project II (RDP) (34); potentially chimeric sequences were not given further consideration. Non-chimeric sequences were aligned to the 16S rDNA sequences of the closest cultured organisms from the GenBank and RDP databases using the ClustalW alignment tool in BioEdit. Sequences were

manually corrected using the MacClade software (version 3.0; Sinauer Associates, Inc., Sunderland, MA) to ensure that only homologous nucleotides were compared between sequences. The edited alignments were evaluated with maximum parsimony, maximum likelihood, and distance methods using the PAUP package (version 4.0b10; Sinauer Associates, Inc.). Trees generated with all three methods were congruent, with only minor rearrangements in branching order. Phylogenetic inference and evolutionary distance calculations were generated using the distance Jukes-Cantor model (gamma parameter equal to 2.0). Ten thousand bootstrap replicates were used to obtain confidence estimates for the phylogenetic trees.

Calculation of diversity indices

The number and frequency of phylotypes identified in the clone libraries were used to estimate the diversity of the different communities using PAST (18). The Shannon diversity index (H) (also referred to as the Shannon-Weaver or Shannon-Wiener index) was calculated using the equation.

$$H = -\Sigma p_i * \ln p_i \quad (3)$$

in which p_i is the frequency of the i^{th} phylotype. A higher H value indicates greater diversity. Equitability (J) was calculated as

$$J = H * \ln S^{-1} \quad (4)$$

where S is the total number of clones. Equitability values vary from 0 to 1 and reflect the ratio of the observed diversity (H) to the maximum diversity within a sample, where maximum diversity equals 1. The Simpson dominance index (D) was calculated using equation 5.

$$D = \Sigma p_i^2 \quad (5)$$

An increase in 1/D is indicative of an increase in diversity.

Accession numbers

All clone sequences have been deposited in the GenBank/DDBJ/EMBL databases under accession numbers AY622227-AY622271.

Results

Comparison of sediment and groundwater communities to those captured on coupon-associated minerals within biofilm coupons

T-RFLP analysis of PCR-amplified 16S rRNA genes was used to compare microbial communities associated with natural sediments and groundwater with those that developed on coupon-associated mineral surfaces contained within a biofilm coupon in the Background Area. Sufficient PCR products for T-RFLP analysis were obtained from all genomic DNA extracts with the exception of the hematite. To overcome this limitation, a method that directly amplified the genomic DNA extracted from the mineral surface-associated community in the presence of other sample components was developed. Intact biofilm PCR (IB-PCR) of hematite particles provided concentrations of PCR products similar to those obtained from the bead-beating extractions. All T-RFLP patterns were aligned manually and used to calculate Jaccard similarity coefficients (based on the presence or absence of a T-RF) (Table 2.2).

The Jaccard similarities were further used to perform cluster analysis (Figure 2.1). Clusters were formed that reflected, to some degree, community origin. The groundwater

and sediment communities were clearly distinct from one another, sharing in common only 11 of their 70 combined T-RFs. The communities from three replicate groundwater samples were most similar to one another, as were the communities from the three sediment samples. A distinct cluster contained all of the communities from the coupon-associated minerals with the exception of the community from the illite shale, all of which were more similar to the groundwater than to the sediments. Nearly identical dendogram topology was obtained when T-RF fluorescence intensity was considered using Euclidean distances in place of Jaccard similarities (data not shown). Notably, the communities associated with the coupon-associated minerals were again more similar to the groundwater communities than to the sediment communities.

The glass wool- and quartz sand-associated communities were most similar to each other (Jaccard value range of 0.458-0.600). With the exception of the relatively high similarity of the hematite-associated community to the community from groundwater sample 1 (Jaccard similarity coefficient of 0.400), the quartz-associated community was marginally more similar to the communities recovered from groundwater (Jaccard value range of 0.382-0.419) than were communities associated with the other coupon-associated minerals (Jaccard coefficient range of 0.263-0.393) (Table 2.2). The hematite-associated community was slightly more similar to the saturated sediment community (sediment sample 3; Jaccard coefficient of 0.174) than were the communities associated with any other coupon-associated media tested (Jaccard coefficient range of 0.100-0.170) (Table 2.2).

Table 2.2. Jaccard similarity coefficients comparing the T-RFLP profiles of communities from natural groundwater and sediments from the Background Area with those formed on surrogate geological media incubated in the same well.

	Hematite	*Glass 1*	*Glass 2*	*Illite*	*Saprolite*	*Quartz*	*Sediment 1*	*Sediment 2*	*Sediment 3*	*Water 1*	*Water 2*	*Water 3*
Hematite	1	0.500	0.360	0.250	0.370	0.345	0.154	0.152	0.174	0.400	0.370	0.379
Glass 1		1.000	0.458	0.367	0.407	0.600	0.148	0.121	0.100	0.333	0.357	0.323
Glass 2			1.000	0.448	0.444	0.464	0.185	0.118	0.120	0.370	0.393	0.355
Illite				1.000	0.406	0.469	0.188	0.189	0.170	0.303	0.286	0.263
Saprolite					1.000	0.419	0.167	0.139	0.135	0.379	0.355	0.324
Quartz						1.000	0.156	0.132	0.109	0.400	0.419	0.382
Sediment 1							1.000	0.417	0.182	0.222	0.207	0.188
Sediment 2								1.000	0.381	0.182	0.171	0.158
Sediment 3									1.000	0.140	0.135	0.148
Water 1										1.000	0.905	0.720
Water 2											1.000	0.800
Water 3												1.000

[a] Glass 1, glass wool separating saprolite and illite shale.
[b] Glass 2, glass wool separating hematite and sand.
[c] Sediment 1, unsaturated sediment.
[d] Sediment 2, variably saturated sediment.
[e] Sediment 3, saturated sediment.
[f] Water 1, Water 2, Water 3, triplicate 4-liter groundwater samples.

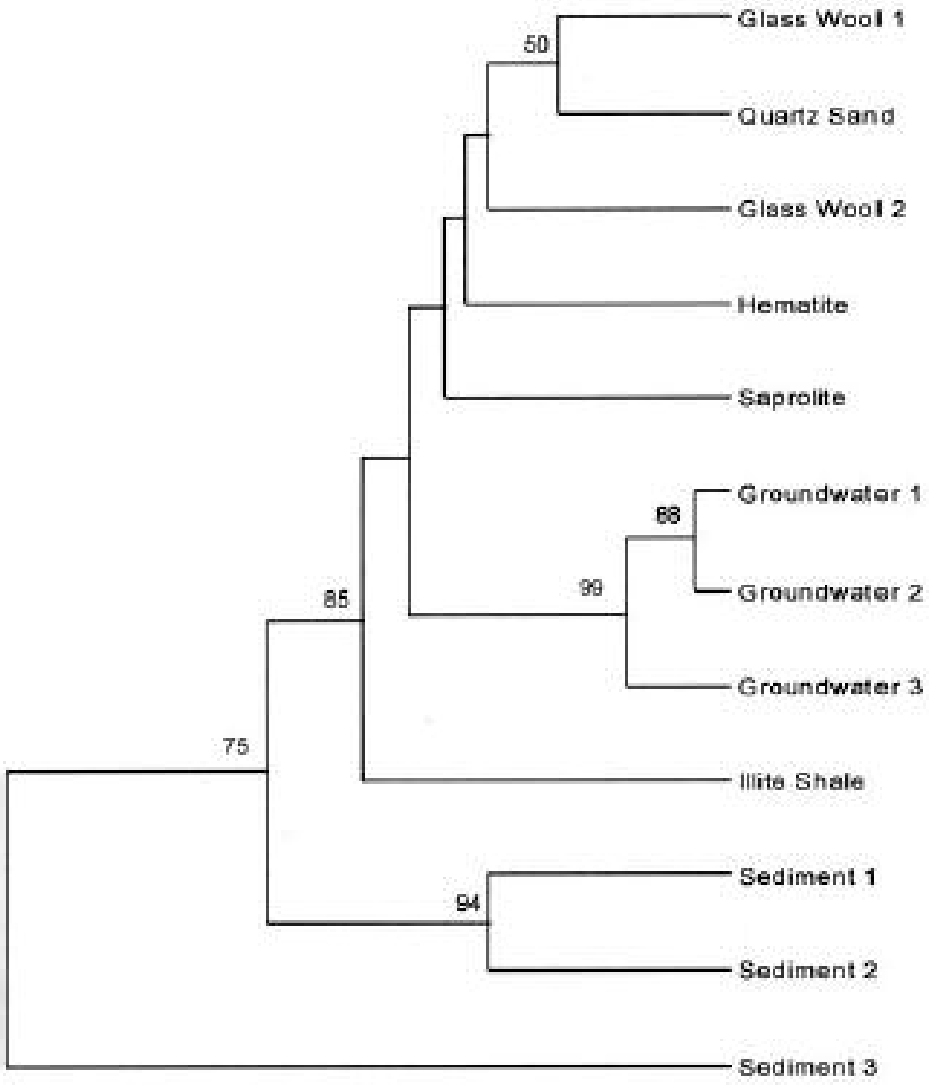

Figure 2.1. Cluster analysis of 16S rRNA-based T-RFLP patterns from communities associated with various surrogate geological media and natural sediment and groundwater in a pristine region of the FRC aquifer, Oak Ridge, Tenn. The UPGMA algorithm was used to cluster patterns based on Jaccard similarities. Bootstrap values above 50 are indicated at the appropriate nodes (based on 1,000 bootstrap replicates).

Table 2.3. Bacterial 16S rDNA clones from communities formed on hematite in FRC Area 3 well FW026.

Clone ID	***GenBank no.***	***Frequency[a]***	***Affiliation[b] (% similarity)***	***Putative Division***
C-CG17	AY622228	59	*Alcaligenes* sp. strain L6 (95)	β-Proteobacteria
C-CS3	AY622233	24	*Frateuria* sp. NO-16 (96)	γ- Proteobacteria
C-CF16	AY622227	4	*Methylobacterium radiotolerans* (99)	α-Proteobacteria
C-CU62	AY622234	3	*Pseudomonas straminea* (99)	γ-Proteobacteria
C-CJ32	AY622229	2	*Beutenbergia cavernosa* (96)	Actinobacteria
C-CY80	AY622237	1	*Herbaspirillum seropedicae* (96)	β-Proteobacteria
C-DA88	AY622239	1	*Burkholderia* sp. A6.2 (98)	β-Proteobacteria
C-CZ82	AY622238	1	*Duganella zoogloeoides* (98)	β-Proteobacteria
C-CL42	AY622230	1	*Pseudomonas syringae* (89)	γ-Proteobacteria
C-CX74	AY622236	1	*Sphingobacterium antarcticum* (99)	γ-Proteobacteria
C-CO51	AY622232	1	*Microbacterium* sp. VKMAc-2050 (99)	Actinobacteria
C-CV63	AY622235	1	*Nocardioides* sp. MWH-CaK6 (98)	Actinobacteria
C-CM46	AY622231	1	Clone MTAC17 (92%)	Unknown

[a] Frequency of a given RFLP-type out of 100 total clones.
[b] Closest identified match in the GenBank database followed by percent similarity. Clone C-CM46 did not match any identified sequences in the database.

With the exception of quartz sand, each of the coupon-associated media captured populations that were detected in sediment samples but remained undetected by analysis of groundwater (data not shown). For example, the T-RFLPs for illite, saprolite, and one of the two glass wool communities all possessed a T-RF that was also present in sediment sample 3, but absent from the groundwater samples. Whether samples prepared from larger volumes of groundwater would improve the detection of these populations is unknown. Similarly, the coupon-associated media captured 22 populations (T-RFs) that were not detectable in T-RFLPs of either the sediments or groundwater. Thus, coupon-associated media incubated in the subsurface provided appreciable quantities of a number of native populations that remained indiscernible when sediments or groundwater served as the source of community DNA.

Comparison of microbial community diversity in the
saturated zone of pristine and contaminated regions of the FRC aquifer.

16S rDNA clones from biofilm coupon-associated hematite. Biofilm coupons containing specular hematite were used to capture and compare surface-associated community diversity in the saturated zone of Area 3 well FW026 and Background Area well FW303. Hematite media was selected over other media based on the evidence from T-RFLP analysis that of the various media evaluated, the community captured on hematite was most similar to the (saturated) sediment-associated community at the Background Area. Unfortunately, we have not yet been able to relate community composition based on IB-PCR to that obtained by conventional DNA extraction and PCR methodologies, since the presence of hematite compromised the recovery of DNA when the latter extraction procedure was used.

Thirteen distinct phylotypes were identified from 100 non-chimeric clones generated from IB-PCR-derived products from hematite particles incubated in Area 3 contaminated well FW026 (Table 2.3, Figure 2.2). Members of the Proteobacteria dominated the library in Area 3, with four phylotypes affiliated with the β-Proteobacteria (62% of the library), another four affiliated with the γ-Proteobacteria (29% of the library), and one affiliated with the α-Proteobacteria (4% of the library). No δ-Proteobacteria were found associated with hematite incubated in the contaminated groundwater. In addition to the Proteobacterial clones, three phylotypes affiliated with the Actinobacteria (4% of the library) and one unidentified lineage (1% of the library) were also present. The library was dominated by a clone similar to *Alcaligenes* sp. (95% similarity) and

another most similar to *Frateuria* sp. (96% similarity), which together contributed 83% of the library. Clone C-CL42 displayed particularly low similarity to its closest known relative, a *Pseudomonas* sp (89% similarity). Clone C-CM46 was not significantly related to any known lineage (Figure 2.2).

Hematite particles incubated in Background Area well FW303, when subjected to IB-PCR, yielded 34 distinct phylotypes (of 95 clones screened), 10 of which were potentially chimeric and thus disregarded (Table 2.4, Figure 2.2). Like the hematite-associated community from the Area 3 contaminated site, members of the Proteobacteria dominated the library from the Background Area, with a particularly high frequency of those belonging to the β subdivision (Table 2.4). The Background Area hematite associated community was composed of 13 phylotypes affiliated with the β-Proteobacteria (69% of the library), six affiliated with the γ-Proteobacteria (21% of the library), two affiliated with the α-Proteobacteria (3.5% of the library), and one affiliated with the δ-Proteobacteria (1.2% of the library). Two non-Proteobacterial clones were also present, one affiliated with the Bacteroidetes (2.4% of the library) and another affiliated with the Verrucomicrobia (1.2% of the library). A clone most similar to the dissimilatory Fe(III)-reducing β-Proteobacterium *Rhodoferax ferrireducens* (13) (98% sequence similarity) made up 45% of the Background Area library. Other clones observed more than once included relatives of *Pseudomonas mandelii*, *Oxalobacter* sp., *Pseudoxanthomonas mexicana*, *Herbaspirillum seropedicae*, *Aquamonas gracilis,* *Novosphingobium* sp., and *Flavobacterium columnare*. Clone B-AB39, while clearly

belonging to the β-Proteobacteria, was not highly related to any known microorganism (Figure 2.2).

Table 2.4. Bacterial16S rDNA clones from communities formed on hematite in FRC Background Area well FW303.

Clone ID	GenBank no.	Frequency[a]	Affiliation[b] (% similarity)	Putative Division
B-Y34	AY622248	38	*Rhodoferax ferrireducens* (98)	β-Proteobacteria
B-B3	AY622240	6	*Pseudomonas mandelii* (98)	γ-Proteobacteria
B-BH93	AY622261	5	*Oxalobacter* sp. p8E (97)	β-Proteobacteria
B-BD81	AY622259	5	*Pseudoxanthomonas mexicana* (98)	γ-Proteobacteria
B-C4	AY622241	4	*Pseudoxanthomonas mexicana* (95)	γ-Proteobacteria
B-AA37	AY622249	4	*Herbaspirillum seropedicae* (97)	β-Proteobacteria
B-E7	AY622242	3	*Aquamonas gracilis* (95)	β-Proteobacteria
B-BF84	AY622260	2	*Novosphingobium* sp. TUT562 (96)	α-Proteobacteria
B-AQ60	AY622255	2	*Flavobacterium columnare* (96)	Bacteroidetes
B-L17	AY622245	1	*Methylocella tundrae* (92)	α-Proteobacteria
B-BI94	AY622262	1	*Aquamonas gracilis* (97)	β-Proteobacteria
B-AG46	AY622253	1	*Gallionella ferruginea* (91)	β-Proteobacteria
B-AX74	AY622258	1	*Aquaspirillum arcticum* (95)	β-Proteobacteria
B-AB39	AY622250	1	*Burkholderia phenazinium* (90)	β-Proteobacteria
B-H11	AY622243	1	*Acidovorax* sp. UFZ-B517 (94)	β-Proteobacteria
B-AW71	AY622257	1	*Zoogloea* sp. strain DhA-35 (91)	β-Proteobacteria
B-N19	AY622246	1	*Imtechium assamiensis* (97)	β-Proteobacteria
B-O21	AY622247	1	*Ideonella* sp. B513 (96)	β-Proteobacteria
B-AU68	AY622256	1	*Pseudomonas rhodesiae* (96)	γ-Proteobacteria
B-AF45	AY622252	1	*Pseudomonas putida* (90)	γ-Proteobacteria
B-AC40	AY622251	1	*Pseudomonas* sp. NZ111 (92)	γ-Proteobacteria
B-BK96	AY622263	1	*Haliangium tepidum* (92)	δ-Proteobacteria
B-I12	AY622244	1	*Opitutus* sp. VeGlc2 (93)	Verrucomicrobia

[a] Frequency of a given RFLP-type out of 85 total clones.
[b] Closest identified match in the GenBank database followed by % similarity.

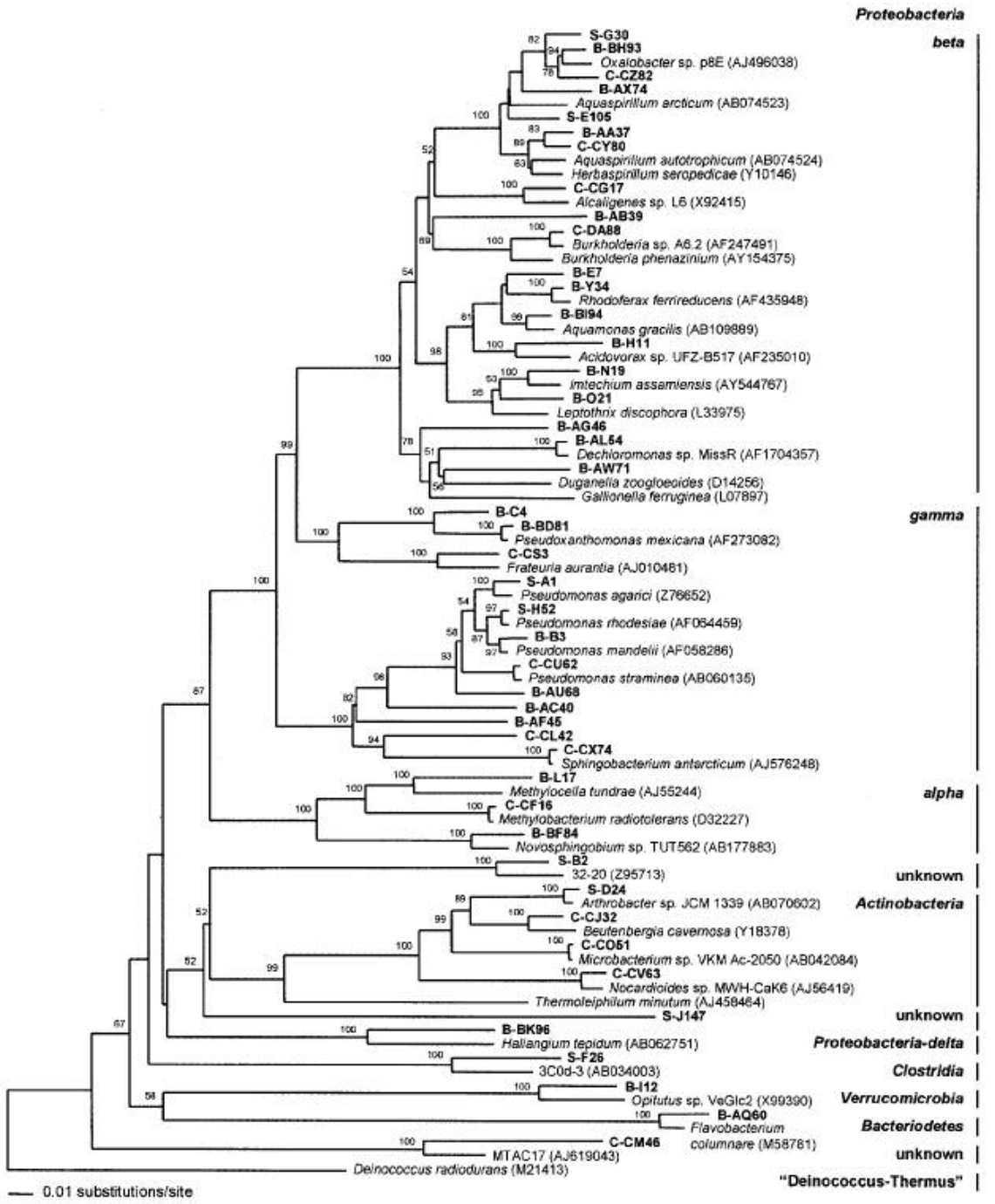

Figure 2.2. Phylogenetic relationships of nearly full-length 16S rDNA sequences. The phylogenetic tree was constructed using PAUP based on maximum-distance analysis with Jukes-Cantor correction. The values at the nodes are bootstrap probabilities (percentages) based on 10,000 replicates; only values greater than 60% are shown. The scale bar indicates 0.01 nucleotide substitutions per site. Clone designations beginning with B, C, or S indicate the library of origin: S, sediment core from background area well FW303; B, biofilm coupon from background area well FW303; C, biofilm coupon from contaminated Area 3 well FW026. Sequences with accession numbers were obtained from the RDP or GenBank databases.

16S rDNA clones from sediment extracts. Microbial communities captured on surrogate minerals in biofilm coupons were compared with those recovered from cores of natural geological material retrieved during drilling of Background Area well FW303 and Area 3 well FW026. A clone library was constructed from DNA extracted by bead-beating of core material from well FW303. The library yielded 8 unique phylotypes after screening 100 total clones (Table 2.5, Figure 2.2). No chimeric sequences were detected in this library. The library was dominated by two *Pseudomonas* phylotypes, clones S-A1 and S-H52, together making up 90% of the library. Clone S-A1, most similar to *Pseudomonas agarici* (98% sequence similarity), comprised 54% of the library, while clone S-H52, most similar to *Pseudomonas rhodesiae* (99% sequence similarity), comprised another 36% of the library. These two phylotypes shared 97% similarity with one another, but we chose to leave them separate because two distinct clusters of clones emerged when they were aligned with one another. The next most frequent phylotypes, each contributing 3% to the total library, were clones S-E105 and S-D24, most similar to strains of the genera *Ultramicrobacterium* (97% sequence similarity) and *Arthrobacter* (98% sequence similarity), respectively. Clone S-G30, representing only 1 clone of 100 in the library, shared 98% sequence similarity with *Duganella zoogloeoides*. The remaining three phylotypes, S-B2, S-F26, and S-J147, each only encountered once in the library, branched deeply and did not group with any characterized lineages (Figure 2.2).

Attempts to amplify 16S rRNA genes from DNA extracts of contaminated sediment collected from well FW026 in Area 3 were unsuccessful.

Table 2.5. Bacterial 16S rDNA clones from sediments at of the FRC Background Area borehole FWB303.

Clone ID	GenBank no.	Frequency[a]	Affiliation[b] (% similarity) (Accession)	Putative Division
S-A1	AY622264	54	*Pseudomonas agarici* (98)	γ-Proteobacteria
S-H52	AY622270	36	*Pseudomonas rhodesiae* (99)	γ-Proteobacteria
S-E105	AY622267	3	*Aquaspirillum autotrophicum*	β-Proteobacteria
S-D24	AY622266	3	*Arthrobacter* sp. JCM 1339 (98)	Actinobacteria
S-G30	AY622269	1	*Duganella zoogloeoides* (98)	β-Proteobacteria
S-B2	AY622265	1	Clone 32-20 (95)	Unknown
S-F26	AY622268	1	Clone 3C0d-3 (93)	Clostridia
S-J147	AY622271	1	*Thermoleiphilum minutum*[d]	Actinobacteria

[a] Sediments were stored at 4°C for approximately 3 weeks prior to analysis.
[b] Frequency of a given RFLP type out of 100 total clones.
[c] Closest identified match in the GenBank database followed by percent similarity.
[d] The sequence of clone S-J147 did not appear to be chimeric; however, it did not significantly match anything in the GenBank database, cultured or uncultured.
Portions of the gene shared significant similarity with that of Thermoleiphilum minutum, but across the entire gene similarity was only 76%.

Diversity indices. Measures of diversity were calculated from the 16S rRNA gene clone library data (Table 2.6). By nearly all accounts, the library generated from sediment-extracted DNA suggested the lowest diversity. Richness, dominance, and the Shannon index were all lower than those calculated for the two hematite biofilm communities. Only the equitability of the sediment extract library was similar to one of the biofilm coupon libraries.

Table 2.6. Diversity of microbial communities in the FRC Background Area well FW303 and contaminated Area 3 well FW026 based on data from 16S rDNA clone libraries of sediments and exogenous hematite.

Library Source	Clones[a]	Coverage[b]	Richness[c]	Dominance	Shannon	Equitability
Background Area sediment	100	96%	8	0.42	1.1	0.53
Background Area hematite	85	81%	25	0.22	2.3	0.71
Area 3 hematite	100	92%	13	0.41	1.3	0.52

[a] The number of individual nonchimeric clones that were grouped by RFLP and ≥97% sequence similarity.
[b] The percentage of clones that were at least duplicated in the library (34).
[c] The number of distinct phylotypes in the library based on RFLP analysis.
[d] Shannon-Weaver diversity index (see "Materials and Methods").

The Background Area hematite biofilm coupon community displayed the greatest overall diversity. This community was marked by rather high richness, and an even distribution of phylotypes, indicated by the dominance index, Shannon index, and equitability. The hematite-associated community from the contaminated Area 3 well was far less diverse than that from the Background Area well, with only half of the number of unique clones and a more skewed distribution of individuals within a few dominant phylotypes. It should be noted that these indices do not take into account phylogenetic distances among community members. Thus, while the Background Area hematite-associated community appears to be the most diverse of those studied here, the observation that nearly all clones belonged to a limited number of lineages in the Proteobacteria is not taken into account.

Discussion

Collection of complex communities on the surfaces of exogenously introduced substrata offers a rapid and inexpensive approach to investigating microbial diversity in the subsurface. Biofilm coupons may be especially beneficial for studying polluted environments where contaminants can interfere with analyses and secondary wastes must be carefully considered. Comparison of the various communities formed on different substrata incubated concurrently in pristine groundwater revealed that care must be exercised when interpreting the results from such studies. The communities that formed on the different substrata were not only distinct from one another, they were also distinct from the groundwater and sediments from the same site (Figure 2.1); none of the communities associated with the surrogate media in the biofilm coupons was perfectly congruent with any of the groundwater or sediment communities. However, the communities that colonized coupon-associated media were more similar to groundwater samples than sediments. This result may not be surprising given the fact that the coupon-associated media were exposed to the local groundwater for only eight weeks, providing a relatively limited inoculum and a short period for successional changes to occur compared to the native sediments. More work is needed to determine the significance of coupon incubation time on community diversity and structure associated with surrogate minerals.

The sediment and groundwater T-RFLPs were also highly distinct from one another, sharing fewer than 16% of their T-RFs in common. This calls into question the common practice of using either groundwater or sediments, but not both, to characterize

the microbial diversity of a subsurface site. More work is clearly needed to understand the distribution of populations and their activities between the attached and planktonic phases. Lehman et al. (32) reported that uncontaminated basalt core material recovered from the Snake River Plain Aquifer was nearly devoid of biomass (1-g samples), while the surrounding groundwater boasted nearly 10^5 cells per mL. Conversely, in a region of the same aquifer contaminated with various organic pollutants, the attached cells (aerobic heterotrophs) outnumbered those in the planktonic state. Large-pore-diameter dialysis chambers filled with either crushed basalt or water were used in a similar manner as the biofilm coupons in this study. The investigators found that the composition of the community on the crushed basalt was very similar to that in the water, but that the metabolic activities of the two were distinct (32).

In recent years, investigators have reported that some microorganisms preferentially attach to different solid substrata. Caccavo et al. (4) demonstrated that the Fe(III)-reducing bacterium *Shewanella alga* attached more rapidly and to a higher cell density to goethite than to either ferrihydrite (HFO) or hematite. Lower and colleagues (33) used atomic force microscopy with living cells of *Shewanella oneidensis* MR-1 to demonstrate its preference for goethite over diaspore (an Al-bearing analog of goethite) under anaerobic conditions. Curiously, under aerobic conditions the cells were more attracted to the diaspore than to the goethite, indicating a change in adhesive/attractive forces between the cell and the mineral due to oxygen tension and a likely change in surface protein expression. The T-RFLPs from the two glass wool media in our study were most similar to that from the quartz sand medium, suggesting that similar physico-

chemical properties (*e.g.*, SiO_2 content) may play a role in selection of the attached communities. By the same token, we cannot be sure that the hematite medium used to compare microbial communities in the pristine and contaminated regions of the FRC aquifer did not select for a disproportionate density of Fe(III)-reducing *Rhodoferax ferrireducens*-like organisms (Table 2.4). Mineral phase surface properties clearly influence microbial colonization and activities (40). Use of biofilm coupons containing relevant geological media offers an excellent experimental framework for such studies in the field.

In the present study, DNA extracted directly from sediments of an acidic U-contaminated aquifer was not readily PCR amplifiable, possibly due to co-extraction of PCR inhibitors (26, 47, 54), or low biomass in the sample. However, when sterile particles of hematite were incubated in the well for eight weeks and analyzed without direct DNA extraction (IB-PCR), considerable diversity was uncovered that would have otherwise remained cryptic. Thus, whereas a bead-beating method failed to provide a means for comparing microbial community diversity at these particular subsurface sites, the biofilm coupon combined with IB-PCR enabled such an analysis.

The structure and diversity of a microbial community adapted to a particular environment should reflect conditions of the surrounding milieu. This concept appears to hold across a variety of different microbial habitats, including those contaminated by anthropogenic activities (3, 27, 35, 37, 41). For instance, acid mine drainage harbors acid-tolerant and acidophilic *Bacteria* and *Archaea* (1, 11), and metal-tolerant microbes are readily isolated from metal-contaminated media (38, 39). Many of the hematite-

associated *Bacteria* detected in the Background Area were common soil and water microorganisms. Most were affiliated with aerobic *Bacteria*, consistent with the redox status of the aquifer. By contrast, the structure of the microbial community from Area 3 may be the result of strong selective pressures exerted by the contaminants. The three predominant populations in the hematite-associated community in Area 3 appear to be adapted to the high-metals and low-pH conditions of this region of the aquifer. Nearly 60% of the clones were from an organism most similar to the genus *Alcaligenes*, which contains metal-tolerant representatives (28). Another 24% of the clones were affiliated with the genus *Frateuria*, a group of strictly aerobic acidophiles (51). The presence of a phylotype with 99% sequence similarity to *Methylobacterium radiotolerans* (4% of the library) is consistent with the high radionuclide content based on the reported radiotolerance of *M. radiotolerans* and other *Methylobacterium* spp. (25). None of these was found in the Background Area libraries. The remaining 13% of the clones, like those from the Background Area, were affiliated with common environmental taxa.

Although β- and γ-Proteobacteria dominated all three libraries, most of the specific phylotypes identified were unique to a single library. There were, however, some phylotypes that appear to have been present in more than one library. Dominant sediment extract clones S-A1 and S-H52 (Table 2.2), highly related to *Pseudomonas* spp., both shared 97% sequence similarity with *Pseudomonas* sp. clone B-B3 (Table 2.4) from the hematite-associated community in the Background Area. Two clones most similar to *Herbaspirillum seropedicae*, B-AA37 from the Background Area (Table 2.4) and C-CY80 from Area 3 (Table 2.3), were 97% similar to one another and 97% similar to clone

S-E105 from the sediment extract library (Table 2.5). Three β-Proteobacterial clones related to the genera *Duganella* and *Oxalobacter*, S-G30 from the sediment extract (Table 2.5), B BH93 from the hematite-associated community in the Background Area (Table 2.4), and C-CZ82 from Area 3 (Table 2.3), were also 97% similar to one another.

The use of IB-PCR to amplify genes of interest from surface-associated populations eliminates many of the biases inherent in DNA extraction (14, 36). However, it likely favors those populations that lyse readily at 99°C. Notably, no Gram-positive organisms, with the exception of the three clones from the community in Area 3 associated with the Actinobacteria (Table 2.3), were identified in IB-PCR-generated libraries. The dominating presence of Proteobacteria in the IB-PCR-generated libraries may be a result of a more readily lysed cell wall among organisms in this division. However, it is worth considering that half of the phylotypes identified by bead-beating extraction of sediments in the Background Area (comprising 94% of the individual clones examined; Table 2.5) were also Proteobacteria, suggesting that the Proteobacterial dominance seen in the hematite-associated community by IB-PCR may not be an artifact of the method, but may in fact reflect actual community composition.

Peacock and colleagues (43) recently used a similar biofilm coupon apparatus loaded with glass wool or powder activated carbon beads to sample the microbial communities stimulated by electron donor amendment at the FRC. These investigators found that, while the type of solid substratum influenced the overall biomass and community composition, the communities were generally predominated by members of

the Proteobacteria. Similar to our study, ribosomal RNA genes were discovered that were affiliated with the genera *Alcaligenes* and *Frateuria*.

Fields et al. (12) reported considerably greater diversity in nearby groundwater at the FRC, possibly because they examined a larger number of clones than were examined in this study. These investigators identified nearly 80 distinct populations in the Background Area groundwater, and between 19 and 34 populations in groundwater from three different wells in Areas 1 and 3 (contaminated area). While diversity and equitability were greater for all samples relative to ours, the authors did observe diminished diversity in the contaminated wells relative to the Background Area, as we also reported here. Again, Proteobacteria were the numerically dominant clones at both sites. Distinct from our study and that of Peacock et al. (43) described above, however, the most abundant populations in the contaminated groundwater were *Azoarcus* and *Pseudomonas* spp. Most notably, Fields did not detect *Alcaligenes* or *Frateuria* spp. in the contaminated groundwater.

Petrie and co-workers (45) found that Fe(III)-reducing communities were also quite distinct at both sites in the FRC, and that pH and nitrate played a constraining role. Iron-reducing enrichment cultures from the Background Area were almost exclusively affiliated with the *Geobacteraceae*, while those from the contaminated sediments were predominated by a population most similar to *Anaeromyxobacter dehalogenans* (45), neither of which was observed in our studies of surface-associated communities.

Several studies from subsurface (23, 31, 32, 44), freshwater (8, 48), and marine environments (8, 9, 53) have indicated differences in microbial activity and diversity

between attached and planktonic communities, and some investigators have concluded that the majority of biomass and activity is dominated by attached microorganisms (2, 15, 19, 22, 44, 52). The limitations of enrichment and isolation methods for uncovering true microbial diversity in these habitats are well known. While the method described here using coupon-associated media incubated in a well certainly has its own limitations, it provides a new approach to improve our understanding of microbial diversity in subsurface environments.

Ecological theory holds that strong selective pressures decrease microbial diversity. We hypothesized that the high metals content and low pH of the Area 3 aquifer would exert pressure on the microbial community, selecting populations that can tolerate or even take advantage of the contaminants. Based on our analysis of the microbial communities formed on hematite particles in Area 3, diversity of the indigenous community was indeed considerably lower than that in the Background Area. Furthermore, the predominant populations in the contaminated geologic media were closely related to metal-tolerant (*Alcaligenes* sp.), acidophilic (*Frateuria* sp.), or radiation-resistant (*Methylobacterium radiotolerans*) microorganisms, consistent with the prevailing conditions. Informed management and remediation efforts should take into account the influence of the environmental conditions on the extant microbial community, and attempt to either capitalize on or mitigate the strong selective pressures exerted by the contaminants.

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CHAPTER 3

HYDROGENASE ACTIVITY AND GENE EXPRESSION BY MINERAL SURFACE-ASSOCIATED CELLS OF THE SULFATE-REDUCING BACTERIUM *DESULFOVIBRIO DESULFURICANS* ESSEX 6

Abstract

The activities of sulfate-reducing bacteria (SRB) have been evaluated primarily using suspended populations cultivated in aqueous media initially free of solid phase minerals. A recent study showed that the Fe-sulfide mineral pyrrhotite formed on hematite (α-Fe_2O_3) surfaces colonized by SRB, but not in the presence of SRB suspended in aqueous medium containing soluble Fe (38). In this study, cells of the sulfate-reducing bacterium *Desulfovibrio desulfuricans* Essex 6 using sulfate as a terminal electron acceptor for anaerobic respiration displayed higher hydrogenase enzyme activity when growing on a hematite surface than when suspended in aqueous medium. When grown fermentatively on pyruvate, hydrogenase activity of hematite-associated cells was not significantly different from that of the suspended cell population in the same culture. Reverse transcription polymerase chain reaction (RT-PCR) of RNA extracts indicated that both [NiFe] and [Fe] hydrogenase genes were expressed by sulfate-respiring hematite-associated and suspended cell populations. *In situ* RT-PCR demonstrated that virtually all cells associated with the hematite surface in sulfate-respiring cultures expressed the gene encoding the [NiFe] hydrogenase. The formation of a secondary

mineral phase such as pyrrhotite on hematite surfaces colonized by sulfate-respiring microbial populations may be due to pH and pE conditions established by hydrogenase and other enzyme activities at the bacterial cell-mineral interface.

Introduction

In water-saturated subsurface environments, microorganisms associated with surfaces far outnumber those suspended in the aqueous phase (20, 22, 25, 40). These particle-associated microbial populations often exhibit higher metabolic activities than the surrounding suspended populations (13, 28, 31, 48). Furthermore, microbial populations growing on mineral surfaces appear to promote formation of secondary mineral phases that are different from those formed in the presence of the same population growing as a suspended culture. Neal et al. (38) demonstrated that the sulfate-reducing bacterium *Desulfovibrio desulfuricans* G20, when growing as a biofilm on the surface of the Fe(III)-oxide mineral hematite promoted formation and precipitation of pyrrhotite ($Fe_{1-X}S$). In contrast, mackinawite (FeS_{1+X}) and/or greigite (Fe_3S_4) are formed by this bacterium when cultivated as a suspended population in aqueous medium containing soluble ferrous iron (5, 26, 45).

In an effort to understand the biochemical basis for the formation of pyrrhotite by SRB associated with hematite surfaces, an investigation of hydrogenase gene expression and enzyme activity was undertaken. Hydrogenase enzymes have the potential to influence redox conditions at or near the cell surface, and thus could affect geochemical

reactions at the bacterial cell-mineral interface. Hydrogenase enzymes catalyze the reversible reaction

$$H_2 \leftrightarrow 2H^+ + 2e^- \quad (6)$$

in the cytoplasm and cell envelope of many microorganisms, and are important mediators of electron and energy flow in sulfate-reducing bacteria (1, 17, 51). Hydrogenase allows sulfate-reducing bacteria to generate reducing equivalents from molecular hydrogen or remove protons from the cell through the formation of molecular hydrogen.

Sulfate-reducing bacteria express three different forms of hydrogenase based on the type of metal(s) at the active site: [Fe], [NiFe], and [NiFeSe] hydrogenases. The [NiFe] hydrogenase is the only hydrogenase expressed in all known SRB (53). A number of studies have measured hydrogenase activity in suspended batch cultures containing soluble iron with sulfate serving as the terminal electron acceptor for respiration (8, 12, 23, 39, 41, 50). Hatchikian et al. (23) showed, however, that hydrogenase activity of suspended cell cultures of *Desulfovibrio vulgaris* strain Groningen varied depending upon the type of electron donor and acceptor used to promote growth. A dependence of hydrogenase activity on the terminal electron acceptor for respiration has also been reported for suspended cell cultures of bacteria that do not respire sulfate such as *Escherichia coli* and *Alcaligenes eutrophus* (30, 44, 47). In spite of the fact that SRB commonly associate with solid phases in nature (32, 42, 55) few studies have evaluated hydrogenase activity in cells growing on redox-active mineral surfaces using different energy-yielding metabolic pathways.

The objective of this study was to evaluate hydrogenase gene expression and enzyme activityby a population of cells of *Desulfovibrio desulfuricans* Essex 6 growing as a suspended batch culture or on the surface of the Fe-oxide mineral hematite using fermentative or anaerobic respiratory pathways. The results offer insight to the dependence of geochemical reactions on the mode of cell growth.

Materials and Methods

Growth of organism

Desulfovibrio desulfuricans strain Essex 6 (NCIB 8307, ATCC 29577) was cultured in modified Postgate Medium C (9, 43) with different electron acceptors supporting sulfate reduction or pyruvate fermentation. Sulfate medium contained the following components (per liter distilled water): 0.06 g $CaCl_2 \cdot 2H_2O$, 0.5 g KH_2PO_4, 1.0 g NH_4Cl, 4.5 g $NaSO_4$, 2.0 g $MgSO_4 \cdot H_2O$, and 5.6 g Na-lactate (60% syrup) and pyruvate medium contained 0.06 g $CaCl_2 \cdot 2H_2O$, 0.5 g KH_2PO_4, 1.0 g NH_4Cl, 1.6 g $MgCl_2 \cdot 6H_2O$, and 3.5 g Na-pyruvate. Both media were adjusted to pH 7.2, purged of O_2 by flushing with N_2 gas and maintained under a N_2 headspace in a glass serum vial (160 mL) sealed with butyl rubber stoppers. Media was autoclaved followed with amendments with anaerobic, filtered (0.2 μm) sterilized vitamin solution (final concentration 0.02 mg L^{-1} of biotin and folic acid, 0.1 mg L^{-1} riboflavin, *p*-aminobenzoic acid, pantothenic acid, and cyanocobalamin, 0.2 mg L^{-1} of thiamine and niacin, and 0.5 mg/L of pyridoxine) (49) and an amino acids solution (final concentrations of 0.02 g L^{-1} of L-arginine, L-serine, and L-glutamic acid). Crushed, jewelry-grade hematite (α-

Fe_2O_3) was combusted at 700°C for 1 hr followed by 4 hrs at 587°C. Particles were added to 10-mL serum vials, sealed with butyl rubber stoppers, purged with N_2 and autoclave sterilized. Five-mL of sterile, anaerobic medium was aseptically and anaerobically transferred to 10 mL serum vials containing particles and sealed with butyl rubber stoppers, purged with N_2 and autoclaved.

Enzyme Assay

To determine hydrogenase activity hematite-associated cells were measured spectrophotometrically using the methyl viologen hydrogen consumption assay (10, 57). One mL of 20 mM Tris buffer (pH 7.8) containing 50 µM methyl viologen (Sigma-Aldrich) and 5 mM glucose was purged with hydrogen for 20 min in a capped cuvette. Sodium dithionite (100 µM, final conc.) was added as a reductant and the solution was purged continuously for 10 min. Glucose oxidase (1 U) was then added to consume any residual oxygen and cuvettes were sealed and incubated at 30°C for 10 min. Suspended cells were harvested in mid-logarithmic phase of growth and 200 µL of cell culture was transferred to the assay cuvette containing 2 mL of reaction mixture. Hematite particles retrieved from the same culture as the suspended populations were aseptically rinsed in sterile, anaerobic culture medium and immediately added to the cuvette containing the assay mixture. Cuvettes were again purged with hydrogen during the addition of suspended cells or colonized hematite. Negative controls were carried out with sterile hematite particles or sterile culture medium. Methyl viologen oxidation was monitored spectrophotometrically at 604 nm (ε_{604} =13.9 mM cm^{-1}) in which 1 U of hydrogenase activity resulted in the consumption of 1 µmol of methyl-viologen per min (11, 57).

Hydrogenase inhibition assays were carried out on suspended populations cultured in the absence of hematite. Anaerobic cultures were supplemented with a final concentration of 1mM $CuCl_2$ (pH 7.0) 15 min prior to measuring hydrogenase activity.

Hydrogenase activity was normalized to total cellular DNA. DNA content of the cells assayed for hydrogenase activity was determined by the PicoGreen dsDNA Quantification Assay (Molecular Probes, Inc.). Briefly, hydrogenase assay mixture was decanted from the cuvette and hematite particles were transferred to PCR tubes containing 50 µL of nuclease-free water. Cells were heat-lysed by incubation at 99°C for 15 min in a thermocycler. Volumes of 30, 15, and 5 µL of cell lysate were transferred to new tubes and nuclease-free water was added to a final volume of 30 µL. PicoGreen dsDNA Quantitation Reagent was added at 1X final concentration and the mixture transferred to 5 x 31-mm glass cuvettes (Wheaton) for analysis.

Suspended cells (1 mL) were harvested from culture vials by centrifugation at 4°C and resuspended in 1 mL of nuclease-free H_2O. Cells were diluted 2-fold in nuclease-free water to a final volume of 50 µL and heat lysed at 99°C for 15 minutes. Cell lysates were then treated identical to lysates generated from hematite-associated cells. Fluorescence was measured on the TBS-380 Fluorometer (Turner BioSystems) using the minicell adaptor according to manufacturers protocol.

Statistical Analysis

The mean and standard deviation of hydrogenase activity were calculated from rates obtained from duplicate cultures harvested at the same optical density. The coefficient of variation (CV) was calculated according to equation 3 (16).

The Student T test was used to determine the difference between the means and analyses were set at P values <0.05.

$$CV = [(\text{standard deviation})/(\text{mean})] \times 100 \quad (7)$$

PCR Amplification and Gene Sequencing

DNA was extracted from cultures using the phenol-chloroform extraction method (15). The [NiFe] hydrogenase gene was amplified from DNA extracts using the primers Hyd1F (5'-CGCGACGCCCAGCACTTCACCCAG-3') and Hyd7R (5'-CGCAGGCGATGCA(G/C)GGGTC-3') (54) specific for a 1500-bp region. A smaller fragment (440-bp) of the [NiFe] enzyme was generated with the primers Hyd1F and Hyd5R (5'-GCAGGGCTTCCAGTAGTGGGCGGTGGCGA-3') (54). The [Fe] hydrogenase was amplified using Fe3F (5'-ACCTCGTGCTGCCCCGGC-3') (24) and Fe7R (5- CGCAGGCGATGCASGGGTC -3') which were specific for a 580-bp region of the gene encoding the large subunit of the enzyme. The primer set was designed complementary to aligned gene sequences of *D. vulgaris*, *D. fructosovorans,* and *D. desulfuricans* G20 to yield a fragment of similar length to that of the smaller amplicon generated for the [NiFe] hydrogenase gene (respective NCBI accession numbers M27212, Y11759, AF331719). PCR reactions were performed in 50 µL volume with the following components (final concentrations): 1X PCR buffer (Invitrogen), 2 mM $MgCl_2$ (Invitrogen), 200 µM of each deoxynucleotide triphosphate (Eppendorf), 0.4 µM of each primer (Integrated DNA Technologies), and 1.25 U Taq DNA Polymerase (Invitrogen). Amplification was performed with a Mastercycler Gradient thermocycler (Eppendorf) with an initial denaturation at 94°C (4 min) followed by 30 cycles of denaturation at 94°C

(1 min), annealing at 60°C (1 min), primer extension at 72°C (1.5 min), and a final elongation step at 72°C (7 min).

RNA extraction

To extract RNA from cells mid-log phase culture was treated with the RNeasy-Mini kit (Qiagen) in conjunction with QiaShredder (Qiagen). Hematite-associated cells were briefly rinsed with sterile anaerobic medium prior to cell lysis. RNA extracts were then treated with RNase-free DNase (0.1 U DNase per 1 μL RNA, Promega) for 30 min and the DNase was heat-inactivated according to the manufacturer's protocol. The quality of the extracts was verified using denaturing agarose gel electrophoresis (3).

Reverse transcription-PCR

To amplify the transcripts reverse transcription PCR (RT-PCR) amplification was performed as a coupled reaction (Access RT-PCR System, Promega) with 1X AMV/*Tfl* 5X reaction buffer, 0.2 mM dNTP mix, 1 μM Hyd1F or Fe3F forward primer and Hyd5R or Fe7R reverse primer, 1 mM $MgSO_4$, 5 U AMV reverse transcriptase, and 5 U *Tfl* DNA polymerase. Reverse transcription was performed at 45 °C (45 min.) and followed by reverse transcriptase inactivation step at 94°C (2 min) and PCR amplification continued with 40 cycles of denaturation at 94°C (1 min), primer annealing at 60°C (1 min), and primer extension at 68°C (2 min) followed by a final extension step at 68°C (7 min). RNA extracts were subjected to RT-PCR with the exclusion of the RT enzyme to confirm contamination DNA was not amplified.

In situ reverse transcription-PCR

Mid-log phase cells associated with hematite particles were fixed in 4% paraformaldehyde overnight, and treated as previously reported (33, 36). Briefly, fixed cells were washed three times with 1X nuclease-free phosphate buffered saline (PBS, pH 7.2) and permeablized in 500 μg mL^{-1} lysozyme at room temperature for 30 min. Following three additional PBS washes, cells associated with hematite particles were treated with 20 U of RQ1 RNase-free DNase (Promega) at a final volume of 200 μLand stored at -20°C in nuclease-free H_2O until further analysis.

Hematite-particle associated-cells were subjected to RT-PCR protocol similar to that for RNA extracted from suspended cells as described above with the fluorescent labeled primers (1 μM; IRIS-Fuchsia labeled dUTP;Cyanine Technologies). Fluorescent amplicons associated with cells and released into the PCR supernatant were evaluated using microscopy and agarose gel electrophoresis.

Sequence Analysis

IS RT-PCR supernatant PCR products without labeled dUTP were sequenced following QiaQuick PCR Purification (Qiagen). The [NiFe] hydrogenase PCR generated amplicon using the Hyd1F/Hyd7R primer set was sequenced using the flanking and internal primers (Hyd900R, 5'-GGGAGCCTTGAGCCAGGAATAA-3' and Hyd860F, 5'-GCGAAACCAAACCCTTCTTCACCA-3') designed from the DNA sequence to yield 2X coverage of the full fragment. PCR products generated from RNA template RNA template were sequenced with Hyd1F and Hyd5R or Fe3F and Fe7R. Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing Ready

Reaction Kit (Applied Biosystems) and an ABI 3700 automated DNA sequencer (Applied Biosystems). Sequences were aligned and edited with Bioedit sequence alignment editor freeware (version 7.0.1; www.mbio.ncsu.edu/BioEdit/bioedit.html). Nucleotide sequences were compared to nucleotide and amino acid sequences deposited in the NCBI database using the Blastn and Blastx tools (http://www.ncbi.nlm.nih.gov/blast/).

Microscopy

Hematite particle-associated cells subjected to IS RT-PCR were stained with 10 μg mL^{-1} 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma Chemical Co., St. Louis, MO) for 15 min and destained in fresh, nuclease-free H_2O for 10 min. Cells were viewed using an Olympus BX60 system microscope equipped with 100 W Hg-vapor discharge lamp and 40X and 100X oil immersion objectives. The MWU filter cube (Olympus America Inc.) was used to view DAPI-stained cells and MWIG filter cube (Olympus America Inc.) for IS RT-PCR-labeled cells. Images were captured using ImagePoint monochrome, Peltier cooled (+10°C) CCD camera (Photometrics Ltd., Tuscon, AZ) and Image-Pro Plus software (Media Cybernetics, Silver Springs, MD). Images of identical fields of DAPI-stained cells and IS RT-PCR-labeled cells were superimposed in ImageJ software (http://rsb.info.nih.gov/ij/index.html). The percentage of cells expressing the [NiFe] hydrogenase gene was evaluated by dividing the total number of cells by the number of cells expressing the gene and multiplying by 100."

Results

Enzyme Activity

Hematite-associated cells cultured in sulfate-containing medium (where sulfate was the electron acceptor) displayed significantly greater (P = 0.03) enzyme activity (1.075 ± 0.200 mmol MV min^{-1} μg^{-1} DNA; 19 % CV) than suspended cells in the same culture (0.193 ± 0.035 mmol MV min^{-1} μg^{-1} DNA; 18 % CV; Figure 3.1). Hydrogenase activity (0.170 ± 0.058 mmol MV min^{-1} μg^{-1} DNA; 34 % CV) of attached populations of pyruvate-grown cells were not significantly different (P = 0.14) than suspended populations of the same culture (0.073 ± 0.004 mmol MV min^{-1} μg^{-1} DNA; 5 % CV). Both attached and suspended populations cultured with sulfate displayed greater hydrogenase activity than pyruvate-grown cells with 84% increase for attached populations (P = 0.03) and 62% increase for suspended populations (P = 0.04).

The whole-cell hydrogenase activity of suspended cultures was also evaluated in the presence of $CuCl_2$, which reportedly inhibits the activity of those enzymes located outside of the cytoplasm (19). All hydrogenase activity was inhibited in both sulfate and pyruvate-grown cultures under these conditions, suggesting that the enzyme(s) responsible for the hydrogenase activity resides outside the cytoplasm in the membrane or periplasm of these cells (Figure. 3.1).

The hydrogenase activity of cells grown in the absence of hematite under sulfate-reducing conditions (0.142 ± 0.019 mM MV min^{-1} $O.D.U.^{-1}$, 14% CV) was not significantly different (P = 0.054) from cells fermenting pyruvate in the absence of hematite (0.130 ± 0.012 mM MV min^{-1} $O.D.U.^{-1}$, 9% CV) (Figure 3.2). These data imply

that the presence of hematite likely affects the hydrogenase activity of the suspended cells since the activity of the suspended populations cultured in the two media in the presence of hematite were significantly different from one another.

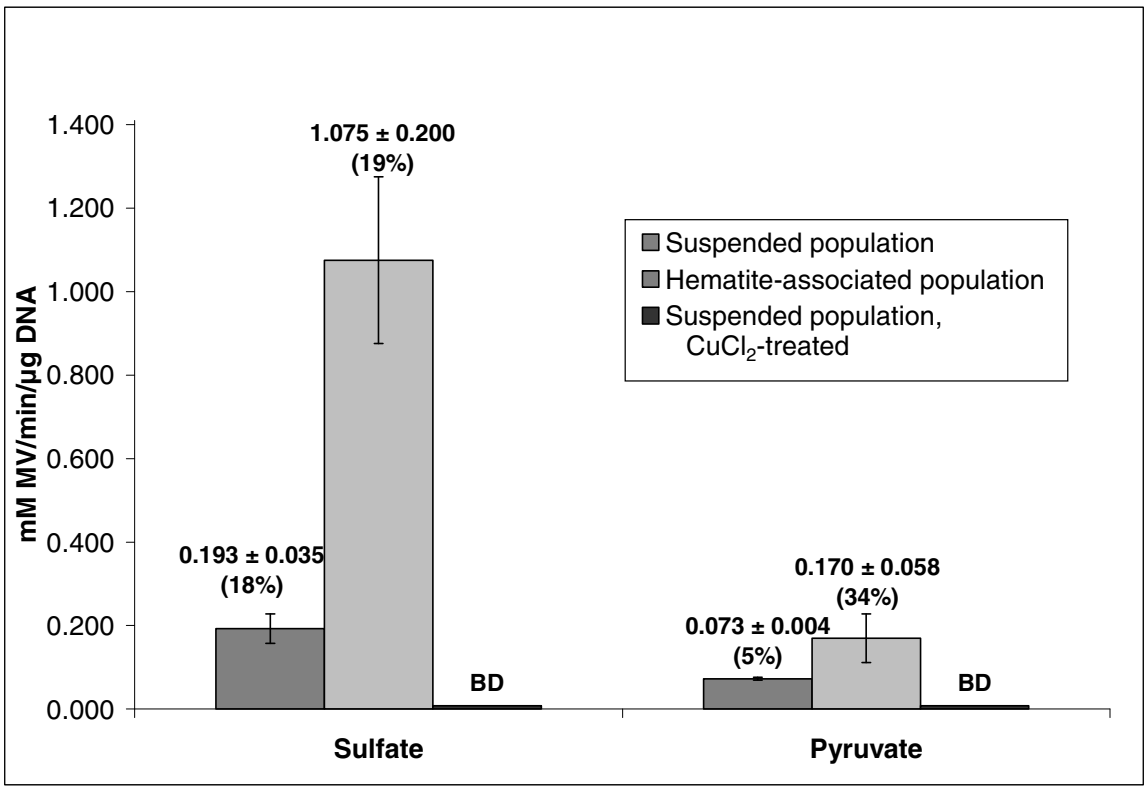

Figure 3.1. Hydrogenase activity of hematite-associated, suspended, and $CuCl_2$-incubated cells cultured with sulfate or pyruvate. Values are displayed as mean ± standard deviation, n = 2, (coefficient of variation). BD indicates measurements below detection.

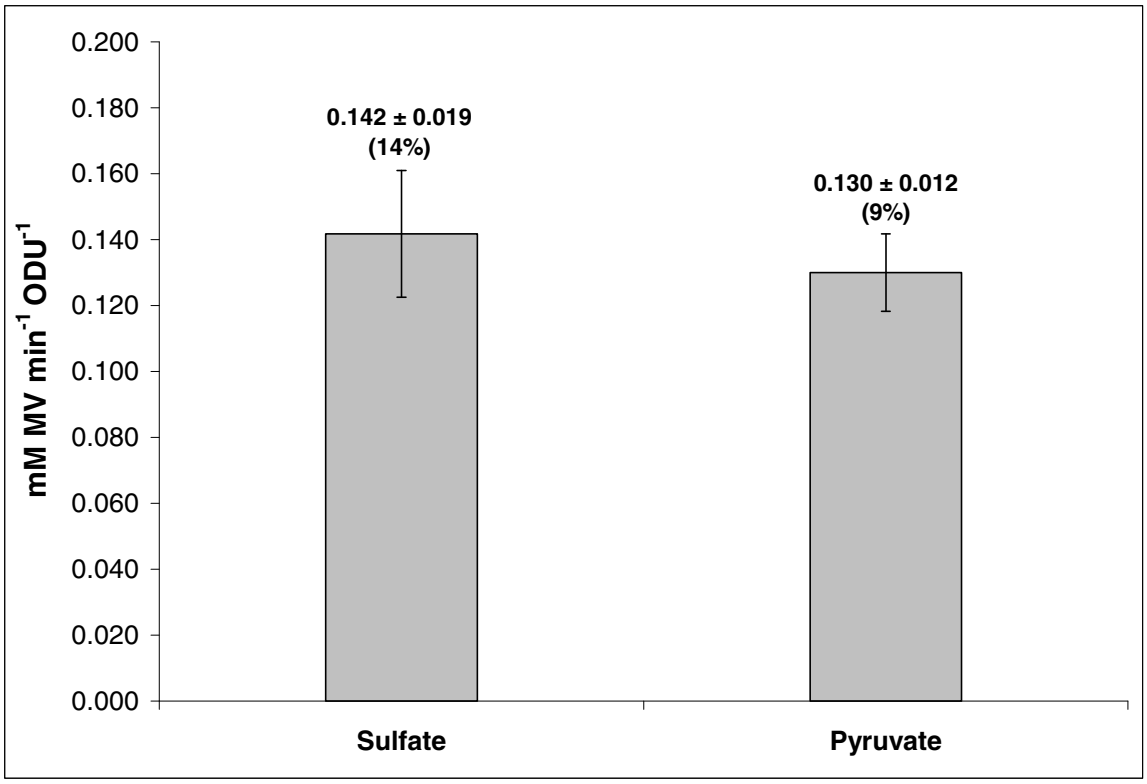

Figure 3.2. Hydrogenase activity of suspended cells cultured in the absence of hematite with sulfate or pyruvate. Values are displayed as mean ± standard deviation, n = 2, (coefficient of variation). Values are normalized to the optical density of the cultures (600 nm).

Attached cell gene expression

The portion of DAPI-stained, hematite-associated cells that expressed the gene encoding the [NiFe] hydrogenase gene was determined by a combination of IS RT-PCR and epifluorescent microscopy. Essentially all of the DAPI-stained cells expressed the [NiFe] hydrogenase (Figure 3.3, panel A). Agarose gel electrophoresis of IS RT-PCR reaction supernatant confirmed the expected size of the amplicon when compared to product generated from RNA extracts of attached cells (Figure 3.4).

The specificity of IS RT-PCR-labeling was determined by subjecting fixed, permeabilized, lysozyme and DNase-treated particle associated cells to IS RT-PCR in the absence of reverse transcriptase. Microscopic analysis of the DAPI-stained particles revealed that cells were attached to the surface, however no cells were visible by IS RT-PCR labeling thus indicating that DNA was completely digested and non-specific labeling did not occur (Figure 3.3, panel B). When the supernatant of the IS RT-PCR reactions was subjected to agarose gel electrophoresis, amplicons were detected indicating that PCR products had leaked out of the hematite-associated cells. To determine if the amplicons leaking into the reaction supernatant had adhered to nearby cells not expressing the [NiFe] hydrogenase gene, fixed, permeabilized, lysozyme and DNase-treated particle-associated cells were incubated with dUTP-labeled PCR product. Microscopic analysis of the DAPI-stained particles showed cells colonizing the surface, but none was labeled by the fluorescent dUTP. This result indicated that labeled DNA released from cells expressing the gene did not become associated with other cells on the surface (Figure 3.3, panel C). Thus, only cells actively expressing hydrogenase were labeled by IS RT-PCR.

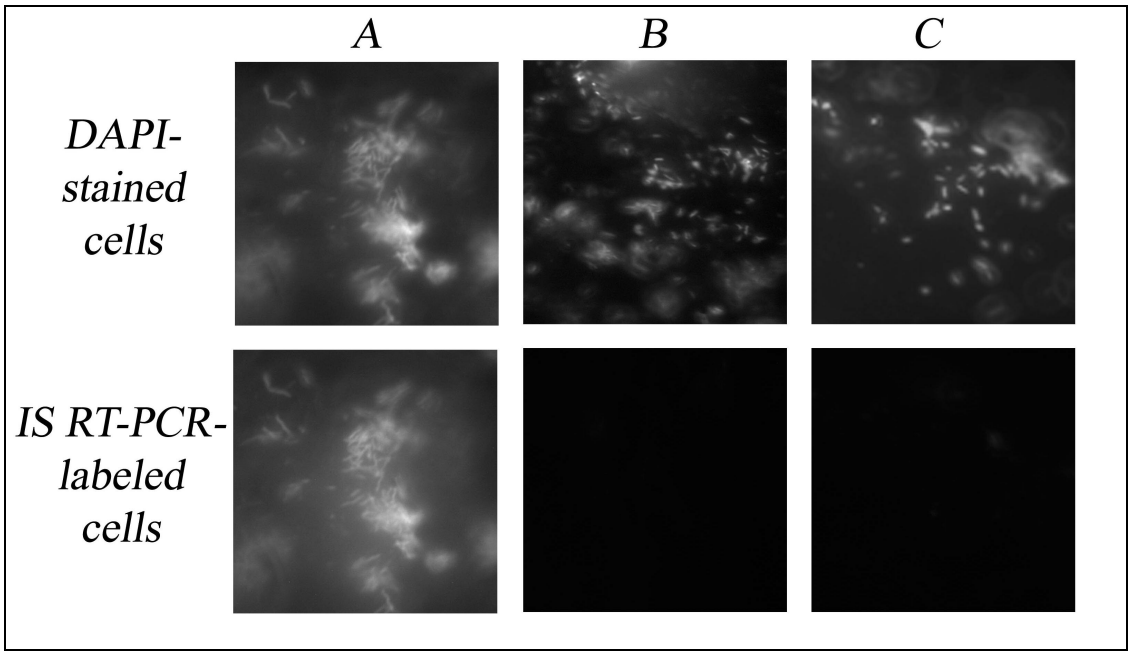

Figure 3.3. Microscopic analysis of [NiFe] hydrogenase gene expression of hematite-associated cells cultured with sulfate. A) IS RT-PCR; B) RT negative IS RT-PCR; C) Incubation with labeled amplicons.

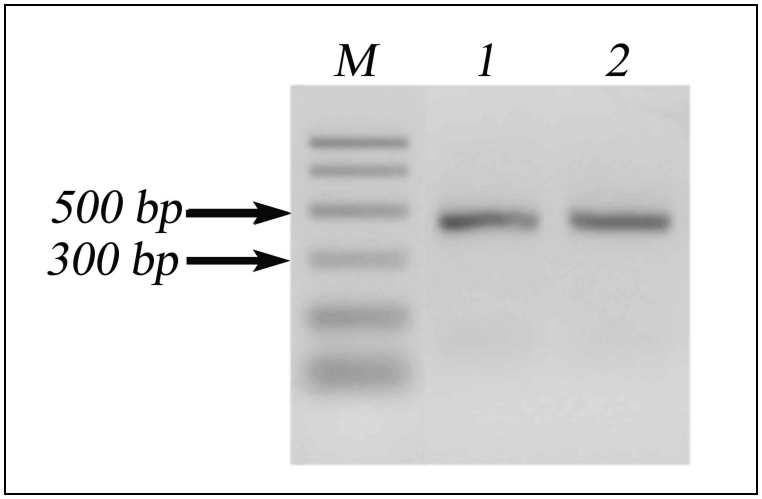

Figure 3.4. Analysis of [NiFe] hydrogenase expression in cells cultured with sulfate. Lane M, molecular marker (PCR marker, Promega); lane 1, RT-PCR of RNA extracted from attached populations; lane 2, IS RT-PCR of RNA from intact, attached populations.

Evaluation of expression of [Fe] hydrogenase gene

D. desulfuricans strain Essex 6 is known to express an [Fe] hydrogenase in addition to the [NiFe] hydrogenase. To determine whether the [Fe] hydrogenase contributed to the hydrogenase activity under the conditions of this study, RT-PCR was performed on RNA extracts from both surface-associated and suspended populations cultured under sulfate-reducing conditions. Products with the predicted size were resolved by agarose gel electrophoresis for both populations.

Comparison of gene sequences generated from nucleic acid extracts and intact cells

The [NiFe] hydrogenase transcript sequences of the RT-PCR and IS RT-PCR products generated from RNA extracts of suspended cultures and intact cells associated with particles, respectively, were aligned and compared to the closest nucleotide sequences deposited in the GenBank database. The [NiFe] sequence was most closely representative of the [NiFe] hydrogenase of *D. desulfuricans* DSM 1942 (96%), *D. desulfuricans* G20 (83%) and *D. vulgaris*Hildenborough (77%). The [Fe] hydrogenase sequence displayed similarity to the [Fe] hydrogenases of *D. vulgaris* Hildenborough (84%) and *D. fructosovorans* (93%).

Discussion

The majority of studies evaluating the effects of culture conditions on hydrogenase enzyme activity have employed suspended populations of SRB (8, 12, 23, 39, 41, 50). The results of the present study demonstrate how association with the surface of the mineral hematite enhances hydrogenase activity in *D. desulfuricans* Essex 6. This surface-enhancement of hydrogenase activity offers a possible explanation for the formation of pyrrhotite on hematite surfaces colonized by *D. desulfuricans* Essex 6 respiring sulfate, but not in cultures of this bacterium growing as a suspended population in an aqueous medium containing soluble ferrous iron (37). Under the latter conditions, the formation of the metastable Fe-sulfides mackinawite and gregeite are favored before stoichiometric conversion to pyrite (6).

The pE(Eh)-pS^{2-} relationship demonstrates the different conditions that favor the formation of pyrrhotite and pyrite (Figure 3.5). Pyrrhotite is favored under highly reducing conditions when S^{2-} concentration is relatively low (6). The hydrogenase activity associated with hematite-associated cells likely created a more reducing condition through the production of electrons at the hematite surface than could be achieved in the aqueous phase by hydrogenase activity of the suspended cell population. The electrons are available to reduce sulfite to sulfide in the dissimilatory sulfate reduction pathway (34). Although not evaluated in the present study, the production of sulfide via sulfate respiration likely promotes reducing conditions immediately surrounding the bacterial cells at the hematite surface (38). The proximity of the

bacterially-produced sulfide to Fe at the hematite surface likely favors the rapid formation of insoluble FeS species, resulting in a lower soluble sulfide concentration at the bacterial cell-mineral surface than that achieved in the bulk aqueous phase of a suspension of sulfide-producing cells, a condition that favors pyrrhotite formation over mackinawite and gregeite.

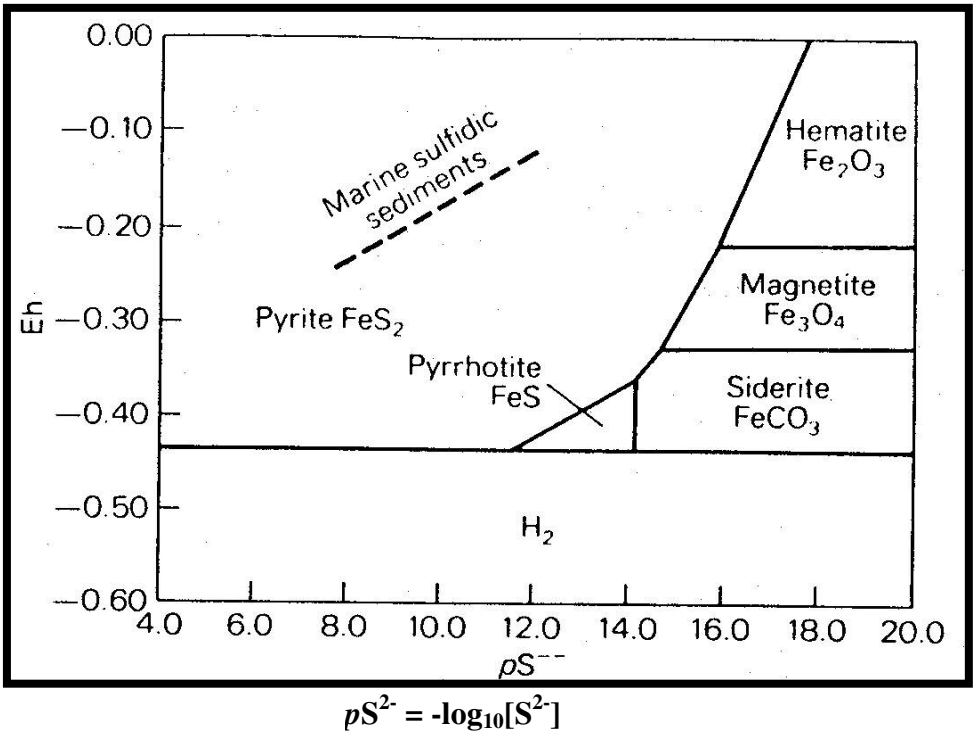

Figure 3.5. Eh-ρS^{2-} diagram for pyrite, pyrrhotite, hematite, magnetite, and siderite (pH 7.37, log P_{CO2} = *-2.40,* T = 25 °C, P = 1 atm). The Eh value for SRB growth is marked on the Eh axis. (Adapted from Berner, 1971 (6))

The hydrogenase-catalyzed conversion of H_2 to protons and electrons also reduces the pH of the environment surrounding the cells. Stoichiometric pyrrhotite is slightly more stable than mackinawite and gregeite in weakly acidic conditions (2). Thus, the

greater hydrogenase activity by cells associated with the hematite surface likely favors the formation of pyrrhotite over gregeite or mackinawite.

The difference in hydrogenase activity displayed by sulfate-respiring cells in cultures containing hematite and pyruvate fermenting cells in cultures without hematite may have been related to the amount of Fe in the cultures. Ferrous iron at concentrations of 0.1 g L^{-1} have been shown to inhibit hydrogenase activity (7). Ferric ions released from hematite into solution and reduced to ferrous ions during pyruvate fermentation may have accumulated to concentrations that inhibited hydrogenase activity, whereas, soluble ferrous iron derived from the hematite was likely scavenged from solution and precipitated by the sulfide produced during sulfate reduction. This explanation is supported by the results that both cultures displayed similar rates of hydrogenase activity reducing sulfate or fermenting pyruvate in the absence of hematite indicating that ferrous iron likely imposed an inhibitory effect on hydrogenase activity in the pyruvate-grown culture. Since hydrogenase activity of pyruvate-fermenting cells of *D. desulfuricans* Essex 6 was similar irrespective of whether cells were suspended in aqueous medium or associated with hematite surfaces, the activity differences observed during pyruvate fermentation does not appear to be a "surface effect", *per se*. Thus, the lower hydrogenase activity in hematite-containing, pyruvate-fermenting cultures compared to hematite-containing, sulfate-reducing cultures was likely the result of ferrous iron inhibition in the former.

D. desulfuricans Essex 6 expressed two hydrogenases which were previously reported to be located intracellularly (18); however the ability to measure enzyme activity

in whole-cell assays contradicts a cytoplasmic location. Methyl viologen is thought to be impermeable to the cytoplasmic membrane (29), thereby restricting measurements to enzymes with periplasmic and extracellular locations. Moreover, $CuCl_2$, an inhibitory compound to hydrogenase, is also thought to be unable to passively cross the cytoplasmic membrane, therefore limited to inhibiting enzymes located outside of the cytoplasm (14, 18, 21, 46). Inhibition by 1 mM $CuCl_2$ resulted in loss of 100% of the detectable hydrogenase activity, suggesting that all activity measured under our assay conditions was generated from enzymes located outside of the cytoplasm. The previous study which determined intracellular localization of hydrogenase in Essex 6 used 0.25 mM $CuCl_2$ concentration in which the activity was not inhibited (18). Other localization studies of whole cells have utilized inhibitory concentrations ranging from 0.75 µM $CuCl_2$ for *Dehalococcoides* sp. (21) up to 1 mM $CuCl_2$ for *Desulfotomaculum orientis* (14). Our results are consistent with other studies which have determined a periplasmic location for both the [NiFe] and [Fe] hydrogenase in other investigated *Desulfovibrio* strains (reviewed in (51, 52)).

Gene expression of microorganisms is typically reported at the population level (34, 35, 56); however, cell-to-cell variations in activity have been observed in populations growing on surfaces but not in suspended cells. Baty et al. (4) demonstrated location-dependent differences in the level of expression of genes encoding chitinase by cells of a marine bacterium growing on a chitin surface (4). This cell-to-cell variation in the level of expression of genes often translates to differences in gene product activity (4). Since many microbially-mediated transformations of minerals appear to be initiated

at the single-cell or microcolony level (15), it is important to be able to detect and measure specific, surface-associated microbial activities at this resolution. While few enzyme assays are capable of resolving specific activities of single cells on surfaces, expression of specific genes by individual cells associated with surfaces has been demonstrated (33, 36).

IS RT-PCR has the ability to resolve individual cells within a population that are expressing specific genes. When the cells are attached to a surface, the technique can also relate gene expression to gene product activity if the activity leads to a detectable chemical or physical change in the surface (33, 36). Neal et al. (37) and Magnuson et al. (33) were the first to demonstrate the application of IS RT-PCR to bacteria growing on insoluble Fe(III)-oxide surfaces. These studies related expression of a gene likely involved in iron reduction to the dissolution of the Fe-oxide surface. In this study, we exploited the ability of IS RT-PCR to probe microbial populations associated with a surface for expression of specific genes at the individual cell level in order to determine what portion of the hematite-associated population expressed the [NiFe] hydrogenase gene. That virtually all of the DAPI-stained cells co-fluoresced with IRIS-Fuchsia is consistent with earlier reports that hydrogenase is expressed by SRB over a wide range of cultivation conditions, and that a condition has yet to be identified where this gene is not expressed in batch cultures (50). While the method of direct incorporation of fluorescent nucleotides by amplicions has been reported to result in higher levels of non-specific fluorescence compared to other IS RT-PCR methods (27), there was no evidence of non-

specific labeling of amplicons or other cellular molecules or background fluorescence on the hematite surface in the studies presented here.

In summary, this study demonstrated that cells of the SRB *D. desulfuricans* Essex 6 exhibit significantly greater hydrogenase enzyme activity when respiring sulfate on a hematite surface than when suspended in the aqueous phase. The hydrogenase activity associated with the sulfate-reducing, hematite-associated population likely created conditions at the mineral surface different from those in the surrounding bulk aqueous phase. The conditions at the mineral surface likely favor the formation of pyrrhotite over mackinawite and greigite as a secondary mineral phase associated with the hematite as has been previously observed (33). Formation of the latter two iron sulfide minerals are reportedly favored in Fe oxide-free cultures containing soluble iron and suspended SRB cell populations. Hematite-associated populations exhibited significantly greater hydrogenase activity when respiring sulfate with lactate as electron donor than when growing fermentatively on pyruvate. The difference was attributed to an inhibitory effect of ferrous ions derived from the ferric iron of hematite in the pyruvate-fermenting culture. The results emphasize the importance of evaluating geo-microbial activities using microbial populations directly associated with mineral surfaces to better understand microbially-mediated geochemical transformations in subsurface environments.

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CHAPTER 4

SUMMARY

This dissertation demonstrated the importance of investigating both the surface-associated and suspended microbial populations in order to gain a more representative view of the true microbiology of the subsurface. The goal of this thesis was to compare suspended populations to the surface-associated populations based on diversity (indigenous subsurface communities recovered from groundwater, sediment and surrogate substrata incubated in the groundwater) and activity (enzyme activity of a hematatite-associated sulfate-reducing bacterium). The goal was addressed in two separate chapters addressing the diversity of a contaminated and pristine region of an aquifer (Chapter 2) and the hydrogenase enzyme activity and gene expression of *D. desulfuricans* Essex 6 (Chapter 3). This chapter summarizes the key results of each study and the future directions of this research.

The microbial community of the suspended population recovered from the groundwater of a pristine region of the aquifer underlying the Oak Ridge Reservation were compared to the attached microbial communities recovered from the sediment and those recovered from different substrata incubated down-well (hematite, illite, saprolite, glass wool) based on 16S rDNA analysis. All of the communities recovered from the different media including the sediment and groundwater were distinct from one another. The communities recovered from each of the surrogate substrata were more representative of the groundwater community than the sediment-associated communities.

Of communities which developed on the surrogate substrata, the community recovered using hematite was most closely representative of the communities recovered from the sediment. The microbial community recovered using hematite particles incubated in the contaminated region of the aquifer was reflective of the highly acidic, metal-, and radionuclide-contaminated conditions. The dominant populations were most closely related to acidophilic (*Frateuria* spp.), metal-tolerant (*Alcaligenes* spp.) and radiotolerant microorganisms (*Methylobacterium radiotolerans).* This chapter was the first demonstration of the use of minerals as surrogate substrata to recover subsurface microbial populations and the application of gradient intact biofilm PCR for analysis of surface-associated cells.

The goal of Chapter 3 was to try to determine a the biochemical basis for the formation of a secondary mineral formed in the presence of *Desulfovibrio* spp. growing on an Fe-oxide surface but not in the presence of suspended populations. Hydrogenase is an enzyme likely to influence the geochemistry immediately surrounding the cell by changing the redox conditions and pH by the production and/or consumption of protons and electrons. The hydrogenase activity of *D. desulfuricans* Essex 6 when reducing sulfate was greater in cells growing on a hematite surface as compared to suspended cells of the same culture. Interestingly, surface-associated growth of pyruvate-fermenting cells did not enhance the hydrogenase activity in comparison to the suspended populations. This phenomenon was attributed to inhibition by ferrous iron released from the hematite surface. *In situ* RT-PCR analysis of the surface-associated cells cultured with sulfate as the terminal electron acceptor determined that all cells were expressing the [NiFe]

hydrogenase gene. The [NiFe] hydrogenase and [Fe] hydrogenase were expressed in both the suspended and attached populations of sulfate-reducing cultures as analyzed by RT-PCR of RNA extracts. This chapter is the first demonstration that hydrogenase activity is enhanced in mineral-associated populations of *Desulfovibrio desulfuricans* cultured under sulfate-reducing conditions thus indicating a plausible explanation for the formation of a different mineral phase in attached population compared to suspended cells.

Specifically, the results presented in this dissertation demonstrated that the microbial diversity and activity of surface-associated populations is distinct from the suspended populations. These results indicate that caution must be taken when interpreting results obtained from only one of the populations. Geo-microbial processes are controlled by the conditions immediately surrounding the cell (e.g. pH and pE) thus changes in enzyme activity (such as hydrogenase) as a result of surface-associated growth likely influence the geochemical reactions in the subsurface. Investigations into geo-microbial processes should include the use of attached populations since the activity of the suspended cells may not be representative of the activity expressed in attached populations. In order to gain a robust understanding of subsurface microbial ecology and geo-microbial processes both the suspended and attached populations should be analyzed since each may be contributing to the overall diversity and activity.

Future Work

Future work in the area of this dissertation includes a broader analysis of the effects of surface-associated growth on hydrogenase activity and functional gene analysis of the communities recovered from the subsurface. Chapter 2 addressed the effects of the surface properties on the diversity of the developing biofilms by comparing the communities recovered on different mineral substrata. Chapter 3, however, only investigated the effect of surface-associated growth on enzyme activity using a redox-active mineral. It is currently unknown how the surface properties of the mineral substrata affects enzyme activity and whether different minerals can enhance the rates of activity. In order to better determine the effect of surface properties on activity, investigations of enzyme activity should be carried out on populations attached to different substrata including glass beads and other Fe(III) oxide surfaces.

The bulk aqueous conditions are not necessarily representative of the conditions experienced by cells attached to surfaces. The formation of a microniche at a particle surface can dictate the microbial physiologies expressed under conditions immediately surrounding the cell. Phylogenetic and functional gene analyses of the bulk sediment or groundwater populations can dilute and obscure microniche communities therefore our analysis of these communities needs to target the microenvironments of these organisms. Hematite particles incubated in the groundwater harbored enough biomass for PCR analysis of the 16S rDNA, thus the next step for analyzing the diversity and activity of subsurface communities is to probe the functional genes and/or functional gene expression (such as cytochromes, dissimilatory sulfite reductase, nitrite reductase and

hydrogenase) of the attached populations in order to better understand the microbial ecology of a site.

The molecular techniques applied here have been modified to analyze surface-associated populations (microbial diversity [GIB-PCR], enzyme activity [hydrogenase assay], and gene expression [IS RT-PCR]). These methods are widely applicable in other fields including the biocorrosion of steel, medical device and implant colonization, and waste-water treatment facilities. Biofilm coupons can be used to sample populations in very low biomass environments such as Antarctic effluent channels and pristine springs. The ability to side-step DNA purification techniques using intact biofilm PCR simplifies the analysis of samples which require extensive purification or sediment washing techniques. The analysis of gene expression of surface-associated cells can be applied to steel coupons for monitoring enzymes like hydrogenase which are involved in biocorrosion or colonized medical devices to probe for antibiotic resistance genes. The hydrogenase assay of surface-associated cells can be applied to bio-corroding surfaces or even extended to analyze the activity of other enzymes that utilize methyl viologen as an artificial electron donor such as dehalogenase or nitrate reductase. The techniques used in this thesis represent a powerful suite of molecular tools which target populations attached to surfaces.

APPENDICES

APPENDIX A

HYDROGENASE LITERATURE REVIEW

Hydrogenase Distribution

Hydrogenase enzymes act as catalysts for the production and/or consumption of hydrogen according to the reaction $H_2 \leftrightarrow 2H^+ + 2e^-$ (1, 15, 58). Although initially discovered in bacteria (10), hydrogenases have been found in all domains of life with representatives in Archaea (18) and Eukaryal subcellular organelles (including protozoan hydrogenosomes (32) and green algal chloroplasts (20, 49, 54)). Three classes of hydrogenase have been constructed based on the metal content of the active site. The two major groups are metalloenzymes with Fe- or NiFe- containing catalytic sites (the NiFe group also includes a NiFeSe group in which a cysteinyl ligand is substituted by selenocysteine) (15, 17, 58). The third group, a metal-free hydrogenase, lacks a metal center and so far appears to be conserved mainly to methanogens (19, 21, 67). Both groups of metalloenzymes are most abundant in the domain Bacteria; however, their presence in other domains is unequally distributed whereas the [Fe] hydrogenases appears to be restricted to Bacteria and Eukarya and the [NiFe] enzymes to Bacteria and Archaea (25, 58).

Hydrogenase in Sulfate-Reducing Bacteria

Hydrogenases are integral to the microbial metabolism of hydrogen, a key intermediate in the consumption of organic compounds in anoxic environments (50). Sulfate-reducing bacteria (SRB) are comprised of a very diverse group of ubiquitous microorganisms capable of dissimilatory sulfate reduction (51). Although initially

described as a group of strict anaerobes with limited physiologic capabilities apart from sulfidogenesis (42), the metabolic versatility and environmental impact of these organisms are now appreciated. One group of SRB, *Desulfovibrio* sp., are able to grow using diverse metabolic pathways including sulfate reduction, nitrate reduction, and fermentation (42). SRB have even been found to carry out oxygen respiration coupled to the formation of ATP, although growth with oxygen has not yet been demonstrated (4, 14, 27). Many of these organisms are able to couple the reduction of sulfate to the oxidation of H_2, organic acids, and even some environmental contaminants including hydrocarbons, alcohols, and aromatic compounds (42).

Hydrogenases are key enzymes in the metabolic activities of SRB and the most extensively studied enzyme within the group (61). Hydrogen is an important intermediate in sulfate reduction and hydrogen production/consumption are integral features of SRB metabolism. Three different hydrogenases have been characterized within the genera *Desulfovibrio*: the [Fe]-, [NiFe]-, and [NiFeSe] hydrogenase. The distribution of the enzymes varies within the genus *Desulfovibrio* and even between strains. For instance, *D. desulfuricans* str. Teddington R encodes only the [NiFe] hydrogenase, *D. desulfuricans* str.Essex 6 encodes both the [NiFe]- and [Fe] hydrogenases, and *D. desulfuricans* str. G200 encodes all three hydrogenases (61). Although the distribution of the genes varies between different strains, only the [NiFe] hydrogenase is expressed in all studied strains of *Desulfovibrio* (15, 58, 61). Numerous studies have focused on the elucidation of the structure, function, and regulation of the [NiFe]- and [Fe]- hydrogenases, however detailed information regarding the [NiFeSe]

enzyme is limited. This review will focus namely on the two main hydrogenases, the [NiFe]- and [Fe] hydrogenase.

Hydrogenase is sensitive to different concentrations of inhibitory compounds. Both the [NiFe]- and [Fe] enzymes are highly sensitive to CO, NO, and Cu, which appear to interfere with either the metal catalytic centers or electron shuttling chains (26, 53). Different levels of inhibition are seen with CO, NO and NO_2^- but all appear to be more inhibitory toward the [Fe] than [NiFe] hydrogenase (15). For example, the [Fe] hydrogenase of *D. vulgaris* is highly sensitive to 40 µM NO_2^- with an 84% reduction in activity, but the [NiFe] hydrogenase of *D. gigas*, *D. multispirans*, and *D. vulgaris* are unaffected with concentrations up to 100 µM (5). The same trend is seen with CO where the [Fe] hydrogenase of *D. vulgaris* is inhibited by 50% with the addition of 0.1 µM CO, yet the addition 20-30 µM are required for the same level of inhibition in the [NiFe] hydrogenase of *D. multispirans, D. gigas,* and *D. vulgaris*(Hildenborough) (15).

Inhibition studies with CO and Cu have been used to determine whether the hydrogenase is located in the cytoplasm (Figure A.1). CO is able to permeate the cytoplasmic membrane, therefore inhibiting both cytoplasmic and periplasmic hydrogenases (16). On the other hand, Cu is unable to permeate the cytoplasmic membrane, therefore only interacting with periplasmic hydrogenases (13, 16). Treatment of whole cells and crude extracts with Cu or CO prior to activity measurements has been used as method to elucidate the location of the enzyme (16, 35).

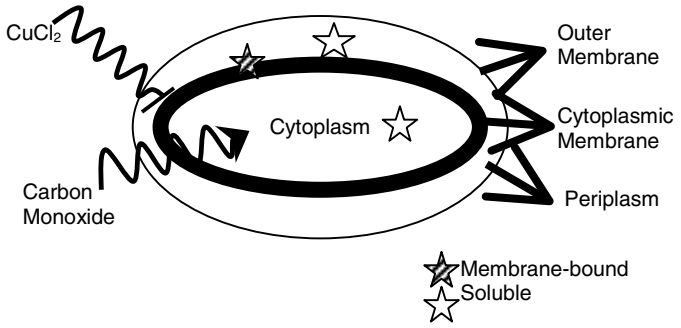

Figure A.1. Location of hydrogenase and permeability of inhibitory compounds to the cytoplasmic membrane. $CuCl_2$ is inhibitory to hydrogenases located outside of the cytoplasmic membrane whereas CO is inhibitory to all hydrogenase.

The cellular location of both enzymes is very different in strains of *Desulfovibrio* as compared to other bacteria. For instance, the soluble periplasmic localization of the [Fe] hydrogenase in *Desulfovibrio* is contrasted by the cytoplasmic location in a number of organisms including *Clostridium pasteurianium* (31)*, Thermotoga maritime* (57) and *Megasphaera elsdeni* (2). The [NiFe] hydrogenase is most commonly located in the periplasmic space in *Desulfovibrio* sp., with the exception of*D. vulgaris*where strains Hildenborough and Miyazaki F express a membrane-bound, periplasmically oriented [NiFe] hydrogenase (43) which is more similar to the location in of other bacteria including *Escherichia coli*, *Rhodobacter capsulatus, Wolinella succinogenes* and *Alcaligenes eutrophus* (43, 58, 60). The differences in location of hydrogenase between *Desulfovibrio* and other bacteria most likely stem from the periplasmic location of the redox partner and the metabolic requirement for energy transduction and a proton gradient in which the exact mechanisms are not yet clearly understood (36).

The primary redox partner for the [NiFe] and [Fe] hydrogenases in all studied strains of *Desulfovibrio* is a periplasmic cytochrome, namely cytochrome c_3 (3, 7, 37, 42). The close interaction of the two molecules was first discovered by co-purification (39). Further structural analysis of the cytochrome supported the interaction by revealing a crown of acidic residues surrounding the recognition site of the cytochrome which could then interface with the complementary crown of basic residues surrounding the hydrogenase electron transferring site (34).

Energy Transduction Pathways of SRB

The hydrogen cycling model, proposed by Odom and Peck (37), suggests a mechanism for energy transduction in *Desulfovibrio* spp. during sulfate reduction. This model outlines a fundamental role of hydrogenase during periplasmic hydrogen oxidation and subsequent transmembrane electron transfer which results in the formation of a proton gradient (36). Briefly, the model states that electrons derived from the cytoplasmic oxidation of lactate are consumed by a cytoplasmic hydrogenase yielding molecular hydrogen. The hydrogen can then diffuse through the cell membrane to the periplasm for utilization by a periplasmic hydrogenase resulting in the formation of a proton gradient which can be used for additional ATP synthesis via ATP synthase (36). The electrons generated from the periplasmic oxidation of hydrogen are then transferred by electron carriers, including cytochrome, to the cytoplasm for sulfate reduction (3).

One challenge of the hydrogen cycling model lies in the requirement of cytoplasmic and periplasmic hydrogenases in which only the latter has been identified in

all studied strains of *Desulfovibrio* sp. (15, 58, 61). The discovery of a membrane-spanning *hmc* cytochrome in *D. vulgaris* provides one possibility for electron transport from the periplasm to the site of sulfate reduction (41, 46). Other interesting possibilities for energy transduction are being uncovered with genome annotations. Information from the recent annotation of *D. vulgaris* Hildenborough has alluded to the presence of two previously undiscovered cytoplasmically oriented, membrane-bound hydrogenases (*ech*ABCDEF and *coo*MKLXUHF operons) which may be able to participate in energy transduction (19). The *ech* hydrogenase (*E. coli*-like hydrogenase) of *Methanosarcina barkeri*, is a multi-subunit [NiFe] hydrogenase capable of reversible oxidation of molecular hydrogen (28). The CO oxidation system (*coo operon*) encodes a CO-tolerant CO dehydrogenase which may be able to generate hydrogen from CO according to the reaction $CO + H_2O \rightarrow CO_2 + H_2$ (23, 64).

Variations in hydrogenase gene expression throughout the genera *Desulfovibrio* greatly hinder the development of a universal model for the mechanism of electron transfer and hydrogenase activity. The purpose of expressing only the [NiFe] hydrogenase as compared to two or all three of the hydrogenases is unclear, but these discrepancies may reflect environmental advantages. Studies have shown the [Fe] hydrogenase to catalyze the consumption and production of hydrogen at much higher rates as compared to the [NiFe] hydrogenase; however the affinity for hydrogen is much greater in the [NiFe] than the [Fe] hydrogenase (22, 38, 61). The difference in enzyme activity and affinity suggests to possible advantages of each enzyme in different environments. For instance, the [NiFe] hydrogenase might be more advantageous in

hydrogen-limiting conditions due to the higher hydrogen affinity and the [Fe] hydrogenase where hydrogen is non-limiting but rapid hydrogen consumption is required (61). Hydrogen affinity among the different enzymes adds an interesting facet to enzyme regulation and mechanism of energy translocation in *Desulfovibrio*, however, our understanding of this system is not yet satisfactory. Advances in molecular techniques, especially the construction of hydrogenase-depleted mutants will add a great amount of clarity to our knowledge.

Molecular Biology of SRB Hydrogenase

The [NiFe] hydrogenase, a heterodimeric enzyme, is encoded by the *hyn*B,A operon in which the gene for the small subunit (β), *hyn*B, precedes the large subunit (α), *hyn*A. The catalytic NiFe center of the enzyme is located within the large subunit in close proximity to an [Fe-S] cluster and the other two [Fe-S] electron shuttling clusters are located on the small subunit . [Fe] hydrogenase is also a heterodimer and encoded by the *hydA,B* operon where the larger α subunit precedes the smaller β subunit (62, 63). Interestingly, unlike the [NiFe] hydrogenase, the Fe catalytic center of the [Fe] hydrogenase and the electron shuttling compounds are both located within the large subunit. The small subunit of both the [Fe] and [NiFe] hydrogenase encodes the signal sequence for periplasmic translocation (reviewed in (61)). Initially, location of hydrogenase was questioned based on the lack of a signal sequences for the general secretory pathway (GSP) (62, 63). However, alignment of [NiFe] hydrogenase genes from various organisms and the small subunit [Fe] hydrogenase genes revealed a

conserved RRXFXK motif in a 30-70 amino acid peptide at the N-terminus (59, 66). The conserved motif, referred to as the "twin arginine leader" operates independently of the GSP via the twin arginine translocation (TAT) pathway which transports partially or fully folded proteins (8, 24, 47, 48, 65). Mutagenesis of theTAT pathway have shown accumulation of catalytically active hydrogenase in the cytoplasm of *E. coli* (45) and *Ralstonia eutropha* (6) thus indicating transport of the fully assembled protein.

Mutagenesis, and other molecular tools, have greatly contributed to understanding of the role of hydrogenase in electron transduction pathways. Molecular analysis has revealed that gene expression is dependent on many factors including growth phase (55), iron availability (9), electron donor (52, 56), yeast extract concentrations (40, 56), and attached vs. suspended growth (44). Mutagenesis is powerful tool for investigating the influence of each hydrogenase on growth in different conditions. Before successful attempts at hydrogenase gene deletion, anti-sense RNA was used in *D. vulgaris* to significantly reduce synthesis of the enzyme (56). The study provided evidence for an essential role of [Fe] hydrogenase in hydrogen production when cultured on lactate and sulfate; however, this finding was contradicted by subsequent gene mutagenesis studies. The construction of an [Fe] hydrogenase deletion mutant in *D. vulgaris* determined the role of the enzyme to be more likely of hydrogen uptake as compared to production when cultured under sulfate-reducing conditions with hydrogen and lactate as electron donors (11). Single ([Fe] hydrogenase only), double ([Fe]- and [NiFe] hydrogenase), and triple hydrogenase ([Fe]-, [NiFe]- and NADP-reducing hydrogenase) deletion mutants of *D. fructosovorans* revealed that the deletion of one hydrogenase may be compensated by

other hydrogenases regardless of enzyme location (NADP-reducing hydrogenase is located in the cytoplasm) (11). The triple mutant also led to the discovery of a fourth hydrogenase in *D. fructosovorans* (11). Further mutagenesis studies will be essential for elucidation of the function of different hydrogenases in *Desulfovibrio* sp. especially if hydrogenases are able to play a compensatory role regardless of cellular location.

Mutagenesis studies provide a wealth of information regarding the broad-scale environmental factors of gene expression. The mutant strains are frequently studied as suspended populations, therefore the obscuring the impacts of gene deletions within individual cells, microcolonies, and/or mixed populations. Molecular techniques are being used with increasing frequency for analysis of gene expression in microorganisms that resist genetic manipulation. RT-PCR is a very sensitive method for analyzing gene expression and is commonly employed in nucleotide extracts of laboratory cultures and environmental samples. One limitation of RT-PCR, however, has been the use of RNA extracts as template precluding detection of cell-to-cell differences in gene expression in a population. *In situ* RT-PCR combines the detection limits of RT-PCR with whole-cell analysis of FISH. The cellular preparation of FISH samples (fixation and permeabilization) in concert with the reverse transcription and amplification of target genes using a fluorophor incorporation method allows for microscopic analysis of cellular gene expression. This method has been applied to suspended bacterial cultures (29), mixed populations (12), and more recently to surface-associated bacteria (30, 33, 44). Despite extensive investigations into energy translocation and electron coupling in the genera *Desulfovibrio*, many questions still remain and the exact pathways have yet to

be elucidated. While mutagenesis studies are beginning to reveal the essential roles of hydrogenase in energy transduction, the application of IS RT-PCR to mutants will help identify the effects on an individual cell basis.

Summary

Hydrogenase is an integral enzyme for energy generation in SRB. The [NiFe] hydrogenase is universally distributed throughout *Desulfovibrio* sp. whereas the [Fe] and [NiFeSe] hydrogenases are less evenly distributed. The distribution of hydrogenase through different species of *Desulfovibrio* raises interesting question regarding the pathways of energy generation. The hydrogen cycling model requires a cytoplasmic hydrogenase however not all strains contain the cytoplasmic [NiFeSe] enzyme but genome sequencing is revealing other hydrogenases previously undiscovered in the genus. Molecular techniques such as mutagenesis and RT-PCR are being employed to try to understand the mechanism for energy generation with different electron donors under sulfate-reducing conditions. The use of IS RT-PCR can be employed to elucidate the mechanisms on a cell to cell basis.

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APPENDIX B

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August 1, 2005

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