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Microbiological Enhancement of Phosphorus Sequestration in Iron-Enriched Sediments

Lindsay Marie Sandersfeld

MICROBIOLOGICAL ENHANCEMENT OF PHOSPHORUS SEQUESTRATION IN IRON-ENRICHED SEDIMENTS

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Lindsay Marie Sandersfeld

University of Northern Iowa

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Dr. Edward J. Brown, Honors Thesis Advisor

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Jessica Moon, Director, University Honors Program

ABSTRACT

An overabundance of phosphorus, primarily due to agricultural run off, is the source of increases in unwanted algae in Iowa's surface waters. Oxidized iron found in mine tailings will sequester phosphorus in sediments and prevent it from entering surface waters. Therefore, adding mine tailings to wetlands is a cheap and easy way to eliminate phosphorus pollution. This phosphorus removal process is enhanced by a microbial component that biochemically solubilizes iron tailings which allows them to efficiently react with free phosphorus in the water. The purpose of this project was to characterize the microbial component in this process. Ferric citrate medium was made and sealed in anaerobic bottles to enrich for and isolate iron-reducing bacteria from sediment samples enriched with iron. Multiple transfers and relatively long (three week) incubation periods were necessary to ensure pure cultures were isolated. Bacteria were then harvested from enrichments and DNA was obtained. The 16s rRNA gene was amplified using PCR and primers specific for metal reducing bacteria, and then the product was sequenced using the DCTS Quick Start Kit. Final sequencing was confirmed by Laragen Inc. (Los Angeles, CA). The sequence obtained from the isolate was compared to known 16s sequences in Genbank and shown to be 99% identical *Desulfotomaculum sp.* The results demonstrate that aquatic sediments supplemented with iron mine tailings sequester phosphorus and harbor bacteria known to reduce iron.

CHAPTER 1

INTRODUCTION

Iowa's surface waters are heavily polluted with the nutrient phosphorus. Excess phosphorus is considered a water pollutant because it can lead to the eutrophication of aquatic environments (Clayton et al., 2004). Eutrophication leads to increases in algal blooms and decreases the amount of oxygen in the water, which in turn is very harmful to aquatic life and may even sustain the growth of pathogenic bacteria (McLellan, 2004). Phosphorus enters water naturally through soil erosion and rock weathering; however, agriculture has greatly increased the concentration of phosphorus in water. The excessive use of fertilizer applies much greater amounts of phosphorus than needed. When the crops and soil don't retain all of this phosphorus it becomes dissolved in water and drains into Iowa's streams and lakes, which then feed into the Mississippi River causing even more destruction such as contributing to the formation of the Dead Zone in the Gulf of Mexico (Clayton et al., 2004).

Previous studies in this laboratory found that adding oxidized iron, found in mine tailings, to phosphorus-laden sediments greatly reduced the amount of phosphorus released into the water, even under anaerobic conditions. This process was enhanced even more when a carbon source, acetate, was added. The major mechanism in this sequestration of phosphorus was hypothesized to be the reduction of ferric iron by microbial respiration. Ferrous iron (reduced iron) is then released into the water and binds with free phosphorus to form a precipitate that binds the phosphorus into the sediment. Phosphorus can also physically sorb to sediments but can also be released

back into the water if the sediment is disturbed or becomes too saturated with phosphorus (Pasco, 2005).

The goal of this project was to identify the key microbial component involved in this process of phosphorus sequestration. Because sediments contain a diverse microbial flora, many genera can be isolated, grown in pure culture and identified using phenotypic and/or genotypic taxonomic indices. To accomplish the goal of this study, i.e. to identify the key microbial component involved in phosphorus sequestration, it was first necessary to use sediments in which this phenomenon was occurring and to select growth conditions suitable only to the microorganisms of interest. Thus, I removed small aliquots from several phosphorus-laden sediment samples that had been previously amended with oxidized iron. These sediments were collected from the Beaver Valley Wetland in Cedar Falls, Iowa. I inoculated the aliquots from the treated sediments into culture media under anaerobic conditions in order to stimulate the growth of only those bacteria capable of iron reduction. When, after several transfers, a pure isolate was obtained, the isolate was characterized phenotypically and a portion of its DNA was sequenced using 16s rRNA analysis for genotypic identification.

CHAPTER 2

LITERATURE REVIEW

Wetland Function and Eutrophication

Phosphorus is a nonpoint source pollutant that is a major contributor to the eutrophication of Iowa's surface waters. Eutrophication is defined as the accelerated nutrient enrichment of water due to accumulation of excess nutrients. This process results in algal blooms that decrease the oxygen levels in aquatic environments, putting a lot of stress on aquatic organisms. One way to help control this phosphorus pollution problem is to use wetlands to buffer and filter areas near surface waters. The biomass of wetlands can sorb phosphorus to its sediments making it unavailable to algae in the water column (Pasco, 2005). Wetlands have often been referred to as the "kidneys of the landscape", due to their ability to retain and transform nutrients in their large amounts of biomass (Mitsch & Gosselink, 1993).

Phosphorus Sorption

While wetlands can be very effective in filtering out excess nutrients from run off that is heading into a water body, most of the wetlands are becoming saturated with phosphorus and can no longer function as a filter. Phosphorus in the sediments can actually desorb and act as a source rather than a sink for phosphorus, contributing more phosphorus to water bodies downstream, especially under anaerobic conditions which is common in wetlands. A second way to control phosphorus pollution levels is to use oxidized iron mine tailings that have been shown to bind the phosphorus, transforming it into a mineral and bringing it out of the water column. Both ferric, Fe(III), and ferrous,

Fe(II), forms of iron can bind with phosphate, precipitating as solid minerals vivianite and strengite, and making phosphorus unavailable for use by algae or other aquatic plants in the water column. Ferric iron, Fe(III), is the most common of the two metals and is found in soils, rocks, in aerobic water, and many minerals; however, most forms of Fe(III) are insoluble (Hutchinson, 1957). Most water treatment methods reduce ferric iron to ferrous iron, the soluble form, under anaerobic conditions to sequester or precipitate phosphorus (Freeze and Cherry, 1979). In anoxic conditions this process is carried out by the activities of iron-reducing bacteria (Straub et al., 1996). The phosphorus sequestration process has a key microbial component involved in reduction of the iron as well, allowing it to become soluble so it can bind with phosphorus in the water column. This oxidized iron treatment method is an innovative way to control phosphorus pollution and could replace the expensive process of aeration as well (Pasco, 2005).

Iron Reducing Bacteria

Iron respiration by microbes plays a key role in the development and metabolism of anoxic subsurface environments. Iron reducing bacteria compose a large majority of bacterial flora in aquatic sediments. While these organisms are responsible for coupling oxidation of organic matter to the reduction of Fe(III), secondary minerals formed from their respiration have also been shown to reduce harmful chemicals also present in the water, such as pesticides, chromium, and chlorinated solvents (Williams et al., 2005 and Kourtev et al., 2006). They also produce other minerals vital to the geology of their

environment, such as magnetite, are capable of remediation of uranium and other toxic chemicals, and can transfer electrons to cathodes to create electricity (Bond et al, 2005).

These anaerobic organisms use humic substances found in the soil for energy by donating electrons to the electron acceptors they are growing on, such as fumarate and nitrate, and obtain carbon from acetate and other available sources (Coates et al., 2002). While these bacteria are capable of reducing Fe(III), most Fe(III) is resistant to microbial reduction and is found deep in the sediments (Lovely and Phillips, 1986). The most predominant members of the iron-reducing bacteria in most environments are of the genera *Geothrix* and *Geobacter*, and are found in almost every sediment environment. Both groups constitute strict anaerobic, gram-negative rods that are capable of conserving energy by oxidizing organic matter to carbon dioxide along with the reduction of Fe(III) (Coates et al., 2001).

The genus *Geobacter* was the first to be identified to conserve energy for growth in this way (Mahadevan et al., 2006). This group belongs in the delta proteobacteria division and has since had several species identified, including *Geobacter metallireducens* (Lovely et al., 1993). Geobacteraceae is also the largest group of Fe(III)-reducing bacteria in the deltaproteobacteria (Lovely et al, 2004). This family of bacteria is of special interest in current research because of its ability to harvest energy from aquatic sediments using electrodes. (Bond et al., 2002; Holmes et al., 2004; Tender et al., 2002). The closest relative to *G. metallireducens*, as identified by 16s rRNA analysis, is *Desulfuromonas acetoxidans*, which has also been shown to oxidize acetate and reduce Fe(III).

Although it was isolated and known as an acetate-oxidizing S^0 reducer, *D. acetoxidans* was also found to grow on acetate with Fe(III) as the electron acceptor (Roden, and Lovely, 1993). Sulfate reducing bacteria inhabit the same sediments as iron-reducing bacteria but are often outcompeted. Fe(III) reduction occurs before sulfate reduction in almost all circumstances (Lovely, 1991). Fe(III) reduction by sulfate reducing bacteria is not considered to be a primary growth-supporting process, but rather a means of survival when sulfate is depleted (Lovely, 2000); however, the mechanism through which sulfate reducing bacteria reduce Fe(III) has not been well studied yet. Fe(III)-reducing bacteria from the phylum Firmicutes, which also contain sulfate reducing bacteria, generally use Fe(III) as an electron sink rather than for growth as other iron-reducing bacteria do (Lin, 2007).

Enrichment, Isolation, and Identification of Bacteria

Enriching for a microorganism from an environment is the process of drawing the organism out of its community and then isolating the organism in a pure culture. Isolation of organisms is vital for controlled laboratory studies and applications in environmental microbiology. Using the enrichment culture technique, specific medium and incubation conditions that are selective for the organism of interest and counterselective for other members of the bacterial community, an organism can be isolated. The medium and conditions for growth must mimic the ecological niche as closely as possible in order for a successful enrichment to occur. Another method of isolating a microorganism is growing the organism on solid media and streaking for isolation. A single colony can be obtained from this method and used to inoculate other

media to obtain a pure culture. A pure culture is also desired in order to assess and classify the organism.

Taxonomy is the science of classification, which generally focuses on phenotypic analyses of organisms. Classifying microorganisms provides a systematic way to analyze their relationships with each other. Several phenotypic characteristics are assessed and used to group organisms by performing morphological and physiological tests. Newer, more advanced methods of classification and identification utilize bacterial RNA to identify microorganisms. With the scientific advances in molecular biology and computer technology, rRNA sequences can be sequenced and compared. 16s ribosomal RNA is obtained from a cell culture and amplified with polymerase chain reaction (PCR) and relevant primers. Dideoxy sequencing is then used to sequence the product, which can then be compared using various rRNA sequence databases. Sequences can then generate phylogenetic trees that best describe the evolutionary relationships of the microorganism (Madigan and Martinko, 2006).

Purpose of the Project

The goals of this project were:

1. To isolate and culture the microorganism responsible for Fe(III) reduction in the process of phosphorus sequestration.
2. To identify this microorganism.

CHAPTER 3

MATERIALS AND METHODS

Sediment Source

The wetland sediment samples used for isolation of bacteria were those previously studied (Pasco, 2005); and some had been treated with oxidized iron. These sediments were collected from the Beaver Valley Wetland (Cedar Falls, IA) in May 2004. Both iron treated and untreated samples were stored at 4°C (Pasco, 2005). Total phosphorus (TP) concentrations for wetland water and sediment samples were measured using the ascorbic acid method (Clesceri et al., 1998). The average total phosphorus concentration in sediments was 217 ± 25 μg PO_4^{3-} -P/g dry weight and TP concentration in wetland water was 454 ± 175 μg PO_4^{3-} -P/l (Pasco, 2005).

Oxidized Iron Source

The oxidized iron used in treating the sediments was a mineral residue of ore extraction from the Cuyuna range in East-central Minnesota. The ore and tailings contain many minerals including hematite, a source of ferric iron. Ferric iron is capable of chemically precipitating with anions as well as sorbing anions to hydrous ferric oxides. The tailings were crushed for treatment of sediments (Pasco, 2005). Total phosphorus concentrations in the tailings were also measured and found to be 82 μg PO_4^{3-} -P/g tailing material (Clayton et al., 2004).

Glassware and Syringes

Fifty milliliter serum bottles sealed with septa caps and aluminum crimps were purchased from Fisher Scientific (Chicago, IL), and used for isolating and transferring cultures. Five-milliliter syringes fitted with 22 ½ gauge needles, purchased from Becton Dickson (Franklin Lakes, NJ), were used for transferring sediments and cultures anaerobically.

Medium

American Type Culture Collection Medium 1768-Anaerobic Ferric Citrate Medium (Lovely et al, 1993) was used to successfully culture and isolate bacteria from the sediments. The media was amended with mineral micronutrient solutions (Brown, 1975) and magnesium chloride. A vitamin solution used to supplement the medium is described in Table 1.

Table 1. Vitamin mixture components.

Vitamin	Concentration mg/ 100 ml
Thiamine	50 mg
Riboflavin	50 mg
Pyridoxine·HCl	50 mg
Calcium pantothenate	50 mg
Nicotinic acid	10 mg
Biotin	10 mg
Folic Acid	10 mg
p-Aminobenzoic acid	10 mg
Choline	10 mg
Vitamin B_{12}	50 mg

Sample Preparation and Growth Conditions

The medium was prepared (pH 7.0) and approximately 20 ml were sealed into each of several serum bottles. Bottles were then purged with a nitrogen gas mixture (93% N_2, 7% CO_2) for twenty minutes and autoclaved. The bottles were then purged a second time for twenty more minutes to remove any remaining oxygen in the medium or headspace of the bottles. The medium was then inoculated using a syringe and 22 1/2-gauge needles with two drops of polluted sediments that had been used in previous experiments (Pasco, 2005).

Several sediment samples were used for enrichments to increase the probability of isolation, but all had been previously amended with acetate and treated with oxidized

iron tailings. After inoculating fresh medium with sediment enrichments, they were then incubated at 30°C for two to three weeks or until evidence of iron reduction and sufficient microbial growth (microscopy) was observed. Two drops from each bottle were then transferred to a new bottle of the same medium to continue the goal of isolating a single bacterium capable of growth on iron. Transfers were carried out for several months until a pure culture of isolated bacteria was obtained.

Pure Culture Growth Conditions

A pure culture of *Geobacter metallireducens* was also grown under the same conditions as the sediment samples to ensure the conditions were suitable for growth and isolation of an iron-reducing bacterium.

DNA Extraction

DNA from the isolated bacteria was obtained by adding 20 ml of oxalate solution (ammonium oxalate 14g, oxalic acid 7.5g, 500 ml H_2O, pH 7.0) to a culture bottle showing growth and iron reduction, and then letting that react for ten minutes to convert all the iron in the medium to a soluble form so only bacterial cells would pellet when centrifuged. The culture bottle was shaken by hand every two to three minutes to ensure proper mixing. Then, using a 5 ml syringe, 1.5 ml of the contents were removed from the bottle and put into a 1.5 ml microcentrifuge tube. The cells were centrifuged for 5 minutes at 12000 rpm. The supernatant containing soluble iron was poured off and if needed another 1.5 ml of the culture was added to the tube for another centrifugation.

Once a good-sized cell pellet was obtained, the supernatant was discarded and 40 µl of distilled water was added to resuspend the cells. The tube was then centrifuged and the water was removed using a pipette. Forty µl of distilled water and 5 µl of chloroform were then added to the tube and the cell pellet was resuspended using a pipette. The tube was then placed in boiling water for 10 minutes to extract DNA. Following boiling, the tube was centrifuged one last time and the supernatant was removed to a sterile 200 µl microcentrifuge tube. Both the cell debris pellet and the DNA supernatant were placed in the freezer (4°C) for future use.

Polymerase Chain Reaction (PCR)

The following components were mixed together in the following order for 50 µl PCR preparations: 5 µl PCR Buffer (100 mM Tris (pH 8.3), 500 mM KCl, 17.5 mM $MgCl_2$, 5% DMSO, and 0.5% Triton X-100), 5 µl dNTPs (8mM), 5 µl forward primer (2.5 mM), 5 µl reverse primer (2.5 mM), 2 µl BSA (10X), 1 µl DNA lysate, 0.5 µl Taq polymerase (2.5 U), and 26.5 µl distilled water. Various primer sets were used (Table 2). The preparations were mixed in either 200 µl microcentrifuge tubes or PCR plates on ice and then loaded into Genemate Genius Thermocycler from Techne (Burlington, NJ).

Amplifications occurred in the thermocycler as followed: 94°C for 3 minutes, then 30 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 2 minutes, followed by a ten minute incubation at 72°C. Results were analyzed by loading 2 µl of the samples mixed with 2 µl loading dye onto a 0.7% agarose gel (100 ml 1X TAE buffer, 0.7g agarose, 5 µl Ethidium bromide), along with 5 µl 100 base pair standard. The gel

was run for 20 minutes at 200 volts and visualized using a Dual intensity UV Transilluminator (UVP; Upland, CA).

Table 2. Primer sets used together and their corresponding sequences.

Primer name	Sequence
8f	5'(AGAGTTTGATCCTGGCTCAG)3'
1525r	5'(AAGGAGGTGATCCAGCC)3'
8f	5'(AGAGTTTGATCCTGGCTCAG)3'
1492r	5'(GGTTACCTTGTTACGACTT)3'
Dup1_*Geobacter metallireducens*	5'(ACGGGTGAGTAACGCGTGGATAAT)3'
Dup2_*Geobacter metallireducens*	5'(ACGCGCTTTACGCCCAATAATTCC)3'
Dup3_*Geobacter metallireducens*	5'(AGTAACGCGTGGATAATCTGCCCA)3'
Geobacter metallireducens Set	5'(CGCCCAATAATTCCGAACAACGCT)3'

Cleaning the Gel-purified Product

After gel electrophoresis, the DNA band was cut out of the gel using a spatula. The gel slice was weighed and placed in a microcentrifuge tube for Gene Clean treatment. The Gene Clean kit was purchased from Qbiogene (Carlsbad, CA). To determine the amount of template in the purified product, 5 µl λ standards were loaded onto a 0.7% agarose gel and run for 30 minutes at 100 volts. Then 2 µl of the Gene clean product and yellow dye were added to the gel and it was run for 15 more minutes at 100 volts. The gel was observed using a transilluminator. The fluorescence of the band was

determined using Kodak 1D 3.6.1 software (Rochester, NY), which yielded the template amount in ng/µl.

Cloning and Transformation

The purified DNA from the Gene clean reaction was cloned into a plasmid in order to obtain more copies of the DNA for sequencing. The PGem-T Easy vector System, from Promega (Madison, WI) was used for cloning. Ten µl of the ligation was then added to JM109 competent cells, also from Promega, and incubated for 20 minutes on ice, flicking the tube lightly to mix. This tube of cells and ligation was then heat shocked for 45-50 seconds at exactly 42°C, and then returned to ice for 2 more minutes. Room temperature S.O.C medium (950 µl) was then added to the tube and incubated at 37°C on a shaker for 1.5 hours. This reaction was plated on four LB+amp+IPTG+x-gal plates, 100 µl per plate, and incubated at 37°C overnight. The plates were taken out and refrigerated until needed.

Culturing the Transformed Bacteria

Four transformed colonies that grew from the transformation plates were scraped off individually using pipette tips and used to inoculate four test tubes of LB+amp media (300 µl Ampicillin, 150 ml LB medium). The tubes were incubated overnight at 37°C on a shaker.

Hurricane DNA Miniprep Purification

One and a half ml of each transformed culture grown in the LB+amp medium were transferred to microcentrifuge tubes. After centrifugation for 1 minute at 12000 rpm, cell pellets were purified using the Hurricane DNA Miniprep Purification Kit, Gerard Biotech (Oxford, OH). To ensure DNA was obtained, 2 µl of the products with 2 µl loading dye were run on a 0.7% agarose gel for 15 minutes on 200 volts. The gel was observed using a UV transilluminator. An Eco R1 Digestion was prepared to confirm the inserted DNA and part of the plasmid were both intact. This was done by adding 2 µl of the purified DNA; 1 µl Eco R1 restriction enzyme; 1 µl restriction digest buffer #2, both from New England Biolabs (Ipswich, MA); and 6 µl sterile water, and incubating the reaction for 1 hour at 37°C. This mixture was run on a 0.7 % agarose gel for 20 minutes at 200 volts. Two fragments were visualized for each of the four purified preparations.

Sequencing Preparation

DNA sequencing was completed using the DTCS Quick Start Kit from Beckman Coulter (Fullerton, CA), using M13 forward and reverse primers purchased from IDT (Coralville, IA). The PCR products were first cleaned using a kit from Edge Biosystems (Gaithersburg, MD). After the samples were dried in the Edge Biosystems plate, 40 µl of sample loading solution was added to each sample, as well as an overlay of mineral oil. The plate and the capillary gel arrays were loaded into the sequencing machine and run.

Analysis of Bacterial Growth

Microscopy was used to monitor growth in the serum bottles and to determine when to transfer cultures to new media. Cultures were plated on tryptic soy agar plates and streaked for isolation after several transfers. Some plates were incubated anaerobically while others were allowed to grow aerobically. This was used to confirm there was no contamination and the bacteria isolated were anaerobes.

Confirmation Sequencing

A frozen cell pellet was sent to Laragen Inc. (Los Angeles, CA) for independent sequence identification.

CHAPTER 4

RESULTS

Ferric citrate medium and anaerobic culturing technique successfully enriched for and allowed the isolation of iron-reducing bacteria from wetland sediment samples treated with oxidized iron mine tailings and was also capable of culturing the pure culture of *G. metallireducens*. As microbial growth increased inside the serum bottles particle precipitation occurred (Figure 1). This is phenotypic evidence that the bacteria are capable of reducing ferric iron to ferrous iron. The resulting iron species are then capable of binding with the free phosphorus in the water to form mineral precipitates and amorphous iron hydroxides, which sorb phosphate anions. Through successive transfers a pure culture was obtained. Under the microscope the isolated bacteria were rod shaped, Gram-negative, produced spores, and often appeared motile.

Inoculation of other types of media with the isolate was difficult. With time the bacteria seemed to adapt to the culture conditions and began to grow faster and were easier to culture, even on solid media. A sulfite containing media was successful at growing the bacteria and even turned black, indicating sulfite reduction to sulfide. Thiolglycolate containing medium also supported the growth of the isolate.

Polymerase Chain Reaction and primers specifically designed for *Geobacter* (Figure 2) did not amplify the 16s rRNA gene of the isolate. One amplification reaction appeared to be successful (Figure 3); however, comparison of the sequencing product with Genbank records resulted in the identification of *Pseudomonas* sp., rather than a typical iron reducer. The *Pseudomonas sp.* may indeed have been growing in a microbial consortium with iron reducing bacteria, or it may have simply been a contaminant during

the amplification procedure. Subsequently, the isolate was sequenced by Laragen Inc. (Los Angeles, CA). I then matched the sequence (Figure 4) to known 16s rRNA sequences using Genbank and found the isolate had 99% similarity with *Desulfotomaculum* sp. (Figure 5).

Figure 1. Particle precipitation in four sediment inoculated serum bottles and one uninoculated bottle, all filled with ferric citrate medium and incubated at 30°C for two weeks.

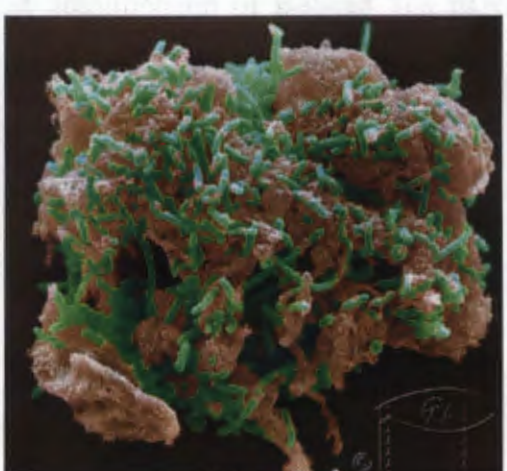

Figure 2. *Geobacter metallireducens* (green rods) sticking to a particle of metal.

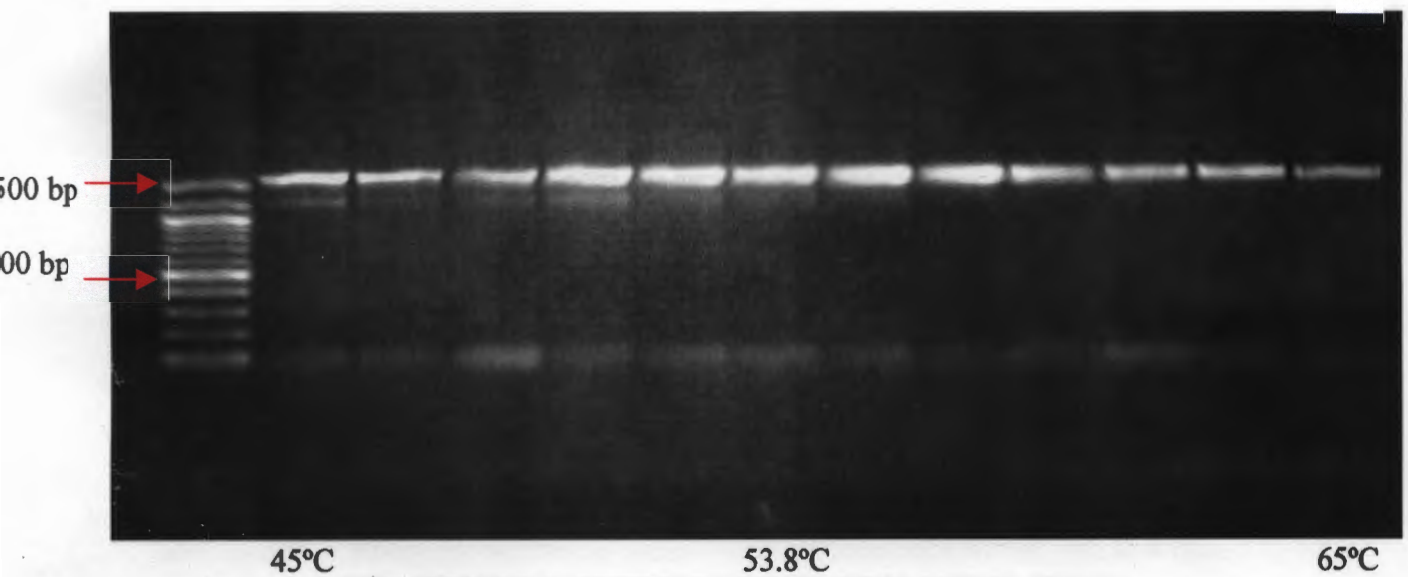

Figure 3. Successful PCR amplification of isolated 16s rRNA run in a temperature gradient Thermocycler.

AGATTGNTCAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCATGC
TCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTAAGGAGGTGATCCAGCCGCAGGTTCCCCTACGGCTACC
TTGTTACGACTTCACCCCAGTCATGAATCACACCGTGGTAACCGTCCTCCCGAAGGTTAGACTAGCTACTTC
TGGTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTGACATTCT
GATTCACGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAGACTGCGATCCGGACTACGATCGGTTT
TATGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCAGGC
CGTAAGGGCCGTGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGC
CCACCATGACGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCTCACGACA
CGAGCTGACGACAGCCATGCAGCACCTGTCCAATGTTCCCGAAGGCACCATCCATCTCTGGAAAGTTCATGG
GATGTCAAGGCCTGGTAAAGGTTCTTCGCGTTGCTTCGAAATTAAAACACATGCCTCCACCGCTTGGTGCGG
GGCCCCCGTCATTTCATTTGAGTTT

Figure 4a. 16s rRNA forward sequence for the isolate.

GCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGC
GGCCGCGGGAATTCGATTAAGGAGGTGATCCAGCCGCAGGTTCCCCTACGGCTACCTTGTTACGACTTCACC
CCAGTCATGAATCACTCCGTGGTAACCGTCCCCCCGAAGGTTAGACTAGCTACTTCTGGAGCAACCCACTCC
CATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTGACATTCTGATTCACGATTACTAG
CGATTCCGACTTCACGCAGTCGAGTTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCA
CCTCGCGGCTTGGCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCAGGCCGTAAGGGCCATGATG
ACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACCTTAACGTGCTG
GTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCC
ATGCAGCACCTGTGTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCTGCATGTCAAGGCCTG
GTAAGGTTCTCCGCGTTGCCTCGAATAAAACCCCATGCCTCACCCGCTTGTGCGGGGCCCCGGTCATTCAAT
TTGAGTTTTAACCCTGCCGGCCGTACTCCCCAGGCGGTCGACTTTA

Figure 4b. 16s rRNA reverse sequence for the isolate.

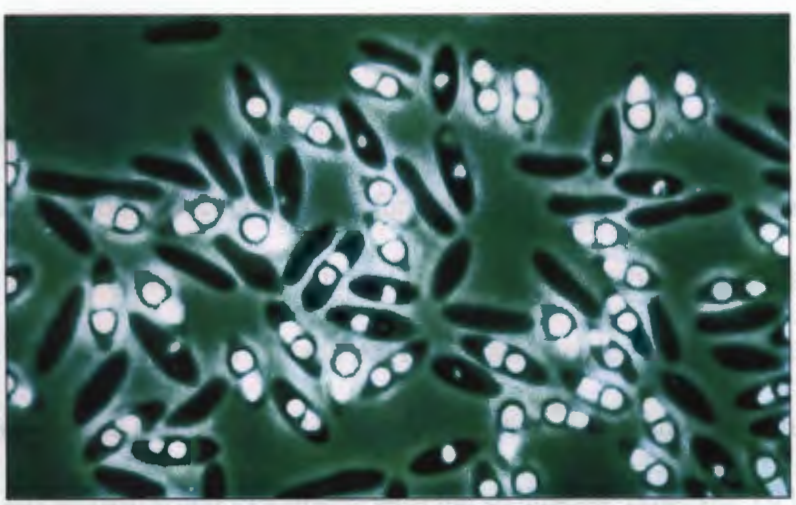

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Figure 5. *Desulfotomaculum sp.* with visible spores.

CHAPTER 5

DISCUSSION

Iron reducing bacteria compose a large majority of soil flora. There is a wide diversity of iron-reducing bacteria among this natural flora. *Geobacter* was the first anaerobic genus found to couple oxidation of organic matter to Fe(III) reduction, and are also the most predominant species found in most environments. This genus has also been shown to clean up ground water polluted with metals and organics, reduce toxic and radioactive chemicals, and to produce electricity from waste organic matter, without using oxygen as the terminal electron acceptor (Mahadevan et al., 2006).

We identified the bacteria in our polluted sediments that were amended with iron to see if such iron-reducing bacteria do exist in these soil samples, and to also describe other characteristics of the isolate. The mechanism by which bacteria reduce iron is not very well understood. It is not known for sure which pathway each species of bacteria uses to shuttle electrons. Microbial reduction of iron has been recognized as a vital process in the ecology of subsurface environments; however, (Figure 6) and new species of bacteria that have this ability are continuously being identified (Lonergan et al., 1996).

Determining the correct conditions for enriching for iron reducing bacteria was challenging at first. Several types of nutrient rich media were tried as well as basal salts supplemented with fumarate and acetate. All media also needed to be purged with a mixture of nitrogen and carbon dioxide to create an oxygen free environment. None of these showed any growth when inoculated with the sediments; however, lack of growth under certain conditions is a phenotypic trait of the isolate though. Eventually, ferric citrate medium was discovered and after two weeks of incubation a sufficient amount of

growth had developed. The amount of growth could be visualized through particle precipitation inside the serum bottles. This phenomenon is phenotypic evidence iron reduction was occurring and also an indicator that conditions were appropriate for ample growth of an iron-reducing bacterium. Growth of the commercially-purchased pure culture of *Geobacter metallireducens* also showed the conditions were suitable for an iron-reducing bacterium leading me to surmise that my isolate was a member of the genus *Geobacter*. Because my isolate and *Geobacter metallireducens* would only grow in liquid media that was purged with nitrogen and carbon dioxide, sequential transfers, rather than streak plates were initially used to ensure a pure culture.

The morphological characteristics of our isolate were very similar to those of *Geobacter*. The cell was a medium sized thin rod that stained Gram negative. It was capable of growing in acetate and based on particle precipitation, appeared capable of iron reduction. *Geobacter* therefore couldn't be ruled out as the identity of the isolate. However, there were a few unusual characteristics that didn't really seem to fit with this identity. The cells often appeared to be producing spores, sometimes appeared motile, and began growing on solid media after several tries in previous months. Once this occurred, a single isolated colony could be used to inoculate media and obtain a pure culture for sequencing.

Identification of our isolate answered our question as to whether it was from the ever-popular genus *Geobacter*. To our surprise it was not and was actually a bacterium known to reduce sulfur and not iron. *Desulfotomaculum* are naturally found in estuarine sediments but can occur in freshwater sediments and in the intestines of animals. The bacterial cells are straight to slightly curved rods that are motile by peritrichous or polar

flagella. They are strict anaerobes that produce endospores and grow in areas where the temperature range is 25-40°C. *Desulfotomaculum acetoxidans* will completely oxidize organic substrates to carbon dioxide. They can utilize acetate and butyrate as sole electron sources and reduce sulfate to sulfide (Williams et al., 1994). While these bacteria haven't been known to reduce iron, new research has indicated that sulfur-reducing species closely related to them do have the ability (Lonergan et al., 1996). These findings offer new applications in microbial ecology and phosphorus treatment in wastes containing sulfur, such as manure (See patent #20070059815). Because our sediment samples were a consortium of bacteria, *Geobacter* cannot be ruled out as having a large role in the iron-reduction in the sediments of the wetland; however, culture conditions that worked for the ATCC *G. metallireducens* did not results in isolation of a *Geobacter* from our treated sediments.

Utilizing bacteria already present in their natural environment and adding oxidized iron mine tailings that are very inexpensive would be a cost effective solution to excess phosphorus pollution entering our surface waters. Future work in the lab will focus on a more complete evaluation of the growth and iron chemistry of pure cultures of *Desulfotomaculum*, as well as an exploration of the bacterial community found in the wetland sediments to see what other iron-reducing bacteria play a part in the process of phosphorus removal.

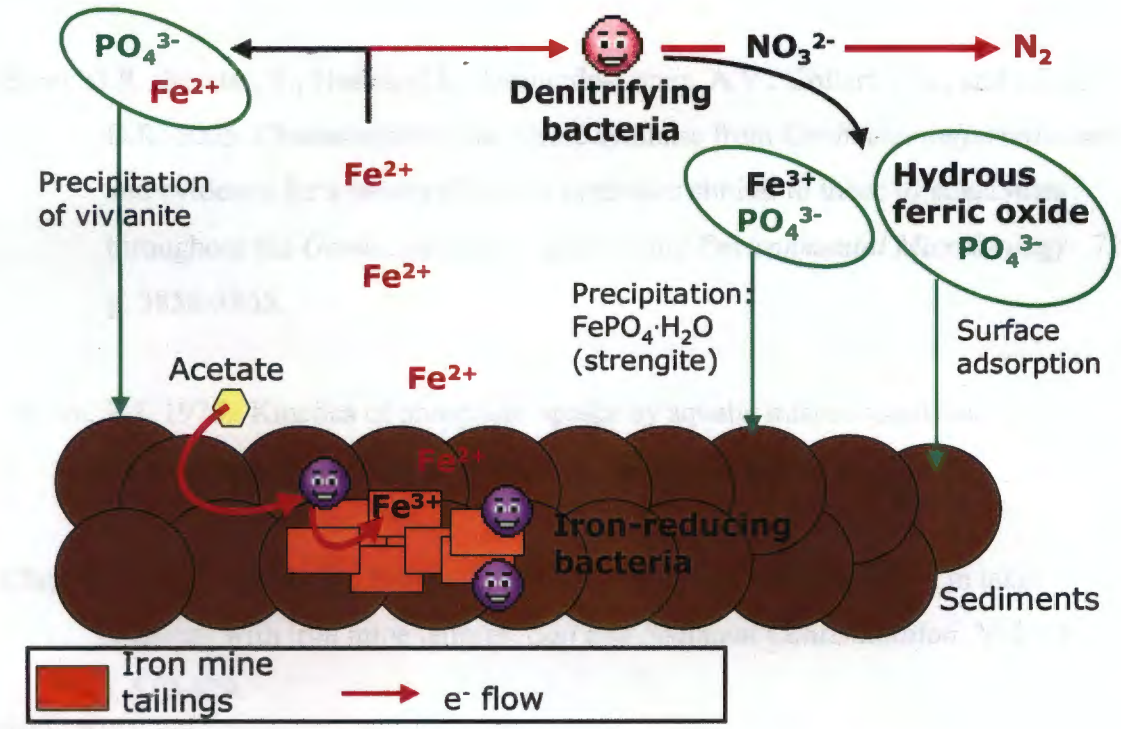

Figure 6. The model of phosphorus sequestration in wetland sediments treated with iron mine tailings shows bacteria reducing Fe(III) in sediments, allowing Fe(II) iron to bind to free phosphorus in the water column.

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