A Functional Gene Assay for Acidithiobacilli in Acidic Coal Mine Drainage

A Thesis in
Soil Science
by
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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

December 2008
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ABSTRACT

Acid mine drainage (AMD) is the single greatest pollutant of waterways in Appalachia. In Pennsylvania alone, it is estimated that over 2,500 miles of stream have been impacted by AMD. Acid mine drainage is formed when sulfide-containing minerals are exposed to air and water, with pyrite (ferrous disulfide) being the most problematic. The sulfur in pyrite oxidizes first to produce sulfuric acid so that emergent mine drainage has high concentrations of Fe(II) and acid. Fe(II) is soluble at low pH, but when mine drainage enters neutral stream waters, the Fe(II) oxidizes chemically to form insoluble iron (oxy) hydroxide precipitates which have a characteristic yellow color. AMD-impacted streams therefore are highly acidic and their streams beds are coated with ‘yellow boy’ precipitates which smother aquatic organisms.

The overall goal of this research is to develop indicators for effective Fe(II) oxidation during passive AMD remediation, which would help mitigate pollution of waterways by AMD. Most passive treatment systems involve passing AMD across limestone beds to neutralize the acidity. However, these systems become inefficient when the treatment bed becomes coated with metal hydroxide precipitates, a condition known as “armoring.” Biological low-pH iron removal from AMD before neutralization could minimize armoring in these passive systems. Since members of the Acidithiobacillus genus are the bacteria most frequently implicated in low-pH iron oxidation in AMD, the objective of this study was to develop a molecular tool for assessing the relative abundance of
acidithiobacilli in AMD under different environmental conditions. Specifically, a functional gene assay for the iron oxidase enzyme known to be present in *Acidithiobacillus ferrooxidans* was tested on AMD sediments incubated under different growth conditions.

Two AMD sites in Northern PA with similar mine water and mineralogical characteristics but contrasting rates of microbial Fe(II) removal were identified for this study; one exhibited a high rate (Gum Boot) and the other a lower rate (Fridays-2) of Fe(II) removal. We compared iron oxidase gene copy numbers in sediments incubated in ambient air with sediments exposed to different gas compositions. Primers specific for the iron oxidase gene, designated *iro* and found in *Acidithiobacillus ferrooxidans*, were used with Q-PCR to determine iron oxidizing gene copy numbers in the sediments.

This study showed that the *iro* gene assay could be used to differentiate Fe(II)-oxidizing communities in AMD sediments incubated under contrasting growth conditions. Specifically, our results from incubation trials using sediments from the two experimental sites indicated that microbial populations capable of Fe(II) removal were abundant at both sites, despite contrasting removal rates in the field. Furthermore, AMD sediments from the Fridays-2 site, which showed low Fe(II) removal rates in the field, were capable of higher rates in the laboratory when provided with the right growth conditions. Our findings with the *iro* gene assay also indicated that other iron-oxidizing bacteria which do not possess the *iro* gene appeared to be responsible for a significant amount of Fe(II) oxidation.
The findings of this study suggested that the *iro* assay is useful in understanding microbial community structure and composition of AMD-impacted sites and for tracking changes in iron oxidizing genes under different environmental conditions. Our improved understanding of microbial communities at Gum Boot and Fridays-2 could be important for remediation of other sites with similar characteristics. Knowing the community composition could aid in selecting suitable solid phase amendments to stimulate remediation. Since communities may also respond to variations in AMD flow rates, altering residence times could be another way to enhance microbially mediated low-pH Fe(II) removal at AMD sites.
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ACKNOWLEDGEMENTS

First I would like to acknowledge my Advisor Dr. Mary Ann Bruns for all her guidance and support through out my masters’ journey. Her mentoring has allowed me to grow tremendously as student and as a person. I would also like to acknowledge the members of my committee Dr. Bill Burgos, Dr. Carmen Enid Martinez and Dr. Dawn Luthe for their insights, and Dr. Marvin Risius for his help with statistical analysis.

I would like to thank Melanie Lucas and Dr. John Senko for their contributions to this research. To my wonderful lab mates past and present: Morgan Minyard, Maina Martir-Torres, Claudia Rojas, Matt McCoy, Emily Fleming, Yonghua Luo, Carolina Yanez-Prieto, I couldn’t have done this without you. To all my friends who have been there to share the ups and downs of it all, I am deeply indebted to you. To my wonderful family, you have been my rock, this one is for you.
To mom and dad who have sacrificed so much so that the dreams of their children could come true, and to Jim and Norma Helderson without whose generosity this feat would not have been possible
CHAPTER 1
LITERATURE REVIEW AND RESEARCH BACKGROUND

1.1. ACID MINE DRAINAGE FROM COAL MINING

Coal mining in the Appalachian coal belt has been a common practice since the 1800’s. The Appalachian coal belt spans several states including Pennsylvania, West Virginia, Kentucky, Tennessee and Ohio (Figure 1.1). The increased demand for coal during the periods of settlement and industrial development in the United States made it almost inevitable that the abundant coal deposits in Appalachia would be mined extensively. By the 1900s it is estimated that up to 30 million tons of coal was being mined in the Appalachian region on an annual basis (Zinc et al., 2005). Today, most of these mines have been abandoned. However, the environmental effects of decades of mining are still being felt in terms of vast areas of unproductive abandoned mined lands. In Pennsylvania alone, up to 250, 000 acres of abandoned mine lands exist (PA DEP, 1999).

Figure 1.1. The Appalachian coal mining region of the USA showing known mines past and present. This region spans several states including Pennsylvania, Ohio, West Virginia and Kentucky (Zinc et al., 2005)
One of the most serious environmental impacts of abandoned mines has been acid mine drainage (AMD) from underground mine networks or runoff from strip mines. Acid mine drainage is generated when sulfide minerals in underground coal seams or exposed rocks come into contact with air and water. As long as the sulfide minerals are buried underground or contained within the rock matrix, they pose no problem to the environment. However, once they are disturbed by mining operations they are exposed to atmospheric conditions (air and water) and thereby undergo oxidation, which generates acidity, sulfates and Fe(II) ions (Stumm and Morgan, 1981).

Although coal ores contain various sulfide minerals including chalcopyrite (CuFeS$_2$), arsenopyrite (FeAsS), and marcasite (orthorhombic pyrite), cubic pyrite (FeS$_2$) is the predominant sulfide mineral implicated in AMD generation (Gagliano and Bigham, 2002; Singer and Stumm, 1970; Madigan and Martinko, 2006). The acidity generated from the oxidation of pyrite can also subsequently leach out other heavy metals into solution, such as Cu, Pb, Zn, and Cd. For that reason, acid mine drainage is characterized by low pH and high concentrations of dissolved metals and sulfates which have been associated with serious environmental pollution in soil and aquatic habitats.

Environmental problems from AMD begin when acidic, soluble, metal-rich effluents encounter neutral stream waters leading to the rapid oxidation and hydrolysis of metals that are soluble at acidic pH but insoluble at higher pH. Upon oxidation and hydrolysis, the metals precipitate out as metal (oxy) hydroxides, thereby coating stream beds, discoloring stream water, and reducing the availability of $O_2$ to aquatic life (Figure 1.2). As AMD flows across land, it covers the soil with acidic precipitates, creating creates bare stretches of land which support little or no vegetation (AMD kill zones).
With more than 2,500 miles of streams, AMD is the single greatest cause of pollution to water ways of Pennsylvania. It is estimated that up to 2500 miles of stream in PA are impacted by AMD (PA DEP, 1999).

![Map showing impact of AMD on aquatic habitats in the Appalachian region.](image)

**Figure 1.2**: Impact of AMD on aquatic habitats in the Appalachian region. The grey regions represent the Appalachian Coal Belt while the red areas represent streams without fish due to the effects of AMD (Zinc et al., 2005).

1.2. ACID MINE DRAINAGE GENESIS

Pyrite in exposed rocks creates most of the problems associated with AMD. Although a single reaction is often used to describe the pyrite dissolution process (eq.1.1), pyrite dissolution actually takes place in several stages as described in equations 1.1-1.5 below (Gagliano and Bingham, 2002).
Typically the dissolution reaction is initiated by the presence of oxygen and water. To begin with, FeS$_2$ is oxidized to generate Fe(II), sulfate and hydrogen ions (eq. 1.2). At the neutral pH values and dissolved oxygen concentrations of most surface waters, Fe(II) generated in equation 2 is rapidly oxidized by oxygen to form Fe(III). Subsequently, if the pH of the AMD remains above 4, Fe(III) hydrolyses rapidly to yield Fe(OH)$_3$ (eq. 1.4). Fe(OH)$_3$ is responsible for the yellow color of the precipitate (yellow boy) seen in streams impacted by AMD.

During the dissolution of pyrite, four equivalents of acidity are generated for every mole of iron pyrite (Stumm and Morgan, 1981). Half of the acidity is generated during the oxidation of the sulfide in FeS$_2$ to sulfate (eq. 1.2), while the other half is
generated during the oxidation and hydrolysis of Fe(II) (equation 1.3 and 1.4). If the
acidity generated during the two reactions exceeds the system’s buffering capacity, the
pH of the system decreases. Typically, most AMD discharges have been found to be
between pH 2-4. However, lower pH values have been reported in slightly high-
temperature environments that support thermophilic microorganisms (Baker et al., 2006,

Below pH 3, the solubility of Fe(III) increases and thus another mechanism of
pyrite oxidation, whereby Fe(III) serves as a primary oxidant for pyrite (Figure 1.2),
becomes important (eq 5.1) (Gagliano and Bigham, 2002). Equation 5.1 illustrates how
Fe(III) in solution is reduced by pyrite to generate Fe(II), additional protons and Fe(II)
ions. Fe(III) has been shown to be a more effective pyrite oxidant than oxygen since the
rate of pyrite oxidation by Fe(III) is more rapid than oxidation by oxygen (Neculita et al.,
2007). Therefore, this reaction makes a significantly larger contribution to large-scale
AMD generation than the oxidation of pyrite by oxygen from air (Gagliano and Bigham,
2002, Stumm and Morgan, 1981). For example, the oxidation of one mole of pyrite by
Fe(III) generates 16 moles of H\(^+\) as opposed to 2 moles of H\(^+\) by oxygen (Stumm and
Morgan, 1981). As a result, dissolution of pyrite by Fe(III) creates a positive feedback
cycle that leads to constant acidification of acid mine waters. The rates of dissolution
depend on a number of factors including rock and mineral type, water chemistry and
temperature (Baker and Banfield, 2003).
Figure 1.3: Pyrite dissolution at acidic and neutral pH. At acidic pH (below pH 4), the rates of pyrite dissolution are primarily controlled by Fe(III). However, above pH 4, the rates of dissolution are controlled by oxygen (Rimstidt, 2004)

1.3. FE(II) OXIDATION: THE RATE LIMITING REACTION

As described in eq. 1.1-1.5, there are several steps involved in the formation of AMD. However, the oxidation of Fe(II) (eq.1.3) seems to exert the greatest effect on the kinetics of AMD generation. Although the hydrolysis of Fe(III) (eq. 1.4) has been shown to occur rapidly even at pH as low as 2.8 (Singer and Stumm, 1970), the oxidation of Fe(II) to Fe(III) is highly dependent on pH. Therefore, the oxidation of Fe(II) to Fe(III) (eq. 1.2) is the rate-limiting reaction during AMD generation. For example, the oxidation of Fe(II) is rapid at pH>5 but quite slow at pH 3 as shown in Figure 1.3.

According to Stumm and Lee (1961), the rate law for the oxygenation of Fe(II) at pH>5.5 is first order with respect to oxygen and Fe(II) and second order with respect to OH⁻. For a constant partial pressure of oxygen, the rate law can be simplified to
Rate = \(-\frac{d\ ln\ [\text{Fe}^{2+}]}{dt}\) = \(k'\ [\text{OH}^-]\)

Where \(k'\) = rate constant = \(8 (+/- 2.5) \times 10^{13}\) liter\(^2\)/(atm-min-mole\(^2\))

According to Snoeyink and Jenkins (1980), a graph of this rate law (log rate against pH) would yield a straight line with a slope of 2, intersecting the x-axis at pH 2.9 (Figure 1.4). This would indicate that the rate of Fe(II) oxidation is slow below pH 2.9. However, this is contrary to what has been observed in the field in AMD environments where Fe(II) is oxidized relatively fast below pH 2.9 to form Fe(III). The explanation for this is that in field environments, the \textit{abiotic} processes described eq. 1.1-1.5 and shown in the graph in Figure 1.3 are accompanied by \textit{biotic} processes carried out by acid-tolerant microorganisms capable of catalyzing Fe(II) oxidation at pH below 2.9 (Figure 1.5).
Figure 1.4. Rate of oxidation of Fe(II) by oxygen. The continuous line represents experimental points obtained from a study by Singer and Stumm, (1970). The circles and squares represent values when exposed to light and when the experiment was run in the dark, respectively (Snoeyink and Jenkins, 1980).

Indigenous acidophilic microorganisms have been implicated in the oxidation of Fe(II) to Fe(III) at low pH, where they are thought to increase the Fe(II) oxidation rates as much as 6 fold (Singer and Stumm, 1970). This explains why rapid Fe oxidation can occur in AMD environments with low pH.
Figure 1.5. Abiotic vs biotic Fe(II) oxidation. At pH>4, Fe(II) oxidation is dependent on pH and occurs rapidly. Below pH 4, Fe(II) oxidation is independent of pH and primarily controlled by acidophilic microorganisms such as *Acidithiobacillus ferrooxidans*. (Rimstidt, 2004)
1.4. ACIDOPHILIC MICROORGANISMS INDIGENOUS TO AMD

Acidithiobacillus ferrooxidans and Leptospirillum ferrooxidans are the most commonly studied Fe(II) oxidizing bacteria in AMD environments. A. ferrooxidans was first described by Temple and Colmer (1951) as Thiobacillus ferrooxidans and later reclassified by Kelly and Wood (2000). A. ferrooxidans is a rod-shaped, gram negative, chemolithoautotroph with optimum activity at pH 2.5 and 30-35 °C (Kelly and Wood, 2000). L. ferrooxidans was first identified in 1972 as a small, curved, rod-shaped bacterium. It is aerobic, gram negative, chemolithoautotrophic and mesophilic with optimum activity at pH 2.5-3.0 (Hippe, 2000). Although both organisms are ubiquitous in AMD environments, L. ferrooxidans is thought to have a higher tolerance for warmer temperatures compared to A. ferrooxidans while A. ferrooxidans is thought to be tolerant of lower pHs (Kelly and Wood, 2000).

Various strains of A. ferrooxidans have been described in the literature (Karavaiko et al., 2003; Qing-ni et al., 2007). Most exhibit similar phenotypic traits; however they show some genetic heterogeneity based on 16S rRNA genes and DNA:DNA hybridization studies (Karavaiko et al., 2003; Qing-ni et al., 2007). Studies done using different A. ferrooxidans strains recovered from different AMD impacted regions have shown that these strains tend to cluster into four different genomovars (groups based on genome similarity) (Karavaiko et al, 2003), whereas three phylogenetic clusters were identified based on 16S rRNA sequences (Karavaiko et al, 2003) and intergenic spacer (ISR) regions (Qing-ni et al., 2007).
Although *A. ferrooxidans* and *L. ferrooxidans* are the most widely recognized iron oxidizers, other groups of Bacteria and Archaea (discussed below) have also been recovered from AMD environments (Johnson, 1998; Baker and Banfield, 2003).

1.4.1 Characterizing acidophilic prokaryotic microorganisms

According to Johnson (1998), one of the most useful ways to characterize acidophilic microorganisms is on the basis of their temperature regimes. Lower temperatures (<40°C, mesophilic) tend to be dominated by gram negative, rod shaped bacteria (Johnson, 1998). Moderately thermophilic (40-60°C) and extremely thermophilic (>60°C) temperatures tend to be dominated by gram positive bacteria and archaea (Euryarchaeota), respectively (Johnson, 1998, Baker and Banfield, 2003). Johnson (1998) pointed out that chemolithoautotrophic bacteria such as *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* are indeed the most studied bacteria from mesophilic AMD systems.

Prokaryotes that are physiologically suited to acidophilic environments in general have also been characterized extensively based on their 16S rRNA relationships. The 16S rRNA approach involves using highly conserved 16S ribosomal RNA gene sequences to determine community composition as well as species richness. Due to the harsh conditions in most AMD-impacted environments, fewer phylotypes tend to be recovered from AMD environments compared to other terrestrial environments (Baker and Banfield, 2003; Johnson, 1998). As a result, it is thought that organisms from a relatively few selected phylogenetic groups account for the most important biogeochemical processes taking place in acidophilic environments. Different bacterial phylotypes have
been detected from AMD environments either by culturing or by culture independent techniques. These phylotypes include actinobacteria, nitrospira, firmicutes and actinobacteria and various divisions within the Proteobacteria, (Johnson, 1998; Baker and Banfield, 2003). Baker and Banfield (2003) report that only a few putative phylogenetic divisions have been recovered from AMD environments (Table 1.1). This contrasts with more pH-neutral soil environments, where representatives of 36 divisions of bacteria alone are commonly detected (Hugenholtz et al., 1998).

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Lineages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinobacteria</td>
<td>Acidimicrobium ferrooxidans,</td>
</tr>
<tr>
<td></td>
<td>Ferromicrobium acidophilus</td>
</tr>
<tr>
<td>Acidobacteria</td>
<td>Acidobacterium capsulatum</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Sulfo bacterillus disulfidoxidans</td>
</tr>
<tr>
<td></td>
<td>Sulfo bacterillus acidophilus</td>
</tr>
<tr>
<td>β-proteobacteria</td>
<td>Thiomonas spp.</td>
</tr>
<tr>
<td>Nitrospira</td>
<td>Leptospirillum (groups 1-111)</td>
</tr>
<tr>
<td>α-proteobacteria</td>
<td>Acidisphaera rubrifaciens</td>
</tr>
<tr>
<td>δ-proteobacteria</td>
<td>IM clones</td>
</tr>
<tr>
<td>α-proteobacteria</td>
<td>Acidiphilum spp.</td>
</tr>
<tr>
<td>γ-proteobacteria</td>
<td>Acidothiobacilli</td>
</tr>
</tbody>
</table>

Table 1.1. Phyla that have been recovered from acidophilic environments. Table is based on Baker and Banfield, 2003.

The most commonly studied iron-oxidizing bacteria in AMD are the chemolithotrophs *A. ferrooxidans* and *L. ferrooxidans*, which belong to the γ-proteobacteria (phylum Proteobacteria) and nitrospira (phylum Nitrospira), respectively. However, other iron oxidizers e.g *Ferromicrobium acidophilus* and *Acidimicrobium ferrooxidans* (phylum Actinobacteria) have also been recovered from AMD environments (Johnson, 1998; Baker and Banfield, 2003). So far, archaea detected in
AMD sediments are limited to the orders of Thermoplasmatales (within the phylum Euryarchaeota) and Sulfolobales (within the phylum Crenarchaeota) (Johnson, 1998; Baker and Banfield, 2003). Out of these, *Ferroplasma acidiphilum* Ferroplasma *acidarmanus* (in Thermoplasmales) and *Metallosphaera sedula* (in Sulfolobales) are known to oxidize Fe(II) (Baker and Banfield, 2003).

Perhaps the most characterized AMD system is located within the Richmond Mine at Iron Mountain near Redding, California. This site is slightly thermophilic, with temperatures ranging from 30°C to 50°C during the year. The site is also extremely acidic (pH of AMD effluent ranges between 0.5 and 1.0). Studies by Bond et al. (2000b) and Druschel et al., (2004) characterized microbial diversity at this site and found that the most dominant 16S rRNA genes recovered were related to *Leptospirillum* species while other sequences clustered with Archaea (Thermoplasmales) and *Acidimicrobium* (some closely related to *Ferromicrobium acidophilus*). However, they also detected sequences affiliated with the delta subdivision of the Proteobacteria (which would be gram negative). This would indicate that even though specific acidophiles tend to occur in certain temperatures and acidic regimes, there is still much work to be done when it comes to understanding the diversity and niches of prokaryotes in various acidophilic environments.

1.4.2. Physiology of commonly studied acidophilic microorganisms

While AMD environments have been documented to contain a limited supply of organic substrates and nutrients, they are still able to support several species that are physiologically adapted to these conditions. As mentioned above, most AMD systems are characteristically low in organic inputs and high in inorganic substrates (reduced metals).
This is another reason why, besides extreme pH, AMD environments tend to have less genetically diverse microbial communities than other materials, like surface soils, which have more organic matter (Johnson, 1998, Baker and Banfield, 2003).

Although various genera of bacteria and archaea have been implicated in Fe(II) oxidation, chemolithotrophic bacteria and archaea that derive their energy from oxidizing Fe(II) to Fe(III) and their cellular carbon from CO₂ are considered to be the most abundant in AMD systems (Madigan and Martinko, 2006). Specifically, *A. ferrooxidans* and *L. ferrooxidans* are the most studied organisms with these physiological traits. Other inhabitants of AMD environments include heterotrophs and phototrophs. Both phototrophs and chemolithotrophs may play significant roles during primary production in AMD environments (Johnson, 1998).

Heterotrophic microorganisms compete for oxygen, which may create conditions that are more favorable for microaerophilic Fe(II) oxidizers. Some heterotrophs may also oxidize Fe(II) to Fe(III) directly and consume dissolved organic carbon which may be toxic to some autotrophic Fe(II) oxidizers (Johnson, 1998). Indeed studies have shown that mixed cultures containing heterotrophic as well as autotrophic iron oxidizers are more efficient at pyrite dissolution than pure cultures (Norris, 1990). Carbon dioxide fixation in chemolithotrophic bacteria such as *A. ferrooxidans* and *Leptospirillum ferrooxidans* is thought to occur via the Calvin reductive pentose phosphate cycle, using the enzyme ribulose 1,5-biphosphate carboxylase (RuBPCase or Rubisco) (Tyson et al, 2004). It has been suggested that when *A. ferrooxidans* is growing on ferrous iron it can adapt to CO₂ limitations by increasing the concentration of CO₂ fixation enzymes (Rawlings, 2005). *Acidithiobacillus ferrooxidans* has also been shown to possess several
nitrogen fixation genes indicating that it is capable of fixing atmospheric nitrogen (Rawlings and Kusano, 1994 and references therein). However, because the energy requirement for nitrogen fixation is large and the fact that nitrogenase activity is limited by the presence of oxygen, this process is thought to occur on a limited basis (Rawlings, 2005; Rawlings and Kusano, 1994). _Leptospirillum ferrooxidans_ has also been found to possess nitrogen fixing genes nifHDK and the nif operon (Norris et al, 1995; Parro and Moreno-Paz, 2003; Parro and Moreno-Paz, 2004). However, a metagenomic study on the genome of Leptospirillum type II by Tyson et al. (2004) did not yield any nitrogen-fixing genes.

The oxidation of Fe(II) to Fe(III) or the oxidation of inorganic sulfur to sulfuric acid are the major energy yielding processes in _A. ferrooxidans_ under oxic conditions. However, under limited oxygen conditions, ferric iron also can be used as an electron acceptor (Rawlings and Kusano, 1994). Studies have shown that several iron oxidizers including _Acidithiobacillus ferrooxidans_ and _Thiobacillus thioxidans_ and _Acidimicrobium ferooxidans_ can couple the oxidation of organic or inorganic electron donors to the reduction of ferric iron (Pronk et al., 1992; Brock and Gustafson, 1976).

The oxidation of Fe(II) to Fe(III) does not generate substantial amounts of energy for growth (Madigan and Martinko, 2006). Thus, chemolithotrophic microorganisms such as _A. ferrooxidans_ must oxidize large amounts of Fe(II) in order to generate enough energy for CO₂ fixation and growth. The redox potential for the Fe(III)/Fe(II) redox pair is relatively high (+770mV at pH 2) (Madigan and Martinko, 2006). Thus, oxygen is the most thermodynamically suitable terminal electron acceptor for this organism (Johnson, 1998, Madigan and Martinko, 2006).
1.4.3. CO\textsubscript{2} and O\textsubscript{2} optimum growth requirements

Since \textit{A. ferrooxidans} is a chemolithoautotroph, CO\textsubscript{2} is necessary for optimum growth. Given that CO\textsubscript{2} has very little solubility at low-pH, it can be a limitation for growth in \textit{A. ferrooxidans} (Nemati et al., 1998). For cultures sparged with CO\textsubscript{2}, several studies reviewed in Nemati et al. (1998) have suggested that the optimum CO\textsubscript{2} range for growth is between 5\% and 8\%. Increasing CO\textsubscript{2} above 8\% resulted in growth inhibition of the cultures (Barron and Luecking, 1990). A study by McDonald and Clark (1970) testing the minimum CO\textsubscript{2} requirement for growth of \textit{A. ferrooxidans} found that sparged gas ranging from 0.03\% (ambient air) to 10\% was not limiting for growth. Other studies reviewed in Nemati et al., (1998) have looked at the minimum oxygen requirement for growth of \textit{A. ferrooxidans}. Oxygen was only found to be limiting when cultures were grown with dissolved O\textsubscript{2} concentrations less than 0.2 mg/L. Typical, non-limiting growth conditions involve growing \textit{A. ferrooxidans} cultures in 500 ml flasks containing 200 ml broth shaken at 240 rpm and incubated at 30\degree C in ambient O\textsubscript{2} conditions (Nemati et al., 1998).

1.4.4. Iron oxidizing mechanisms in bacteria

In most iron-oxidizing bacteria, iron oxidation is thought to be mediated by several proteins including cytochromes which are part of the ATP-generating electron transport chain (Madigan and Martinko, 2006). With the exception of \textit{A. ferrooxidans}, the enzymes involved in iron oxidation pathways in acidophilic bacteria remain unknown.

In \textit{A. ferrooxidans}, before the entire mechanism of iron oxidation can be completed, ferrous iron must be oxidized outside the cell membrane at low pH (pH 2.0),
followed by the reduction of oxygen inside the cell at much higher pH (pH 6.5) (Nemati et al., 1998). A proton motive force resulting from the difference in pH between the periplasm and the cell membrane and the difference in the charge (positively charged periplasm vs negatively charged cytosol), facilitates the movement of electrons from the cell surface (after oxidation of ferrous iron) to the terminal electron acceptor (Oxygen) (Nemati et al., 1998). It is estimated that *A. ferrooxidans* has as many as 12 different cytochrome c proteins (Yarzabal et al., 2004), as well as a variety of cytochrome oxidases which are thought to play different functions depending on whether iron or sulfur is being oxidized (Brasseur et al, 2004).

Several components thought to play an integral role in the electron transport chain of *A. ferrooxidans* have been recognized (Rawlings and Kusano, 1994; Brasseur et al., 2004). Of these, a blue copper protein (rusticyanin, encoded by the gene *rus*) and the enzyme Fe(II) oxidase (encoded by the gene *iro*) have been suggested as the most likely integral components in the iron oxidation pathway (Kusano et al., 1992, Cox and Boxer, 1978; Rawlings and Kusano, 2004, Ramirez et al., 2004).

1.4.5. Iro: its homology with the high-redox potential iron-sulfur proteins (HiPIP)

Fe(II) oxidase (Iro) has been shown to have a high level of homology to the high-redox potential iron-sulfur proteins (HIPIP) (Kusano et al., 1992; Bruscella et al., 2005; Bruscer et al., 1997). High-redox potential iron-sulfur proteins are common in purple photosynthetic bacteria whereby they act as electron transporters between the bc1 reaction center (encoded by the *petII* operon) (Levican et al., 2002) and a terminal
oxidase (Bonora et al., 1999; Pereira et al., 1999). For this reason, Iro has also been referred to as HIPIP in some studies.

Some of the purple photosynthetic bacteria known to possess HiPIPs include *Allochromatium vinosum* (Bruser et al., 1997), *Rhodocyclus tenuis* and *Rhodopila globiformis* (Bruscella et al., 2005). Kusano et al. (1992) presented an alignment of the Iro and HiPIP protein sequences to determine the degree of homology (%) between Iro and HiPIP in several purple photosynthetic bacteria. Their study found that the Iro protein of *A. ferrooxidans* had the highest homology to the HiPIP protein of the bacteria *Rhodocyclus tenuis* 2741 (55.8%) and *Rhodocyclus tenuis* 3761 (50.0%) (Figure 1.6).

The highlighted regions associated with the [4Fe-4S] cluster in Figure 1.6 represent four cysteine residues that are thought to be conserved in the HiPIPs (Kusano et al., 1992). These cysteine residues are thought to bind a single, redox-active, four-iron-four-sulfur cluster [4Fe-4S] that is found in HiPIPs (Kusano et al., 1992).

![Figure 1.6 Iro protein alignment from *A. ferrooxidans* and several purple non-sulfur bacteria that contain the HiPIP. The dots represent HiPIP residues that are homologous to the *A. ferrooxidans* Iro residues (top bar). Three of the bars (joined together) highlight the cysteine residues supposedly used to bind the four iron four sulfurs cluster. The other three bars represent other residues that are conserved in Iro and HiPIP (Kusano et al., 1992).](image-url)
Other studies have also compared the homology of Iro and HiPIP sequences (Bruser et al., 1997; Bruscella et al., 2005). Table 1.2 summarizes the percent homology between Iro in \textit{A. ferrooxidans} and HiPIP in different purple non-sulfur bacteria reported in various studies.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Type of sequence</th>
<th>% homology</th>
<th>study</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Rhodocyclus tenuis} 2741</td>
<td>protein</td>
<td>55.8</td>
<td>Kusano et al., 1992</td>
</tr>
<tr>
<td>\textit{Rhodococcus tenuis} 3761</td>
<td>protein</td>
<td>50.0</td>
<td>Kusano et al., 1992</td>
</tr>
<tr>
<td>\textit{Paracoccus sp}</td>
<td>protein</td>
<td>25</td>
<td>Kusano et al., 1992</td>
</tr>
<tr>
<td>\textit{Rhodopseudomonas gelatinosa}</td>
<td>protein</td>
<td>36.5</td>
<td>Kusano et al., 1992</td>
</tr>
<tr>
<td>\textit{Thioploca pfennigii}</td>
<td>protein</td>
<td>23.1</td>
<td>Kusano et al., 1992</td>
</tr>
<tr>
<td>\textit{Chromatium gracile}</td>
<td>protein</td>
<td>25</td>
<td>Kusano et al., 1992</td>
</tr>
<tr>
<td>\textit{Thioploca roseopersicina}</td>
<td>protein</td>
<td>30.8</td>
<td>Kusano et al., 1992</td>
</tr>
<tr>
<td>\textit{Allochromatium vinosium}</td>
<td>protein</td>
<td>32.7</td>
<td>Kusano et al., 1992</td>
</tr>
<tr>
<td>\textit{Rhodopila globiformis}</td>
<td>protein</td>
<td>67.2</td>
<td>Bruscella et al., 2005</td>
</tr>
</tbody>
</table>

Table 1.2. Summary of the % homology between Iro in \textit{A. ferrooxidans} and HiPIP in various purple non-sulfur bacteria reported in the literature.

1.4.6 Suggested iron-oxidizing pathways for \textit{A. ferrooxidans} and \textit{L. ferrooxidans}

Different configurations of pathways for electron flow in \textit{A. ferrooxidans} have been proposed. Some studies have suggested that Iro is the first electron acceptor in the
electron transport chain whereby it is linked to cytochrome c552 (Fukimori et al., 1988; Yamanaka and Fukimori, 1995) (Figure 1.7). However, recent studies by Brasseur et al. (2004), Bruscella et al. (2005) and Bruscella et al., (2007) have suggested a different pathway whereby Iro serves as an electron shuttle linking the bc₁ electron transfer complex (encoded by the petA2B2C2 gene cluster) with the terminal reductase (Figure 1.8). The petA2B2C2 gene cluster is part of the petII operon which encodes a cytochrome (cycA2), a short chain dehydrogenase (sdrA1) and Iro(Hip) (Figure 1.9) (Bruscella et al., 2007).

The iron oxidation pathway in *L. ferrooxidans* still remains to be deciphered. Using genome data, Banfield and co workers (2004) proposed genes coding for components of an electron transport chain in *L. ferrooxidans*. However, they did not identify genes with any homology to those in *A. ferrooxidans* which encode rusticyanin or iron oxidase (Tyson et al., 2004), suggesting that *L. ferrooxidans* does not contain either *iro* or *rus* genes.
Figure 1.7. Proposed electron transport chain as suggested by Yamanaka et al., (1998) and Yamanaka and Fukimori (1995) where Fe(II) oxidase (Iro) coupled to c552 (c) serves as the first electron acceptor. In this Figure, Cu represents rusticyanin while a, a3,CuA, CuB are likely components of a terminal oxidase. Fo and F1 are the membrane integral and membrane-associated portions of ATP synthase (Rawlings and Kusano, 1994)
Figure 1.8. Iron oxidation pathway as suggested by Bruscella et al., 2007. According to this study, *A. ferrooxidans* has two functional bc1 complexes. The two complexes could play different roles during the oxidation of iron and sulfur.

Figure 1.9 The petII operon of *A. ferrooxidans* showing the genes encoding the bc1 gene cluster (petA2B2C2). The *iro(hip)* gene also referred to as hip is located downstream of the petA2B2C2 gene cluster in this operon (Bruscella et al., 2007).
1.4.7 Expression of *iro* and *rus* genes in growth studies

Monitoring the level of expression or gene copy number of iron oxidizing bacteria in batch incubations can be a way to determine how their abundance changes under different growth conditions. Therefore, genes such as *iro* and *rus*, which encode important enzymes involved in iron oxidizing oxidation, can serve as a tool to track changes under different growth conditions in iron-oxidizing bacteria which possess these genes.

It is known that the expression of proteins involved in iron oxidation by such acidophilic bacteria as *A. ferrooxidans* can vary depending on the amount of substrate present (Ramirez et al, 2004). The expression of rusticyanin protein has been shown to be five fold higher when *A. ferrooxidans* is grown in the presence of available iron (Yarzabal et al., 2003). Bruscella et al. (2007) found that the petII gene cluster (containing the Iro gene) was transcribed when cells were grown in iron as well as sulfur. Interestingly, they found that *iro* transcription was higher during growth in sulfur medium than in iron medium. This raises the possibility that *iro* could also be involved in electron transfer during sulfur oxidation.

1.5. MITIGATING ACID MINE DRAINAGE

Several approaches have been put in place to remediate AMD-impacted sites depending on the speed of flow and the severity of AMD. Acid mine drainage remediation approaches include active systems, which involve incorporation of alkaline
chemicals, and passive systems, which involve diversion of flow into wetlands or limestone beds to add alkalinity and cause metal oxides (Fe, Al, and Mn) to precipitate. The following is a brief review of active and passive treatment systems for mitigating AMD.

1.5.1. Active treatment systems

Active treatment systems are recommended for severe and high flow AMD effluents (Zinc et al., 2005). They rely on the addition of strong bases such as calcium oxide (pebble quick lime) to neutralize the acidity of AMD (Zinc et al., 2005, Kirby et al., 1999, Johnson, 1995). The rise in pH causes rapid oxidation and precipitation of Fe(II) as well as other metals in the AMD. As a rule, active methods are very effective at bringing about neutralization of acid waters. In the longer term, however, Johnson (1995) suggests that the effectiveness of an active system depends on how fast the complete oxidation and precipitation of Fe(II) from the AMD waters occurs once neutralization is initialized.

For complete oxidation and neutralization of Fe(II) to occur at neutral pH, sufficient amounts of oxygen must be present in the AMD system. In most AMD systems the amount of dissolved oxygen present is not enough to meet the oxygen demand required for complete oxidation (Hustwit et al, 1992). Therefore, ferrous iron still in solution might still be carried further downstream where it undergoes oxidation to generate more acidity, thus prolonging the AMD problem. In such situations, the addition of an aeration procedure or a chemical oxidant becomes important to facilitate complete oxidation and precipitation of Fe(II).
Figure 1.10 (Zinc et al., 2005) is an example of an active system whereby calcium oxide is being added directly to the stream flow. Calcium oxide is stored in a silo connected to a wheel. As the water flows downstream, it powers the wheel to dispense the pebble quick lime into the flow. As the water is routed downstream, metal precipitates are generated. Typically near the silo facility, settling or separation ponds are created to collect the sludge generated after neutralization. Once the AMD reaches the settling ponds, sludge is removed and the treated water is released into streams. Although active methods are effective at neutralizing acidity, they often involve high running costs and result in bulky precipitates that require constant removal (Johnson, 1995).
1.5.2 Passive treatment systems

Passive remediation approaches have been proposed as more cost-effective ways of mitigating acid mine drainage. They have mostly been suggested for use in AMD systems with medium to slow AMD flow (Zinc et al., 2005). Since they do not involve high start up costs, passive treatment systems are easier to maintain. Almost entirely, passive remediation systems utilize chemical strategies (limestone beds) and/or biological treatment (wetlands) to bring about alkalinity and decrease metal concentrations (Johnson, 1995; Neculita et al., 2007).

A typical passive system involves the passage of AMD waters over a series of settling ponds or wetlands (Figure 1.11) that may or may not contain limestone. The alkalinity generated by limestone or by microbial activity (sulfate reducers) neutralizes the acidity of the mine drainage, facilitating metal oxides to precipitate out of solution (Neculita et al., 2007).

Although passive systems have been found to be helpful in neutralizing AMD waters, their effectiveness can be limited by various factors. For example in passive systems the efficiency of metal removal depends on the microbial pathways taking place and the organic substrates present (Neculita et al., 2007). When neutralization is primarily dependent on limestone to neutralize and precipitate metals from AMD, coating of limestone surfaces by metal precipitates can interfere with the release of alkalinity and reduce the effectiveness of this approach. This is common in limestone-lined or wetland-based passive remediation approaches, where rapid precipitation of Fe(III) oxides at neutral pH on solid surfaces causes the phenomenon known as “armoring”. Therefore, for
limestone beds to become a successful AMD treatment option, the complete oxidation of Fe(II) to Fe(III) and subsequent precipitation of Fe hydroxides prior to neutralization (at low pH) must be achieved (Gazea et al, 1996).

As discussed earlier, the kinetics of Fe(II) oxidation and precipitation at low pH are very slow unless catalyzed by acidophilic microorganisms. Based on this, removal of Fe(II) from solution by biological low-pH oxidation would appear to be an effective way to catalyze iron removal from AMD and reduce armoring on limestone surfaces.
Figure 1.11: Different types of passive remediation strategies (Skousen, 1998)
1.6. SITE DESCRIPTIONS

Personnel from the Pennsylvania Department of Environmental Protection, Bureau of Abandoned Mineland Reclamation (BAMR) Cambria office, Clearfield County, have identified two AMD discharges exhibiting contrasting rates of Fe(II) removal. Site 1 (Gum Boot) is located in McKean County (41° 41’ 02” N; 78° 29’ 37” W). Site 2 (Fridays-2) is located in Clearfield County (41° 14’ 34” N; 78° 32’ 28” W) (Figure 1.12).

Figure 1.12: Map of PA showing locations of the Gum Boot (A) and Fridays-2 (B) discharge.

1.6.1 Site 1 (Gum Boot)

At the Gum Boot mine, acid mine discharge emanates from the top of a hill and flows downwards for a distance of ~127 m before entering an adjacent creek (Gum Boot Run). For the first 15 m, the discharge at the Gum Boot site flows as a thin sheet of fluid (~5cm deep) over a large build-up of AMD sediments (~ 1 m depth), which has a hard surface crust of iron oxide precipitates (Figure 1.13a). At about 15 m downhill, the
discharge then flows underground and then reemerges as a series of channels that flow further downhill into Gum Boot Run (Figure 1.13b).

The initial discharge from the underground seep at Gum Boot mine contains very little dissolved oxygen and has a flow rate of 50 l min$^{-1}$. However, it is quickly oxygenated within the first 2 m of flow. Within the first 15 m after emergence, 50 mg l$^{-1}$ is removed from solution and a concurrent pH decrease is observed with the removal (Figure 1.15). This would suggest that Fe(II) is being removed by being oxidized and hydrolyzed, causing it to fall out of solution (this reaction is accompanied by a decrease in pH, as shown in eq. 1.4.) At the pH at which the oxidative precipitation of Fe(II) is occurring ($< 4$), the kinetics of abiotic Fe(II) oxidation are not favored (Singer and Stumm, 1970). Thus it is presumed that microbially mediated Fe(II) oxidation is catalyzing this process. Indeed a study by Senko et al. (2008) found that bio-oxidative precipitation of Fe(II) was occurring at the Gum Boot location.

Senko and co workers’ study involved collecting sediment samples at discrete locations along the flow path of the Gum Boot discharge (Figure 1.13b) and then measuring iron oxidation rates and numbers of culturable iron oxidizing bacteria in the sediments. Their study found a strong correlation between culturable iron oxidizers and rates of Fe(II) removal, supporting the earlier hypothesis that oxidative precipitation is microbially mediated (Figure 1.15).
1.6.2. Site 2 (Fridays-2)

The discharge at the Fridays-2 mine emerges at a flow rate of 136 l min\(^{-1}\) and flows as a 5 cm-thick sheet in a manner similar to flow at the Gum Boot site. The mine effluent flows for a distance of ~10 m before entering into the adjacent creek (Figure 1.14a&b). Although the two discharges (Gum Boot and Fridays-2) have been identified to have similar source water characteristics (flow rates, mineralogy) (Table 1.3), little Fe(II) is oxidatively removed at the Fridays-2 site (Figure 1.15).
Figure 1.14 a & b: Picture of the Fridays-2 site (left panel, a) and a schematic representing how the site appears (right panel, b). Discrete sampling locations at each site have been marked with an X on the schematic.
Figure 1.15: Summary of characterization of water chemistry, abundance of culturable Fe(II) oxidizing bacteria (Fe(II)OB), and microbial activities from discrete sampling points at the Gum Boot and Fridays-2 AMD sites. Dissolved Fe(II) concentrations (□) are shown in panels A and E; pH (□) and dissolved oxygen concentrations (○) are shown in panels B and F; numbers of culturable Fe(II)OB (●) (as indicated by colony forming units CFU) are shown in C and G; first order rate constants (k) of Fe(II) oxidation (■) observed in sediment incubations and starting (♦) and ending (◇) pH of the incubations are shown in panels D and H. In the Fridays-2 panels, upstream and downstream sampling points in the unnamed creek are pointed out using white arrows and black arrows, respectively. Error bars represent one standard deviation (Senko et al., 2008).
<table>
<thead>
<tr>
<th></th>
<th>High-rate site (Gum Boot)</th>
<th>Low-rate site (Fridays-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al (µM)</td>
<td>52</td>
<td>4</td>
</tr>
<tr>
<td>Ca (µM)</td>
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<td>232</td>
</tr>
<tr>
<td>K (µM)</td>
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<td>18</td>
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<tr>
<td>Mg (µM)</td>
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<td>103</td>
</tr>
<tr>
<td>Mn (µM)</td>
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<td>BDL</td>
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<tr>
<td>Na (µM)</td>
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</tr>
<tr>
<td>Si (µM)</td>
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<td>129</td>
</tr>
<tr>
<td>Fe(II) (µM)</td>
<td>869</td>
<td>1150</td>
</tr>
<tr>
<td>Fe(III) (µM)</td>
<td>197</td>
<td>142</td>
</tr>
<tr>
<td>SO₄²⁻ (µM)</td>
<td>987</td>
<td>3961</td>
</tr>
<tr>
<td>DO. (µM)</td>
<td>BDL</td>
<td>BDL</td>
</tr>
<tr>
<td>pH</td>
<td>4.10</td>
<td>4.50</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Flow rate (l/min)</td>
<td>50</td>
<td>136</td>
</tr>
</tbody>
</table>

Table 1.3. Dissolved constituents and physical characteristics of emergent AMD at the Gum Boot site and at the Fridays-2. BDL = below detection limit. (Senko et al., 2008).
The Gum Boot site: potential as a natural remediation site

The work done by Senko et al., (2008) at the Gum Boot (high rate of Fe(II) oxidation) and Fridays-2 (low-rate) sites suggests that the former may be responsible for natural AMD remediation whereby Fe(II) is being removed from solution without any human intervention. If indeed the Gum Boot site is supporting natural remediation, the biogeochemical processes taking place there could be applied at other sites with similar discharges but lower rates of removal.

We need to understand the microbial diversity and interactions taking place at the Gum Boot site so as to understand the dynamics of how the microbially mediated Fe(II) removal can be stimulated at other sites. Molecular studies by Senko et al (2008) have characterized bacterial communities at the two sites using Ribosomal RNA Intergenic Spacer Analysis (RISA) based on variable lengths between the 16S rRNA and the 23S rRNA genes (Castillo-Gonzalez and Bruns, 2005). Their study found that there were discrete locations which exhibited the highest rates of Fe(II) removal and culturable iron oxidizing bacteria counts (Figure 1.15). These locations also had relatively few RISA bands compared to locations upstream or downstream of the AMD flow and exhibited lower Fe(II) iron oxidation rates (Figure 1.16). This indicates that bacterial diversity at these two AMD sediments is lower than at sites not impacted by mine drainage as found in other similar studies (Baker and Banfield, 2003).

The clone libraries generated from AMD sediments at the two sites by Senko et al. (2008) indicated that phylotypes affiliated with actinobacteria, chloroflexi, acidobacteria, firmicutes, nitrospirae, proteobacteria and candidate divisions TM7 and WS6 were recovered from these two locations. Phylotypes affiliated with actinobacteria
were more abundant at the Fridays-2 (low-rate) site while phylotypes affiliated with chloroflexi were more abundant at the Gum Boot (high-rate) site. Senko and co-workers noted that the abundance of chloroflexi at the high-rate site was surprising since are no known iron-oxidizers in the phylum chloroflexi.

Figure 1.16. Ribosomal RNA intergenic spacer analysis (RISA) (top panels) and division-level distribution of 16S rDNA sequences observed in clone libraries recovered from the RISA gel (bottom panel) of microbial communities present at discrete sampling points in the Gum Boot and Fridays-2 systems (Senko et al, 2008)

1.6.4. Applying the knowledge from Gum Boot to other AMD-impacted sites

The ability for acidophilic microorganisms to oxidize Fe(II) at low pH could be utilized to accelerate Fe(II) removal from AMD discharges with similar characteristics as the Gum Boot mine prior to treatment passive systems. Particularly, microbially mediated Fe(II) oxidation could be used to stimulate complete oxidation and precipitation
of Fe(II) at low pH where it tends to precipitate on limestone surfaces leading to clogging and reduction of the neutralization capacity of the limestone.

Bio-oxidative precipitation of Fe(II) at low-pH would occur as a two-step process. First, Fe(II) would be oxidized microbially to Fe(III) at low pH, then the resulting Fe(III) would be hydrolyzed to form iron (oxy)hydroxides (insoluble precipitates). Hydrolysis of Fe(III) is said to occur even at pH as low as 2.8 (Singer and Stumm, 1970). The Fe(II)-free effluent could then be neutralized with limestone with less potential for armoring, thus maintaining the capacity of the limestone to neutralize acidic waters for longer periods of time.

To exploit the activity of indigenous acidophilic communities to catalyze Fe(II) oxidation and precipitation, it is important to determine which microorganisms are responsible for microbially mediated low-pH iron oxidation and the factors that promote or control their activity in field and in lab settings. AMD-dwelling communities derive their energy for carbon fixation and growth from a variety of sources including organic (heterotrophic), light (phototrophic) and inorganic sources (chemolithoautotrophic) (Madigan and Martinko, 2006; Sylvia et al, 1999). Since microbial metabolic requirements are different for chemolithoautotrophs vs heterotrophs, acidophiles might respond differently after addition of carbon sources or other electron acceptors such as nitrate. As a result, the kinetics of biological low-pH oxidation could vary after such substrates are added. It is thus important to identify the populations responsible for Fe(II) oxidation at the two AMD sites in order to establish which conditions or solid phase amendments might stimulate increased biological low-pH Fe oxidation.

1.6.5 Approaches used to characterize AMD prokaryotes
Both culture-dependent and culture-independent approaches have been used to characterize microbial populations in low-pH environments. Culture-dependent techniques are effective but only on organisms that can be easily grown in liquid or solid media (less than 1% of global genetic diversity) (Amann et al., 1990; Hugenholtz and Pace, 1996). Culture-independent techniques targeting highly conserved 16S rRNA genes have helped identify other microorganisms that cannot be cultured by traditional approaches. Even so, culture-independent techniques, e.g. 16S rRNA based approaches which use universal PCR primers for highly conserved regions, might only recover genes from populations that have no mismatches to the "universal" primers (Janssen, 2006). Studies have shown that clone libraries generated using universal 16S rRNA primers do not capture accurately the abundance of biogeochemically important populations that are perhaps serve critical functions in the environment (Janssen, 2006).

In our case, in the entire clone library generated by Senko et al. (2008) (Figure 1.16), none of the sequences were affiliated with Acidithiobacillales, the order that includes \textit{A. ferrooxidans}. Interestingly, Senko et al. (2008) were able to obtain \textit{A. ferrooxidans} from the AMD sediments by using enumeration plates of acidic mineral media. This suggests that recognized groups of acidophilic iron-oxidizing bacteria are present at these two sites but that their rRNA genes may have mismatches with the RISA primers, thus producing a bias against amplifying genes from these biogeochemically important bacteria.

Functional gene assays have been used to study microbes involved in other biogeochemically important pathways such as nitrification. The main goal of such approaches is usually to capturing more fully the populations that occur in relative small
proportions relative to other dominant populations. For that reason, assessing functional
genes would be the most appropriate approach for studies seeking to establish a
quantitative link between function and diversity in an environment.

In this study an approach based on functional genes for iron oxidation will be
used to evaluate the presence of known iron oxidizers in AMD sediments from these two
sites. Specifically, PCR primers will be developed from conserved regions in iro genes
from different A. ferrooxidans strains obtained from GENBANK. PCR amplification of
microbial community DNA using these primers will enable assessment of changes in the
abundance of iro gene copies in sediments incubated under different growth conditions.
Although the gene iro is not found in all iron-oxidizing bacteria, we hypothesize that it
will serve as quantitative estimate of the relatively small but biogeochemically important
populations (A. ferrooxidans and close relatives) in the context of other more numerically
dominant populations.

1.7. OBJECTIVES

This study is part of a larger interdisciplinary research project investigating the
potential for acidophilic microorganisms to improve biological low-pH iron removal in
AMD systems prior to passive neutralization treatment with limestone. The goals of this
project are to understand the biogeochemical processes that are taking place at the Gum
Boot and Fridays-2 sites and to determine why iron is being removed more efficiently at
the Gum Boot site. Understanding factors which lead to faster Fe(II) removal at Gum
Boot could then be applied to other sites with similar mine discharges.

To learn whether different rates of Fe(II) removal at Gum Boot and Fridays-2 are
associated with differences in microbial community composition and activity, I will
apply a functional gene approach for evaluating communities in AMD sediments.

Although many studies have used 16S rRNA gene diversity to characterize microbial community DNA obtained from environmental samples, rRNA-based approaches are capable of amplifying microorganisms from diverse taxa, many of which may not be involved in iron oxidation. A more specific approach for studying AMD communities, therefore, is to target functional genes known to be involved in iron oxidation pathways. As discussed earlier, *A. ferrooxidans* is the only iron oxidizer that has a characterized pathway for electron transport chain during iron oxidation. A previous study, which indicated that *A. ferrooxidans* can be cultured from Gum Boot sediments, supports the use of this functional gene in characterizing these AMD communities.

In this study, my first task was to determine whether the functional gene for *Iro* could be detected in AMD sediments. Although analysis of microbial RNA would be the preferred way to study activity and gene expression of *iro*, environmental RNA is highly unstable and difficult to work with. As a first step in evaluating the use of the *Iro* functional marker, therefore, I focused on quantifying the numbers of *iro* gene copies in the microbial community DNA extracted from AMD sediments. Chapter 2 of this thesis describes the development of a PCR-based assay for the *iro* gene of *Acidithiobacillus* spp.

To confirm the utility of the functional gene marker *iro* in understanding how variable environmental conditions in AMD influence *Acidithiobacillus* populations, copy numbers of *iro* genes were measured in sediments from batch reactor experiments conducted by Melanie Lucas during her M.S. research in Civil and Environmental Engineering at Penn State (Lucas, 2008). My findings on *iro* gene copy changes in the
batch reactors are described in Chapter 3 of this thesis. Since microbial groups with different metabolic needs (e.g. chemolithotrophs or heterotrophs) can inhabit acidophilic environments, different gas mixes containing varied concentrations of CO₂ and O₂ were used in batch reactor studies. Lower than ambient O₂ and higher than ambient CO₂, were used to assess the contribution of autotrophic communities at the two sites. Furthermore, since it is not known how sediment communities would respond to organic amendments, sediments were incubated in batch reactors sediments with glucose at ambient air conditions. These latter experiments were conducted to learn more about the contribution of heterotrophic acidophilic populations to iron oxidation in these sediments. This study therefore used the \textit{iro} gene as a functional marker for abundance of \textit{Acidithiobacillus ferrooxidans} in incubation experiments containing variable gas head spaces to mimic environmental variations in a field setting.

Thus this study will accomplish two specific objectives:

1. To design a functional gene assay for monitoring numbers of bacteria possessing the \textit{iro} gene, which is involved in iron oxidation pathway in \textit{A. ferrooxidans} and related organisms (Chapter 2). For this part of the study, primers specific to the enzyme iron oxidase (encoded by the gene \textit{iro}) will be designed from gene sequences obtained from different strains of \textit{Acidithiobacillus ferrooxidans}. The primers obtained will be tested using strain \textit{Acidithiobacillus ferrooxidans ATCC# 55720}, as well as cultures of other iron and sulfur oxidizers.
To use the *iro* gene assay on batch reactor sediments incubated under different conditions using Q-PCR to determine AMD community responses to varied levels of O$_2$, CO$_2$, and glucose (Chapter 3).


correctly processed and translocated in *Escherichia coli*, in spite of the periplasm pH difference between these two micro-organisms. *Microbiology, 15*: 1421–1431.


Rimstidt J.D. (December 2004). Factors controlling rates of pyrite weathering.


CHAPTER 2

A PCR-BASED ASSAY FOR IRON OXIDASE GENES OF Acidithiobacillus spp.

1. INTRODUCTION

Acid mine drainage (AMD) environments are characterized by low concentrations of dissolved organic carbon, high acidity and high concentrations of dissolved metals (Johnson, 1998). As expected from the relatively few recognized species capable of using reduced Fe and S as energy sources, these environments do not support a diverse range of microorganisms when compared to other terrestrial systems (Baker and Banfield, 2003).

The chemolithotrophic iron-oxidizer Acidithiobacillus ferrooxidans is the most extensively studied AMD microbe. This bacterium can thrive in AMD environments because of its chemolithotrophic abilities, obtaining cellular carbon from atmospheric CO₂ and electrons for energy from reduced forms of S and Fe (Ingledew, 1982). The processes of energy generation occurs in electron transport pathways associated with the cell membrane and periplasm, with O₂ as the terminal electron acceptor. The oxidation of Fe(II) to Fe(III), which is considered to take place outside the cell (Ingledew, 1982), is mediated by several cytochromes and proteins that have been described in various studies (Blake and Shute, 1994; Appia-Ayme et al., 1998; Appia-Ayme et al., 1999; Yamanaka and Fukimori, 1995; Fukimori et al., 1988; Bruscella et al., 2005; Bruscella et al., 2007)

The pathways by which these cytochromes and proteins interact, and the mechanisms by which they transfer electrons from Fe(II) oxidation to the terminal
electron acceptor ($O_2$) are subjects of current studies by several groups. Fukimori et al. (1988) and Yamanaka et al. (1995) have suggested a pathway whereby Fe(II) oxidation takes place in the periplasm with the enzyme Iro (bound to cytochrome c552) as the first electron acceptor (Figure 1.7). Fukumori et al., (1988) found that Iro readily reduced cytochrome c552 in the presence of Fe(II) ions under acidic conditions suggesting that it was acting as an iron oxidase enzyme. Other workers (Bruscella et al., 2005; Bruscella et al. 2007) have proposed a different location for Iro in the electron transport chain of A. ferrooxidans, whereby it acts as an electron transporter between the bc1 reaction center (encoded by the petII operon) (Levican et al., 2002) and a terminal oxidase, as suggested for other high redox potential iron-sulfur proteins, or “HiPIPs” (Bonora et al., 1999; Pereira et al., 1999). Such a location for Iro in the periplasm is supported by studies of the iro gene in A. ferrooxidans ATCC 19859, ATCC 23270 (type strain) and ATCC 33020, where it was located immediately after petC2 (Figure 1.9). Since the petC2 gene encodes cytochrome c1 from the second A. ferrooxidans bc1 complex located in the periplasm (Bruscella et al., 2005; Brasseur et al., 2002), this suggests that Iro also is located in the periplasm. In contrast, studies of iro genes found in two other strains of A. ferrooxidans, strain Fe-1 in Kusano et al., (1992) and strain BRGM (Cavazza et al. (1995), have reported that iro was located immediately after the purA gene in a different operon, rather than petC2.

Although Iro has been implicated in the iron oxidation pathway of A. ferrooxidans strains commonly detected in AMD sediments, no study has reported on efforts to detect iro or rus genes in AMD sediments. Banfield and coworkers, who used metagenomics to propose genes that could encode components of an electron transport chain in L.
*ferrooxidans*, did not identify any genes with homology to rusticyanin or iron oxidase in *A. ferrooxidans* (Tyson et al., 2004). Furthermore, it is not known if other bacteria associated with AMD contain the *iro* genes. This study will design and test oligonucleotide probes for a functional gene assay for the gene *iro* with the purpose of using it as a DNA marker for the presence of *Acidithiobacillus* spp. or other prokaryotes possessing this gene in environmental samples.

2. MATERIALS AND METHODS

2.1. Primer design for the *iro* gene

Sequences of five *A. ferrooxidans* strains (accession nos. ATCC 33020 (AJ32026), strain Tf49 (AJ621389), ATCC 19859 (AJ621387), strain BRGM1 (AJ621388) strain CC1 (AJ621386) and one *iro* sequence belonging to the type strain ATCC 23270, whose genome has been completely sequenced, was downloaded from the TIGR Genome database ([http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi](http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi)) (TIGR locus: AFE_iro_0373). These sequences were aligned using Clustal X ver. 1.83 software (Thompson et al., 1997) to determine the most highly conserved regions (Figure 2.2.1). Three primers were designed using the Primer 3 software (Rozen and Skalesky, 2000) from the conserved regions with each primer targeting a region with a GC content of 48-50 % and a Tm of about 55-60. Two other primer sequences were obtained from the report by Bruscella et al., 2007.

Numbering for the primers was based on the sequence of the *iro* locus (1378 bp partial *petC2* region) (Table 2.1) of strain ATCC 33020, which was sequenced and annotated by Bruscella et al. (2005) as part of the *petII* operon. The *petII* operon, which contains the *iro* gene, is thought to contain several genes which are co-transcribed
together. These genes include a *petABC* gene cluster, a *cycA2* encoding a cytochrome C, a *sdrA2* encoding a putative short chain dehydrogenase, and a *HiPIP*, also referred to as *iro* (Figure 2.2) (Bruscella et al., 2005). The *petII* operon sequence also was used in the present study to design primers complementary to each region flanking the *iro* gene. Use of these latter primers resulted in amplicon length between 700-825 bp long (Table 2.1).

2.2 Primer testing

The newly designed primers were initially tested with genomic DNA from cultures of *A. ferrooxidans* ATCC 55720 to ensure that amplicons of appropriate length and sequence were obtained. Once amplification with *A. ferrooxidans* genomic DNA was validated, selected primer pairs were used to test for the presence of the *iro* gene in genomic DNAs extracted from cultures known to oxidize Fe or S. These included *L. ferrooxidans* ATCC 53992; *Rhodocyclus tenuis* ATCC 25093; *Sulfobacillus acidophilus* ATCC 20253; *Allochromatium vinosum* ATCC 35206; *Acidothiobacillus albertensis* ATCC 35403; *Ferritrophicum radicola* BAA-1016; *Sideroxydans, paludicola* BAA-1017; *Sideroxydans lithotrophicus* BAA-1018; and *Acidithiobacillus thioxidans* ATCC 19377. Other sources of DNA which were tested included raw acid mine drainage sediments that had been collected from the surface (0-5 cm) at two AMD discharges described in section 1.6; colonies plated from AMD sediments on Fe(II)-enrichment medium (Senko et al., 2008); and batch reactors slurries of incubated AMD sediments described in section 2.3.
2.3. Culture growth conditions

The bacterial strains listed above were obtained from ATCC and grown in the media recommended for each strain. *Acidithiobacillus ferrooxidans* ATCC 55720 and *L. ferrooxidans* ATCC 53992 were cultured at 25°C in ATCC Medium 2039 (pH 2.3) containing per L 0.8 g of (NH₄)₂SO₄; 0.4 g of K₂HPO₄; 20 g of FeSO₄. 7H₂O; 2.0 g of MgSO₄.7H₂O supplemented with 5 mL Wolfe’s mineral solution (ATCC catalog no. MD-TMS). *Rhodocyclus tenuis* ATCC 25093 was cultured anaerobically at 25°C in ATCC medium 550 R 8 A H (pH 6.9) containing per L 2.5g Malic acid; 1.0 g Yeast Extract; 1.25 g (NH₄)₂SO₄; 0.2 g MgSO₄. 7H₂O; 0.07 g CaCl₂. 2H₂O; 0.01 g Ferric citrate; 0.02 g EDTA; 0.6g KH₂PO₄; 0.9 K₂HPO₄, supplemented with 7.5 mL Wolfe’s mineral solution (ATCC catalog no. MD-TMS) and 1 mL Trace element solution containing per 100 mL 0.3 g Ferric citrate; 0.002 g MnSO₄. H₂O; 0.001 g H₂BO₃; 0.001 g CuSO₄. 5H₂O; 0.02 g (NH₄)₆Mo₇O₂₄. 4H₂O; 0.001 g ZnSO₄; 0.05 g EDTA; and 0.02 g CaCl₂. 2H₂O. *Sulfbacillus acidophilus* ATCC 200253 was cultured at 45°C in ATCC Medium 2045 (pH 2.0) containing per L 0.5 g MgSO₄. 7H₂O; 0.4 g (NH₄)₂SO₄; 0.2 g K₂HPO₄; 0.1 g KCl; 0.25 g Yeast Extract; and 10 mg FeSO₄. 7H₂O. *Allochromatium vinosium* ATCC 35206 was cultured anaerobically at 25°C in ATCC Medium 1370 (pH 2.0) containing per L 1.0 g K₂HPO₄; 0.5 g MgSO₄; 10.0 g yeast extract; and 25 g NaCl. *Acidothiobacillus albertensis* ATCC 35403 was cultured at 25°C in ATCC Medium 1353 (pH 4.5) containing 5.0 g NaSO₃. 5H₂O; 3.0 g KH₂PO₄; 0.4 g (NH₄)₂SO₄; 0.5 g MgSO₄. 7H₂O; 0.25 g CaCl₂. 2H₂O; and 10.0mg FeSO₄. 7H₂O. *Ferritrophicum radicola* BAA-1016, *Sideroxydans, paludicola* BAA-1017 and *Sideroxydans lithotrophicus* BAA-1018 were cultured at 25°C in ATCC Medium 7266, or Modified Wolfe’s Mineral Medium.
(MWMM) gradient plate medium which contained two layers of medium. The over layer of each plate contained 16 mL MWMM, 5mM NaHCO$_3$; 16 uL ATCC trace minerals (ATCC catalog no. MD-TMS); and 16 uL of ATCC trace vitamins (ATCC catalog no. MD-VS). The under layer of each plate contained 4.25 mL MWMM; 42.5 mL FeS; and 1.2% high melt agarose. The MWMM was prepared to contain per L 1.0 g NH$_4$Cl; 0.2 g MgSO$_4$. 7H$_2$O; 0.10 g CaCl$_2$. 2H$_2$O; and 0.05 g K$_2$HPO$_4$. *Acidithiobacillus thioxidans* ATCC 19377 was grown in ATCC Medium 125 containing per L 0.2 g (NH$_4$)$_2$SO$_4$; 0.5 g MgSO$_4$. 7H$_2$O; 0.25 g CaCl$_2$; 3.0 g KH$_2$PO$_4$; and 5.0 mg FeSO$_4$.

2.4. DNA extraction from ATCC cultures

Cell growth was determined visually based on the presence of turbidity in the cultures. For DNA extraction, five mL of medium containing cell material was collected and centrifuged for three minutes at 10 000 rpm at room temperature to obtain a pellet. DNA was extracted directly from the cell pellet using the MoBio Power Soil DNA Extraction kit (MoBio Laboratories, Inc., Carlsbad, CA).

2.5. DNA extraction from AMD sediments, colonies and batch reactor slurries

DNA was extracted from 0.5g of fresh AMD sediment using the MoBio Power Soil DNA Extraction kit (MoBio Laboratories, Inc., Carlsbad, CA). Genomic DNA was also obtained from colonies picked from plates containing iron-oxidizing enumeration medium described in Senko et al., (2008). These colonies had been plated from an iron-oxidizing enrichment culture that had been previously inoculated with AMD sediment and maintained through ten transfers. To obtain genomic DNA, growth from a single, well-isolated colony was suspended in 20 µL of water. Two µL of this mixture were used for DNA extraction with a microLysis PCR-Ready DNA kit (The Gel Company, San
Francisco, CA) according to manufacturer’s instructions. To obtain DNA from the batch reactor slurries, 10 mL of slurry was obtained from batch reactor incubations that contained AMD sediments incubated in artificial AMD water (SAMD) described by Silverman and Lundgren (1964) (see Table 1 of Appendix A). Concentrated AMD solids containing cells (pellet) were harvested from the 10 mL samples by centrifugation at 10,000 g for three minutes. Genomic DNA was extracted directly from the entire pellet using the MoBio Power Soil DNA Kit. All the DNA samples obtained were stored at -20°C pending further analysis with PCR.

2.6. Testing of iro-specific primers using PCR

Primers identified in this study were tested using DNA from A. ferrooxidans ATCC 55720 to identify the optimal primer combinations. Suitable primer pairs were selected and used to test for the presence of the iro in the in the different DNA sources (cultures, AMD sediments, colonies and batch reactor slurries). Before amplification with iro primers, DNA templates from different sources were first amplified with bacteria-specific primers based on Escherichia coli positions 16S-27f (5’-AGAGTTTGATCMTGGCTCAG-3’) and 1492r (5’-TACGGYTACCTTGTTACGACTT-3’) (Lane, 1991) to ensure that the DNA was amplifiable (i.e., no interference with PCR).

PCR amplification mixtures for both the 16S rRNA and iro primers contained 1 µl of genomic DNA; 5 µl of 10x HotMaster PCR buffer with 25 mM MgCl₂ (Eppendorf Corp., Westbury, NY); 1 µl of 10 mM dNTPs; 2 µl (each) of 10 mM primer; 0.5 µl of 50 mg/ml bovine serum albumin; 0.25 µl of 5 u/µl HotMaster Taq polymerase (Eppendorf Corp., Westbury, NY); and 38.25 µl of molecular biology grade water. PCR cycling was
carried out in a 2400 Perkin-Elmer thermocycler. The cycling conditions consisted of an initial denaturation step for 5 min at 94°C and 35 cycles of 94°C for 0.5 min, 54°C for 0.5 min, and 72°C for 1 min, followed by a final extension step at 72°C for 7 min. After PCR completion and checking for correct *iro* amplicon length by agarose gel electrophoresis, fresh amplicons were directly cloned into TOPO-TA vector (Invitrogen) following the manufacturer’s instructions. DNA sequencing of cloned inserts was performed in both directions at The Pennsylvania State University’s DNA sequencing facility using an ABI Hitachi 3730XL DNA Analyzer.

2.7. Phylogenetic analysis

The sequences obtained from the cloned amplicons were subjected to BLAST searches ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)) (Altschul et al., 1997) to determine their similarities to published *iro* gene sequences. Sequences from this study along with several other sequences related to the *iro* gene (obtained from GenBank) were downloaded into a MEGA 4.1 software environment (Tamura et al., 2007) and aligned within the MEGA environment using the ClustalW algorithm (Thompson et al., 1994). Evolutionary distance trees (neighbor joining algorithm) also were produced using MEGA 4.1. The GenBank sequence for the *HiPIP* gene from *Allochromatium vinosium* was used an out-group. The *iro* gene sequences obtained in this study have been deposited under GenBank accession numbers X to Y.
3. RESULTS

3.1. Testing *iro* gene-specific primers on different sources of DNA

All the primers tested in this study, including those designed for the flanking regions (Table 2.1), were found to work equally well when tested on genomic DNA from *A. ferrooxidans* strain ATCC 55720. Amplicons generated in this study were between 100 and 315 bp long (Table 2.1) Primer pair Iro_for1-Iro_rev1 was used to test for the presence of the *iro* gene in the different sources of DNA (cultures, AMD sediments, colonies and batch reactor slurries). DNA from AMD sediments, colonies and batch reactor slurries yielded PCR amplicons of correct size and sequence when tested with these *iro* primers (Figure 2.3). Genomic DNA obtained from all cultures amplified with the 16S rRNA bacterial primers 27f and 1492r. However, DNA from *A. ferrooxidans* cultures was the only genomic DNA to amplify with *iro* gene primers (iro_for1-iro_rev1). No DNA from cultures other than *A. ferrooxidans* amplified with the *iro* primers, even after another (nested) PCR (Figure 2.4).
**Figure 2.1:** Alignment of the *iro* gene from several *A. ferrooxidans* strains ATCC 33020 (AJ32026), strain TF49 (AJ621389), ATCC 19859 (AJ621387), strain BRGM1 (AJ621388), strain CC1 (AJ621386) and strain ATCC 23270 (AJ320262) generated using *Clustal X* ver 1.83 showing primers that were designed in this study. Primers *iro_for1* and *iro_for2* overlap slightly; only one arrow has been used to represent them. Conserved nucleotide positions are marked with asterisks at the bottom.
Table 2.1. *iro* gene oligonucleotide primers tested in this study. Numbering is based on the partial *petII* operon sequence of Bruscella et al (2005) (Figure 2.2). Primer pair *iro_for1-* *iro_rev1* (213 bp) was used to amplify the DNA sources used in this study. Primers with the ‘flk’ notation were designed using the sequence for the partial *petII* operon to amplify the region flanking the *iro* gene.

<table>
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<th>Numbering position</th>
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<tr>
<td><em>iro_for1</em></td>
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<td>578-598</td>
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<td><em>iro_rev1</em></td>
<td>5'-ACCACCTTACAGCAGCATTCCG-3'</td>
<td>770-790</td>
<td>This study</td>
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<tr>
<td><em>iro_rev2</em></td>
<td>5'-GCCGGCATCGGCCTGAAC-3'</td>
<td>826-844</td>
<td>(Bruscella et al., 2007)</td>
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<tr>
<td><em>iro_rev3</em></td>
<td>5'-ACCTTACAGCAGCTCCGGGGC-3'</td>
<td>872-892</td>
<td>(Bruscella et al., 2007)</td>
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<tr>
<td><em>iro_flk_1f</em></td>
<td>5'-GGACGAATCTCCCCAGAAAT-3'</td>
<td>260-280</td>
<td>This study</td>
</tr>
<tr>
<td><em>iro_flk_2f</em></td>
<td>5'-GGCCTATTTTCTCAAGAAGG-3'</td>
<td>409-426</td>
<td>This study</td>
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<td><em>Iro_flk_1r</em></td>
<td>5'-ATGGTGTGGCCGATGTAC-3'</td>
<td>1040-1060</td>
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<td><em>Iro_flk_2r</em></td>
<td>5'-GACCTGAAATCAACCTTCGC-3'</td>
<td>1062-1082</td>
<td>This study</td>
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Designation
Nucleotide sequence
Numbering position
Reference

**petC2**

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<td>5'-GGACGAATCTCCCCAGAAAT-3'</td>
<td>260-280</td>
<td>This study</td>
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**Iro_flk_2f**

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<td>This study</td>
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**Iro_flk_2r**

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<th>Numbering position</th>
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<td><em>Iro_flk_2r</em></td>
<td>5'-GACCTGAAATCAACCTTCGC-3'</td>
<td>1062-1082</td>
<td>This study</td>
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</tbody>
</table>
Figure 2.2. DNA sequence of the *iro* locus of strain ATCC 33020 showing nucleotides and proteins encoded by the *petC2* gene, the *iro* gene and the putative downstream regions adapted from the annotated sequence reported in Bruscella et al., 2005. Oligonucleotides used in this study have been underlined. Primers labeled with the ‘iro_flk’ notation were designed for developing a plasmid for Q-PCR.
Figure 2.3. Genomic DNA from different DNA sources amplified with 16S rRNA bacterial primers 27f and 1492r (top panel) and with the iro gene primer pair iro_for1 and iro_rev1 (bottom panel). Lane 1: DNA marker, Lane 2, enrichment (GB-E3), Lane 3, direct AMD (GB-1m), Lane 4: direct AMD (FR5), Lane 5: batch sediment (FR5 gas mix 4), Lane 6: enrichment (GB-E3), Lane 7: negative control.

Figure 2.4. Genomic DNA from different cultures were amplified with 16S rRNA bacterial primers 27f and 1492r (top panel) and also tested with the iro gene primer pair iro_for1 and iro_rev1 (bottom panel). Except for A. ferrooxidans (lane 9, bottom panel), these genomic DNAs did not amplify with the iro primers. Lane 1: DNA Marker, lane 2: negative control, lane 3: Allochromatium vinosum ATCC 35206 lane 4: Ferritrophicum radicola BAA-1016, lane 5: Sideroxydans, paludicola BAA-1017, lane 6: Sideroxydans lithotrophicus BAA-1018, lane 7: L. ferrooxidans ATCC 53992, lane 8: Sulfolobacillus acidophilus ATCC 200253, lane 9: A. ferrooxidans ATCC 55720, lane 10: Rhodocyclus tenuis ATCC 25093 lane 11; Acidithiobacillus thioxidans ATCC 19377 lane 12: Acidithiobacillus albertensis ATCC 35403.
3.2. Phylogenetic analysis

To determine how the *iro* sequences obtained in this study were related to other *iro* sequences found in GenBank, phylogenetic analysis was performed using sequence alignments. The phylogenetic tree revealed 3 major clusters, with the sequences from this study grouping within cluster A (Figure 2.5). Within cluster A, sub-clusters similar to those identified by Karavaiko et al. (2003) and Ni et al. (2007) were found. In cluster A, sequences tended to cluster together according to source. No clustering patterns were observed in terms of sites. Cluster A mainly consisted of sequences related to the *iro* found in *A. ferrooxidans* strains, ATCC 23270 and 33020, which were reported by Bruscella et al. (2005). Cluster B and C consisted of *iro* related to *A. ferrooxidans* strains Fe-1 and BRGM. None of the sequences from amplicons obtained in this study were found in this cluster.
Figure 2.5. Neighbor joining tree showing relationship between *iro* sequences recovered in this study and known HiPIP and *iro* sequences from GenBank. Three major clusters (A, B & C) were identified. The sequences recovered in the present study all clustered within cluster A. *Allochromatium vinosum* (U81381) which also contains the HiPIP was used as an out-group. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Bar represents 0.1 changes per site. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007)
4. DISCUSSION

4.1. Potential use of the *iro* gene as a functional biomarker for iron oxidation

The main objectives of this study were to design PCR primers for the gene *iro* in *A. ferrooxidans* and to test their efficacy on DNA from several AMD sources as well as on genomic DNAs from various cultures of bacteria known to oxidize Fe or S. We designed several primers using conserved regions based on the homology of *iro* genes from several *A. ferrooxidans* strains, namely ATCC 33020 (AJ32026), strain Tf49 (AJ621389), ATCC 19859 (AJ621387), strain BRGM1 (AJ621388), strain CC1 (AJ621386) and ATCC 23270 (AJ320262). We also tested two primers from published sequences. All the primer combinations tested in this study worked equally well when tested on genomic DNA of *A. ferrooxidans* ATCC 55720. Our study has shown that the *iro* primers tested in this study can be used to detect the presence of *iro* genes in DNA obtained from different AMD-related sources (enrichments, direct AMD and batch reactor slurries).

Our phylogenetic analysis revealed that the *iro* sequences obtained in this study clustered with other sequences. Specifically, within the three major clusters that were identified, all our sequences fell within cluster A, which included *iro* sequences from ATCC accessions 23270 and 33020. None of our sequences grouped within clusters B and C (*iro* sequences from strains BGRM and Fe-1). Bruscella et al. (2005) compared the sequence of the mature Iro proteins representing Cluster A with sequences for Fe-1 and BRGM (Cluster B and C) and found that they were only 58.3% similar. Not only do the protein sequences differ, the genome locations of the *iro* genes from Cluster A and from Clusters B/C may differ. In strains Fe-1 and BRGM, *iro* is located downstream from
purA, but in the ATCC strains 23270 and 33020, it is located downstream from the petII operon. Orthologs of the iro present in ATCC23270 and 33020 are also present in strains CC1, BRGM-1, ATCC 19859 (Bruscella et al., 2005). When performing the phylogenetic analysis in this study, iro sequences from other GenBank accessions of A. ferrooxidans (deposited by groups in China and Japan from unpublished studies) also grouped with those of Fe-1 and BRGM (Fig. 2.4).

Interestingly, the iro gene from strain BRGM-1 grouped in Cluster A in our phylogenetic tree, even though this strain was a clonal derivative of BRGM, whose iro gene grouped in Cluster B/C (Bruscella et al., 2005). Bruscella et al. (2005) designed degenerate primers from conserved regions in the two Clusters of iro genes and used these to amplify genomic DNA from strain 23270 strain Fe-1. Although these primers amplified the iro gene in strain ATCC 23270, they failed to amplify the iro gene present in strain Fe-1.

Due to the obvious differences between the Iro in strain Fe-1 and the Iro of strains 23270 and 33020, it is possible that the two Iros are not orthologous and that they encode proteins with different functions. A. ferrooxidans strains have been known to show phylogenetic differences based on 16S rRNA gene regions (Karavaiko et al., 2003 and Ni et al., 2007). For that reason, it is plausible that the differences in the iro sequences represent other phylogenetic differences at the strain level.

The iro gene has been shown to contain high homology with the high potential iron-sulfur proteins (HiPIPs) which are found in purple non-sulfur bacteria (Figure 1.6). In this study, we tested the hypothesis that primers targeting the iro gene would also amplify potential homologs from other bacteria with HiPIP which are known to oxidize
Fe or S. To determine whether the *iro* primers were specific to *A. ferrooxidans*, we tested our primers with genomic DNAs from various cultures reported to contain the HiPIPs (*Allochromatium vinosium* ATCC 35206, *Rhodocyclus tenuis* ATCC 25093), or from cultures known to oxidize Fe(II) or S (*Ferritrophicum radicola* BAA-1016, *Sideroxydans, paludicola* BAA-1017, *Sideroxydans lithotrophicus* BAA-1018, *L. ferrooxidans* ATCC 53992, *Sulfobacillus acidophilus* ATCC 200253, *Acidithiobacillus albertensis* ATCC 35403, *Acidithiobacillus thioxidans* ATCC 19377). No genomic DNAs from these cultures amplified with the *iro* primers pairs *iro_for1*-iro_rev1, even those known to contain a HiPIP (*Allochromatium vinosium* ATCC 35206 and *Rhodocyclus tenuis* ATCC 25093). These two cultures contain a HiPIP with 52.3% and 32.7% similarity to the *iro* in strain 33020 and Fe-1 respectively (Bruscella et al., 2005; Kusano et al., 1992).

Lack of amplification with others cultures which oxidize Fe(II)/S or which have been found in AMD could be an indication that these strains do not contain the *iro* gene. This is consistent with the fact that no study has reported the presence of *iro* or a homologous gene in any of these bacteria. In a metagenomic study of microbial community DNA from an AMD impacted site in Iron Mountain California, no genes with homology to the *iro* were found (Tyson et al., 2005).

Our study suggests that *iro* is a reliable biomarker to assay for some but not all strains of *A. ferrooxidans*, e.g in engineered systems such as commercial bioreactors. Since *iro* was not detected in other AMD bacteria besides *A. ferrooxidans*, it would not be appropriate to use as a general marker for iron-oxidizing populations in heterogeneous AMD communities. Furthermore, although *iro* has been suggested to be involved in the
electron transport pathway of *A. ferrooxidans*, no genetic study has directly linked *iro* to iron oxidation. Bruscella et al. (2007) have suggested that *iro* may be more involved in the sulfur oxidation pathway than in the iron-oxidation pathway. Rusticyanin (encoded by *rus*) may be a more suitable candidate for a functional, “iron-oxidation” biomarker, since its role in iron oxidation is supported by genetic studies (Ramirez et al., 2004, Yarzabal et al., 2003, Yarzabal et al., 2004, Bruscella et al., 2007).

4.2 Prospects for developing a functional gene assay for Rusticyanin (encoded by *rus*)

Attempts were made in this study to develop a functional gene assay for *rus*, which encodes the blue copper protein Rusticyanin. Like the *iro* gene, *rus* has also been thought to be involved in the iron oxidation pathway of *A. ferrooxidans* (Cobley and Haddock, 1975; Cox and Boxer, 1978, Appia-Ayme et al., 1999; Bengrine et al., 1998). In contrast to the *iro* gene, several genetic studies have been done to elucidate the role of *rus* in the iron oxidation pathway of *A. ferrooxidans*. Rus expression has been shown to be higher in cells grown in iron media compared to cells grown in sulfur media (Cobley and Cox, 1983; Cox and Boxer 1978; Ramirez et al., 2004). Specifically, Cox and Boxer (1978) reported that Rus constituted up to 5% of the total soluble protein when *A. ferrooxidans* was grown autotrophically in iron media. Additionally, the gene *rus* and several other genes associated with the *rus* operon were shown to be up-regulated when *A. ferrooxidans* cells were grown in iron media (Yarzabal et al., 2003, Yarzabal et al., 2004; Bruscella et al., 2007). In the latter study by Bruscella et al. (2007), *iro* was shown to be up-regulated during sulfur oxidation but not during Fe(II) oxidation. These studies confirmed that *rus* plays an integral role in the iron-oxidation pathway, and that *rus*
would be a more reliable functional marker than *iro* for iron oxidation activity by *A. ferrooxidans*.

Although *rus* may hold more promise as a functional marker for *A. ferrooxidans* compared to *iro*, our work with the *rus* gene encountered several different technical difficulties, ranging from inconsistent primer amplification, failure to clone the *rus* gene into the TOPO-TA (Invitrogen) vector, and poor amplification of DNA during Q-PCR.

To amplify the *rus* gene in this study, primers for *rus* were designed from the sequence of *A. ferrooxidans* ATCC 23270 and several other published sequences (Sasaki et al., 2003 and Yarzabal et al., 2004). Primers (see table 1 of Appendix A) were then tested on genomic DNA from *A. ferrooxidans* ATCC 55720, but successful amplification was achieved with only four of the eight primer combinations (Rus_3f-5r, 1f-8r, 1f_9r, 3f-1r.) Primer combination Rus_3f-1r was picked to develop a plasmid spanning the region flanking the *rus* gene. After amplifying this primer pair with PCR, we were unable to obtain transformants with the appropriate inserts. Similar difficulties with cloning *rus* were reported by Bengrine et al., 1998. Further work must be done to overcome these difficulties before the *rus* gene is assessed as a valid functional marker.

In summary, our study has indicated that *iro* is not an appropriate functional marker for iron-oxidation by heterogeneous AMD communities. Understanding the correlation between *iro* genes and iron-oxidation rates in AMD sediments, however, could still provide insights on the relative contributions of *iro*-containing *A. ferrooxidans* strains to iron oxidation activity.


Pereira M. M., Carita J. N. and Teixeira M. (1999). Membrane-bound electron transfer chain of the thermohalophilic bacterium *Rhodothermus marinus*: characterization of the iron-sulfur centers from the dehydrogenases and investigation of the high-potential iron-


CHAPTER 3

CHANGES IN COPY NUMBERS OF IRON OXIDASE GENES IN ACID MINE DRAINAGE SEDIMENTS INCUBATED UNDER VARIABLE GROWTH CONDITIONS

1. INTRODUCTION

Acid mine drainage (AMD) is the biggest pollutant of water ways in Pennsylvania (PA-DEP, 1999). It is estimated that more that 2,500 miles of stream and 250,000 acres of abandoned mine lands in Pennsylvania have been impacted by AMD (PA DEP, 1999). Since soluble metals and low pH account for the most detrimental characteristics of AMD, one of the main goals of AMD remediation is to neutralize and remove soluble metals from AMD in a cost-effective manner. Methods for treating AMD are categorized as either active (direct neutralization with strong alkali), or passive (passing AMD effluent over limestone and or wetlands). Passive methods are considered more cost effective than active methods since they require less oversight (Zinc et al., 2005). However, passive systems that involve limestone can have limitations. For example, neutralization of AMD with limestone promotes the rapid precipitation of Fe(III) (hydroxy)oxides which clog limestone surfaces limiting further dissolution. This process is known as ‘armoring’ (PA-DEP, 1999)

An alternative approach to improve the efficacy of passive treatment would be to remove soluble Fe(II) from AMD at low-pH prior to passage of AMD across limestone. Since chemical oxidation of Fe(II) is extremely slow at the pH values of most AMD discharges (2.5-4.5) (Cravotta et al., 1999), biological oxidation is needed to achieve
faster Fe(II) oxidation at low pH. Indigenous AMD microorganisms are capable of oxidizing soluble Fe(II) to Fe(III) at low-pH (<4) (Stumm and Lee, 1961 and Kirby et al., 1999). Hydrolysis (eq. 1.4) and subsequent precipitation of the resulting Fe(III) can then occur even at pHs as low as 2.8 (Stumm and Morgan, 1981). However, little is known about how indigenous Fe(II) oxidizers at AMD sites might respond to different environmental conditions, and greater understanding of the kinetics of biological iron-oxidation is needed.

In a previous study of Fe(II) oxidation during passive AMD treatment, two sites in McKean and Clearfield Counties in northern PA exhibited contrasting rates of Fe(II) removal by oxidation and precipitation (Senko et al., 2008). At the Gum Boot site, so named because treated AMD is discharged into Gum Boot Run, 50 mg/L Fe(II) was removed over a distance of 15 meters, and a concomitant decrease in pH indicated that the Fe(II) removal was microbially mediated. At the Fridays-2 site, no Fe(II) removal was observed over a distance of 10 meters. To understand the microbial and geochemical processes that contributed to these differences, the resident microbial communities and sediment chemical properties at the two sites were characterized (Senko et al., 2008). Despite the observed differences in Fe(II) removal in the field, isolates of *Acidithiobacillus ferrooxidans* were cultured from sediments from both sites. Furthermore, sediments from both sites were incubated in the laboratory and found to be capable of efficient Fe(II) removal. Therefore, the differences in Fe(II) removal from AMD at the two sites were proposed to be due to differences in dissolved oxygen availability along the flow paths.
To understand how environmental factors such as O$_2$ might influence the kinetics of low-pH Fe(II) oxidation at the two sites, a 21-day batch reactor incubation study was carried out with sediments from both sites. Sediments in the reactors were purged with different gas mixtures during incubation, with O$_2$ concentrations ranging from ambient (20%) to 1.1% and CO$_2$ concentrations ranging from 0.003-7.3%. Varied CO$_2$ concentrations were tested because autotrophic bacteria, such as _A. ferrooxidans_, are thought to be primarily responsible for Fe(II) oxidation at low pH. One incubation study also incorporated glucose as a supplement in the reactors to evaluate the possible contribution of heterotrophic populations to Fe(II) oxidation.

As a means of tracking _A. ferrooxidans_ populations in these AMD sediments, a functional gene assay was designed based on the iro gene encoding the iron oxidase enzyme of _A. ferrooxidans_. This gene was a reasonable choice for biomarker development in this study, because _A. ferrooxidans_ could be cultured from both of the AMD sediments. We recognized, however, that the iro gene might not serve as a comprehensive biomarker for Fe(II)-oxidation, because the iro gene is not found in some known Fe(II)-oxidizing bacteria such as _Leptospirillum ferrooxidans_. Nevertheless, we considered iro to be the best functional gene candidate to investigate as an alternative to using 16S rRNA-based approaches. Indeed, most diversity studies of low-pH AMD communities have relied on 16S rRNA (Bond et al., 2000a; Bond et al., 2000b; Nicomrat et al., 2006a; Nicomrat et al., 2006b; Senko et al., 2008). Although 16S rRNA based approaches can be effective at characterizing community diversity, the highly conserved nature of 16S rRNA genes (Lane, 1991; Hugenholtz et al., 1998) can interfere with detection of populations which make up very low proportions of the total community
(Janssen, 2006). Given the high diversity of these AMD communities as revealed by 16S rRNA clone libraries in the study by Senko et al. (2008), we hypothesized that a functional gene assay would be a more sensitive indicator of changes in the small but biogeochemically important populations of *A. ferrooxidans* in these AMD sediments.

In Chapter 2, we developed PCR primers that could amplify the *iro* gene in several strains of *A. ferrooxidans*, as well as in DNA extracted from Gum Boot and Fridays-2 AMD sediments. We also demonstrated that these primers did not yield amplicons from the genomic DNA of several other Fe(II)- and S-oxidizing bacteria. Although these primers were shown to detect *iro* genes from *A. ferrooxidans* in the AMD sediments, we recognized that the correlation between iron-oxidation rates and *iro* genes could be low if microorganisms other than *A. ferrooxidans* were responsible for Fe(II) oxidation in these sediments.

The objective of this study was to evaluate the efficacy of the *iro* gene as functional marker for Fe(II) oxidation in AMD sediments. To our knowledge, this is the first attempt at applying a functional gene assay in AMD sediments undergoing Fe(II) oxidation under varied conditions.

2. MATERIALS AND METHODS

2.1. Site description

Two AMD discharges exhibiting contrasting rates of Fe(II) removal were identified by personnel in the Pennsylvania Department of Environmental Protection, Bureau of Abandoned Mineland Reclamation (BAMR). The Gum Boot site is located in
McKean County (41° 41’ 02” N; 78° 29’ 37” W) while the Fridays-2 site is located in Clearfield County (41° 14’ 34” N; 78° 32’ 28” W) (Figure 1.12).

2.2. Sample collection

Samples of AMD sediments were collected from the surface (0-5 cm) at discrete points along the flow path at each site. Two samples were collected at points that exhibited the highest rates of Fe(II) removal and colony forming units (CFU) (Senko et al., 2008). At the Gum Boot site, this sample was located near the point of AMD emergence from the abandoned mine (2 m downstream) (Figure 3.1). This sample, referred to as “GB2,” was considered as the “high-rate” sample from the Gum Boot site. At the Fridays-2, the “high-rate” sample was taken 10 m downstream from the point of AMD emergence and referred to as “FR5.” Another sample at the Fridays-2 site, “FR2”, was collected near the point of emergence (3 m), where the lowest rates of Fe(II) removal were observed (Figure 3.1). Upon collection, the sediments were transported in dry ice and then stored at -80°C until the time of sediment incubation or microbial DNA extraction.
2.2. Sediment incubations

Sediment incubations were carried out by Melanie Lucas, M.S., Department of Environmental Engineering, Pennsylvania State University (Lucas, 2008). The three sediments were incubated under five different gas mixtures in continuously stirred 1-L HDPE batch reactors containing 25 g fresh sediment in 500 mL synthetic acid mine drainage (SAMD). The SAMD, modified after Silverman and Lundgren (1964), was adjusted to a starting pH of 3.2-3.4, with an initial Fe(II) concentration of approximately 5 mM (medium composition described in Table 1 of Appendix 2). Reactors were wrapped in aluminum foil to exclude light and incubated for 21 days. The headspace above the sediment slurry was sparged constantly with the respective gas mixtures to maintain positive pressure during incubation (see Fig. 3.2, taken from Lucas (2008).
Figure 3.2: Diagram of experimental reactor setup. Reactors were continuously stirred by stir bar and headspace air was constantly purged at positive pressure. SAMD is the synthetic acid mine drainage media used for the incubations (Lucas, 2008).
CO₂ concentrations in these gas mixtures varied from ambient conditions (0.03%) to 7.3%, while O₂ concentrations varied from ambient conditions (21%) to 0.7%. The remainder of each gas mixture consisted of N₂ gas. Two separate reactor trials were run by sparging ambient air in the headspace with and without glucose in the SAMD. Specific gas combinations of O₂, CO₂ and N₂ that constituted the five gas mixes are listed in Table 3.1.

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<tr>
<td>2</td>
<td>1.5 %, 7.3%, 91.2%</td>
</tr>
<tr>
<td>3</td>
<td>10 %, 1%, 89%</td>
</tr>
<tr>
<td>4</td>
<td>Ambient air (21 %, 0.03 %, 79%)</td>
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<tr>
<td>5</td>
<td>Ambient air (21 %, 0.03 %, 79%) with glucose</td>
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</tbody>
</table>

Table 3.1. Gas compositions (O₂, CO₂ and N₂) that constituted the various gas mixes used for the batch reactor incubations.

Abiotic control reactors were prepared for each sediment and gas mixture combination by adding 5 ml of formaldehyde, to track net changes in Fe(II) concentrations under abiotic conditions (Lucas, 2008). Fe(II) and pH were measured at least once daily during incubation. Fe(II) was spiked into the reactors after 7 days and after 14 days to bring up the Fe(II) concentration to initial levels (~5 mM) for a total of 3 Fe(II) additions. The pH was adjusted accordingly to keep the reactors at or above 2.5. At the end of the 21 day incubation period, reactors were shaken to homogenize the slurry prior to taking 10 ml sub-samples for storage at -80°C prior to microbial community DNA extraction. For each gas mixture, DNA was extracted from samples after the 21 day incubation period using Power Soil DNA kit (MoBio Laboratories, Inc., Carlsbad,
CA) as described in section 2.5. DNA samples were stored at -20°C pending further analysis.

In addition to extracting DNA from the incubation slurries, DNA was also obtained from fresh sediments used to inoculate the batch reactor incubations. The procedure used for extracting DNA from fresh AMD sediments has also been described in section 2.5.

Gene copy numbers in this study have been reported based on g/ fresh sediment (each reactor contained 25 g of sediment in 500 mL of SAMD, therefore each 10 mL sub-sample contained 0.5 g of sediment and each MoBio DNA extract generated 100 µL of DNA, but only 1 µL of DNA of each extract was used). Therefore, the final gene copy numbers/ g of wet sediment were as follows:

\[
(q\text{-PCR gene value}) \times (1g / 0.5g) \times (100\mu L /1 \mu L) = \text{gene copy/ g wet sediment}
\]

2.3. Assembly of a plasmid for Q-PCR standard curve

For use in generating Q-PCR standard curves, we constructed a plasmid containing the entire iro gene and its flanking regions, using PCR amplification with primer pair iroflk_1f- 2r (Table 2.1) and genomic DNA from \textit{A. ferrooxidans} ATCC 55720. Plasmid inserts were sequenced in both directions for validation.

2.4. Plasmid copy number determination

The concentration of the resulting plasmid DNA was determined using (Perkin-Elmer Lamda 40 UV/VIS Spectrometer) and the corresponding copy number of the iro gene insert was determined. The equation given below was used to determine the plasmid copy number per µL of plasmid DNA (Whelan et al., 2003). The plasmid was then linearized using PstI restriction enzyme.
Plasmid copy number/µl of DNA =

Size of the plasmid (insert + plasmid) in bps * 330 Da * 2 nucleotides/bp = X g/mole plasmid


\[
(\frac{X}{\text{g/mole plasmid}}) / \text{Avogadro’s constant (6.02 \times 10^{23} \text{ (copy/mole plasmid)})} = \frac{g/\text{plasmid molecule (i.e., copy number)}}{
\]

Number of plasmid copy numbers in a single reaction:

\[
\text{Concentration of plasmid (g/µL)/g/plasmid molecule} = \text{copy number/µL of plasmid}
\]

2.5. Q-PCR amplification

A ten fold serial dilution series of the *iro* linearized plasmid ranging from $10^{-2}$ to $10^{-9}$ copy numbers/µL was created and 1 µL of each dilution was used to create a Q-PCR standard curve. Q-PCR cycling was performed in an Applied Biosystems 7500 Fast Real-Time PCR System thermocycler. Experimental Q-PCR runs consisted of no template control samples (NTC), relative standard curve samples (serial dilution) and unknown DNA samples (experimental DNA). Three replicates were run for each unknown and relative standard sample while eight NTC samples were run for each 96 well plate. 25 µL Q-PCR mixtures were set up using 12.5 µL Power SYBR Green PCR Master Mix, 1.5 µL (each) of 10 mM primer, 1 µL of DNA and 8.5 µL of nuclease-free water. Power SYBR Green PCR Master Mix contained SYBR Green 1 dye, AmpliTaq Gold DNA Polymerase, dNTPs with dUTP, optimized buffer components, MgCl₂ and Passsive Reference (ROX dye). The Q-PCR cycling conditions consisted of three stages. The first stage was an initial denaturation step for 10 min at 95°C. The second stage
consisted of 40 cycles of 95°C for 0.5 min, 56°C for 0.5 min, and 72°C for 1 min. The third stage consisted of a dissociation stage that consisted of one cycle of 95°C for 0.25 min, 60°C for 1 min, 95°C for 0.25 min, and 60°C for 0.25 min. During the Q-PCR run, data collection was done during the third step of the second stage (72°C for 1 min).

2.6. Statistical analysis

_ird_ gene copies obtained from triplicate runs during the Q-PCR experiments were averaged and then analyzed further using ANOVA (SAS Analyst-mixed model procedure) (SAS Institute Inc., Cary, NC). We obtained results from one location from site 1 (GB2) and from 2 locations in site 2 (FR5, FR2). Since we did obtain DNA from all gas mixes in location FR2, our analysis with ANOVA focused on the GB2 and FR5 sediments. _ird_ gene copies obtained from the FR2 location have been reported in Figure 3.1 of Appendix 2.

Two different ANOVA tests were run for this study. The first one was a 2-way (gas x site) ANOVA to determine if the _ird_ gene copies obtained at two sites at the end of the 21-day incubation from the five gas mixes differed. For this test, all effects were considered fixed except the sampling variation. Sampling variation here represents _ird_ gene samples that were obtained from triplicate Q-PCR runs.

The second ANOVA test was done to establish changes in _ird_ gene copies with time over a four point time series (0, 7, 14 and 21 days). For this test, _ird_ gene copies from different time points in gas mixes three (10 % O₂, 1% CO₂, 89% N₂), and five (21 % O₂, 0.03 % CO₂, 79% N₂) were analyzed using 3-way ANOVA (site x gas x time).
Site, gas and time were considered fixed while sampling was considered random. There were no differences in the site x gas x time and the site x time interactions, therefore these components was added to the error term. Since there were no differences between the sites (the two interactions above were not different), samples from similar sites were also pooled together for each gas mix. For this study, we will discuss the increase in each gas mixes independently since there were different starting *iro* gene copy values. Levels of significance were kept at \( p = 0.05 \) for both tests.

To determine the relationship between *iro* gene copies and iron-oxidation rates in the sediments, the correlation between *iro* genes from the GB2 and FR5 locations and the respective iron-oxidation rates, (Figure 3.2)(Lucas, 2008) were determined using SAS software (descriptive statistics). Correlation tests were run for both sites separately and then for the sites combined.

3. RESULTS

3.1. *iro* gene copy numbers from initial sediments

Initial *iro* gene copy numbers from all the three locations were within one order of magnitude of each other (Table 3.2). GB2 location had the most gene copy numbers while FR2 had the least amount. This finding is consistent with what has been reported for culturable iron-oxidizers (CFU) and first order rate constants for iron-oxidation at the two sites by Senko et al., 2008 (Figure 1.15).

<table>
<thead>
<tr>
<th>Sediment</th>
<th>Gene copy number/ g wet sediment</th>
</tr>
</thead>
<tbody>
<tr>
<td>GB2</td>
<td>( 3.97 \times 10^4 )</td>
</tr>
<tr>
<td>FR5</td>
<td>( 3.74 \times 10^4 )</td>
</tr>
<tr>
<td>FR2</td>
<td>( 2.35 \times 10^4 )</td>
</tr>
</tbody>
</table>
3. iro gene copy numbers after 21 day incubation (2-way ANOVA test)

Results from the 2-way ANOVA test on the GB2 and FR5 sediments after the 21-day incubation showed that there were no significant differences in the iro gene copy numbers between the sites and the treatments (gas mixes) except in gas mix 3 (10 %, 1%, 89%) (Figure 3.3).
Figure 3.3: Graphs showing *iro* gene copy numbers from various gas mixes after the 21-day incubation period. Gene copy numbers from the GB2 sediments are shown in the top panel while those from FR5 are shown in the bottom panel. Bars marked an (a) were not found to be significantly differently from each other at $p = 0.05$ while p-values represented with a (b) were different. Error bars represent the standard error of the means. Test of significance was determined using 2-way ANOVA.

3.2 Changes in *iro* gene copy numbers with time (3-way ANOVA test)

Results from the 3-way ANOVA test evaluating changes in gene copy numbers with time in gas mixes 3 and 5, showed that there was no significant increase in gene copy numbers with time when sediments were incubated under gas mix 5 conditions (21 % O$_2$, 0.03 % CO$_2$, 79% N$_2$ + glucose). In contrast, there was a significant increase at the third and fourth time points when sediments were incubated under gas mix 3 conditions (10 % O$_2$, 1% CO$_2$, 89% N$_2$) (Figure 3.4).
Figure 3.4. Graph showing increase in *iro* gene copy numbers with time (Time zero = 0-days, spike 1 = 7-days, spike 2 = 14-days, final = 21-days). The top panel shows gene copy numbers from gas mix 3 (10% O₂, 1% CO₂, 89% N₂) while the bottom panel shows those from gas mix 5 (21% O₂, 0.03% CO₂, 79% N₂ + glucose). For both gas mixes, sediments from both sites were pooled together. Means represented with (a) were not found to be significantly different from each other at p = 0.05 while means denoted with (b or c) were different. Error bars represent the standard error of means. 3-way ANOVA was used to determine p-values.
3.3. Correlation between *iro* gene copies and Fe(II) oxidizing rates

Correlation coefficients were calculated for *iro* gene copies after the incubation and Fe(II) oxidation rate constants after the third iron addition using SAS software for the GB2 and FR5. The correlation was determined for each of the sites separately and for the two sites combined. No significant correlation was observed when the sites were tested separately or when they were combined at p= 0.05 (Table 3.3). Site 1 gene copy numbers had a weak positive correlation with rate of 0.25 while site 2 gene copy numbers had a weak negative correlation (-0.43) rate. When both sites where combined a cumulative correlation value of 0.09 was obtained.

To assess if there was any correlation with time during the incubation, M.S excel was used to generate the scatter plot of the Fe(II) oxidation rates and *iro* gene copy numbers at the 7th and 21st time point for gas mix 3 and 5. This figure only had an R² of 0.14 indicating that there was a negligible correlation between rate and *iro* gene copy numbers even at the different sampling times (Figure 3.5).

<table>
<thead>
<tr>
<th></th>
<th>Correlation</th>
<th>P value (level of significance at p=0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site 1 (GB 2 sediments)</td>
<td>0.25</td>
<td>0.67</td>
</tr>
<tr>
<td>Site 2 (FR5 sediments)</td>
<td>-0.43</td>
<td>0.46</td>
</tr>
<tr>
<td>Both sediments combined</td>
<td>-0.09</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Table 3.3: *iro* gene copy numbers for GB2, FR5 locations after the 21-day incubation period correlated with Fe(II) oxidation rates after the third iron addition. Significance level was set at p = 0.05
Figure 3.5: A graph showing the correlation between Fe(II)-oxidation rates and *iro* gene copy numbers for GB2, FR5 locations at the 7 and 21-day time points in gas mixes 3 and 5.
Figure 3.6: Zero-order rates of Fe(II) oxidation for GB 2m, FR 3m, and FR 10 m sediments. Blue and orange represent the abiotic and biological contribution, respectively; biological values were obtained by subtracting the abiotic rate from the overall rate. Different batch experiments are designated by % O\textsubscript{2}: 0, 0.7, 1.5, 10, 21, and 21% + glucose. The 21% + Glucose gas mix represents experiment 5 with breathing air and glucose spikes. FR 3m sediments were omitted from experiments 5 and 6 with 21% O\textsubscript{2} + glucose and 0% O\textsubscript{2}. The panels (left and right) represent the rates for the first and third iron spikes respectively (Lucas, 2008)
4. DISCUSSION

4.1 Quantification of *iro* genes using Q-PCR

In the previous chapter we identified the *iro* gene as a good candidate for a functional marker for tracking the presence of *A. ferrooxidans* in different types of AMD derived sediments. Even though our study was able to detect *A. ferrooxidans* sequences from AMD using *iro*, we could not use these observations to propose that a correlation would exist between iron-oxidation rates and *iro* gene copies in these sediments. The objective of this study was to test the correlation between *iro* gene copy number and iron oxidation in batch reactor AMD sediments from two sites which exhibited contrasting rates of Fe(II) removal described by Senko et al. (2008). Since *A. ferrooxidans* was cultured from these sediments, and since *iro* is specific for *A. ferrooxidans*, our hypothesis was that our primers for *iro* would detect these genes in the batch sediments. On the other hand, since 16S rRNA clone libraries from community DNA in these sediments did not contain *A. ferrooxidans* sequences, we also hypothesized that no correlation would be found between iron oxidation rates and *iro* gene copy numbers due to the likely presence of other Fe(II)-oxidizers in these sediments.

After incubating the batch sediments for 21 days with the five different gas mixes, our results with 2-way ANOVA showed that there were no significant differences in *iro* gene copies between the sites and among the gas mixes except in gas mix 3 (10% O$_2$) where significant differences were present in sediments from both sites (Figure 3.3). In contrast, the highest rates of iron oxidation were observed in the gas mix with 20% O$_2$ (Figure 3.6). This could suggest that *A. ferrooxidans* could be doing well in lower O$_2$ (<10%) and that higher O$_2$ (>21 %) could be favoring other bacteria that could be out-
competing *A. ferrooxidans* for O\(_2\). For this study, we did not perform any incubation whereby either CO\(_2\) or O\(_2\) was kept constant while the other was varied. However from our results, CO\(_2\) seemed to have little effect on *iro* gene copy numbers, in contrast to O\(_2\).

Lack of significant differences in gene copy numbers between the sites after the 21-day incubation suggested that Fe(II)-oxidizing populations other than *A. ferrooxidans* seemed to be abundant. Laboratory results observed with both sediments indicated that their microbial communities were capable of Fe(II) removal when provided with the right conditions, even though the sediments exhibited differences in the field.

So as to understand the changes in *iro* gene copy numbers with time during the incubation, we evaluated *iro* genes from four time points in two gas mixes; gas mix 3 (10% O\(_2\), 1% CO\(_2\), 89% N\(_2\)), and 5 (21% O\(_2\), 0.03% CO\(_2\), 79% N\(_2\) + glucose). Only sediments from gas mixes 3 and 5 were compared because DNA from all the time points was not available for the rest of the gas mixes. For each of the two gas mixes, gene copy numbers were pooled according to site (GB2 and FR5 were combined) since no differences between the sites were found. Our results with 3-way ANOVA showed that there was no significant increase in gene copy numbers for gas mix 5 while there was a significant increase after the third and fourth time points in gas mix 3 (Figure 3.4). These observations were consistent with our earlier conclusion that gas mix 3 seemed to enhance iron oxidation by *A. ferrooxidans* and also suggested that higher O\(_2\) (>21%) with glucose did not appear to enhance iron-oxidation by *A. ferrooxidans*.

In contrast to the findings after incubation with glucose (no significant increase in gene copy numbers after incubation with glucose), the highest rates of iron-oxidation were observed in the gas mix with ambient air and glucose (Figure 3.6). Therefore, it is
possible that glucose could have enhanced iron oxidation by bacteria other than A. *ferrooxidans* which do not contain the *iro* gene, perhaps heterotrophs as was suggested by Leveille et al. (2001) in a reverse sample genome probing study. In their study, Leveille and co-workers found that enrichment of several AMD cultures (*A. ferrooxidans*, *A. acidophilus*, *A. thioxidans*, and *L. ferrooxidans*) in glucose followed by transfer to tetrathionite media, selected for the heterotrophic *A. acidophilus* strains.

Heterotrophs are proposed to play integral role in AMD environments whereby they utilize organic substrates that would otherwise be harmful to chemolithotrophic communities such as *A. ferrooxidans* (Johnson, 1998). Previous molecular analyses of these sediments by Senko et al. (2008) detected no genera of known iron oxidizers, which led to the suggestion that heterotrophic populations could be important players especially at the second site. Moreover, as discussed in section in section 3.3, our study also found no correlation between iron oxidation rates and *iro* gene copies after 21 days of incubation and at different sampling points, suggesting that *A. ferrooxidans* was not the primary iron oxidizer in these sediments. Similar suggestions regarding the lack of importance of *A. ferrooxidans* has been suggested for other AMD sites (Bond et al., 2000a; Bond et al., 2000b). The lack of a strong correlation between *iro* gene copy numbers and biotic iron oxidation rates could suggest that bacteria containing the *iro* gene (*A. ferrooxidans*) are not the most important players during iron oxidation at the two sites, unlike what has been thought for most acidophilic systems (Nicomrat et al., 2006a; Nicomrat et al., 2006b).
4.2 Prospects for Q-PCR amplification using the *rus* gene

In the previous chapter, we described our attempts to design a functional gene probe for rusticyanin. Several primers were identified for amplifying *rus* amplicons (Table 1 of Appendix 1). Recognizing the need for a longer *rus* amplicon for use in a plasmid DNA standard for q-PCR, we subsequently designed another set of primers for *rus* (see Table 6 of Appendix 2) that was more specific for strain ATCC 23270. These primers gave strong amplification results when tested with DNA from ATCC 55720. Primer pair Rus_1f-4r was used to develop a plasmid for the region flanking the *rus* gene. Although we were able to get an insert into the TOPO-TA vector and a plasmid for developing a standard curve for quantitative PCR, we did not get any detectable amplification with target DNA from AMD batch reactor sediments. Further attempts to amplify this DNA with the *iro* and 16S rRNA primers (27f and 1492r) have not been successful.

In summary, this study evaluated the prospects of using *iro* and *rus* as functional gene assays for iron oxidation potential in AMD sediments. Although this study was not based on RNA extraction and analysis, which would have been a more reliable approach for characterizing active populations, the *iro* PCR assay did reveal the presence of *A. ferrooxidans* in these sediments. *Rus* also has potential, but more work involving probe testing is necessary. Based on our findings and those of Senko et al. (2008), we suggest that heterotrophs might be playing important roles at these sites. However, studies have shown that other bacterial as well as archaeal groups constitute the diversity in AMD environments (Johnson et al., 2008; Baker and Banfield, 2003). Therefore, more
community characterization studies are necessary to elucidate the populations that play important roles during iron-oxidation at these sites.
BIBLIOGRAPHY


CHAPTER 4

GENERAL CONCLUSIONS

In this study, our overall objective was to design and test a functional gene assay for Fe(II) oxidation using the gene iro. Our aim was to apply this assay to AMD sediments from two Appalachian sites during incubations under different environmental conditions. We found no correlation between iro gene copy numbers and Fe(II) oxidation rates exhibited by these sediments. We attributed this observation to the presence of diverse Fe(II)-oxidizing organisms in these sediments and to the fact that PCR primers for iro genes were specific for A. ferrooxidans. Our failure to amplify genomic DNA from other Fe(II)- and S-oxidizing bacteria with iro primers indicated that other functional genes are involved in Fe(II) oxidation by these organisms. The major conclusions from the research presented in this thesis are summarized in the following list:

- Primers targeting the iro gene amplified community DNA extracted from AMD sediments, indicating that bacterial populations possessing the gene iro were present. These results were consistent with previous studies by Senko et al. (2008) who were able to culture iro gene-carrying A. ferroxidans from the sediments. The iro gene assay applied to field sediment samples indicated that gene copies ranged from 23,500 to 39,750 per gram.

- Primers targeting the rus gene did not amplify community DNA extracted from AMD sediments. Although several primer sets for the rus genes could amplify genomic DNA from A. ferroxidans, none of the primer sets yielded PCR products
from any sediments. These results suggested that genes for rusticyanin are more variable among acidithiobacilli than the genes for iron oxidase.

- The sequence similarities of all *iro* amplicons obtained from AMD sediments in this study indicated that these *A. ferrooxidans* populations were all closely related (>96% nucleotide similarity). All these *iro* genes grouped within one cluster (A) distinct from other *iro* sequences having only 60% nucleotide similarity (Clusters B/C). These clusters based on *iro* genes were consistent with other studies that were based on 16S rRNA genes.

- In batch reactor studies in the laboratory, there were no significant differences in *iro* gene copy numbers in sediments from the two sites after 21 days of incubation. This suggested that microbial populations possessing the *iro* gene were similar in abundance at the two sites, even though Fe(II) oxidation rates differed in the field. This observation failed to support a correlation between *iro* gene copy number and Fe(II) oxidation rate.

- The highest *iro* gene copies were observed in the gas mix containing 10% O$_2$. On the other hand, the highest rates of Fe(II) oxidation in the batch reactors were achieved with ambient O$_2$ (21%). This suggested that more oxic conditions favored other bacteria besides *A. ferrooxidans* and enabled them to out-compete *A. ferrooxidans* in the presence of higher O$_2$. Moreover, similar high rates of Fe(II) oxidation in the batch reactors were achieved with ambient O$_2$ (21%) in the presence or absence of glucose. The fact that similar Fe(II) oxidation rates were observed with and without glucose indicated that O$_2$ concentrations were more influential than glucose in regulating Fe(II) oxidation activity.
• No significant correlation was observed between *iro* gene copy numbers and iron-oxidation rates measured in the bioreactors suggesting that *A. ferrooxidans* is not the key player during iron-oxidation in these sediments. Other bacteria which do not possess the *iro* gene appear to play more important roles in Fe(II) oxidation.

• This study demonstrated that the use of *iro* as a functional marker for Fe(II) oxidation is limited to environments where *A. ferrooxidans* is known to be responsible for this activity. However, *iro* appears to be a useful genetic marker for *A. ferrooxidans* within microbial communities, so it could be applied to such systems as metal leaching reactors that employ *A. ferrooxidans* as the treatment agent.

Future work:

• Because this study quantified *iro* genes in community DNA extracts, rather than in cDNA derived from mRNA extracts, our data provided information only on presence of *iro*-containing populations but not on their Fe(II)-oxidizing activity. Therefore, future work employing PCR primers for functional genes will be more informative if they are based on mRNA quantification.

• Various bacterial and archaeal genera can also be involved in Fe(II) oxidation. Therefore, it appeared that microorganisms other than *A. ferrooxidans* were responsible for Fe(II) oxidation in these sediments. More work is needed to elucidate the populations and pathways involved in Fe(II) oxidation at the two sites.
APPENDIX 1: Additional materials for chapter 2

Rusicyanin primer set 1

<table>
<thead>
<tr>
<th>designation</th>
<th>Nucleotide</th>
<th>Location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rus_1f</td>
<td>5’-TGGAAAGAGGCGACGCTTC-3’</td>
<td>117-137</td>
<td>This study</td>
</tr>
<tr>
<td>Rus_1r</td>
<td>5’GAACATACCGGTGGGCGCGCAT-3’</td>
<td>524-544</td>
<td>This study</td>
</tr>
<tr>
<td>Rus_3f</td>
<td>5’-GGATAAACATATGTATACACAGAACACGATG-3’</td>
<td>1-21</td>
<td>Sasaki et al., 2003</td>
</tr>
<tr>
<td>Rus_5r</td>
<td>5’-AGTCTTGCCCCTGTAGGTAA-3’</td>
<td>188-207</td>
<td>Sasaki et al., 2003</td>
</tr>
<tr>
<td>Rus_8r</td>
<td>5’-GACCAAATCCCTTGTTGGTG-3’</td>
<td>332-352</td>
<td>This study</td>
</tr>
<tr>
<td>Rus_9r</td>
<td>5’-AAGGTCACGTCTACGTTGC-3’</td>
<td>305-325</td>
<td>This study</td>
</tr>
</tbody>
</table>

Table 1. Primers 1f and 1r were designed by aligning by aligning several rus sequences obtained from GenBank: Strain JCM 3863-AB094637, T. ferrooxidans rus-X95624, Strain 33020-AJ006456, and Strain 23270-TIGR locus-AFE_3186. Primers 8f and 9r were designed from strain ATCC 23270 (TIGR locus-AFE_3186). Combinations that worked: Rus_3f-5r, 1f-8r, 1f_9r, 3f-1r. Target flanking primer combination that worked but did not insert into TOPO-TA: 3f-1r
Appendix 2 – Additional Information for chapter 3

Table 1: Composition of the synthetic acid mine drainage (SAMD) used for the batch reactor sediment incubations, given as final concentrations.

<table>
<thead>
<tr>
<th>Component</th>
<th>mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{Al}_2(\text{SO}_4)_3 \cdot 17\text{H}_2\text{O}$</td>
<td>0.5</td>
</tr>
<tr>
<td>$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$</td>
<td>5.0</td>
</tr>
<tr>
<td>$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$</td>
<td>4.0</td>
</tr>
<tr>
<td>$\text{MnSO}_4 \cdot \text{H}_2\text{O}$</td>
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</tr>
<tr>
<td>$\text{Na}_2\text{SO}_4$</td>
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</tr>
<tr>
<td>$(\text{NH}_4)_2\text{FeSO}_4 \cdot 6\text{H}_2\text{O}$</td>
<td>0.1</td>
</tr>
<tr>
<td>$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$</td>
<td>4.5</td>
</tr>
<tr>
<td>$\text{KH}_2\text{PO}_4$</td>
<td>0.07</td>
</tr>
</tbody>
</table>
Figure 1: *iro* gene copy numbers for FR-2 sediments with different percentages of O\(_2\). Error bars represent standard error of the means.
Table 2: Gene copy numbers for all the gas mixes 1, 2 & 4 (gene copy number/ g wet sediment)

<table>
<thead>
<tr>
<th>Gas Mix 1</th>
<th>GB 2</th>
<th>FR5</th>
<th>FR2</th>
</tr>
</thead>
<tbody>
<tr>
<td>rep1</td>
<td>4554</td>
<td>18654</td>
<td>47052</td>
</tr>
<tr>
<td>rep2</td>
<td>73140</td>
<td>31914</td>
<td>114691</td>
</tr>
<tr>
<td>rep3</td>
<td>21590</td>
<td>41116</td>
<td>70868</td>
</tr>
<tr>
<td>Mean</td>
<td>33095</td>
<td>30561</td>
<td>77537</td>
</tr>
<tr>
<td>SE</td>
<td>20618</td>
<td>6519</td>
<td>19808</td>
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<table>
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<tr>
<td>rep1</td>
<td>30939</td>
<td>60596</td>
<td>75637</td>
</tr>
<tr>
<td>rep2</td>
<td>11874</td>
<td>49882</td>
<td>52733</td>
</tr>
<tr>
<td>rep3</td>
<td>30864</td>
<td>69523</td>
<td>57478</td>
</tr>
<tr>
<td>Mean</td>
<td>24559</td>
<td>60000</td>
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<tr>
<td>SE</td>
<td>6343</td>
<td>5678</td>
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<table>
<thead>
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<th>Gas Mix 4</th>
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<th>FR2</th>
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<td>rep3</td>
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Table 3: Gene copy numbers for gas mix 4 (gene copy number/ g wet sediment)

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<th></th>
<th>Time zero</th>
<th>spk 1</th>
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<th>final</th>
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Table 4: Gene copy numbers for all the gas mix 5 (gene copy number/ g wet sediment)

**Gas Mix 5**

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Table 5: Gene copy numbers for initial sediments used for incubation

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<tr>
<td></td>
<td>rep2 3.85E+04</td>
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<td>rep3 4.28E+04</td>
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<td>mean 3.97E+04</td>
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<td></td>
</tr>
<tr>
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<td>rep1 4.28E+04</td>
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<tr>
<td></td>
<td>rep2 3.77E+04</td>
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<td>mean 3.74E+04</td>
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<td></td>
<td>rep3 2.08E+04</td>
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Rusticyanin primers set II

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<td>Rus_1r</td>
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<td>Rus_2r</td>
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<td>Rus_4r</td>
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Table 6: Designed from type strain ATCC 23270 (TIGR locus: AFE_3186) using Primer 3 software (Rozen and Skalesky, 2000). All combinations in this set worked. Primer pair 1f-4r (395 bps) was used to amplify a plasmid for quantitative PCR. Primer pair 3f-6r (293 bps) was used for Q-PCR amplification of DNA.