MICROBIAL DIVERSITY IN A CONSTRUCTED WETLAND SYSTEM FOR TREATMENT OF ACID SULFATE WATER

by

YONG JIN LEE

(Under The Direction of Juergen Wiegel)

ABSTRACT

Acid sulfate waste waters are produced from industrial and mining activities, which have serious impacts on public health and our environment. A constructed treatment wetland system has been developed to treat low pH, ferric iron-dominated acid sulfate water drained off from two coal storage facilities at the DOE Savannah River Site, near Aiken, SC. Since microbial sulfate reduction plays a pivotal role in raising pH and removing metal contaminants, it is necessary to obtain microbial profiles especially sulfate-reducing bacteria (SRB) to understand their ecological niches and roles in the processes. A large number of sequences obtained from PCR-based culture-independent molecular method were identified as uncultured or unidentified bacteria. The sequences retrieved with a universal primer set were distributed to several main clades, such as: the *Cytophaga-Flexibacter-Bacteroides* group, OP11, *Chloroflexi*, and the delta-subclass *Proteobacteria*. The sequences identified using six sets of SRB-specific primers fell into each target species, but the specificity and diversity of each primer group varied. To identify the predominant sulfate-reducing bacterial community, 16S rDNA sequence analysis was performed with the highest positive MPN dilution tubes. The obtained data suggested *Desulfovibrio*-like species are the predominant SRB and play a major role in the sulfate

reduction process. Heterotrophic bacteria were also abundant and may play a fundamental role in the anaerobic community of the system. The sequencing data showed the addition of acetate to enhance microbial sulfate reduction altered the composition of the predominant community from Gram-type positive spore-forming bacteria to Gram-type negative sulfate-reducing bacteria. Spatial and temporal changes of the predominant microbial communities in the system were also detected using denaturing gradient gel electrophoresis. A parallel approach was directed to isolate, identify, and characterize known and novel bacteria obtained from the highest MPN cultures to determine their roles in various biogeochemical processes in such acidic and metal-contaminated environment. The majority of isolates belonged to low G+C Gram-type positive bacteria including sulfate-reducing *Desulfotomaculum* and *Desulfosporosinus* species. Among the isolates, a novel heterotrophic bacterium proposed as *Gracilibacter thermotolerans* gen. nov. sp. nov. has been described.

INDEX WORDS: Acid sulfate water, Constructed treatment wetland, Microbial diversity, Molecular phylogenetics, Most probable number, Bioremediation, Sulfate-Reducing bacteria, Heterotrophic fermentative bacteria

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DEDICATION

This dissertation is dedicated to God who has guided me through the study and let me have this wonderful academic family.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

What Is Acid Mine Drainage

Acid sulfate waste waters are produced from industrial and mining activities. Such high sulfate-containing waters, especially acid mine drainage (also referred to as acid rock drainage) from mining, mine tailings and coal storage piles have serious impacts on public health and our environment (Davis and Boegly, 1981; Gray, 1997). Acid mine drainage occurring from sulfide oxidation in rocks is the most difficult one to address among the types of mine waste problems (Durkin, 1995). In many areas of the U.S., acid mine drainage is formed naturally when sulfides come into contact with water, air, and bacteria (Nordstrom and Alpers, 1999) and slowly releases acids (mainly sulfoxide acids), toxic metals, and high concentration of sulfates into rivers, lakes, streams, wetlands and groundwater (Atlas and Bartha, 1998). Acid mine drainage can be characterized by its low pH (typically less than 4), elevated heavy metals, high concentration of sulfate (100-5,000 mg/dm^3) and total dissolved solids (Table 1.1). Furthermore, the low pH water can solubilize heavy metals contained within the waste rocks, lower the pH, and cause more severe effects of acid mine drainage on aquatic life, the environment, and man-made structures such as pipes and dams (Gray, 1997). Therefore, when industry does not take the proper precautions to protect the environment, this process may speed up the acid generation and increase the amounts of acids to the point that the environment contains more acid than it can neutralize by natural processes.

Acid mine drainage typically contains high concentration of iron and sulfate of which high acidity eventually solubilize other metals and metalloids by mineral dissolution (Johnson, 1995). Among the elevated metals and metalloids are aluminum, copper, lead, zinc, cadmium, nickel and arsenic. Some metals such as iron, manganese, and aluminum have low solubility (below 1 mg/L) in natural water. The drainage from coal mines generally contains these metals (usually the metal cations) and causes severe water pollution problems that are very difficult to clean up (Leduc et al., 2002). Other metals such as copper, zinc, and cadmium in acid water have a higher solubility and frequently occur in concentrations above regulatory limits that may be toxic to human and wildlife (Johnson, 1998). Acid drainage affects hundreds of thousands of miles of streams throughout the U.S. as well as small and large animals, plants, and microorganisms living in or near the stream (Gray, 1997; Kolmert and Johnson, 2001). Acid mine drainage is toxic to most aquatic organisms such as hydras, sponges, flatworms, and vertebrates (Gray, 1996). Besides microorganisms only some insects and plants such as cattail have been found in the acid mine drainage-impacted regions. Although they are the most dominant form of life in acid mine drainage, most microorganisms under this condition are killed, become distorted, or result in devoid of cytoplasm (Wortman et al., 1986). It is estimated that over 19,300 km of rivers and streams and over 730 km^2 of lakes and reservoirs have been impacted by acid mine drainage from abandoned mines (Kleinmann et al., 2000). It was also reported that more than 17,000 km of streams and rivers in the Ohio River Valley and Appalachian regions have been affected by acid mine drainage (Mills, 1985). Over 50% of streams impacted by acid mine drainage fail to maintain diverse aquatic life, which caused more than $67 million dollars loss annually in fishery in Pennsylvania (USEPA, 1995). Furthermore, it was estimated that the cost of restoring all streams within the Slippery Rock Creek watershed in

Pennsylvania was over $ 8.9 million (Brenner, 2001). Therefore, the prevention and remediation of acid mine drainage has higher priority among pollutions affecting the environment and local economy.

Generation of Acid Mine Drainage

Acid Mine Drainage Chemistry

Iron is the fourth most abundant element (~5.1%) in the Earth's crust (Straub et al., 2001). Ferrous iron (Fe^{2+}) forms minerals such as siderite ($FeCO_3$), vivianite ($[Fe_3(PO_4)_2]\cdot 8H_2O$) or ferrous sulfide (FeS) only under anoxic conditions at moderately acidic to neutral pH (Straub et al., 2001). In the presence of oxygen, ferrous iron is stable only at low pH, but is rapidly oxidized to ferric iron at neutral pH (Schwertmann and Fitzpatrick, 1992). Ferric iron minerals are widely distributed in both oxic and anoxic environments. A total of 16 different ferric iron oxides, hydroxides or oxyhydroxides are known, and are collectively referred to as iron oxides (Schwertmann and Fitzpatrick, 1992). At low pH, ferric and ferrous iron exist as dissolved ions, Fe^{3+} and Fe^{2+}, respectively, but at neutral pH, most substrates and products of iron metabolism are insoluble (Straub et al., 2001).

In the presence of oxygen and water, iron sulfide (pyrite, which is the most abundant sulphidic mineral) is oxidized to iron (III) hydroxide, sulfate, and hydrogen ions (eq. 1).

$$4\ FeS_2\ (s) + 15\ O_2 + 14\ H_2O \rightarrow 4\ Fe(OH)_3 + 8SO_4^{2-} + 16H^+ \quad (1)$$

The released hydrogen ions cause more acidity in water (every mole of pyrite yields 4 moles of hydrogen ions) though the acidification of the immediate environment and acidic discharge can be mitigated by the potential neutralizing minerals such as carbonates (Johnson, 1995). Ferrous iron (Fe^{2+}) is released either by simple dissociation or by oxidation of the pyrite (eq. 2, 3, 4; Singer and Stumm, 1970)

$$FeS_2(s) + 7/2\ O_2(aq) + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \quad (2)$$

$$Fe^{2+} + 1/4\ O_2(aq) + H^+ \rightarrow Fe^{3+} + 1/2\ H_2O \quad (3)$$

$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3(s) + 3H^+ \quad (4)$$

$$FeS_2(s) + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \quad (5)$$

Ferrous iron (Fe^{2+}) is oxidized by O_2 to ferric iron (Fe^{3+}) which subsequently reduced by pyrite to reproduce ferrous iron and acidity (eq. 5). This reaction indicates every mole of pyrite results in the production of 16 moles of protons and 2 moles of sulfate, which explains the polluting capability of the oxidation of pyrite. Two possible oxidants for pyrite are oxygen and ferric iron (Garrel and Thomson, 1960). Since oxygen does not affect the reduction of ferric iron by pyrite, and there is no oxidation of pyrite observed without ferric iron, ferric iron must be the major oxidant of pyrite in acidic condition (Singer and Stumm, 1970). Therefore, the oxidation of ferrous iron by oxygen is crucial and the rate-limiting step in the generation of acid mine drainage. Among the many factors showing a catalytic effect on the oxidation of ferrous iron by oxygen, microorganisms appear to play the most important role in enhancing the oxygenation of ferrous iron by a factor larger than 10^6 in acid-leaching environments (Singer and Stumm, 1970; Lundgren and Silver, 1980).

The Role of Microbial Iron and Sulfur Oxidation in Acid Mine Drainage

Even in the severe acidic environment (pH<2.5), microorganisms exist and participate actively in various biogeochemical processes (Tyson et al., 2004). It is considered that most of the neutrophilic bacteria isolated from acid mine drainage inhabit a less acidic micro-niche, exist in inactive forms such as spores (Johnson, 1991), or accidentally migrate in precipitates and run-off (Johnson, 1995). Therefore, most of the bacteria actively involved in acid mine drainage are either acidophilic (pH_{opt} = 0-5.5) or acid-tolerant (pH_{opt} > 5.5, but able to grow below pH 5.5).

Most acidophilic bacteria isolated up to this point are aerobes or aerotolerants (Johnson, 1998). Acid mine drainage contains relatively low concentration (10 mg/L) of dissolved organic carbon (Kolmert and Johnson, 2001), and, as such, is called an oligotrophic environment. Notably most iron- and sulfur-oxidizing bacteria are both acidophilic and autotrophic (Pronk et al., 1991).

Bacterial oxidation of sulfides occurs directly or indirectly (Fig. 1). Whereas indirect oxidation occurs chemically by either ferric iron or proton attack, direct oxidation includes contact between microorganisms and minerals. For instance, *Acidithiobacillus ferrooxidans* (basonym: *Thiobacillus ferrooxidans,* Kelly and Wood, 2000) attaches to sulfide minerals and oxidize either the sulfide moiety or both iron and sulfur (Myerson and Kline, 1983; Mustin et al., 1992). Two indirect mechanisms (via either thiosulfate or polysulfides) are involved in bacterial leaching of metal sulfides (Schippers and Sand, 1999). The acid insoluble metal sulfides such as FeS_2, MoS_2, and WS_2 are chemically attacked by iron(III) hexahydrate ions generating thiosulfate and ferrous iron (eq. 6).

$$FeS_2 + 6Fe(H_2O)_6^{3+} + 3H_2O \rightarrow Fe^{2+} + S_2O_3^{2-} + 6Fe(H_20)_6^{2+} + 6H^+ \quad (6)$$

Thiosulfate is stable at circumneutral conditions (Johnson, 1998), however, further oxidized via tetrathionate, disulfane-monosulfonic acid, and trithionate to sulfuric acid in acidic conditions (eq. 7-12, Sand et al., 1995). The oxidation end products consist of up to 90% sulfate and 1-2% polythionates (Schippers et al., 1996). Elemental sulfur occurs as a byproduct. The sulfite in eq. 12 is unstable in acidic solution and is further oxidized to sulfate, possibly by *A. ferrooxidans*.

$$2Fe^{3+} + S_2O_3^{2-} \leftrightarrow 2FeS_2O_3^{+} \rightarrow 2Fe^{2+} + S_4O_6^{2-} \quad (7)$$

$$S_4O_6^{2-} + H_2O \rightarrow HS_3O_3^{-} + SO_4^{2-} + H^+ \quad (8)$$

$$S_3O_3^{-} + S_2O_3^{2-} + 0.5O_2 + 2H^+ \rightarrow S_5O_6^{2-} + H_2O \quad (9)$$

$$S_3O_3^{-} + S_4O_6^{2-} \rightarrow S_2O_3^{2-} + S_5O_6^{2-} \quad (10)$$

$$S_3O_3^- + 1.5O_2 \rightarrow S_3O_6^{2-} \quad (11)$$

$$4S_3O_3^- \rightarrow S_8 + 4SO_3^{2-} \quad (12)$$

The oxidation of acid soluble metal sulfides such as ZnS, $CuFeS_2$, PbS, MnS_2, As_2S_3, and As_4S_4 begins with a proton attack and is followed by the oxidation of sulfide by ferric iron (eq. 13, Me^{2+} represents a divalent metal cation in solution).

$$MeS + Fe^{3+} + 2H^+ \rightarrow Me^{2+} + H_2S^{\cdot+} + Fe^{2+} \quad (13)$$

Polysulfide formation starts with the dissociation of the strong acid $H_2S^{\cdot+}$ to the $HS^{\cdot}$ radical (eq. 14).

$$H_2S^{\cdot+} + H_2O \rightarrow H_3O^+ + HS^{\cdot} \quad (14)$$

Two $HS^{\cdot}$ radicals may react to yield a disulfide (eq. 15), and chain elongation proceeds further by reactions as seen in equations 16, 17 and 18.

$$2HS^{\cdot} \rightarrow H_2S_2 \quad (15)$$

$$H_2S_2 + Fe^{3+} \rightarrow H_2S_2^{\cdot+} + Fe^{2+} \quad (16)$$

$$H_2S_2 + HS^{\cdot} \rightarrow HS_2^{\cdot} + H_2S \quad (17)$$

$$2HS_2^{\cdot} \rightarrow H_2S_4, \text{ or } HS^{\cdot} + HS_2^{\cdot} \rightarrow H_2S_3 \quad (18)$$

Under acidic conditions, polysulfides eventually decompose to S_8 rings (>99%) (eq. 19).

$$H_2S_9 \rightarrow H_2S + S_8 \quad (19)$$

Consequently, bioleaching of metal sulfides is the function of bacteria that can generate sulfuric acid to supply protons and ferric irons for hydrolysis attack and oxidative attack, respectively (Schippers and Sand, 1999).

The significance of acidophilic iron-, sulfur-oxidizing bacteria has been well studied and discussed. The most studied group is the one comprising of iron- and sulfur-oxidizing chemolithotrophic bacteria such as *Acidithiobacillus ferrooxidans* and *Leptospirillum*

ferrooxidans, known to increase the rate of sulfide oxidation at low pH by oxidizing Fe^{2+} to Fe^{3+} (Nordstrom and Alpers, 1999; Peccia et al., 2000). However, recent studies of the microbial communities in acid-leaching environments by fluorescent in situ hybridization showed that *A. ferrooxidans* and *L. ferrooxidans* were not the predominant microorganisms (Edwards et al., 1999; Schrenk et al., 1998). It has been reported that *A. ferrooxidans* is prominently found at relatively higher pH (2-4) and low temperature (<30 ^{o}C) regions suggested this microorganism may affect ferric iron precipitation, but not acid generation as previously thought (Schrenk et al., 1998). Similarly, group I and II *L. ferrooxidans* made up minor proportions in a biofilm (pH 0-1), whereas group III *L. ferrooxidans* was found to be dominant in that same region (Bond and Banfield, 2001). *Ferroplasma* species have also been detected as a dominant species at lowest pH and highest ionic strength conditions, and *Sulfobacillus* species were prominent in higher temperature regions (~43^{o}C) (Bond et al., 2000). This result was also confirmed by environmental genomics using a whole genome shotgun cloning approach (Tyson et al., 2004). At elevated temperatures (35-55^{o}C), microbial communities are quite different, and moderately thermophilic and acidophilic microorganisms are indigenous (Sharp et al., 1992). Iron-oxidizing acidophilic thermophiles are metabolically diverse, capable of reducing ferric iron at low oxygen level and oxidizing ferrous iron under aerated conditions. It has been reported that an iron-oxidizing archaeon isolated from an acid mine drainage is able to grow at pH 0.5 at 40^{o}C and constitutes up to 85% of the microbial community at acid-generating sites (Edwards et al., 2000). At higher temperatures (> 60^{o}C), most of the acidophilic microorganisms are archaea (eg. *Sulfolobus*, *Acidianus*, *Metallosphaera*, and *Sulfurococcus*) that are responsible for mineral oxidation (Norris, 1990; Ghauri and Johnson, 1991). Therefore, archaea might play more

important roles in pyrite oxidation and acid generation at lower pH (0-1) and high temperature (~40^{o}C) environments than the more familiar bacteria.

At neutral pH, ferrous iron can be oxidized under anaerobic conditions by anaerobic phototrophic bacteria (Widdel et al., 1993) and by nitrate-reducing bacteria (Straub et al., 1996; Benz et al., 1998). These reactions are energetically feasible. Other heterotrophs found in acid mine drainage are the bacteria *Acidiphilium sp*., *Acidocella sp*., *Acidispaera sp*., *Frateuria sp*., *Flavobacterium sp*. and *Bacillus sp*., and the fungi such as *Aspergillus sp*. and *Penicillium sp*. (Dugan et al., 1970; Harrison, 1984; Johnson et al., 2001).

Remediation of Acid Mine Drainage

There have been many attempts to prevent or remediate acid mine drainage pollution. To prevent acid mine drainage formation, either mechanical or chemical treatment can be applied to preclude iron and sulfide oxidation by removing water and oxygen required for acid mine drainage production. Mechanical approaches include sealing off mine shafts and adits, underwater disposal of mine wastes, and using dry covers on spoil heaps. Chemical approaches are based on the use of chemicals such as anionic surfactants such as sodium lauryl sulfate, which are toxic to iron- and sulfur-oxidizing bacteria, or which raise the pH and eventually cause metal ions to precipitate (Hammack et al., 1994; Lanouette, 1977). The latter is called ‘chemical mitigation’ where neutralizing chemicals such as calcium carbonate, calcium oxide, calcium hydroxide or sodium hydroxide are typically added. This chemical mitigation is used once acid mine drainage has already formed, and requires continuous alkali treatment that tends to produce large amounts of precipitates. Remediation of acid mine drainage generally employs either the addition of alkaline materials with aeration to promote ferrous iron oxidation, or natural or constructed wetlands (Kolmert and Johnson, 2001). Due to its efficiency and convenience, many

industries use chemical treatment methods to solve acid mine drainage problems and spend approximately 1 million dollars a day (Evangelou and Zhang, 1995). However, the use of chemicals is expensive and sometimes inappropriate. For example, the use of surfactants adds the problem of water pollution. This high maintenance cost leads to another approach called 'biological mitigation' by using passive treatment systems that may provide an alternative to active treatment (lime precipitation). This biological mitigation method is considered to have less tendencies to consume energy and to pollute (Brierley, 1990). Among the types of passive treatment systems, constructed treatment wetlands employ natural chemical and biological reactions and thus may offer continuous, low-cost and effective ways to solve the problem over conventional chemical treatment systems (Brenner et al., 1993; Thomas, 2003).

Anaerobic Constructed Treatment Wetlands

Constructed treatment wetlands (CTW) have been used to treat wastewater and pollutants from the food industry, agriculture and mine industry (Barton and Karathanasis, 1999; Brenner, 2001; Eger, 1994; Hedin et al., 1994; Mays and Edwards, 2001). Wetlands usually represent highly complicated ecosystems (Kalin et al., 1991). They form distinct geochemical zones ranging from aerobic to anaerobic. These zones have their unique features including pH gradations and metal precipitates. Among the types of constructed wetlands, anaerobic CTW have been thought to be an effective way of neutralizing acid mine drainage and of natural removal of metal ions in wetlands (Thomas et al., 1999). The integrity of anaerobic CTW is greatly impacted by the microbial activity of indigenous anaerobic microbial communities, especially sulfate reducers. Anoxic zones of sediments provide conditions for microbial and chemical reductions and transform metal cations and sulfates to hydrogen and sulfides. Metal cations are kept from being oxidized and coating the limestone present in the compost (Thomas,

2003). This design encourages the generation of bicarbonate alkalinity (HCO_3^-) by both anaerobic microbial sulfate reduction (eq. 20) and limestone dissolution (eq. 21, CH_2O represents organic carbon in solution).

$$2[CH_2O]\ (s) + SO_4^{2-} \rightarrow H_2S\ (aq) + 2HCO_3^- \quad (20)$$

$$CaCO_3 + H^+ \rightarrow Ca^{2+} + HCO_3^- \quad (21)$$

Bicarbonate neutralizes the acidity of the acid mine drainage, thereby raising the pH (eq. 22), which increases the precipitation of acid-soluble metals such as iron (eq. 23). The hydrogen sulfide generated is readily oxidized which consume oxygen.

$$HCO_3^- + H^+ \rightarrow H_2O + CO_2 \quad (22)$$

$$Me^{2+} + H_2S\ (aq) \rightarrow MeS\ (s) + 2H^+ \quad (23)$$

Alternatively, divalent metal cation can precipitate in the form of metal hydroxides at neutral pH (eq. 24).

$$Me^{2+} + 2H_2O\ (aq) \rightarrow Me(OH)_2\ (s) + 2H^+ \quad (24)$$

Despite the expected variation and complexity of microbial communities in CTW, the microbial diversity and ecology associated with these systems has not been well characterized. It is considered that microbiologically-mediated transformation is the major factor in pH increase and metal removal. These transformations include ammonification, denitrification, iron reduction, sulfate reduction, and methanogenesis. Denitrification and methanogenesis are regarded as minor processes compared to iron- and sulfate-reduction. Ammonification can be a major source of microbial alkalinity generation in acid mine drainage amended with nitrogen-rich organic matter (Kalin et al., 1991). Otherwise, soluble nitrogen can be a limiting factor in the system.

Microbial Iron Reduction in the Bioremediation of Acid Mine Drainage

As mentioned above, ferric iron is one of the most abundant electron acceptors in soil (Straub et al., 2001). The redox change between Fe(II) and Fe(III) plays an important role in redox processes in anoxic soils and sediments (Schwertmann and Fitzpatrick, 1992). Iron (III) reduction is one of the most important biogeochemical reactions in various anoxic environments. In the absence of molecular oxygen, various microorganisms can use nitrate, manganese and iron oxides, and sulfate as alternative electron acceptors (Cummings et al., 2000; Senn and Hemond, 2002). Thermodynamically, soluble Fe(III) as the terminal electron acceptor yield more energy through oxidation of organic substrates than either SO_4^{2} or CO_2 as terminal electron acceptors, thus dissimilatory iron reducing bacteria (IRB) outcompete both SRB and methanogens for limiting electron donors when Fe(III) exists (Lovley and Phillips, 1987). Only in marine sediments and some lakes sulfate (~28 mM in seawater) can outcompete ferric iron as an electron acceptor (Straub et al., 2001).

Dissimilatory iron-reducing bacteria obtain energy by coupling the oxidation of organic compounds or hydrogen to the reduction of ferric iron. The bioavailability of various iron compounds for iron reduction is in the following order: ferric citrate > amorphous ferric oxyhydroxides > more crystalline ferric oxides (Kostka et al., 2002). Since iron oxides and oxyhydroxides co-precipitate toxic, ore-associated elements, such as arsenic (Cummings et al., 2000), iron-reducing bacteria are a significant biological component in constructed wetlands treating acid mine drainage.

A large number of microorganisms are able to reduce Fe(III) and are present in many environments (Balashova and Zavarzin, 1980; Johnson, 1998; Lovley, 1991; Lovley and Anderson, 2000). Microbial iron(III) reduction is energetically and kinetically feasible at low pH

(Banks et al., 1997; Brock and Gustafson, 1976), so the use of iron-reducing acidophilic bacteria has been evaluated in acidic environments (Brock and Gustafson, 1976; Johnson and McGinness, 1991). Among these acidophiles are mesophilic chemoautotrophs, mesophilic heterotrophs and moderate thermophiles (Bridge and Johnson, 1998; Brock and Gustafson, 1976; Johnson and Bridge, 2002; Johnson and McGinness, 1991). Although these bacteria are aerobes, they perform iron reduction growing on elemental sulfur as an energy source when oxygen is depleted. The addition of organic materials into wetlands enhances the activity of acidophilic heterotrophic bacteria that lower oxygen levels and lead to iron(III) reduction. The reduction of ferric iron to ferrous iron decreases the redox potential from +600 to +250 mV, so oxygen will be removed by these acidophiles (Johnson, 1995). The reduction of ferric iron by acidophilic heterotrophic bacteria plays an important role in metal mobilization and alkalinity generation (eq. 25, Vile and Wieder, 1993).

$$CH_2O + 4Fe(OH)_3 + 7H^+ \rightarrow 4Fe^{2+} + HCO^{3-} + 10H_2O \quad (25)$$

They also decompose high molecular weight organic materials into small molecular weight organic acids or alcohols that can serve as substrates for sulfate-reducing bacteria (Cummings et al., 2000). Iron-reducing bacteria have been shown to be active in mining environments and co-exist with SRB (Cummings et al., 2000; Küsel and Dorsch, 2000). Sulfate-reducing bacteria may outcompete iron reducing bacteria through production of sulfide. This sulfide abiotically reduces Fe(III) (Berner, 1984), which suppresses microbial iron reducing populations at conditions that favor sulfate reducing bacteria (e.g., high pH, high availability of lactate or acetate) (Küsel and Dorsch, 2000). Therefore, it is obvious that both IRB and SRB participate and play important roles in various mineral cycling in mining environments.

Microbial Sulfate Reduction in the Bioremediation of Acid Mine Drainage

One of major biogeochemical cycles of significance to climate and life is the sulfur cycle. The reductive side of the sulfur cycle, sulfate reduction occurs only biologically, while the oxidative side of the sulfur cycle, sulfide oxidation occurs both biologically and chemically (Fig. 1.2, Okabe et al., 2005). The chemical oxidation reaction is, however, rather slow at natural pHs and temperatures (Chen and Morris, 1972; Janssen et al., 1995). The chemical oxidation reaction proceeds via thiosulfate ($S_2O_3^{2-}$), while the biological oxidation reaction occurs via sulfur (S^0) (Buisman et al., 1990; Janssen et al., 1995). Sulfate is regenerated from reduced sulfur species at the oxic-anoxic transition zone by phototrophs and chemolithotrophs (Hockin and Gadd, 2003).

Microbial sulfate reduction is a very ancient process and dates back to the early Archaean era, more than ~3.47 billion years ago (Shen et al., 2001). The sulfate-reducing bacteria (SRB) are a physiologically very distinct group of bacteria that can use sulfate as a terminal electron acceptor in respiration. Sulfate-reducing bacteria use sulfate through two different mechanisms, assimilation and dissimilation. Sulfate reduction starts with its activation by ATP to form adenosine 5'-phosphosulfate (APS), which can then be further reduced either dissimilatively or assimilatively (Fig. 1.2). Sulfate-reducing bacteria are phylogenetically and physiologically diverse group of bacteria which belong either to one of the four bacterial phyla *Proteobacteria*, Firmicutes, *Nitrospirae*, or *Thermodesulfobacteria* (Table 1.2, Widdel and Hansen, 1992; Rabus et al., 2000). More than 90% of described species are placed within the δ-*Proteobacteria* (ca. 14 genera and 50 species) and Firmicutes (*Desulfotomaculum sp*.). They conserve energy for growth by linking the oxidation of various substrates to the dissimilatory reduction of sulfate (S^6) to sulfide (S^{2-}).

Sulfate-reducing bacteria are important regulators in the mineralization of organic matter in anaerobic environments (Barton and Tomei, 1995; Vester and Ingvorsen, 1998) such as marine sediments, salt marshes, wastewater and anaerobic constructed treatment wetlands (Howarth and Hobbie, 1982; Jørgensen, 1982; Kühl and Jørgensen, 1991; Thomas et al., 1999). They compete for carbon and energy sources with other bacteria that can use different electron acceptors such as O_2, NO_3^-, Mn^{4+}, Fe^{3+} and CO_2. With the exception of CO_2 for methanogens, the other electron acceptors are thermodynamically more favorable than sulfate, however when sulfate concentrations are high, SRB are expected to be the predominant microorganisms (Phifer et al., 2001). For example, in marine sediments, up to 50 % of the organic matter may be oxidized by sulfate reduction (Jorgensen, 1982), and SRB showed the capability to use alternative electron acceptors such as nitrate, iron, manganese and even oxygen (Dannenberg et al., 1992; Lovley and Phillips, 1994; Szewzyk and Pfennig, 1987). Sulfate-reducing bacteria also play a primary role in the sulfur cycle of aquatic systems by serving as the primary means by which sulfate (SO_4^{2-}) is reduced to sulfide (S^{2-}). Sulfate-reducing bacteria grow best in a pH range from 5.5-9.0 (Flauque, 1995) and in an Eh range from 0 to -150 mV (Fenchel et al., 1998). Sulfate-reducing bacteria use a number of volatile fatty acids and hydrogen as electron donors. It has also been reported that some SRB are able to utilize long-chain fatty acids, hydrocarbons and aromatic compounds (Aeckersberg et al., 1991; Beller et al., 1996; Friedrich and Schink, 1995; Harms et al., 1999; Rabus et al., 1993). The number of protons consumed and alkalinity produced varies with the electron donor (eq. 26, 27, 28 and 29). For example, acetate consumes 1 mole of protons and produces 2 moles of alkalinity (eq. 26).

$$CH_3COO^- + SO_4^{2-} + H^+ \rightarrow H_2S + 2HCO_3^- \quad (26)$$

$$CH_3CHOHCOO^- + 3/2SO_4^{2-} + H^+ \rightarrow 3/2H_2S + 3HCO_3^- \quad (27)$$

$$4CH_3CH_2COO^- + 7SO_4^{2-} + 6H^+ \rightarrow 7H_2S + 12HCO_3^- \quad (28)$$

$$4H_2 + SO_4^{2-} + 2H^+ \rightarrow H_2S + 4H_2O \quad (29)$$

Microbial sulfate reduction in mine environments is very important since they can neutralize low pH (utilization of H^+ and conversion of H_2SO_4, a strong acid to H_2S, a weak acid), lower high sulfate, and eventually promote metal precipitation (Fortin et al., 2000a). Microbial sulfate reduction is the sole mechanism responsible for the formation of iron monosulfides and pyrites (Fortin et al., 2000b), and it has been identified as an important reaction in metal removal in wetland systems (Machemer and Wildeman, 1992; Webb et al., 1998). *Since metal sulfides have higher tendency to be produced,* lower solubility and less tendency to resolubilize, microbial sulfate reduction is considered to be a better way to precipitate metals (El Bayoumy et al., 1999). Sulfate-reducing bacteria form an extracellular iron sulfide coating resulting from the reduction of iron sulfate to sulfide. Such iron sulfide minerals play an important role in trace metal adsorption in anoxic sediments (Fortin et al., 1995). Sulfate-reducing bacteria have been shown to metabolize iron [Fe(III)], chromium [Cr(VI)], uranium [U(VI)], manganese [Mn(IV)], and technetium [Tc(VII)] (Lloyd et al., 1998; Lovley and Phillips, 1992; Lovley et al., 1993; Lovley and Phillips, 1994; Tebo and Obraztsova, 1998).

Sulfate-reducing bacteria have been considered to prefer anoxic condition but were isolated from both oxic-acidic and anoxic-neutral environments (Canfield and Des Marais, 1991; Dilling and Cypionka, 1990; Fortin et al., 1996; Fortin and Beveridge, 1997; Widdel, 1988; Wielinga et al., 1999). It has been reported that DcrA, a methyl-accepting protein from SRB senses the oxygen concentration or the redox potential of the environment (Fu et al., 1994). Catalase and superoxide dismutase have also been found in SRB (Abdollahi et al., 1990; Hatchikian et al., 1977; Morris, 1976). The possible strategies of SRB to avoid oxygen stress

include migration to the anoxic zone (negative aerotaxis), clump formation, and oxygen removal by active respiration (Krekeler et al., 1998). Recently, aerotolerant sulfate-reducing bacteria of the family *Desulfobacteriaceae* have been reported to form sulfide compounds with zinc and other toxic metals such as arsenic and selenium (Labrenz et al., 2000). Sulfate-reducing bacteria in the oxic zone may successfully compete with aerobic heterotrophic bacteria for organic substrates but they may also play a significant role in aerobic carbon cycling in phototrophic microbial mats. These effects form the basic strategy of bioremediation of wastewater using sulfate-reducing bacteria. Therefore, sulfate-reducing bacteria can be useful tools for defining the ecology of such systems and function as important regulators of a variety of biogeochemical processes in wetland soils.

Although mixed cultures reported reduced sulfate using sawdust as an electron donor at pH 3.0, no acidophilic bacteria that can reduce sulfate to sulfide have been identified (Fortin et al., 2002; Johnson, 1995). Those isolates including *Desulfotomaculum*-like species reported to reduce sulfate are considered to be acid-tolerant rather than acidophilic. For instance, a pure culture isolated from an acidic pond (pH 3.38) did not reduce sulfate below pH 5.5 (Tuttle et al., 1969). This might be due to the formation of a biofilm of SRB around substances that provide more alkaline microenvironment. Since there have been reports of aerobic sulfate reduction and acid-tolerant SRB, an attempt to isolate and characterize aerotolerant and acidotolerant SRB will provide useful insight into in situ bioremediation of acid mine drainage.

Sulfate-reducing bacteria can reduce iron (III) indirectly via sulfide production that comes from sulfate reduction, which can reduce iron oxyhydroxides and form iron sulfides. It was also reported that some of SRB can reduce iron (III) directly through an enzymatic mechanism producing siderite ($FeCO_3$) (Coleman et al., 1993). Sulfate-reducing bacteria are

abundant in iron-reducing zones in deep aquifers, which indicates that they may survive by reducing iron in such environments (Coleman et al., 1993).

Despite the importance of sulfate-reducing bacteria in this type of environment, most of the biogeochemical studies in the acid mine drainage have been focused mainly on iron and sulfur-oxidizing bacteria (Bond et al., 2000; Bond and Banfield, 2001; Colmer et al., 1950; Edwards et al., 1999; Goebel and Stackebrandt, 1994; Southam and Beveridge, 1992; Wood et al., 2001). Since the chemical conditions in acid mine drainage are not considered suitable to SRB, little is known about the occurrence and distribution of SRB in mining environments (Fortin et al., 1995). However, SRB have been recovered from mining environments receiving acidic leachate and from slightly acidic mine tailings (Fortin et al., 1995; Gyure et al., 1990; Herlihy and Mills, 1985). The formation of ferrous monosulfide (FeS) by microbial sulfate reduction is an important geochemical reaction and it can lead to pyrite formation (Berner, 1984). It can also affect the solubility of other dissolved metals such as Cu, Zn, Pb, Cd, etc. through sorption and co-precipitation (Mores and Luther III, 1999). Therefore, it is essential to address the occurrence and distribution of complex microbial communities, especially of sulfate-reducing bacteria and to elucidate how they respond to and control biogeochemical reactions in the acid mine drainage and in wetland systems.

Other Heterotrophic Bacteria in Acid Mine Drainage

Heterotrophic bacteria are versatile and can be isolated from extremely acidic environments (Johnson, 1998). Many acidophilic heterotrophic bacteria involved in iron cycling in acid mine drainage were isolated and have shown to metabolize organic materials that are toxic to autotrophic iron-oxidizers (Pronk and Johnson, 1992). Some heterotrophic bacteria can immobilize metal by adsorption. This mechanism can be applied for metal removal in

bioremediation and for metal recovery in industrial processes (Brierley, 1990). Some acidophilic bacteria play an important role in dissimilatory iron oxidation and reduction. These include an iron oxidizer, *Ferromicrobium acidophilus* and various *Acidiphilium*-like iron-reducing isolates (Bond et al., 2000). Fermentation of organic materials by obligate anaerobes including *Clostridium* sp. and *Bacteroides* sp. stimulates sulfate reduction in the anoxic zone (Lloyd et al., 2004). For instance, complex organic compounds can be degraded to short chain fatty acids that are available to SRB (Howarth, 1993). Aerobic heterotrophic bacterial populations will limit transport of oxygen, thereby inhibit the growth of aerobic chemolithoautotrophic bacteria and result in a cessation of acid production. There are two possible ways by which this can occur: (i) a fast-growing heterotrophic biofilm covers chemolithoautotrophic iron oxidizers such as *T. ferrooxidans*, preventing oxygen diffusion to the autotrophs; and (ii) heterotrophic bacteria consume all the oxygen. Therefore, a variety of heterotrophic bacteria are involved and contribute directly or indirectly in the biogeochemical reduction processes.

Research Components

After the National Energy Plan was introduced in 1977, the use of coal increased drastically, causing subsequent environmental concerns such as acidic runoff generated by coal piles (Thomas, 2003). There are two coal-storage facilities associated with electric power plants at the Department of Energy's Savannah River Site, Aiken, SC. Acid-sulfate runoff from these coal storage piles is retained in a coal pile runoff basin and have features of low pH and high concentration of sulfate and toxic metals (primarily contaminated with Fe, Al, and Mn but also Cr, Co, Ni, Cu, As, Zn, U etc.), which are comparable to acid mine drainage (Brofft et al., 2002; Thomas, 2003). Two constructed treatment wetland systems receiving acid-sulfate water from the coal pile runoff basin were built for the study of remediation of acid mine drainage. Each

constructed treatment wetland consists of three consecutive tanks including the first anaerobic tank that acidic runoff passes through and various biogeochemical reactions take place.

Since the success of this treatment depends greatly on the indigenous microbial activity, we used scaled columns to survey the microorganisms present in the wetland system and to determine the neutralization potential and metal removal capability of the system. The column used in this study was manufactured of clear polyvinyl chloride (PVC) tubing and was packed with composted horse manure, spent brewery grains, Kricket Krap® and granular limestone (Thomas et al., 1999). There were 3 cores in each cell and in each core four different biogeochemical reaction zones existed as a) *oxide* zone (pH 2.3-3.8), b) transitional (pH ~4.5), c) *sulfide* zone (pH>6.5), and d) unreacted zone (pH ~6.8). Acid-sulfate water (pH<2.4) was pumped into the columns at an average flow rate of 20 ml/min. Samples collected from cores of each non-supplemented (36A) and acetate-supplemented (30A) tank in June and November 2000 were used for microbiological characterizations. Acetate was added to the acetate-supplemented tank in July 2000 to enhance microbial sulfate reduction and to evaluate the alterations in microbial community structures and the extents of the enhancement and stimulation of sulfate-reducing bacterial community.

This project involves work at the interface between geology, chemistry, and biology and is being done in collaboration with Professor Romanek's and McArthur's laboratories at Savannah River Ecology Laboratory, Aiken, SC. A microbial approach of this project has many components, including molecular assessment of the complex microbial communities, profiling SRB, identifying predominant SRB via most probable number (MPN) analysis, culturing, isolation of known and possibly novel microorganisms. Our primary goal was to advance the understanding of sulfate-reducing bacteria, whose diversity and population dynamics are not well

understood in mining environments. In chapter 2, we present bacterial profiles retrieved from the sulfide zone of the acetate-supplemented tank. We used small subunit ribosomal RNA gene sequence analysis to provide a relatively non-biased and powerful approach for evaluating the phylogenetic diversity of environmental samples (Baker et al., 2001; Groflkopf et al., 1998; Whitehead and Cotta, 1999). This method can be applied to most taxa and give an insight into microbial ecology and diversity associated with various biogeochemical processes in environments. Culture-based approach may be biased (Dunbar et al., 1997), but can provide an alternative way to evaluate microbial ecology and functions (Cypionka, 2005). Thereby our research primarily focused on isolation and characterization of known and novel sulfate-reducing bacteria using the most probable number technique. In chapter 3, we present recovered culturable SRB diversity along with other bacterial profiles, and subsequently describe a novel heterotrophic thermotolerant species isolated from the oxide zone in chapter 4.

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Table 1.1. Typical mine drainage compositions and permissive levels for industrial effluents in USA (modified from Gazea et al. (1996) and Johnson (2003)).

Metals (mg/L)	Coal mines	Metal mines	Industrial effluents
pH	< 4, typically 2.3-6.3	2.0-7.9	6-9
Sulfate	100-5,000	250-3,100	20
Fe	1-473	8.5-3,200	3.5
Al	1-58	4.3-128	
Mn	1-130	0.25-20	2
Zn	< 1	0.04-1,600	0.2-0.5
Cu	< 1	0.005-76	0.05
Pb		0.02-90	0.2

Table 1.2. Differentiation of dissimilatory sulfate-reducing bacteria (modified from The Prokaryotes)

Characteristics	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Genus	*Desulfotomaculum* *Desulfosporosinus*	*Desulfobulbus*	*Desulfococcus* *Desulfonema* *Desulfosarcina*	*Desulfobacterium*	*Desulfobacter*	*Desulfovibrio* *Desulfomicrobium*
Phylogenetic affiliation	Firmicutes			*δ-Proteobacteria*		
Spore formation	+	-	-	-	-	-
Sulfate reduction to sulfide	+	+	+	+	+	+
Sulfur reduction to sulfide	-	+ or -	-	-	-	+ or -
Complete oxidation to CO_2	+ or -	-	+	+	+	-

Fig. 1.1. Bacterial oxidation of pyrite (FeS_2) by the direct and indirect mechanisms (modified from Johnson, 1995).

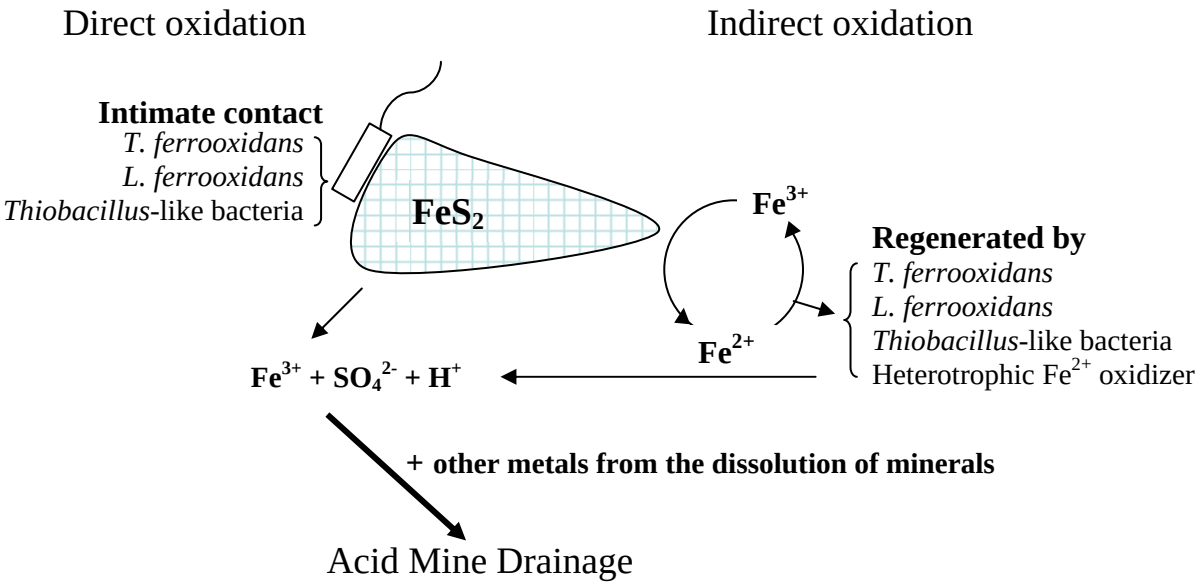
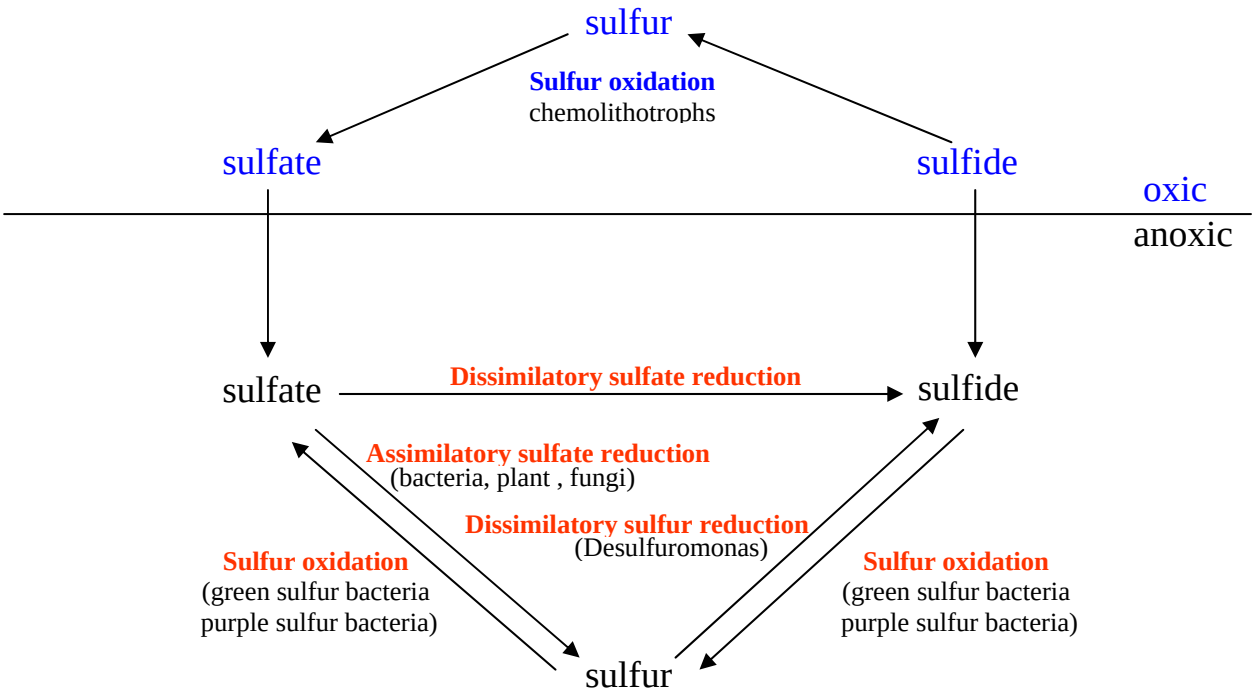

Direct oxidation
Indirect oxidation
Intimate contact
T. ferrooxidans
L. ferrooxidans
Thiobacillus-like bacteria
FeS2
Fe3+
Regenerated by
T. ferrooxidans
L. ferrooxidans
Thiobacillus-like bacteria
Heterotrophic Fe2+ oxidizer
Fe2+
Fe3+ + SO4 2- + H+
+ other metals from the dissolution of minerals
Acid Mine Drainage

Fig. 1.2. The sulfur cycle in mining environments (modified from Fauque, 1995).


sulfur
Sulfur oxidation
chemolithotrophs
sulfate
sulfide
oxic
anoxic
sulfate
Dissimilatory sulfate reduction
sulfide
Assimilatory sulfate reduction
(bacteria, plant , fungi)
Dissimilatory sulfur reduction
(Desulfuromonas)
Sulfur oxidation
(green sulfur bacteria
purple sulfur bacteria)
Sulfur oxidation
(green sulfur bacteria
purple sulfur bacteria)
sulfur

CHAPTER 2

MICROBIAL CHARACTERISTICS OF THE CONSTRUCTED TREATMENT WETLAND SYSTEM RECEIVING ACID SULFATE WATER[1]

[1] Lee, Y.-J., B. C. Thomas, C. S. Romanek, J. V. McArthur, J. Wiegel. To be submitted to *Applied and Environmental Microbiology*.

ABSTRACT

A wetland system modeling a reducing and alkalinity-producing system (RAPS) was constructed to treat acid sulfate runoff from coal piles. This system successfully removed metals (93-99 % of iron and aluminum) and increased pH (2.4 to 6.5) during the course of this study. Four distinct reaction zones developed in the system: oxide, transitional, sulfide and unreacted zones. Each reaction zone possessed unique chemical features and affiliated microorganism-mediated processes. Using PCR-based molecular techniques and bacterial domain-specific primers, retrieved sequences were grouped into 11 bacterial divisions showing extensive diversity. The major divisions of 16S clones recovered in an acetate-supplemented tank were *Chloroflexi*, *Proteobacteria*, OP11, Firmicutes and *Cytophaga-Flavobacterium-Bacteroides* (CFB). Upon feeding with acetate, sequences belonging to the Firmicutes and CFB groups decreased significantly. Sulfate-reducing bacterial diversity was investigated using six SRB group-specific primer sets. Although the specificity of each group-specific primer set varied, all groups were recovered except the *Desulfobacterium* group. Using denaturing gradient gel electrophoresis, the spatial and temporal changes in the microbial diversity were observed even in the same reaction zones of the same tank. It is concluded that microbial communities were tightly coupled with biogeochemical processes taking place in the constructed wetland and were responsible for the generation of alkalinity and metal removals.

INTRODUCTION

After the National Energy Plan was introduced in 1977, the use of coal increased drastically and caused subsequent environmental concerns, such as acidic sulfate runoff formed from coal piles. Such high sulfate-containing waters, especially acid mine drainage (also referred

to as acid rock drainage) have serious impacts on public health and the environment. Acid mine drainage typically contains high concentrations of iron and sulfuric acid, which solubilizes other metals and metalloids from the minerals (Johnson, 1995). There are two major sources of acidity in acid mine drainage (AMD), proton acidity (pH) and mineral acidity (Thomas, 2003). Since mineral acidity is produced primarily from dissolved metal cations and constitutes more than 85% of the total acidity (Hedin et al., 1994), metal removal is fundamental and essential to the neutralization of AMD.

Some metal cations such as iron, manganese, and aluminum have low solubility (below 1 mg/L) in natural water. The drainage from coal mines generally contains these metal cations and often causes severe water pollution problems. Other metal cations, such as copper, zinc, and cadmium, have higher solubility in acid water and frequently occur in concentrations above regulatory limits that may be toxic to human and wildlife. Many soluble metals can be precipitated as insoluble complexes with hydroxides, carbonates, phosphates, and sulfides. Among many approaches to remove metal cations, microbial sulfate reduction is frequently used. It readily precipitates metals as metal sulfides (Fortin et al., 1995; Hard et al., 1997; Webb et al., 1998).

Due to the efficiency and convenience, many industries use chemical treatment methods and spend approximately 1 million dollars a day to solve AMD problems (Evangelou and Zhang, 1995). However, the use of chemicals is not cost-effective and produces wastes and additional water pollution. The high maintenance cost and attendant problems has led to an approach called ‘biological mitigation’ by using the passive treatment system that may provide an alternative to active chemical treatment. Among the types of passive treatment systems, constructed treatment wetlands employ natural geochemical and biological reactions and may offer continuous, low-

cost and effective ways to solve the problem over conventional chemical treatment systems (Thomas, 2003).

Constructed treatment wetlands (CTW) have been used to treat various wastewater and pollution from the food, agriculture, and mining industry (Hedin et al., 1994; Barton and Karathanasis, 1999; Brenner, 2001; Mays and Edwards, 2001). Among the types of constructed wetlands, anaerobic CTW have been thought to be an effective way of neutralizing acid waste waters and of removing metal cations (Thomas et al., 1999). The integrity of anaerobic CTW relies on microbial activity of indigenous anaerobic microbial communities, especially of sulfate reducing bacteria. Anoxic zone of the sediments provide conditions for microbial and chemical reductions, transforming iron and sulfates to iron sulfides, keeping metals from being oxidized, and coating the limestone in the compost (Thomas, 2003). This design encourages the generation of bicarbonate alkalinity (HCO_3^-) by both anaerobic microbial sulfate reduction (eq. 1) and limestone dissolution (eq. 2, CH_2O represents organic carbon).

$$2[CH_2O]\ (s) + SO_4^{2-} \rightarrow H_2S\ (aq) + 2HCO_3^- \quad (1)$$

$$CaCO_3 + H^+ \rightarrow Ca^{2+} + HCO_3^- \quad (2)$$

Bicarbonate neutralizes the acidity of the acid mine drainage, thereby raising the pH (eq. 3), which increases the precipitation of acid-soluble metal cations such as iron (eq. 4, Me represents metal cations).

$$HCO_3^- + H^+ \rightarrow H_2O + CO_2 \quad (3)$$

$$Me^{2+} + H_2S\ (aq) \rightarrow MeS\ (s) + 2H^+ \quad (4)$$

Subsequently, some of these divalent metal cations can be precipitated in the form of metal hydroxides at neutral pH.

$$Me^{2+} + 2H_2O\ (aq) \rightarrow Me(OH)_2\ (s) + 2H^+ \quad (5)$$

Microbiologically-mediated transformations are the major factors in pH increase and metal removal. Despite the expected variation and complexity of microbial communities in CTW, the microbial diversity and ecology associated with this system has not been well characterized. Therefore, the knowledge of the microbial communities and the way they interact with the physicochemical conditions in the system needs to be elucidated.

Two constructed treatment wetlands were built to treat acid runoff from coal piles at DOE's Savannah River Site, Aiken, SC. This constructed treatment wetland system was modeled after the reducing and alkalinity producing system (RAPS) because of its featured capability of long term treatment of acidic water rich in ferric iron (Thomas, 2003). Despite the importance, the microbial communities in CTW are not well characterized with respect to the diversity and ecology. To understand biogeochemical processes in such environments, it is fundamental to obtain microbial profiles. Since microbial sulfate reduction is the key process and plays an important role for neutralization of the low pH water and metal removal in constructed wetland systems, we investigated the occurrence and distribution of sulfate-reducing bacteria within the microbial communities.

METHODS

Acid runoff origin

Two coal-storage facilities are associated with the electric power plants at the DOE's Savannah River Site (SRS), Aiken, SC. Acid sulfate water produced from the coal piles are collected in a coal pile runoff basin (CPRB, Fig. 2.1). The basin water has a low pH (~2.4) and is ferric iron-dominated (>95% of total iron).

Constructed treatment wetland system

Non-supplemented control (36A) and acetate-supplemented experimental (30A) systems were used to treat acid sulfate runoff. These vertical flow reactor systems consisted of a series of three plastic tanks (92 cm diameter and 122 cm height, Fig. 2.2). They were filled with limestone-buffered organic substrates containing organic substrates, bacterial sources, and neutralizing agents (Table 2.1). The organic substrates used were composed of composted stable wastes, spent brewing grains, and Kriket Krap® (composted cricket manure). The composted stable waste was mixed and homogenized with fine-grained limestone screenings (*particle size* mainly $CaCO_3$) at the ratio of 3:1 by volume. Acid sulfate runoff was continuously pumped into the top of the first anaerobic tank of the system with an average flow rate of ~20 ml/min. Once the runoff delivered to the top of the first tank, the water flowed vertically through the composted substrates and limestones by gravity. The acetate-supplemented system received acetate (~120 mM) that was mixed into the acid water stream to enhance microbial sulfate reduction for 4 months after the initial sampling. The first tank of each system, which we studied during this project, is an essentially anaerobic reactor where most of the remediation takes place.

Samples

Four distinct reaction zones developed in the systems during a series of mineral dissolution-precipitation reactions (Thomas et al., 1999). These different reaction zones were identified as a) oxide zone (pH 2.3-3.8), b) transitional zone (pH ~ 4.5), c) sulfide zone (> pH 6.5) and d) unreacted zone (~ pH 6.8) (Fig. 2.3a). The influent and effluent were sampled and analyzed weekly during the operation from April 1999 to December 2000 (Thomas, 2003). The samples for microbial community analysis were collected from three cores (approximately 120^o apart) of

both the non-supplemented (36A) and the acetate-supplemented (30A) tanks in June before starting the acetate supplementation and again in November 2000 (Fig. 2.3b, Table 2.3).

Analytical techniques

Temperature and pH were measured by using an Orion 250A field pH meter (Orion Research Inc., Beverly, MA) with a temperature-pH electrode. For sulfate determination, samples were filtered into a glass vial containing 50 mg silver phosphate, and the filtrate was analyzed using a $BaCl_2$ gravimetric method (APHA, 1998). Dissolved metal ions were analyzed after preservation with nitric acid by an inductively coupled plasma mass spectrometer (ICP-MS, Elan 6000, Perkin-Elmer Co., Norwalk, CT) using U.S. EPA methods 200.8. Acidity and alkalinity were measured by APHA method 2310 and 2320, respectively (APHA, 1998). Ferrous iron was determined as previously described (To et al., 1999). Dissolved sulfide was detected using CHEMetrics^{TM} sulfide test kit (CHEMetrics Inc., Calverton, VA) after filtration of the sample.

Extraction of DNA and PCR amplifications of 16S rRNA genes

To characterize the microbial communities, DNA was extracted directly from 1 g of sediment samples of each reaction zone using a Ultra Clean Soil DNA kit (MoBio Laboratories, Solana Beach, CA). The 16S rRNA genes were amplified by PCR with the bacterial domain-specific primer set, 27 forward and 1392 reverse (Lane, 1991) and 6 SRB group-specific primer sets (Daly et al., 2000) (Table 2.4). The amplification was performed in a PTC-150 MiniCycler (MJ Research Inc., Waltham, MA) using 'touchdown' PCR to increase the specificity of the amplification and to reduce spurious by-products (Don et al., 1991). PCR products obtained with bacterial domain-specific primer set were then used as templates for 'nested' amplification using SRB group-specific primer sets (Daly et al., 2000). Each PCR amplification was carried out using a 'hot start' PCR to fully denature the DNA template (Chou et al., 1992).

Cloning and sequencing

Amplified 16S rDNA was cloned into the shuttle vector, pGEM-T (Promega Inc., Madison, WI) and transformed into *E. coli* strains, JM109 or DH5α. For the PCR products that were bigger than 1 kb, the TOPO TA Cloning Kit (Invitrogen Co., Carlsbad, CA) was used with the pCR2.1 TOPO vector and TOP10 competent cells. Each positive clone was inoculated into Luria-Bertani broth medium containing ampicillin (100 μg/ml). After 12-16 hrs of growth, the 16S rDNA inserts were amplified directly from 3 μl of each culture with the vector primer set, M13 forward and M13 reverse. PCR amplification was performed in a Mastercycler (Brinkmann Instruments, Westbury, NY), using 2 min of denaturation at 95°C, then 30 cycles of 30 s at 95°C, 20 s at 55°C, and 90 s at 72°C, followed by 5 min of extension at 72°C. PCR products were purified by using ExoSAP-IT (USB Co., Cleveland, OH), and further used as templates for subsequent sequencing reactions. The sequencing reaction was set up using ABI's Big Dye Terminator Kit v2.0 (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction and performed in a Mastercycler (Brinkmann Instruments, Westbury, NY), using 2 min of denaturation at 95°C, then 40 cycles of 10 s at 95°C, 5 s at 50°C, and 4 min at 60°C, followed by 10 min of final extension at 60°C. The sequencing reactions were precipitated by adding 1 μl of 1.5 M sodium acetate + 250 mM EDTA (pH 8.0) and 40 μl of 95% ethanol. After incubation at -20^{o}C for 20 min, precipitated sequencing reactions were centrifuged at 1,500 x g for 45 min and, after removing the supernatant, resuspended with 4 μl of formamide dyes and heated at 95^{o}C for 5 min, then further analyzed in an ABI model 377 automated sequencer at the Savannah River Ecology Laboratory (SREL) according to the manufacturer's instruction.

Denaturing gradient gel electrophoresis (DGGE) analysis

DGGE analysis was performed by using a CBS Scientific DGGE system (Del Mar, CA) based on the previous description (Ferrari and Hollibaugh, 1999). The variable 3 region of 16S rDNA was amplified using Ready-to-Go PCR bead (Amersham Pharmacia Biotech, Piscataway, NJ) with the addition of 0.5 µM of each primer, 356 forward with GC clamp and 517 reverse labeled with fluorescein at the 5' end, and 10-100 ng of each template (Murray et al., 1996; Muyzer et al., 1993). PCR products (approximately 300 ng) were loaded on a 6.5% polyacrylamide gel containing a 40 to 65% gradient of denaturant (urea and formamide). A reaction containing genomic DNA from *Clostridium perfringens* and *Bacillus thuringiensis* (Sigma Chemical Co.) was used as a standard and positive control. Gels were run as previously described (Bano and Hollibaugh, 2000). The bands on gels were scanned by a FMBIO II (Hitachi, Alameda, CA) gel scanner and subsequently excised from the gels. DNA was eluted from the excised bands into 50 µl of water by incubation for 90 min at 60°C. The eluted DNA was cloned into pGEM-T vector (Promega Inc., Madison, WI) as described above and sequenced by Agencourt (Beverly, MA).

Phylogenetic analysis

The similarities of partial sequences were determined using the Sequencher™ v4.1.4 (Gene Codes Co., Ann Arbor, MI). Retrieved 16S rDNA sequences were analyzed using BLASTN and then aligned manually using GeneDoc v2.6.02 (http://www.psc.edu/biomed/genedoc) to create a multiple sequence alignment. Sequence data were checked for chimera formation with CHIMERA_CHECK v2.7 of the Ribosomal Database Project II (Cole et al., 2003) and Bellerophon (Huber et al., 2004). Clone sequences were assigned to operational taxonomic units (OTUs) by using DOTUR (Schloss and Handelsman, 2005). A representative sequence of each OTU was used for further phylogenetic analysis. The rarefaction curve for each 16S rDNA clone

library was obtained using Analytic Rarefaction v1.3 (Holland, 2003; http://www.uga.edu/strata/software/Software.html). LIBSHUFF analysis was used to estimate and compare clone libraries (Singleton et al., 2001; http://libshuff.mib.uga.edu/). Phylogenetic trees were inferred by the neighbor-joining method (Saitou and Nei, 1987) using the model of Jukes and Cantor (Jukes and Cantor 1969) with the phylogenetic analysis package PHYLIP v3.6a2.1 (Felsenstein, 2001).

Nucleotide sequence accession numbers

The 16S rDNA sequences of 258 environmental clones have been deposited in the GenBank nucleotide sequence databases under the accession no. DQ137891 through DQ138054 and DQ140004 through DQ140097.

RESULTS

Water chemistry

The water chemistry of the influent and effluent samples was analyzed between April 1999 and December 2000 (Table 2.2). The influent was oxic and had a low pH 2.4 and high acidity (738-2,320 mg/L as $CaCO_3$ equivalent), 92-237 mg/L of total iron, 39-274 mg/L of aluminum, and high sulfate (926-3,385 mg/L). The effluent in each system had higher pH from of 6.5 and lower metal content.

Molecular assessment of the affiliated microorganisms in the sulfide zone

Of the various biogeochemical reaction zones developed in the CTW (Fig. 2.3 and Table 2.3), the sulfide zone was characterized using molecular methods. A total of 164 environmental clones were analyzed (Table 2.5). With the 97% sequence similarity cutoff value and the furthest-neighbor algorithm of DOTUR (Schloss and Handelsman, 2005), 60 clones retrieved from the

sulfide zone of core 1 (before acetate supplementation) were assigned to 33 OTUs. After acetate supplementation, 49 clones obtained from the sulfide zone of core 4 were assigned to 34 OTUs. From nonsupplemented tank, 55 clones from the sulfide zone of core 4 were grouped into 39 OTUs.

The retrieved sequences were classified into 11 groups (Fig. 2.4). The major groups in the pre-acetate supplementation samples were *Chloroflexi* (23 %), *Proteobacteria* (22 %), OP11 (13 %), Low G+C gram-type positive bacteria (Firmicutes, 12 %) and CFB (12 %). After the acetate supplementation (November samples) the major groups were *Proteobacteria* (35 %), *Chloroflexi* (18 %) and OP11 (16 %). Firmicutes and CFB appeared with less frequency in core 4 of the acetate-supplemented tank but were not detected in the non-supplemented tank (36A). *Acidobacteria* were detected in core 4 samples, especially from the nonsupplemented tank. Two 16S clones affiliated with candidate division TM6 and one 16S clone related to candidate division were recovered only from the non-supplemented tank. *Nitrospira* and CFB were only detected in the acetate-supplemented tank. Green sulfur bacteria (*Chlorobi*) and candidate division OP10 were detected only from the core 1 of the acetate-supplemented tank. A total of seven 16S clones could not be clearly assigned to known bacterial divisions based on publicly available 16S rRNA gene sequence information. The *Proteobacteria* is the only major division which increased in numbers after acetate supplementation. Among the members of the *Proteobacteria*, the delta subgroup was the most abundant (29-42 %), and included clones related to *Geobacter* and *Syntrophobacter*.

LIBSHUFF analysis yielded *P* values of <0.025 for the comparison of the clone libraries, indicating that the libraries were significantly different. The clone libraries from the nonsupplemented and the acetate-supplemented tanks were significantly different for both the

XY and YX comparisons (P = 0.001). The clone libraries constructed before and after acetate supplementation from the same acetate-supplemented tank were significantly different for only one comparison (P = 0.001 for $\Delta C_{30A1c/30A4c}$ and 0.522 for $\Delta C_{30A4c/30A1c}$). This result indicated that the community in sample 30A4c was a subset of the community in 30A1c.

Distribution of *Delta-Proteobacteria*

A total of 95 sequences were obtained with six SRB group-specific primer sets from core 1 and 4 of the acetate-supplemented tank (Table 2.6). The retrieved sequences indicated the presence of *Desulfobulbus, Desulfobacter, and Desulfovibrio*-like strains from core 1 before acetate supplementation. However, after acetate was added, sequences related to *Desulfobulbus, Desulfobacter, Desulfococcus, Desulfotomaculum* and *Desulfovibrio*-like species were obtained. No sequences representing the members of *Desulfobacterium* species were observed.

The *Desulfotomaculum*-like sequences were recovered only from the core 4 of the acetate-supplemented tank (Fig. 2.5c). Only two of the six retrieved sequences belonged to *Desulfotomaculum* species (*Dfm. acetoxidans*). The others affiliated with *Chlorobi* (1), Firmicutes (1) and unclassified bacteria (2). The *Desulfobacter*-like sequences were also recovered from the core 4 of the acetate-supplemented tank, however, no sequences affiliated with the group, indicating poor specificity of the primer (Fig. 2.5d). The *Desulfobulbus*-like sequences were recovered from the core 1 and 4. All of eleven sequences retrieved from core 1 were related to *Desulfobulbus propionicus*, whereas *Dbb. elongatus*-like sequences were recovered from core 4 (after acetate supplementation) (Fig. 2.5a). A total of 20 clones from core 1 sequenced with *Desulfococcus-Desulfosarcina-Desulfonema* group-specific primers contained a variety of bacterial sequences, including three sequences belonging to *Desulfonema* spp. (92-93% similarity to *Dsn. magnum*), eleven to *Desulfofaba* spp. (~94% similarity to *Dsf. fastidiosa*)

and three to *Desulfosarcina* spp. (~95% to *Dss. cetonicum*). Two other sequences were affiliated with uncultured *Chlorobi* and *Pseudomonas* spp. (99% to *P. fluorescens*). A total of 19 clones were sequenced from core 4. All belonged to the *Desulfococcus-Desulfosarcina-Desulfonema* group except one *Sedimentibacter hydrobenzoicus*-like sequence (93% similarity) (Fig. 2.5b). For *Desulfovibrio-Desulfomicrobium* group, thirteen and eleven clone sequences were recovered from cores 1 and 4, respectively (Table 2.6, Fig. 2.5e). The sequences retrieved from core 1 included five that were distantly affiliated with *Desulfovibrio ferrophilus* (91-92% similarity), three with *Desulfoarculus baarsii* (89%), two with *Desulfobacca acetoxidans* (90 and 89%), one with the sequence similarity to clone FW106 from a reject coal-impacted forested wetland soil (95%), one with candidate division WS3 (92%) and one with *Desulfonema ishimotoei* (90%). The sequences from core 4 were mainly related to *Desulfovibrio ferrophilus* (~92% similarity) except for one that was related to *Desulfobacca acetoxidans* (89%).

Rarefaction analysis showed the diversity of SRB-like sequences in the system were well presented by the libraries (excluded non-specific sequences), however the 16S rDNA libraries using bacteria domain-specific primers did not cover the microbial diversity (Fig. 2.6).

Spatial and temporal distributions of the microbial communities.

For a rapid and direct visualization of predominant microorganisms in the system, we employed denaturing gradient gel electrophoresis analysis of PCR-amplified 16S rDNA fragments. Banding patterns from DGGE analysis showed highly variable signatures in horizontal (12 sulfide zones) and vertical (core 1 and 4 of each tank) profiles. DGGE profiles from all of the sulfide zones (horizontal profile) showed the spatial changes of the predominant bacteria and revealed the heterogeneity of microbial communities even in the same reaction zones in the same tank (Figs. 2.7 and table 2.7). The spatial differences in microbial profiles also appeared in the

banding patterns of DGGE gel from the same cores taken at opposite sites of the tanks (Fig. 2.8 and table 2.8). The predominant microbial signatures found in DGGE analysis were beta- and gamma-*Proteobacteria* in the vertical DGGE profiles (including three different reaction zones) and Firmicutes, alpha- and beta-*Proteobacteria* in the horizontal DGGE profile (from all sulfide zones) (Tables 2.7 and 2.8). Interestingly, delta-*Proteobacteria* were not dominant in DGGE gels profiling, and the majority of delta-*Proteobacteria* recovered from DGGE were affiliated with *Geobacter* species. One candidate division OD1-like and one ε-*Proteobacteria*-like sequence was detected in samples from the sulfide zone of core 2 of the nonsupplemented tank. These sequences were not recovered in the clone libraries (Table 2.7).

DISCUSSION

In this study, we simulated vertical flow reactor-type constructed treatment wetlands modeling a reducing and alkalinity producing system. This system efficiently treated acid sulfate water and its effluent showed a pH increase from 2.4 to 6.4 and >97% removal of ferric iron. Total alkalinity generated in this system was more than 4 times higher than the traditional reducing and alkalinity-producing system. Based on isotope analysis, half of the alkalinity came from microbial sulfate reduction (Thomas, 2003). To stimulate sulfate reduction, the intake stream of one of the tank systems was supplemented with acetate. Acetate was chosen as the direct carbon substrate over lactate due to its cost-efficiency even though lactate is known as a more widely used substrate for SRB. Via concurrent sulfate reduction and acetate oxidation, 1 mole of proton is consumed, which results in the generation of 2 mole of bicarbonate alkalinity (eq. 6).

$$CH_3COO^- + SO_4^{2-} + H^+ \rightarrow H_2S + 2HCO_3^- \quad (6)$$

Therefore, adding this carbon source may stabilize the microbial sulfate reduction quickly, even though it can be used by other fermentative aerobic bacteria. The limestone-buffered organic substrate also contained various indirect and direct carbon substrates such as wood chips, straw, brewed grains, horse manure and cricket manure. Indirect carbon substrates for sulfate reduction such as wood chips required the symbiotic cooperation of heterotrophic polymer-hydrolytic fermentative bacteria. These heterotrophic fermentative bacteria break down the polymeric substrates into low molecular weight carbon sources that are then available to SRB such as pyruvate, lactate, acetate, ethanol and H_2. Strains belonging to the Firmicutes and *Bacteroides*, whose presence was indicated by 16S rDNA sequences retrieved from the clone libraries, could provide this benefit to the SRB in the system.

The oxide zone was directly impacted by acid sulfate runoff (pH ~ 2.4), so it was assumed that microorganisms associated with this zone could be acidotolerant and aerotolerant. The microbial signatures retrieved from DGGE analysis showed *Proteobacteria*, including alpha, beta and gamma subdivisions, might be dominant in this zone. Many beta-*Proteobacteria* such as *Acidithiobacillus ferrooxidans* can thrive at low pH and oxidize or reduce iron (Harahuc et al., 2000; Sand, 1989). *Thiomonas*-like sequences were recovered from the oxide zone, this organism can metabolize arsenite and arseniate that are common cocontaminants in acidic waste water (Bruneel et al., 2003). Other bacterial signatures, such as those of the floc-forming *Acidovorax delafieldii* and a thermo- and aerophilic *Aneurinibacillus thermoaerophilus*, were also retrieved. The transitional zone developed between the oxide zone and the sulfide zone had aluminum hydroxysulfates as main precipitates. Microbial diversity was not investigated in this zone, but the numbers of SRB were comparable to the sulfide zone (data not shown). Most known SRB are sensitive to acidic pH. However, under laboratory conditions, SRB can be active

at pHs as low as 3 (Kolmert & Johnson, 2001), which suggests that microbial sulfate reduction may be metabolically significant at acidic conditions.

The sulfide zone was characterized by an abundance of iron sulfides and an increased alkalinity (Thomas, 2003). Most of the bacterial sequences identified from the clone libraries were related to uncultured bacteria. The most predominant signatures were recovered as *Chloroflexi* and *Proteobacteria* in the sulfide zone of the acetate-supplemented tank. Koizumi and colleagues (2004) reported that *Chloroflexi* are dominant in the top of lake sediments and coexist with SRB. Although *Chloroflexi* are widespread in various environments, their roles remain uncertain due to the absence of a closely related isolate and information about their physiology. Recently, the physiological studies of two isolates belonging to this group showed that neither of them was photosynthetic, but they were able to grow under anaerobic and fermentative conditions (Sekiguchi et al., 2003). Thus it is possible that some microorganisms related to the *Chloroflexi* may grow heterotrophically by degrading macromolecules under anaerobic conditions instead of performing photosynthesis aerobically. Thus, we assume *Chloroflexi* and other fermentative bacteria such as members of the Firmicutes and *Bacteroides*, degrade polysaccharides and other macromolecules in the system and provide fatty acids (i.e. pyruvate, lactate and acetate) and hydrogen as carbon sources for SRB. However, Brofft and colleagues (2002) reported that *Chloflexi*-like sequences were recovered only from an unaffected site near a reject coal pile, indicating that this group may be sensitive to the acidic pH.

Based on the clone libraries, *Proteobacteria*-like sequences became more abundant in core 4 of both the acetate-supplemented tank and the non-supplemented tank, but sequences belonging to the delta subdivision of *Proteobacteria* did not increased in the acetate-supplemented tank. It was observed that the sulfide zone in the acetate-supplemented tank

expanded to other zones especially to the oxide zone. It is possible that the sulfate in the sulfide zone of the acetate-supplemented tank was consumed resulting in sulfate limitations of the zone. In addition, the clone library analysis and DGGE analysis *Geobacter* were mainly retrieved as a delta subgroup from the sulfide zone of the acetate-supplemented tank. Members of the Geobacter/Pelobacter group can reduce sulfur (S^0), thus they are ecologically related to SRB (Lonergan et al., 1996).

Nested PCR was used to further identify members of the SRB. The results indicate that SRB were not numerically dominant in the system. Only members of the *Desulfovibrio* group were recovered without nested amplification, indicating this group may be the most abundant SRB. *Desulfotomaculum* and *Desulfobacter* groups were not retrieved from nested PCR in the samples taken before acetate supplementation. Since the primers for these two SRB groups turned out not to be very specific, their infrequency may due to the low primer-specificity. Analysis of the clone library confirmed that these two groups were present. Based on the retrieved sequences, it is suggested that the predominant microbial community in the sulfide zone shifted from heterotrophic fermentative bacteria to *Proteobacteria*. In addition, the microbial communities changed within the phylogenetic group retrieved by sequence analysis. For example, *Desulfosporosinus*-like sequences were recovered from core 1 but not from core 4 of both tanks. LIBSHUFF analysis of the clone libraries with *P* values below 0.025 indicated each clone library was considered significantly different from each other, which further suggested that the bacterial compositions were different in both tanks and even locally in the same tank. Various factors such as perturbation by substrates and temporal variations could have affected the obtained bacterial richness detected in this system. It also includes systematic experimental factors such as the size of the clone libraries and PCR bias. However, at higher

taxonomic levels, the phylogenetic analyses indicated that all clone libraries share the same major taxa, which were retained throughout the treatment.

The unreacted zone was located below the sulfide zone possessed a neutral pH (~6.8) and contained no unique precipitates. Although the microbial diversity was not investigated in detail, based on DGGE profiles we assumed that this zone may still have SRB activity and also other microbial reactions such as methanogenesis.

Microbial characterizations of this constructed wetland system suggest that an appropriate supplemental substrate such as acetate will enhance microbial sulfate reduction activity and alter the composition of indigenous sulfate-reducing bacterial communities. This study also indicates that supplemented substrates for SRB, such as the acetate used in this study also enhances the initial growth of heterotrophic bacteria, which later can support the growth of SRB by the degradation of insoluble macromolecules of the tank matrix. Based on the data present here we concluded that heterotrophic fermentative bacteria and SRB syntrophically associate with each other, and play a major role in metal removal and alkalinity generation in constructed treatment wetlands.

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Table 2.1. Composition of the limestone-buffered organic substrates.

Composition of limestone-buffered organic substrates	% dry weight
Composted stable wastes	72
wood shavings	
straw	
horse manure	
Spent brewing grains	3
Kriket Krap (composted cricket manure)	<1
Fine grained limestone screenings (grain size of 1.2 mm, 91.5% $CaCO_3$)	25

Table 2.2. The water chemistry data from influent and effluent samples (averaged from April 1999 to December 2000).

Variable	Influent	Effluent
pH	2.4 (1.6-3.0)	6.5 (5.5-7.1)
Acidity (mg/L, CaCO3 equivalents)	1304 (738-2,320)	35 (4-100)
Alkalinity (mg/ L , CaCO3 equivalents)	ND[a]	619
Total iron* (mg/ L)	142 (92-237)	15 (0-123)
Aluminum (mg/ L)	84 (39-274)	0.05 (0-9.3)
Sulfate (mg/ L)	1,521 (926-3,385)	1201 (263-2732)
Calcium (mg/ L)	52 (23-114)	600 (268-1003)
Sulfide (mg/ L)	ND	0.7-16

[a]ND, not determined

Table 2.3. Samples for this study.

Tank	Non-supplemented (36A[a])			Acetate-supplemented (30A[b])		
Core	1 (4)	2 (5)	3 (6)	1 (4*)	2 (5*)	3 (6*)
Zone	a[1]	a	a	a	a	a
	c[2]	c	c	c	c	c
	d[3]	d	d	d	d	d

Note: Cores 1, 2 and 3 were taken in June 2000.
Cores (4), (5), and (6) are were taken in Nov. 2000.
[a]36A represents a tank without acetate supplementation.
[b]30A represents an acetate-supplemented tank.
* Samples from core 4, 5 and 6 of 30A tank were taken after acetate supplementation only to the acetate-supplemented (30A) tank.
[1]a represents the oxide zone.
[2]c represents the sulfide zone.
[3]d represents the unreacted zone.

Table 2.4. PCR primer sets used in this study.

Primers	Target organisms	Sequences (5' to 3')	Annealing Temp. (^{o}C)	Expected size of product (bp)	Reference
356F	Eubacteria	CCTACGGGAGGCAGCAG	56	192	Lane, 1991
517R	Universal	ATTACCGCGGCTGCTGG			
907R*	Universal	CCGTCAATTCCTTTGAGTTT		1365	
1392R*	Universal	ACGGGCGGTGTGTAC			
M13F*	pCR 2.1 vector	GTAAAACGACGGCCAG	55		
M13R*		CAGGAAACAGCTATGAC			
DFM140*	*Desulfotomaculum*	TAGMCYGGGATAACRSYKG	58	700	Daly *et al*., 2000
DFM842		ATACCCSCWWCWCCTAGCAC			
DBB121*	*Desulfobulbus*	CGCGTAGATAACCTGTCYTCATG	66	1120	Daly *et al*., 2000
DBB1237		GTAGKACGTGTGTAGCCCTGGTC			
DBM169	*Desulfobacterium*	CTAATRCCGGATRAAGTCAG	64	840	Daly *et al*., 2000
DBM1006		ATTCTCARGATGTCAAGTCTG			
DSB127*	*Desulfobacter*	GATAATCTGCCTTCAAGCCTGG	60	1150	Daly *et al*., 2000
DSB1273		CYYYYYGCRRAGTCGSTGCCCT			
DCC305*	*Desulfococcus* *Desulfonema* *Desulfosarcina*	GATCAGCCACACTGGRACTGACA	65	860	Daly *et al*., 2000
DCC1165		GGGGCAGTATCTTYAGAGTYC			
DSV230*	*Desulfovibrio*	GRGYCYGCGTYYCATTAGC	61	610	Daly *et al*., 2000
DSV838*	*Desulfomicrobium*	SYCCGRCAYCTAGYRTYCATC			

* primers used for sequencing.

Table 2.5. Distribution of sequences retrieved using bacterial domain-specific primers.

Phylogenetic group	Non-supplemented (sulfide zone/core 1)	Acetate-supplemented (sulfide zone/core 1)	Acetate-supplemented (sulfide zone/core 4)*
Acidobacteria	5		1
Actinobacteria			2
Candidate division OP10		1	
Candidate division OP11	7	8	8
Candidate division TM6	2		
Candidate division WS6	1		
CFB (mainly Bacteroides)		7	3
Chloroflexi	9	14	9
Green sulfur (*Chlorobi*)		2	
Low G+C Gram-type positives (Firmicutes)			
Desulfosporosinus		4	
Enterococcus			3
Other Firmicutes		3	2
Nitrospira		4	2
Planctomycetes	1	2	2
Proteobacteria			
alpha	5	3	6
beta	5	4	6
gamma	4	1	
delta	10	5	5
Spirochete	1		
Unclassified	5	2	
Total sample size (n)	55	60	49

* Samples taken in Nov. 2000 after acetate supplementation to the acetate-supplemented tank.

Table 2.6. Summary of environmental sequences retrieved from the sulfide zones (c) of core1 and 4 in the acetate-supplemented tank (30A) using SRB group-specific primer sets.

Primers	30A 1[a]c	30A 4[b]c
DFM140 and 842	ND[c]	6
DBB121 and 1237	11	7
DBM169 and 1006	ND	ND
DSB127 and 1273	ND	9
DCC305 and 1165	20	17
DSV230 and 818	13	11

[a]1 represents the core of which sample was taken in June 2000.
[b]4 represents the core of which sample was taken in November 2000.
[c]ND, not detected, no PCR product obtained after nested PCR reaction.

Table 2.7. Distribution of 16S rDNA sequences obtained from the sulfide zones with DGGE analysis. Numbers are band numbers in fig. 2.7.

Phylogenetic groups / Tank core/reaction zone	Non-supplemented (36A4b)						Acetate-supplemented (30A4b)					
	1c	2c	3c	4c	5c	6c	1c	2c	3c	4c*	5c*	6c*
Actinobacteria		5		1				3				
Candidate division OD1		1										
Candidate division TM6	1	4		5, 8		1						
Chloroflexi				10, 4								
Green sulfur (*Chlorobi*)				8								
Low G+C Gram-type positives (*Firmicutes*)				3, 4, 5, 7						2, 2, 2, 3	1, 2, 2, 3, 3, 5, 10, 10	
Proteobacteria												
alpha		3		1, 7, 10			1, 1, 6, 2, 3, 4, 5, 5	1				
beta		1, 3, 2		9, 2, 5, 3, 7, 6			2, 3, 5, 6, 2	1				
gamma				9								
delta										1, 3		
epsilon		1										
Spirochete							4					
Unclassified										4		

* Samples from the sulfide zone of core 4, 5 and 6 of the acetate-supplemented (30A) tank were taken after acetate supplementation.

Table 2.8. Distribution of 16S rDNA sequences obtained from three different biogeochemical reaction zones of both non-supplemented and acetate-supplemented tanks by DGGE analysis. Numbers are band numbers in fig. 2.8.

Phylogenetic groups / Tank core/reaction zone	Non-supplemented (36A)						Acetate-supplemented (30A)					
	1a[1]	1c[2]	1d[3]	4a	4c	4d	1a	1c	1d	4a*	4c*	4d*
Acidobacteria					2							
Actinobacteria				2								4
Candidate division TM6												4
CFB (mainly Bacteroides)			1			1, 1			1			
Chloroflexi					1							1, 2
Low G+C Gram-type positives (*Firmicutes*)				1			2			2, 4, 5, 6	2, 2	
Proteobacteria												
alpha								1		1, 3		2
beta		1, 1, 2	2		1, 2, 3		1, 1	2, 2, 3, 3				
gamma	1, 3, 2, 4	2		1								1, 2, 3, 4
delta		1	1, 2								1	2
Unclassified										3, 7	2	

* Samples from the three different zones of the core 4 in the acetate-supplemented (30A) tank were taken after acetate supplementation.

[1]a represents the oxide zone.

[2]c represents the sulfide zone.

[3]d represents the unreacted zone.

Figure 2.1. Coal pile runoff basin (CPRB) at Department of Energy's Savannah River Site near Aiken, SC.

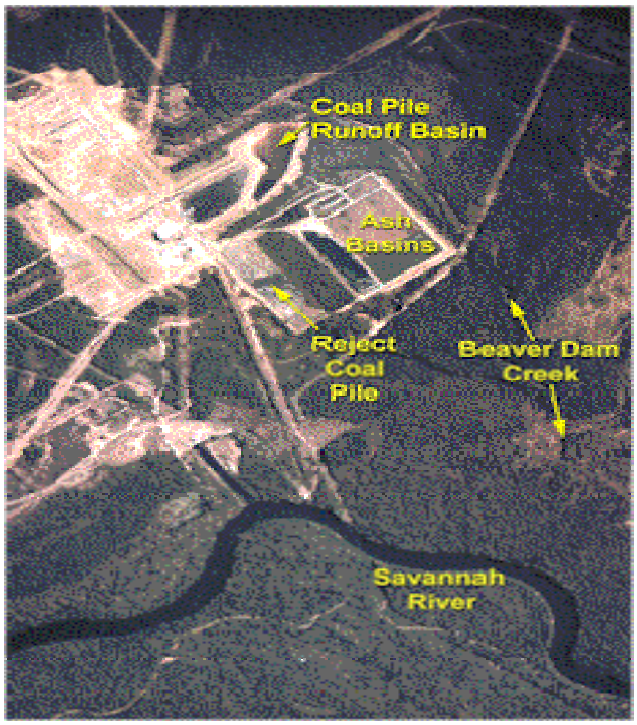
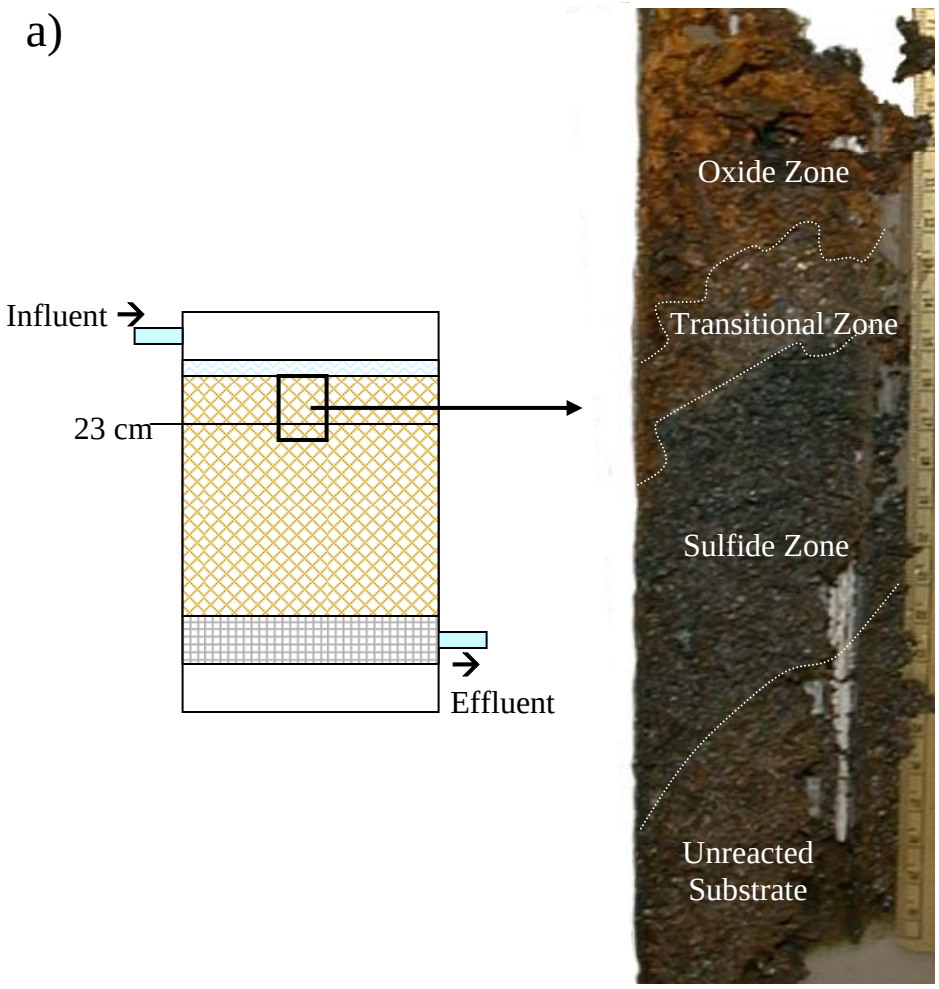

Coal Pile
Runoff Basin
Ash
Basins
Reject
Coal
Pile
Beaver Dam
Creek
Savannah
River

Figure 2.2. Constructed treatment wetland systems receiving acid runoff from coal piles.

Figure 2.3. The schematic view of a constructed wetland system (modified from Thomas, 2003): a) a schematic diagram of a tank and an example of the zonation that developed in a core during the course of this study, b) a schematic view of cores; core 1, 2 and 3 were sampled before acetate supplementation into the acetate-supplemented tank, while core 4, 5 and 6 were sampled after acetate supplementation.

a)


Influent
23 cm
Effluent
Oxide Zone
Transitional Zone
Sulfide Zone
Unreacted
Substrate

b)

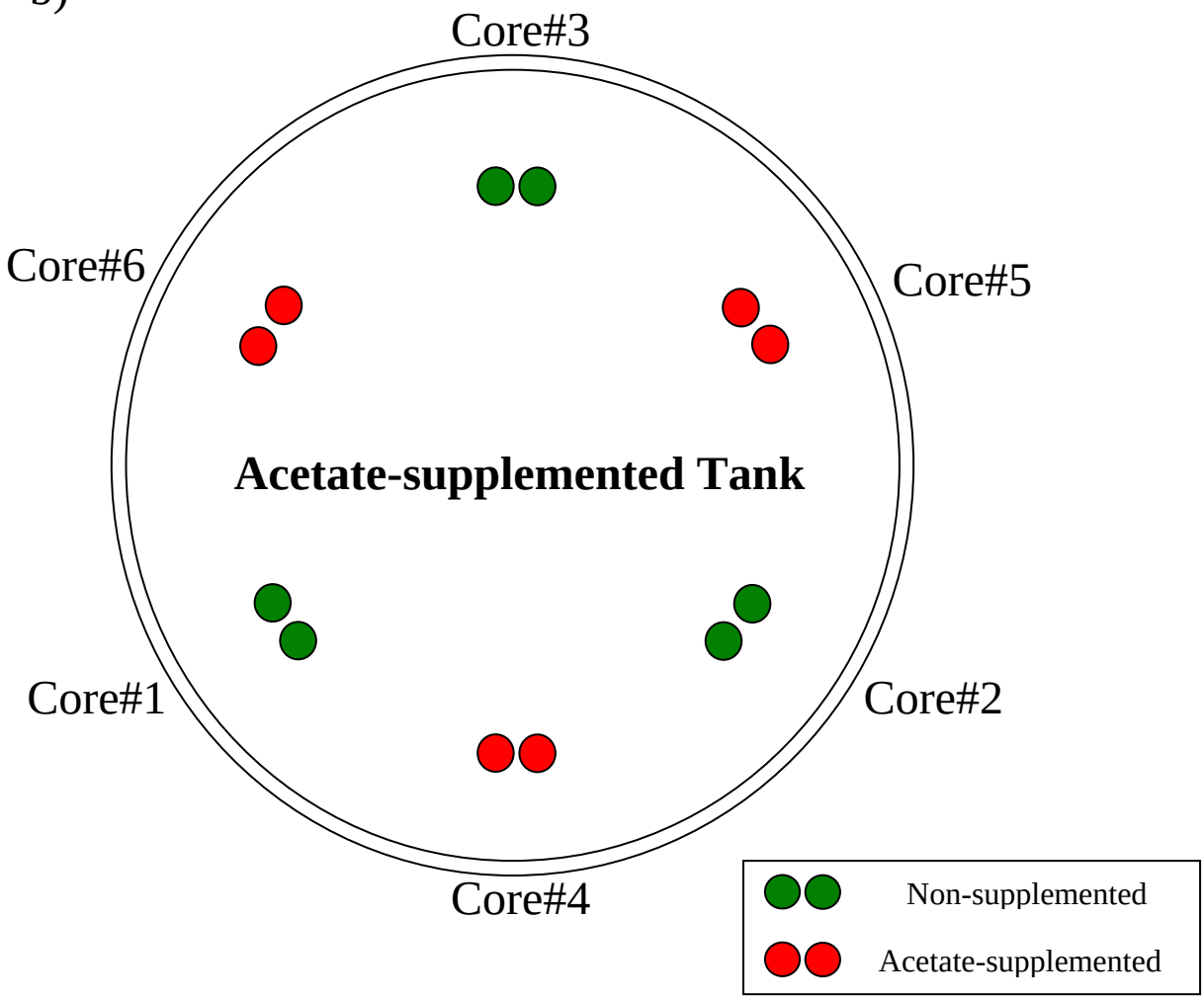

Core#3
Core#6
Core#5
Acetate-supplemented Tank
Core#1
Core#2
Core#4
Non-supplemented
Acetate-supplemented

Figure 2.4. Phylogenetic relationships based on partial 16S rDNA sequences of clones, which retrieved using bacterial domain-specific primers. The tree was constructed using Neighbor-joining method with Jukes and Cantor distance corrections. The scale bar indicates 5 nucleotides substitutions per 100 nucleotides. Bootstrap values above 50% are shown at nodes. The names of sequences recovered after acetate supplementation into the acetate-supplemented tank are colored with green.

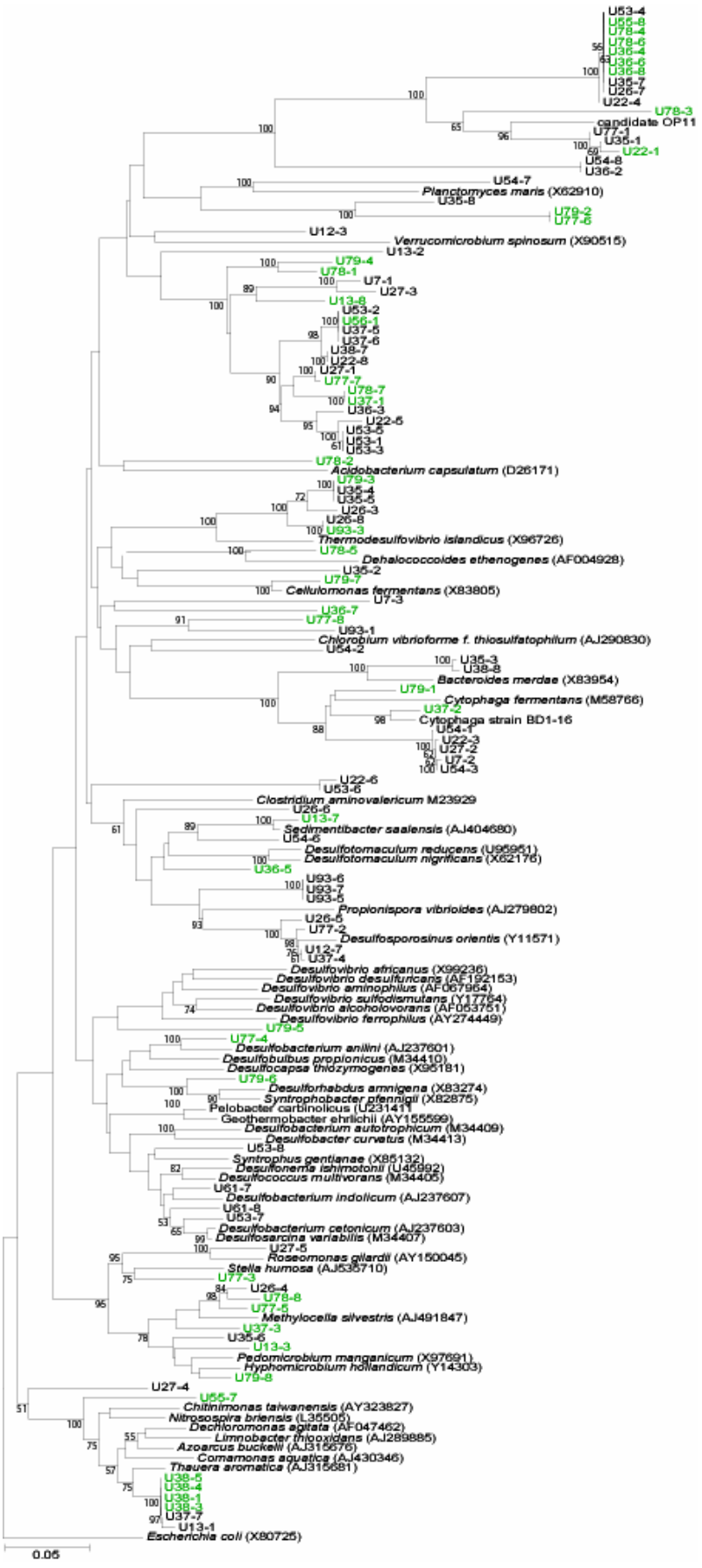
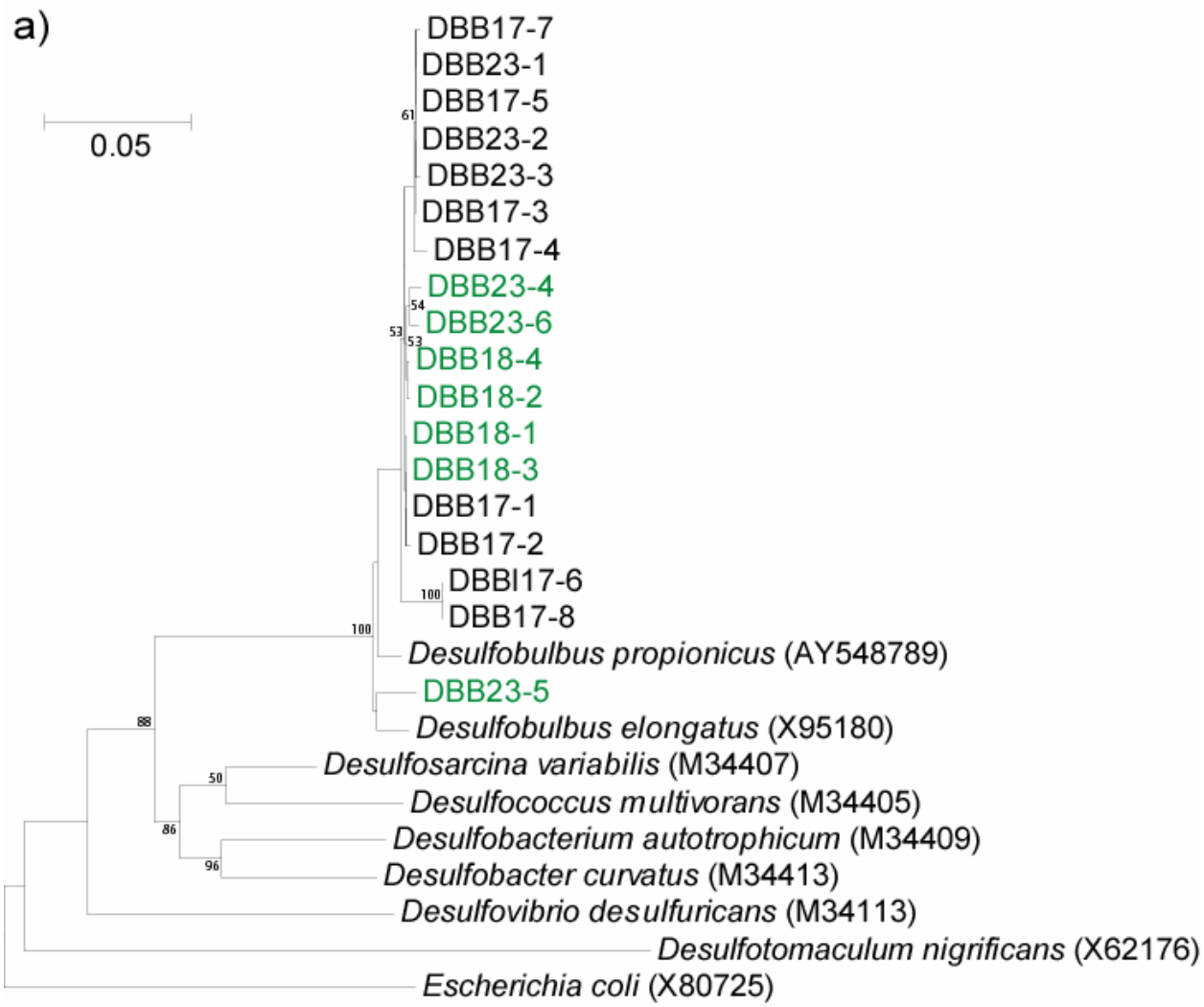
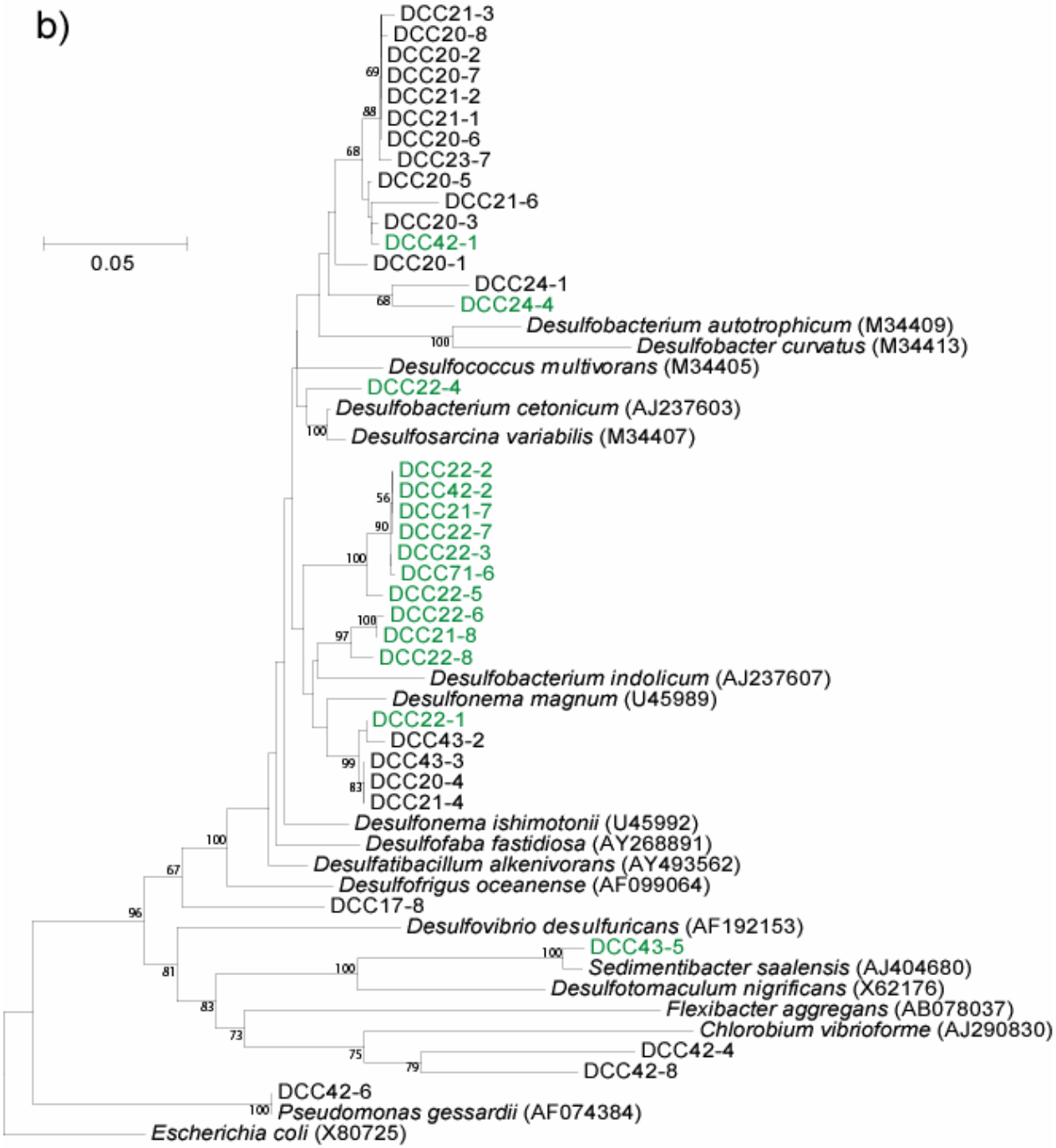
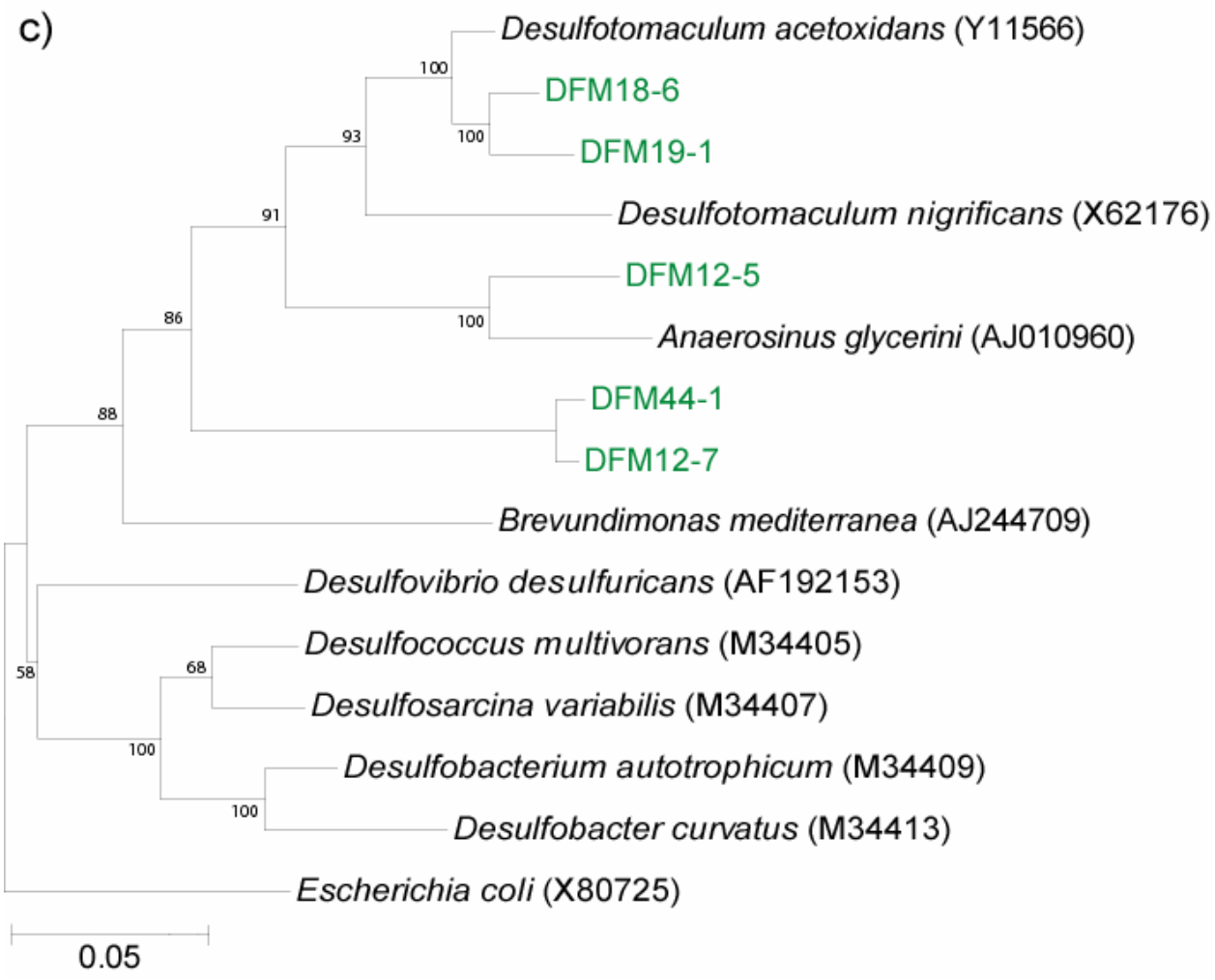
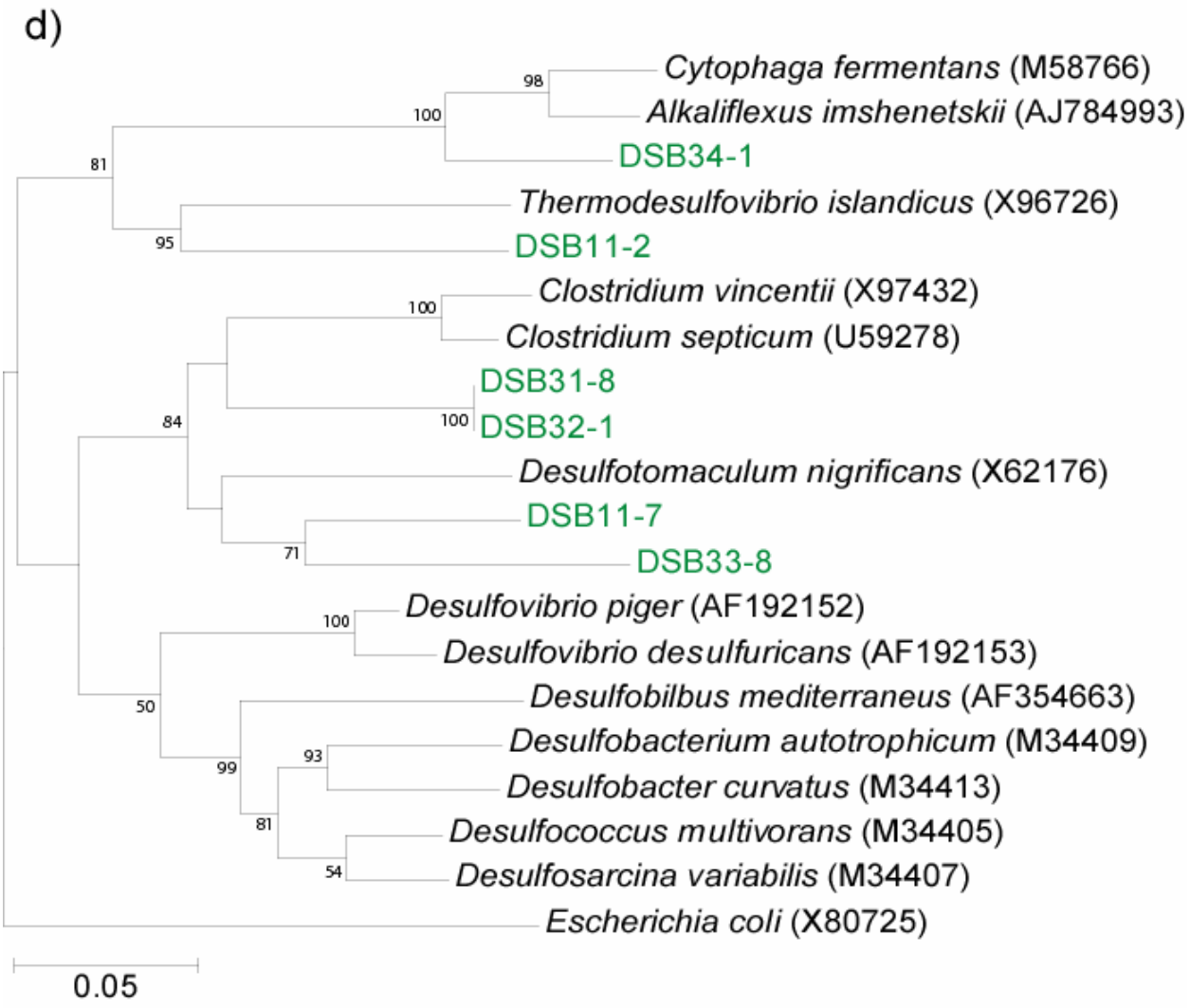

U53-4
U35-8
U78-4
U78-6
U36-4
U36-6
U36-8
U38-7
U26-7
U22-4
U78-3
candidate OP11
U77-1
U35-1
U22-1
U54-8
U36-2
U54-7
Planctomyces maris (X62910)
U35-8
U79-2
U77-6
U12-3
Verrucomicrobium spinosum (X90515)
U13-2
U79-4
U78-1
U7-1
U27-3
U13-8
U53-2
U56-1
U37-5
U37-6
U38-7
U22-8
U27-1
U77-7
U78-7
U37-1
U36-3
U22-5
U53-5
U53-1
U53-3
U78-2
Acidobacterium capsulatum (D26171)
U79-3
U35-4
U35-5
U26-3
U26-8
U93-3
Thermodesulfovibrio islandicus (X96726)
U78-5
Dehalococcoides ethenogenes (AF004928)
U35-2
U79-7
Cellulomonas fermentans (X83805)
U7-3
U36-7
U77-8
U93-1
Chlorobium vibrioforme f. thiosulfatophilum (AJ290830)
U54-2
U35-3
U38-8
Bacteroides merdae (X83954)
U79-1
Cytophaga fermentans (M58766)
U37-2
Cytophaga strain BD1-16
U54-1
U22-3
U27-2
U7-2
U54-3
U22-6
U53-6
Clostridium aminovalericum M23929
U26-6
U13-7
Sedimentibacter saalensis (AJ404680)
U54-5
Desulfotomaculum reducens (U95951)
Desulfotomaculum nigrificans (X62176)
U36-5
U93-6
U93-7
U93-5
Propionispora vibrioides (AJ279802)
U26-5
U77-2
Desulfosporosinus orientis (Y11571)
U12-7
U37-4
Desulfovibrio africanus (X99236)
Desulfovibrio desulfuricans (AF192153)
Desulfovibrio aminophilus (AF067964)
Desulfovibrio sulfodismutans (Y17764)
Desulfovibrio alcoholovorans (AF063751)
Desulfovibrio ferrophilus (AY274449)
U79-5
U77-4
Desulfobacterium anilini (AJ237601)
Desulfobulbus propionicus (M34410)
Desulfocapsa thiozymogenes (X95181)
U79-6
Desulforhabdus amnigena (X83274)
Syntrophobacter sfennigii (X82875)
Pelobacter carbinolicus (U23411)
Geothermobacter ehrlichii (AY155599)
Desulfobacterium autotrophicum (M34409)
Desulfobacter curvatus (M34413)
U53-8
Syntrophus gentianae (X85132)
Desulfonema ishimotonii (U45992)
Desulfococcus multivorans (M34405)
U61-7
Desulfobacterium indolicum (AJ237607)
U61-8
U53-7
Desulfobacterium cetonicum (AJ237603)
Desulfosarcina variabilis (M34407)
U27-5
Roseomonas gilardii (AY150045)
Stella humosa (AJ535710)
U77-3
U26-4
U78-8
U77-5
Methylocella silvestris (AJ491847)
U37-3
U35-6
U13-3
Pedomicrobium manganicum (X97691)
Hyphomicrobium hollandicum (Y14303)
U79-8
U27-4
U55-7
Chitinimonas taiwanensis (AY323827)
Nitrosospira briensis (L35505)
Dechloromonas agitata (AF047462)
Limnobacter thiooxidans (AJ289885)
Azoarcus buckelii (AJ315676)
Comamonas aquatica (AJ430346)
Thauera aromatica (AJ315681)
U38-5
U38-4
U38-1
U35-3
U37-7
U13-1
Escherichia coli (X80725)
0.05

Figure 2.5. Phylogenetic trees constructed with using Jukes and Cantor distance corrections and Neighbor-joining method based on partial 16S rDNA sequences of clones recovered by using six SRB group-specific primer sets: a) *Desulfobulbus*, b) *Desulfococcus*, c) *Desulfotomaculum*, d) *Desulfobacter and* e) *Desulfovibrio* groups. The names of sequences recovered after acetate supplementation into the acetate-supplemented tank were colored with green. The numbers at nodes represent >50% bootstrap support (100 replicates). The scale bar indicates 5 nucleotides substitutions per 100 nucleotides.


a)
0.05
DBB17-7
DBB23-1
DBB17-5
DBB23-2
DBB23-3
DBB17-3
DBB17-4
DBB23-4
DBB23-6
DBB18-4
DBB18-2
DBB18-1
DBB18-3
DBB17-1
DBB17-2
DBBI17-6
DBB17-8
Desulfobulbus propionicus (AY548789)
DBB23-5
Desulfobulbus elongatus (X95180)
Desulfosarcina variabilis (M34407)
Desulfococcus multivorans (M34405)
Desulfobacterium autotrophicum (M34409)
Desulfobacter curvatus (M34413)
Desulfovibrio desulfuricans (M34113)
Desulfotomaculum nigrificans (X62176)
Escherichia coli (X80725)
61
54
53
53
100
100
88
50
86
96


b)
0.05
DCC21-3
DCC20-8
DCC20-2
DCC20-7
DCC21-2
DCC21-1
DCC20-6
DCC23-7
DCC20-5
DCC21-6
DCC20-3
DCC42-1
DCC20-1
DCC24-1
DCC24-4
Desulfobacterium autotrophicum (M34409)
Desulfobacter curvatus (M34413)
Desulfococcus multivorans (M34405)
DCC22-4
Desulfobacterium cetonicum (AJ237603)
Desulfosarcina variabilis (M34407)
DCC22-2
DCC42-2
DCC21-7
DCC22-7
DCC22-3
DCC71-6
DCC22-5
DCC22-6
DCC21-8
DCC22-8
Desulfobacterium indolicum (AJ237607)
Desulfonema magnum (U45989)
DCC22-1
DCC43-2
DCC43-3
DCC20-4
DCC21-4
Desulfonema ishimotonii (U45992)
Desulfofaba fastidiosa (AY268891)
Desulfatibacillum alkenivorans (AY493562)
Desulfofrigus oceanense (AF099064)
DCC17-8
Desulfovibrio desulfuricans (AF192153)
DCC43-5
Sedimentibacter saalensis (AJ404680)
Desulfotomaculum nigrificans (X62176)
Flexibacter aggregans (AB078037)
Chlorobium vibrioforme (AJ290830)
DCC42-4
DCC42-8
DCC42-6
Pseudomonas gessardii (AF074384)
Escherichia coli (X80725)


c)
Desulfotomaculum acetoxidans (Y11566)
DFM18-6
DFM19-1
Desulfotomaculum nigrificans (X62176)
DFM12-5
Anaerosinus glycerini (AJ010960)
DFM44-1
DFM12-7
Brevundimonas mediterranea (AJ244709)
Desulfovibrio desulfuricans (AF192153)
Desulfococcus multivorans (M34405)
Desulfosarcina variabilis (M34407)
Desulfobacterium autotrophicum (M34409)
Desulfobacter curvatus (M34413)
Escherichia coli (X80725)
100
100
93
91
100
86
88
58
68
100
100
0.05


d)
Cytophaga fermentans (M58766)
Alkaliflexus imshenetskii (AJ784993)
DSB34-1
Thermodesulfovibrio islandicus (X96726)
DSB11-2
Clostridium vincentii (X97432)
Clostridium septicum (U59278)
DSB31-8
DSB32-1
Desulfotomaculum nigrificans (X62176)
DSB11-7
DSB33-8
Desulfovibrio piger (AF192152)
Desulfovibrio desulfuricans (AF192153)
Desulfobilbus mediterraneus (AF354663)
Desulfobacterium autotrophicum (M34409)
Desulfobacter curvatus (M34413)
Desulfococcus multivorans (M34405)
Desulfosarcina variabilis (M34407)
Escherichia coli (X80725)
98
100
81
95
100
84
100
71
100
50
99
93
81
54
0.05

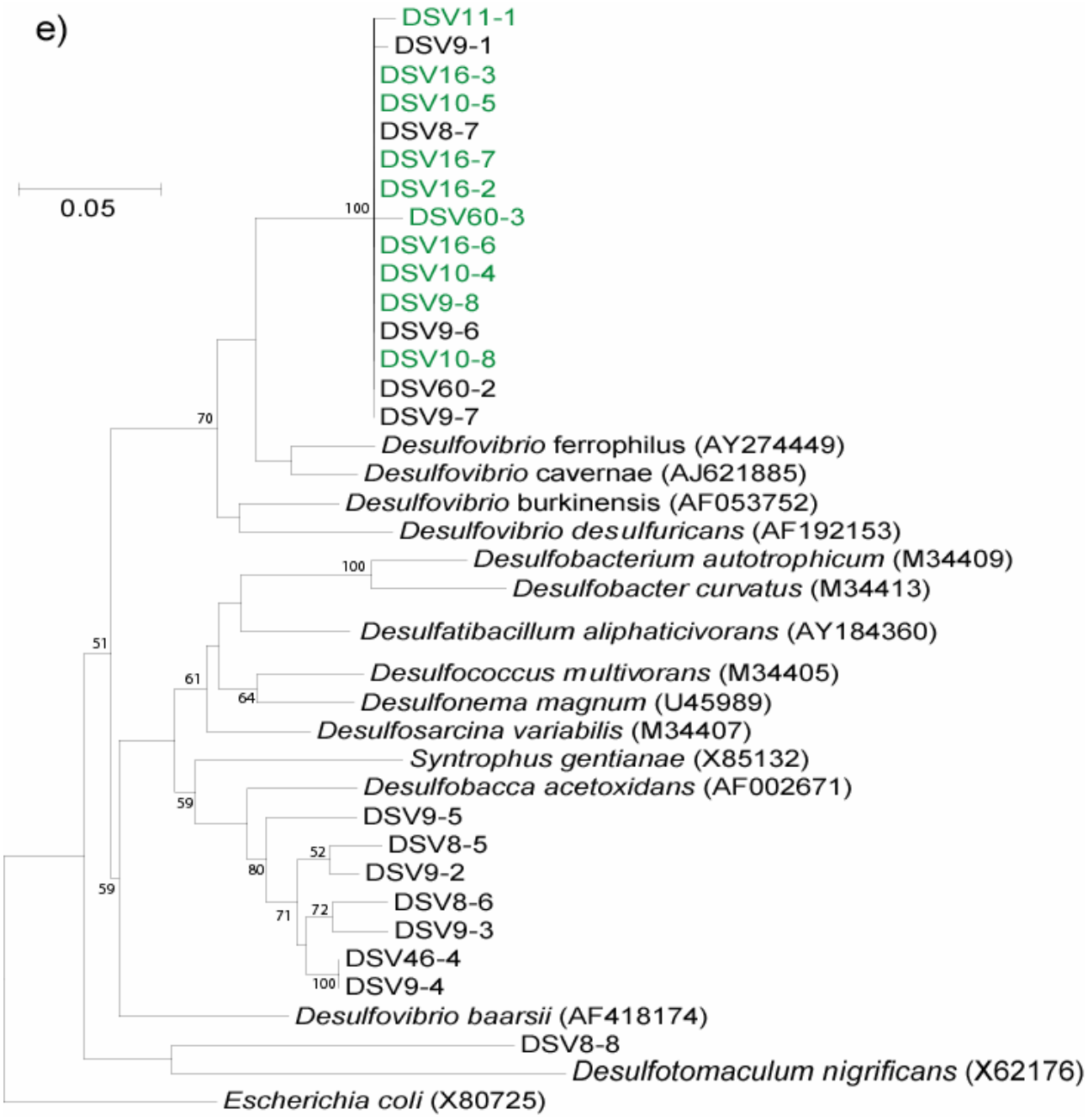

e)
0.05
DSV11-1
DSV9-1
DSV16-3
DSV10-5
DSV8-7
DSV16-7
DSV16-2
DSV60-3
DSV16-6
DSV10-4
DSV9-8
DSV9-6
DSV10-8
DSV60-2
DSV9-7
Desulfovibrio ferrophilus (AY274449)
Desulfovibrio cavernae (AJ621885)
Desulfovibrio burkinensis (AF053752)
Desulfovibrio desulfuricans (AF192153)
Desulfobacterium autotrophicum (M34409)
Desulfobacter curvatus (M34413)
Desulfatibacillum aliphaticivorans (AY184360)
Desulfococcus multivorans (M34405)
Desulfonema magnum (U45989)
Desulfosarcina variabilis (M34407)
Syntrophus gentianae (X85132)
Desulfobacca acetoxidans (AF002671)
DSV9-5
DSV8-5
DSV9-2
DSV8-6
DSV9-3
DSV46-4
DSV9-4
Desulfovibrio baarsii (AF418174)
DSV8-8
Desulfotomaculum nigrificans (X62176)
Escherichia coli (X80725)
100
70
100
51
61
64
59
52
80
72
71
100
59

Figure 2.6. Rarefaction curves of each 16S rDNA clone libraries using Analytic Rarefaction v1.3. Sequences were grouped into OTUs using DOTUR with a furthest-neighbor (complete-linkage) algorithm.

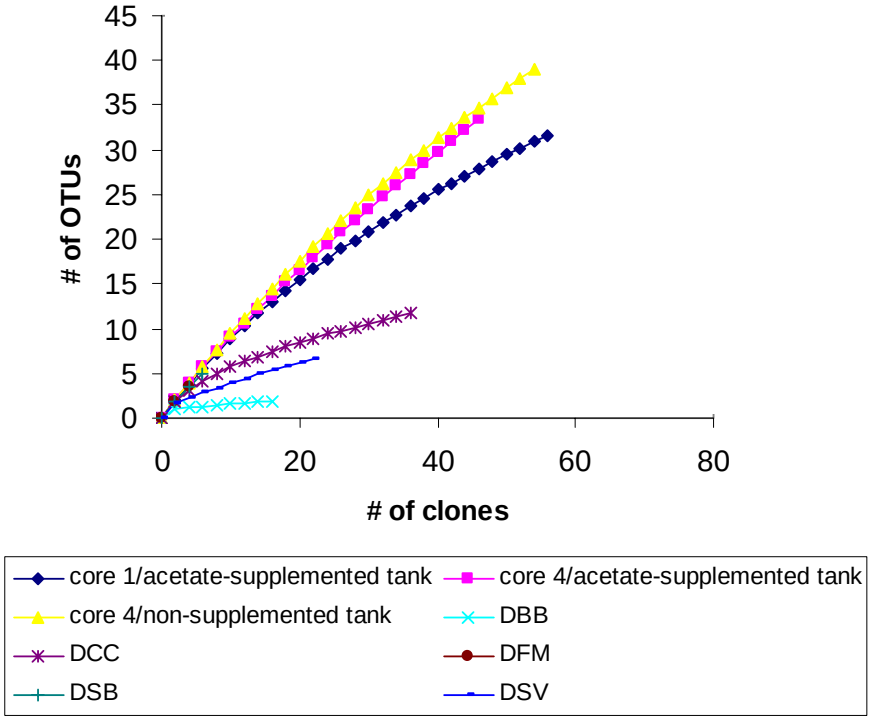

45
40
35
30
25
20
15
10
5
0
# of OTUs
0
20
40
60
80
# of clones
core 1/acetate-supplemented tank
core 4/acetate-supplemented tank
core 4/non-supplemented tank
DBB
DCC
DFM
DSB
DSV

Figure 2.7. DGGE fingerprints of the sulfide zones of both acetate-supplemented and non-supplemented tanks. Cores 1, 2 and 3 were sampled in June 2000, while cores 4, 5 and 6 were sampled in Nov. 2000. Numbers to sides of lanes represent bands excised and sequenced from each lane of the gel. Clone libraries were constructed from the samples marked by numbers, and three clones of each band were sequenced. The phylogenetic affiliations of these bands are given in Table 2.7.

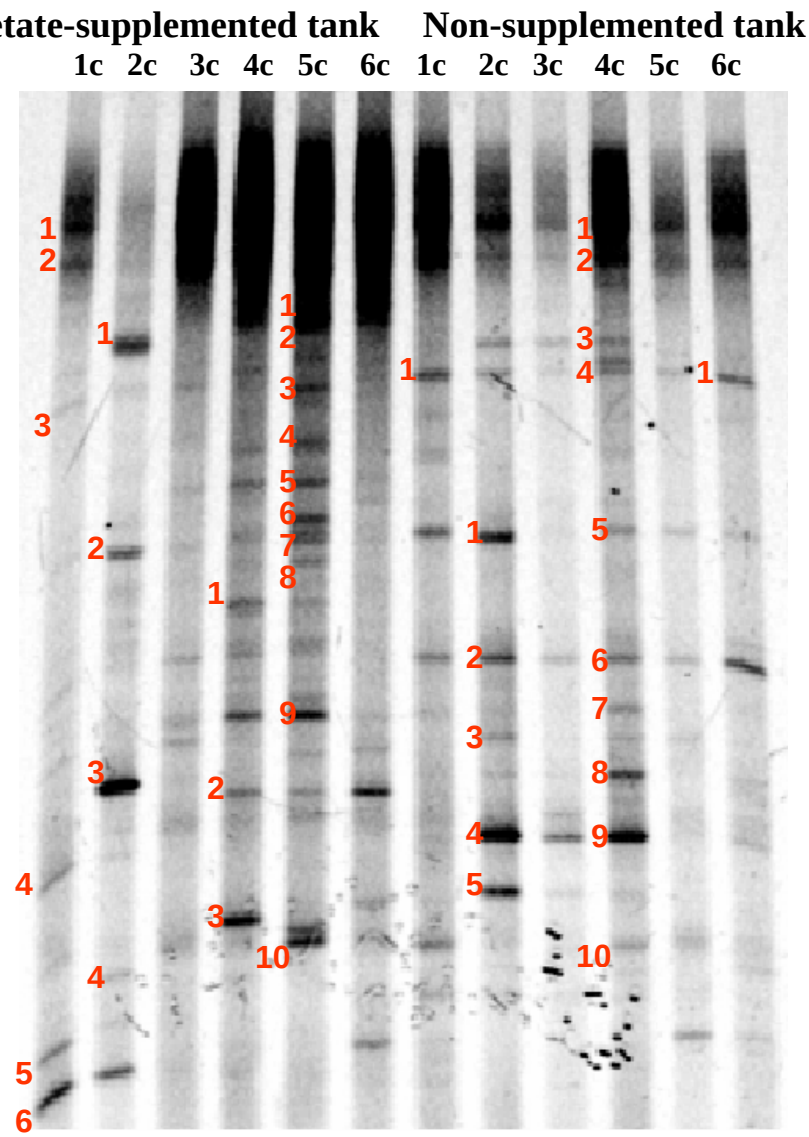
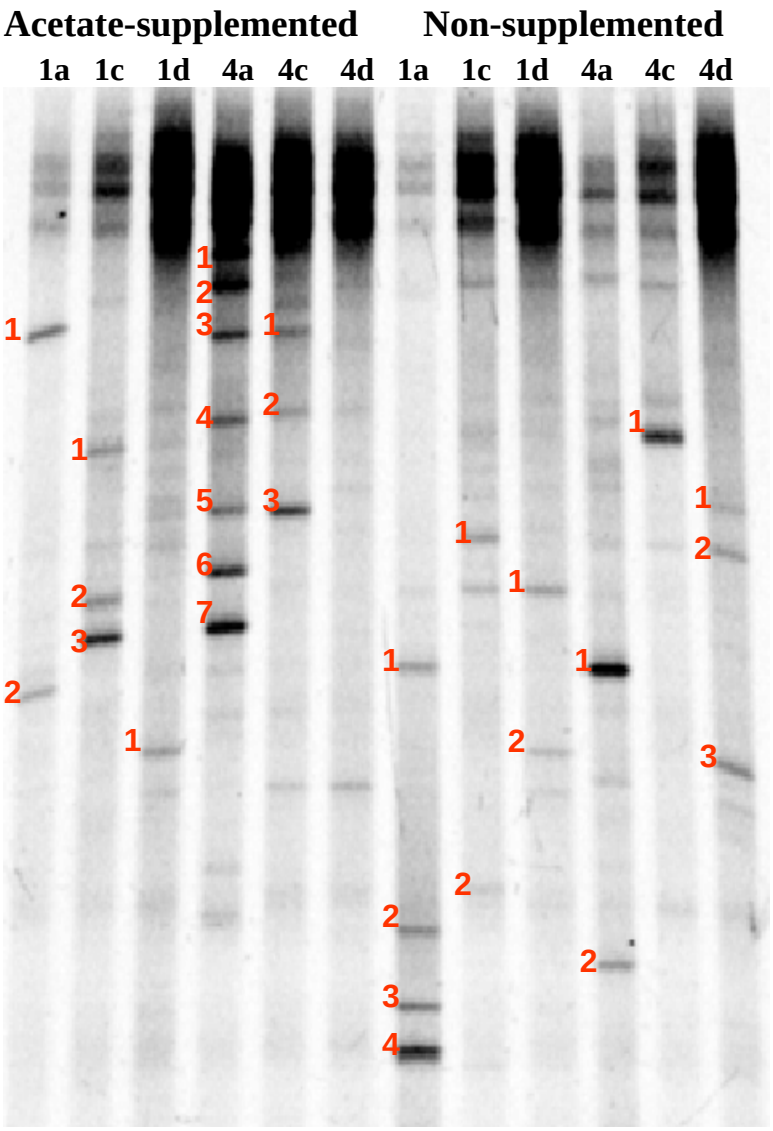

Acetate-supplemented tank
Non-supplemented tank
1c 2c 3c 4c 5c 6c 1c 2c 3c 4c 5c 6c

Figure 2.8. DGGE profiles of core 1 and 4 of both acetate-supplemented and non-supplemented tanks. Core 1 was sampled in June 2000, while core 4 was sampled in Nov. 2000. Numbers to sides of lanes represent bands excised and sequenced from each lane of the gel. Bands indicated by numbers were cloned, and three clones per band were sequenced. The phylogenetic affiliations of these bands are given in Table 2.8.


Acetate-supplemented
Non-supplemented
1a
1c
1d
4a
4c
4d
1a
1c
1d
4a
4c
4d

CHAPTER 3

CULTURABLE DIVERSITY ASSOCIATED WITH DIFFERENT REACTION ZONES FORMED IN A CONSTRUCTED TREATMENT WETLAND SYSTEM RECEIVING ACID SULFATE WATER[1]

[1]Lee, Y.-J, R. C. Thomas, C. S. Romanek, J. V. McArthur, and J. Wiegel. To be submitted to *FEMS Microbiology Ecology*.

ABSTRACT

Investigation on the cultural microbial diversity is fundamental to link genomic data to the ecology and function of in situ microorganisms. To elucidate the sulfate-reducing bacterial community in different reaction zones formed in a constructed wetland system, the most probable number (MPN) technique was employed and coupled with the analysis of 16S rDNA clone libraries constructed using a bacterial domain-specific primer set and two SRB group-specific primer sets. Profiles retrieved from the highest MPN dilutions using a universal primer set for eubacteria indicated that the predominant bacterial community changed from Gram-type positive to Gram-type negative bacteria. The dominant bacterial signatures of core 1 came from the low G+C Gram-type positive bacteria group (Firmicutes, 73%), while *Bacteroides* appeared relatively dominant (43%) in core 4 taken after one system was supplemented with acetate. The population size of *δ-Proteobacteria* remained similar in each system over time. Using six SRB group-specific primer sets (Daly et al., 2000), only *Desulfovibrio*-like amplification products were obtained in 14 months old system, while *Desulfococcus*-like amplification products were also recovered 5 months after the initial sampling. Using the primer set for the group *Desulfovibrio-Desulfomicrobium*, 67 sequences were retrieved and fell into the groups of *δ-Proteobacetria* (67%), Firmicutes (31%), and Spirochetes (1 sequence). The primer set for the *Desulfococcus* group was less specific for the target group because 48% of the sequences identified belonged to the *δ-Proteobacteria*, and only two belonged to the *Desulfococcus-Desulfonema-Desulfosarcina* group. Other groups detected were *Bacteroides* (36%), Clostridia (12%), and a *Chloroflexus*. A total of 101 pure strains were isolated from the highest positive MPN cultures. Based on 16S rDNA analysis, the isolates belonged mainly to two phyla, the low G+C Gram-type positive bacteria and the *δ-Proteobacteria*. Clostridia were the dominant

bacteria isolated. Among the members of *δ-Proteobacteria*, only *Desulfovibrio* species were recovered, which include two species, *Desulfovibrio africanus* and *Desulfovibrio sulfodismutans*.

INTRODUCTION

Acid sulfate waste waters are produced from industrial and mining activities. These sulfate-containing waters, especially acid mine drainage (AMD) from mining, mine tailings and coal storage piles have serious impacts on public health and our environment. To solve this problem, various methods have been employed including passive treatment systems such as constructed wetlands. Constructed treatment wetlands (CTW) have been used to remediate wastewater and pollutants from the food industry, agriculture and the mine industry (Hedin et al., 1994; Barton and Karathanasis, 1999; Brenner, 2001; Mays and Edwards, 2001). Among the types of constructed wetlands, anaerobic CTW have been thought to provide an effective way of neutralizing acid mine drainage and remove natural metals in wetlands (Alexander 1993; Thomas et al., 1999). The integrity of anaerobic CTW is greatly impacted by the activity of indigenous anaerobic microbial communities, especially of sulfate reducers. Sulfate-reducing bacteria (SRB) are important regulators of the mineralization of organic matters in anaerobic environments because they can neutralize low pH, lower high sulfate, and eventually promote metal precipitation (Barton and Tomei, 1995; Fortin et al., 2000a; Vester and Ingvorsen, 1998). SRB have been recovered from mining environments under a wide range of physico-chemical conditions (Benner et al., 2000; Fortin et al., 2000b; Wielinga et al., 1999; Fortin and Beveridge, 1997). The formation of ferrous monosulfide (FeS) by microbial sulfate reduction is an important geochemical reaction and can lead to pyrite formation (Berner, 1984). It also affects the solubility of other dissolved metals such as Cu, Zn, Pb, Cd, etc. through sorption and co-

precipitation (Mores and Luther III, 1999). Therefore, it is crucial to address the occurrence and distribution of complex microbial communities, especially of sulfate-reducing bacteria in a CTW system and to elucidate how they respond to and control biogeochemical reactions in wetland systems.

Molecular phylogenetic analysis using 16S rDNA sequences provides a relatively non-biased and powerful approach for evaluating the genetic diversity in environmental samples (Baker et al., 2001; Groflkopf et al., 1998; Whitehead and Cotta, 1999). Despite the vast information that genomic analysis provides, it is difficult to infer the function of indigenous microbial community in a specific environment. The functions of almost half of the sequenced genes are still unrevealed (Cypionka, 2005). Even among the same species, each individual strain can be metabolically different. Thus the isolation of pure cultures is required for microbial physiological studies and often provides important information linking microbial diversity to ecological roles and functions. Microorganisms have developed various metabolic pathways through evolution enabling them to thrive on chaotic earth and to communicate with the environments by participating in the various biogeochemical challenges. To understand fundamental questions about biogeochemical processes in a certain environment, the understanding of microbial diversity and ecology is fundamental.

Wetlands usually represent highly complicated ecosystems (Kalin et al., 1991). They form distinct geochemical zones ranging from aerobic to anaerobic. These zones have unique features including pH gradients and different metal precipitates. Although microbial sulfate reduction is the key process in the neutralization of the low pH water and metal removal in constructed wetland systems, little is known about the occurrence and distribution of sulfate-reducing bacteria. The aims of this study were to obtain predominant microbial signatures,

especially of sulfate-reducing bacteria that are closely associated with various biogeochemical processes occurring in a constructed treatment wetland system.

METHODS

Constructed treatment wetland system

Two constructed treatment wetland systems were built to treat coal pile runoff at D-area of DOE's Savannah River Site operated from April 1999 to December 2000 (Thomas, 2003). Acid sulfate runoff produced from the coal piles was featured as low pH (~2.4) and ferric iron-dominated (>95%) water. These vertical flow reactor systems consist of a series of three plastic tanks (92 cm diameter and 122 cm height), which were filled with limestone-buffered organic substrates containing carbon and energy sources, bacterial sources, and neutralizing agents (Table 3.1). The first tank is an anaerobic tank where we focused on microbial characterizations.

Environmental samples

Four distinct reaction zones developed in the system during a series of mineral dissolution-precipitation reactions (Thomas et al., 1999). These different reaction zones were identified as a) oxide zone (pH 2.3-3.8), b) transitional zone (pH ~ 4.5), c) sulfide zone (> pH 6.5) and d) unreacted zone (~ pH 6.8) (Fig. 3.2). Sediment samples were taken from each reaction zone twice in June and November of 2000 with three replicates (3 cores) in non-supplemented (36A) and acetate-supplemented (30A) tanks (Table 3.1). Sodium and ammonium acetate (~120 mM) were supplemented continuously into the experimental CTW to enhance bacterial sulfate reduction starting on July 26, 2000. The characteristics of the influent directly from the coal pile runoff basin were as follows: pH 2.4 (1.6-3.0), oxic (near saturation), high acidity (738-2,320

mg/l, average 1,304 mg/l as $CaCO_3$ equivalent), 92-237 mg/l of total iron (average 142 mg/l), 39-274 mg/l of aluminum (average 84 mg/l), high sulfate (926-3,385 mg/l, average 1,521 mg/l).

MPN medium and culture conditions

To determine the predominant SRB, different substrates were used in a most probable number (MPN) analysis. The base-medium was prepared as described by Widdel and Bak (Widdel and Bak, 1992) using the anaerobic Hungate technique (Ljungdahl and Wiegel, 1986). Either acetate (15 mM) or lactate (15 mM) was used as the sole carbon and energy source. An iron nail was added to each tube to detect black precipitation of FeS as a visual indication of sulfate reduction. The pH^{25C} was around 6.8. MPN enumerations were carried out by serial dilution (up to 10^{-11}) of aliquots with four replicates, which were prepared by adding approximately 3 g of soil sample taken from the three reaction zones to 27 ml of sodium pyrophosphate (0.1 M, pH 7.0) followed by sonication after 12 hours incubation at 15^{o}C. The cultures were incubated at 37^{o}C up to one month. The growth of sulfate-reducing bacteria was monitored based on the formation of black precipitates of ferrous sulfide (FeS). A MPN table (Cochran, 1950) was used to enumerate predominant SRB.

DNA extraction and amplification

DNA was extracted from the highest positive MPN cultures obtained from the sulfide zone of core 1 and 4 each in the non-supplemented tank (36A) and from the oxide zone and the sulfide zone of core 1 and 4 each in the acetate-supplemented tank (30A) using a Ultra Clean Soil DNA kit (MoBio Laboratories, Solana Beach, CA). Extracted DNA was used as a template for PCR amplification of 16S rDNA using bacterial domain-specific primer set and 6 SRB group-specific primer sets (Table 3.3). PCR products were ligated into pCR 2.1 TOPO vectors and transformed into *E. coli* TOP10 competent cells using Topo TA Cloning Kit (Invitrogen Inc., Carlsbad, CA)

according to the manufacturer's instructions. Each positive clone was picked and suspended in Luria-Bertani broth medium containing ampicillin (100 μg/ml). After transformants were grown up, 16S rDNA inserts were amplified directly from 3 μl of each culture using the vector primer set, M13 forward and M13 reverse (Table 3.3). PCR amplification was performed in a Mastercycler (Brinkmann Instruments, Westbury, NY), using 2 min of denaturation at 95°C, then 30 cycles of 30 s at 95°C, 20 s at 55°C, and 90 s at 72°C, followed by 5 min of extension at 72°C. PCR products were purified by using ExoSAP-IT (USB Co., Cleveland, OH), and further used as templates for subsequent sequencing reactions.

Sequencing procedure

The sequencing reaction was set up using ABI's Big Dye Terminator Kit v2.0 (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction and performed in a Mastercycler (Brinkmann Instruments, Westbury, NY), using 2 min of denaturation at 95°C, then 40 cycles of 10 s at 95°C, 5 s at 50°C, and 4 min at 60°C, followed by 10 min of final extension at 60°C. The DNA was precipitated by adding 1 μl of 1.5 M sodium acetate + 250 mM EDTA (pH 8.0) and 40 μl of 95% ethanol. After incubation at -20^{o}C for 20 min, precipitated sequencing reactions were centrifuged at 1,500 x g for 45 min and, after removing the supernatant, resuspended with 4 μl of formamide dyes and heated at 95^{o}C for 5 min, then further analyzed in an ABI model 377 automated sequencer according to the manufacturer's instruction.

Phylogenetic analysis

Similarity of partial sequences was determined using the Sequencher^{TM} v4.1.4 (Gene Codes Co., Ann Arbor, MI). Retrieved 16S rDNA sequences were analyzed using BLAST (basic local alignment search tool) and then aligned manually using ClustalX v1.81 (Thompson et al. 1997) to create a multiple sequence alignment. Sequences with less than 85% similarity to any other

known sequence were checked for chimera formation with CHIMERA_CHECK v2.7 of the Ribosomal Database Project II (Cole et al., 2003) and Bellerophon (Huber et al., 2004). Only unambiguous positions were used in subsequent phylogenetic analyses. We used several methods for inferring phylogenetic trees, including parsimony, distance matrix, and maximum likelihood methods, using the phylogenetic analysis package PHYLIP v3.6a2.1 (Felsenstein, 2001). Evolutionary distances for the distance trees followed the model of Jukes and Cantor (Jukes and Cantor, 1969). Bootstrap analysis was performed using SEQBOOT with 100 replicates and used to analyze the robustness of the obtained tree topologies from the neighbor-joining, parsimony, and maximum likelihood methods (Felsenstein, 1985).

Isolation and identification of microorganisms from MPN cultures

From the highest positive MPN dilution tubes, subcultures were made into prereduced media with pH 6.8-7.2 (pH of the sulfide zone) and at pH 3.5, 4.5 and 5.5 to obtain acidophilic or acidotolerant SRB. Subcultures were incubated at 37^{o}C for an extended period (from 10 days up to 4 weeks). To obtain pure cultures, we used the methods of dilution rows of agar-shake-roll-tubes containing above SRB media and 0.5%, 0.8% or 1.5% agar, respectively (Ljungdahl and Wiegel, 1986). After three rounds of purification, DNA was extracted using DNeasy Tissue Kit (Qiagen Inc., Valencia, CA) and amplified using a universal primer set, 27 forward and 1492 reverse (Lane, 1991). PCR amplification was carried out in a PTC-150 MiniCycler (MJ Research Inc., Waltham, MA), using 2.5 min of denaturation at 95°C, then 30 cycles of 30 s at 95°C, 30 s at 58°C, and 45 s (increased 15 s after each 10 cycle) at 72°C, followed by 7 min of final extension at 72°C. PCR products were purified by QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA), then sent for sequencing by Macrogen (Seoul, Korea). Retrieved sequences were

analyzed as described above. For long-term storage, isolates are maintained at -4^{o}C on agar-shake-roll tubes or at -80^{o}C as glycerol stock cultures (containing 15% glycerol).

RESULTS

MPN enumeration of sulfate-reducing bacteria

Sulfate-reducing bacteria were enumerated by a culture-based method, the most-probable-number (MPN) technique using core samples collected from oxide, transitional, and sulfide zones (Table 3.4). Media composed with containing either acetate or lactate yielded little difference in SRB enumeration. The highest MPN results from both non-supplemented and acetate-supplemented tanks showed approximately 10^6 cells per gram of sediment came from the sulfide zone in core 1, while around 10^7 cells came from cultures of the transitional and sulfide zones in core 4. Control and acetate-supplemented tanks showed almost similar enumeration results except the numbers from the oxide zones sampled in core 4. The acetate-supplemented tank showed 10-100 fold higher microbial numbers for the oxide zone than for the oxide zone of the non-supplemented tank (36A). Furthermore, in the acetate-supplemented tank, SRB enumeration results were almost same for both substrates (Table 3.4).

Identification of predominant bacterial community

The microbial populations in the highest positive MPN dilution tubes originating from the oxide and sulfide zones were further investigated with 16S rRNA sequence analysis (Table 3.5). The most dominant bacterial signatures in core 1 (June samples) came from the low G+C Gram-type positive bacteria group (Firmicutes, 22 of 30), while in core 4 (November samples) *Bacteroides* were relatively dominant (12 of 28) suggesting a shift in the composition of the microbial communities (Table 3.6 and fig. 3.2). *Bacteroides* were not detected in core 1 samples

but appeared in the cultures of core 4 samples containing acetate as a sole carbon source. The frequency of *δ-Proteobacetria* remained similar in each system over time. The *δ-Proteobacetria* were detected only in the sulfide zones of core 1 samples in both control and acetate-supplemented tanks, while one *Desulfovibrio*-like sequence (97% similar to *Desulfovibrio alcoholovorans*) was obtained from the oxide zone of core 4 samples in the acetate-supplemented tank. Gram-type positive bacteria such as *Desulfotomaculum* species and *Desulfosporosinus* species were identified from both the oxide and sulfide zone and mainly from the cultures containing acetate as a sole carbon source.

Analysis using SRB group-specific primer sets

The PCR amplification results using six SRB group-specific primer sets showed that the DNA extracted directly from the highest positive MPN cultures was amplified by two SRB primer sets, for the genera *Desulfococcus-Desulfonema-Desulfosarcina* and *Desulfovibrio-Desulfomicrobium*. Only *Desulfovibrio*-like amplification products were obtained from June samples, while *Desulfococcus*-like amplification products were also recovered in November samples (Table 3.4). The 67 sequences retrieved using the primer set for the group *Desulfovibrio-Desulfomicrobium* were assigned to *δ-Proteobacetria* (44), *Firmicutes* (21 including 9 *Desulfotomaculum*-like species), and a spirochete (Fig. 3.3). Based on the BLAST results, the closest known relatives among the sequences belonged to *δ-Proteobacteria* were *Desulfovibrio aminophilus* (20) and *Desulfovibrio alcoholovorans* (11). Phylogenetic analysis of 16S rRNA gene of *Desulfococcus*-like amplification products showed 16 out of 33 identified sequences belonged to *δ-Proteobacteria*, but only two was affiliated with *Desulfococcus-Desulfonema-Desulfosarcina* group (Fig. 3.4). Other groups detected by nested PCR approach

were *Bacteroides* (12), *Clostridium* (4 including a Gram-type positive SRB), and *Chloroflexus* (1), indicating that the specificity of this primer set was not absolute under the protocol used.

Isolation and identification of individual bacterial taxa.

A total of 101 purified isolates were obtained from the highest positive MPN cultures (Table 3.7). Based on the 16S rDNA analysis the isolates belonged to two phyla, low G+C Gram-type positive bacteria and *δ-Proteobacteria*. Although MPN analysis was directed toward SRB's, *Clostridia* were the dominant bacteria among the isolates. Besides 16 different species of Clostridia, *Paenibacillus* species and the sulfate-reducers *Desulfotomaculum*, *Desulfosporosinus* were also isolated. Specifically, *Desulfotomaculum guttoideum*-like species were isolated from the acetate-supplemented tank while *Desulfotomaculum ruminis*-like species were obtained from the non-supplemented tank. *Desulfosporosinus auripigmenti*-like species (~97% similarity) were isolated from the oxide zone of the acetate-supplemented tank while *Desulfosporosinus meridiei*-like isolates (98%) were from the sulfide zone of the non-supplemented tank. All of the low G+C Gram-type positive bacteria isolated belong to the family *Clostridiaceae*. Among the *δ-Proteobacteria*, only *Desulfovibrio* species were retrieved. These were closely related to two known species, *Desulfovibrio africanus* (>99%) and *Desulfovibrio sulfodismutans* (>99%). Most of the isolates from the highest positive MPN cultures showed more than 98% similarity to cultured relatives while three different unknown species were isolated from the oxide zone: based on BLAST search, one strain from the acetate enrichment was affiliated with *Clostridium pascui* (~92%) and two from the lactate enrichment with *Clostridium scatologenes* (~95%). Two strains were isolated from lactate enrichment of the sulfide zone of the acetate-supplemented tank and were related to *Desulfosporosinus auripigmenti* (~97%). One strain affiliated with

Clostridium tetani (~94%) was isolated from the sulfide zone of core 4 in the non-supplemented tank (Table 3.7).

DISCUSSION

The enumeration of the predominant SRB that are responsible for the alkali production and metal removal in the system indicated that there were no significant difference in the population size between non-supplemented tank and acetate-supplemented tank during the course of the study except in the oxide zone (Table 3.4). SRB enumeration from the acetate-supplemented tank showed higher MPN result in the oxide zone by a factor of 10 to 100 compared to the non-supplemented tank (36A). Interestingly, the enumeration in the oxide zone of core 4 (November samples) in the non-supplemented tank showed the same result with the same zone of core 1 (June samples, before acetate supplemented) in the acetate-supplemented tank. However, the changes in the enumeration of SRB from core 4 samples in the acetate-supplemented tank indicate that the addition of acetate in the acetate-supplemented tank increased the sulfate reducer population in the oxide zone of the system. Based on the data for the oxide zone after acetate treatment, the population of metabolically active SRB communities in the sulfide zone expand anaerobic region to the oxide zone. Although the SRB enumeration results presented here showed the similar results compared to the results from other environments such as intertidal mud flat (Muβmann et al., 2005), we expected more richness of SRB signatures in the system. To prevent evaporation of acetate, a cover lid was used, which might have caused an increased toxic level of hydrogen sulfide for some SRB's and led to the limited numbers in the system. Since the acid mine drainage has an acute and chronic toxicity to indigenous microbial communities in AMD-impacted environments (Gray, 1997), which results

in generally limited diversity (Goebel and Stackebrandt, 1994; Tyson et al., 2004), it is assumed that most bacteria in the constructed wetland systems were affected by this toxicity before the systems reached their “steady state”.

Small subunit ribosomal DNA analysis revealed dominance of the low G+C Gram-type positive bacteria including Gram-type positive SRB such as *Desulfotomaculum* and *Desulfosporosinus* species. However, in core 4 samples Gram-type negative bacteria were retrieved more commonly, and *Bacteroides* were relatively abundant (~43%) suggesting that heterotrophic fermentative bacteria are more abundant than SRB and play an important role in the system. Bacteria belonging to *Bacteroidetes* division, previously known as the *Cytophaga-Flavobacteria-Bacteroides* (CFB) group, are widely distributed in various environments (Okabe et al., 2003). Species belonging to this group exhibit enormous phenotypic and metabolic diversity. The change of predominant bacteria communities may be related to the stabilization of the system, not with the addition of acetate in the system. Once microbial sulfate reduction stabilized, acid production through fermentation by heterotrophic bacteria can be exceeded by an increase in alkalinity that led to the shift of predominant microbial communities in the system. It is notable that fermentation of organic matter by obligate anaerobes including both *Clostridium* sp. and *Bacteroides* sp. enhanced sulfate reduction in the anoxic zone (Lloyd et al., 2004). These heterotrophic bacteria decompose various complex organic compounds to short chain fatty acids and thus support the growth of SRB.

A parallel approach was to isolate and identify the individual bacterial taxa to infer their biogeochemical roles in the system. The low G+C Gram-type positive bacteria, especially Clostridia, were isolated predominantly from the highest MPN cultures for SRB and showed relatively high diversity. This dominance is possibly due to their capability to produce spores

and/or their metabolic versatility. Among them were two strains from the oxide zone of the experimental sample in June, which showed 95% similarity to *Clostridium scatologenes* that was isolated from an acidic coal mine pond (Kusel et al., 2000). This indicated that these bacteria might be from the acid sulfate water of coal pile runoff basin (CPRB) and they could grow actively at lower pH (>4.0) (Lee et al., unpublished data). These spore-forming bacteria have many advantages in extreme environments. The acid sulfate water has pH ~2.4, thus these spore-formers might be viable but not active metabolically. If they encounter less acidic microenvironments, they grow and through their metabolisms lower the redox potential, which in turn supports the growth of SRB. Therefore, we propose that these acidotolerant heterotrophic Firmicutes may play an important role in the bioremediation processes using constructed wetlands. Another isolate from the oxide zone was *Desulfosporosinus auripigmenti*. It was isolated from the November sample and grew in culture below 10^{o}C, thus suggesting that this type of bacteria is active and thus maintains sulfate reduction during winter season. Other bacteria observed such as *C. bifermentans* and *C. sartagoforme* are known to be associated with swine manure, which suggested that these bacteria came from the composted substrates containing horse manure as a bacterial source. Furthermore, the isolated/identified thiosulfate reducers and *C. thiosulfatireducens* and *C. subterminale* are also assumed to contribute to the generation of alkalinity in the system. Among the 16 species of Clostridia, *C. amygdalinum, C. celerecrescens* and *C. thiosulfatireducens* were recovered from almost all MPN cultures and indicated these bacteria must play a significant role in the decomposition of high molecular weight substrates to low molecular weight substrate for SRB. Furthermore based on the culture-independent method, *Bacteroides* were dominant in core 4 samples. Although they were not obtained as pure cultures, they have a similar role because these obligately anaerobic and Gram-

type negative bacteria are highly saccharolytic. For this reason they apparently outcompete other bacteria in the system.

Sequences of clones retrieved from the highest MPN cultures indicated *Desulfovibrio aminophilus* and *D. alcoholovorans* were dominant SRB while *D. africanus* and *D. sulfodismutans* were isolated in the sulfide zones. Cultured strains of SRB are sensitive to even mild acidity (Hard et al., 1997), however, microbial sulfate reduction has been inferred in acidic and oxic mine environments (Canfield and Des Marais, 1991; Johnson et al., 1993; Johnson, 1995; Kolmert and Johnson, 2001). Unfortunately, we could not isolate aerotolerant and/or acidotolerant SRB from the MPN cultures of the oxide zone, although other novel strains were retrieved from this oxide zone. Although active in the constructed wetland system, SRB were probably not numerically dominant despite acetate supplementation. The sensitivity of characterized SRB to acidity limits their use in acid mine drainage remediation (Kolmert and Johnson, 2001). A mixed community of acidotolerant SRB and other acidophilic heterotrophic bacteria may grow syntrophically. It is also inferred that the low G+C Gram-type positive SRB may be more important in acidic environments than the Gram-type negative *δ-Proteobacteria* because they are capable of forming spores and are metabolically more versatile.

Subsequent metabolic and biochemical characterization is necessary to understand the role of these heterotrophic bacteria in acidic environments. These metabolic and biochemical studies of isolates are also essential to understand mineral formation and metal cycling and to develop a new strategy for metal removal using a constructed wetland system. A more complete understanding of bioenergetics and biogeochemical reactions at the individual bacteria level may provide various ways to estimate and stimulate microbial communities in such environments.

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Table 3.1. Composition of the limestone-buffered organic substrates.

Composition of limestone-buffered organic substrates	% dry weight
Composted stable wastes	72
wood shavings	
straw	
horse manure	
Spent brewing grains	3
Kriket Krap (composted cricket manure)	<1
Fine grained limestone screenings (grain size of 1.2 mm, 91.5% $CaCO_3$)	25

Table 3.2. Samples for this study. Cores (4), (5), and (6) are defined as cores taken after the system run for weeks. Each core 4, 5 and 6 has 60^{o} difference from the previous cores 1, 2 and 3, anticlockwise.

Tank	Control (36A[1])			Experimental (30A[2])		
Core	1 (4)	2 (5)	3 (6)	1 (4*)	2 (5*)	3 (6*)
Zone	a[3]	a	a	a	a	a
	b[4]	b	b	b	b	b
	c[5]	c	c	c	c	c
	d[6]	d	d	d	d	d

[1] 36A tank is a control without acetate treatment.
[2] 30A tank is an acetate-treated tank.
[3] a is an oxide zone.
[4] b is a sulfide zone.
[5] c is a transitional zone.
[6] d is an unreacted zone
* Samples taken after acetate supplementation to the experimental (30A) tank.

Table 3.3. PCR primer sets used in this study.

Primers	Target organisms	Sequences (5' to 3')	Annealing Temp. (^{o}C)	Expected size of product (bp)	Reference
27F*	Eubacteria	GTTGATCCTGGCTCAG			
907R*	Universal	CCGTCAATTCCTTTGAGTTT	56	1365	Lane, 1991
1392R*	Universal	ACGGGCGGTGTGTAC			
1492R*	Prokaryotes	ACGGYTACCTTGTTACGACTT			
M13F	pCR 2.1 vector	GTAAAACGACGGCCAG	55		
M13R		CAGGAAACAGCTATGAC			
DFM140	*Desulfotomaculum*	TAGMCYGGGATAACRSYKG	58	700	Daly *et al*., 2000
DFM842		ATACCCSCWWCWCCTAGCAC			
DBB121	*Desulfobulbus*	CGCGTAGATAACCTGTCYTCATG	66	1120	Daly *et al*., 2000
DBB1237		GTAGKACGTGTGTAGCCCTGGTC			
DBM169	*Desulfobacterium*	CTAATRCCGGATRAAGTCAG	64	840	Daly *et al*., 2000
DBM1006		ATTCTCARGATGTCAAGTCTG			
DSB127	*Desulfobacter*	GATAATCTGCCTTCAAGCCTGG	60	1150	Daly *et al*., 2000
DSB1273		CYYYYYGCRRAGTCGSTGCCCT			
DCC305	*Desulfococcus* *Desulfonema* *Desulfosarcina*	GATCAGCCACACTGGRACTGACA	65	860	Daly *et al*., 2000
DCC1165*		GGGGCAGTATCTTYAGAGTYC			
DSV230	*Desulfovibrio*	GRGYCYGCGTYYCATTAGC	61	610	Daly *et al*., 2000
DSV838*	*Desulfomicrobium*	SYCCGRCAYCTAGYRTYCATC			

* primers used for sequencing

Table 3.4. Numbers of predominant sulfate-reducing bacteria based on MPN analysis.

	Non-supplemented tank (36A[1])				Acetate-supplemented tank (30A[2])					
	June	November			June			November*		
Zone	C	a[3]	b[4]	c[5]	a	b	c	a	b	c
Acetate	10^6	10^6	10^7	10^7	10^6	10^5	10^6	10^7	10^7	10^7
Lactate	10^6	10^5	10^7	10^7	10^5	10^6	10^6	10^7	10^7	10^7

[1] 36A tank is a control without acetate treatment.
[2] 30A tank is an acetate-treated tank.
[3] a is an oxide zone.
[4] b is a transitional zone.
[5] c is a sulfide zone.
* Acetate treated samples.

Table 3.5. The numbers of clones identified from the highest positive MPN analysis.

Site (tank/core/zone)	Substrate	Primer set		
		27f and 1392r	DCC 305 and 1165	DSV 230 and 818
36A/1/c	Acetate	5	ND	5
	Lactate	5	ND	4
30A/1/a	Acetate	5	ND	5
	Lactate	5	ND	4
30A/1/c	Acetate	5	ND	4
	Lactate	5	ND	5
36A/4/c	Acetate	4	6	5
	Lactate	5	5	9
30A/4/a*	Acetate	7	5	8
	Lactate	2	8	7
30A/4/c*	Acetate	6	4	7
	Lactate	4	5	4

ND, not detected.
* Acetate-treated samples.

Table 3.6. Distribution of clone sequences retrieved from MPN cultures of different biogeochemical reaction zones using bacterial domain specific primers set (27f and 1392r).

Affiliated phylogenetic group	June						November					
	Non-supplemented (36A[1])		Acetate-supplemented (30A[2])				Non-supplemented (36A)		Acetate-supplemented (30A) *			
	sulfide (c)		oxide (a)		sulfide (c)		sulfide (c)		oxide (a)		sulfide (c)	
	A[3]	L[4]	A	L	A	L	A	L	A	L	A	L
Actinobacterium								1				
Bacteroides							2		6		4	
Chlorobi				1								
Chloroflexi						2		1		1	1	
Firmicutes	5	3	5	4	5		1	2			1	2
δ–Proteobacteria		2				3	1	1		1		2
Spirochetes									1			

[1] 36A tank is the control without acetate treatment.
[2] 30A tank is an acetate-treated tank.
[3] A is acetate used as a carbon source for MPN analysis.
[4] L is lactate used as a carbon source for MPN analysis.
* Acetate-treated samples

Table 3.7. Identification of bacterial isolates from highest positive MPN cultures

Closest cultured relative	Numbers of isolates (% 16S rRNA gene sequence similarity)											
	Non-supplemented tank (36A[1])						Acetate-supplemented tank (30A[2])					
	1/c (core/zone)		4/c		1/a		1/c		4/a		4/c	
	A[3]	L[4]	A	L	A	L	A	L	A	L	A	L
Firmicutes:												
Clostridium												
Amygdalinum	3(100)	2(100)		2(98)	1(100)		2(100)	1(100)	1(100)	2(100)		
Bifermentans												1(99)
Celerecrescens	3(99)		1(99)	1(99)		1(99)	4(99)	3(99)				
Diolis				3(99)								
Magnum				4(98)								
Pascui					1(92)							
Propionicum									1(98)			
Sartagoforme												1(98)
Scatologenes						3(95)						
Sporogenes				1(99)			1(99)					
Sporosphaeroides											1(98)	1(98)
Subterminale	3(98)	1(99)										
Tetani				1(94)								
Tetanomorphum	7(99)	6(99)										
thiosulfatireducens			2(99)	4(99)	3(99)	2(99)			8(99)	1(99)		
Tunisiense											1(99)	
Desulfotomaculum												
Guttoideum						1(99)				1(98)		1(99)
Ruminis					2(99)							
Desulfosporosinus												
Auripigmenti										2(97)		
Meridiei			2(99)									

Table 3.7. Identification of bacterial isolates from highest positive MPN cultures (cont.).

Closest cultured relative	Numbers of isolates (% 16S rRNA sequence similarity)											
	Non-supplemented tank (36A[1])						Acetate-supplemented tank (30A[2])					
	1/c (core/zone)		4/c		1/a		1/c		4/a		4/c	
	A[3]	L[4]	A	L	A	L	A	L	A	L	A	L
Firmicutes (cont.):												
Paenibacillus												
Amylolyticus			1(99)									
δ–Proteobacteria:												
Desulfovibrio												
africanus				1(100)								5(99)
sulfodismutans				1(99)								

[1] 36A tank is the control without acetate treatment.
[2] 30A tank is an acetate-treated tank.
[3] A is acetate used as a carbon source for MPN analysis.
[4] L is lactate used as a carbon source for MPN analysis.

Figure 3.1. The schematic diagram of the constructed wetland tank and vertical view of core (modified from Thomas, 2003).

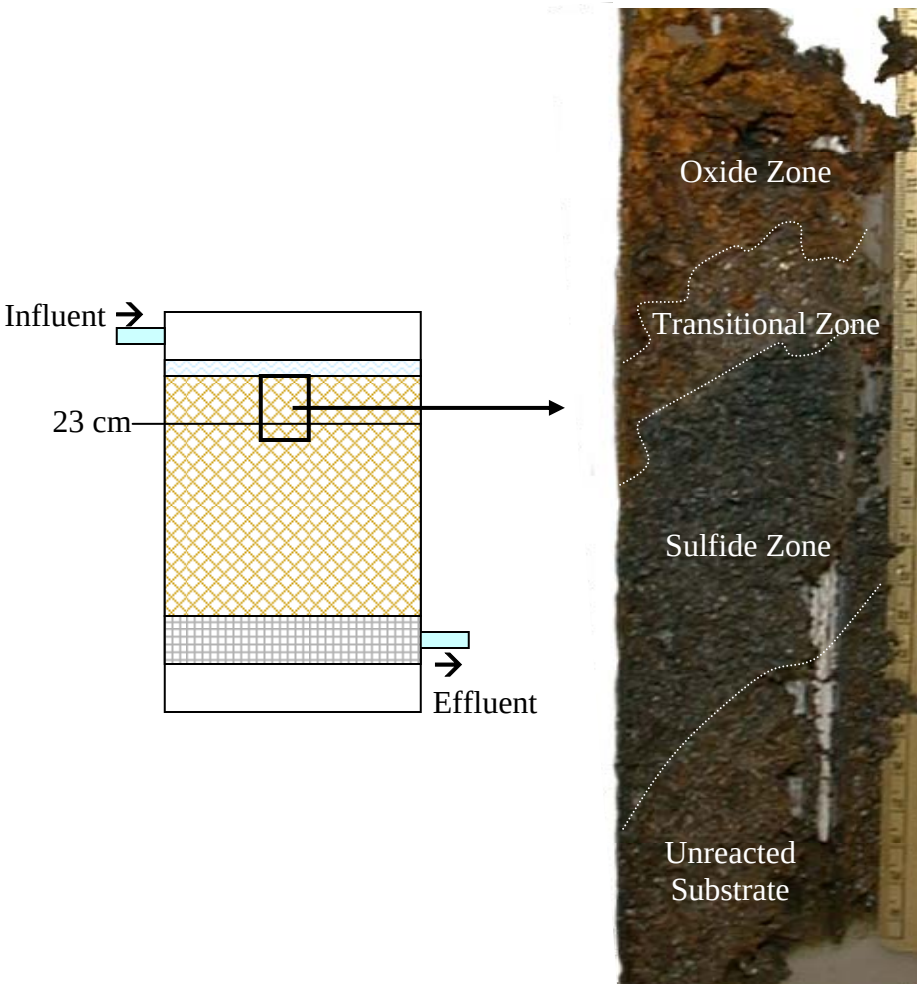

Influent
23 cm
Effluent
Oxide Zone
Transitional Zone
Sulfide Zone
Unreacted
Substrate

Figure 3.2. Phylogenetic relationships based on partial 16S rDNA sequences of clones, which were retrieved using bacterial domain-specific primers. The tree was constructed using neighbor-joining method with Jukes and Cantor distance corrections. The numbers at nodes represent >50% bootstrap support (100 replicates). The scale bar indicates 5 nucleotides substitutions per 100 nucleotides. Sequences recovered after acetate supplementation into the acetate-fed tank was colored with green. The designation of clone sequences in the first parentheses represents (tank/core/reaction zone/substrate for culture). The number in the second parentheses indicates the frequency of occurrence of an OTU.

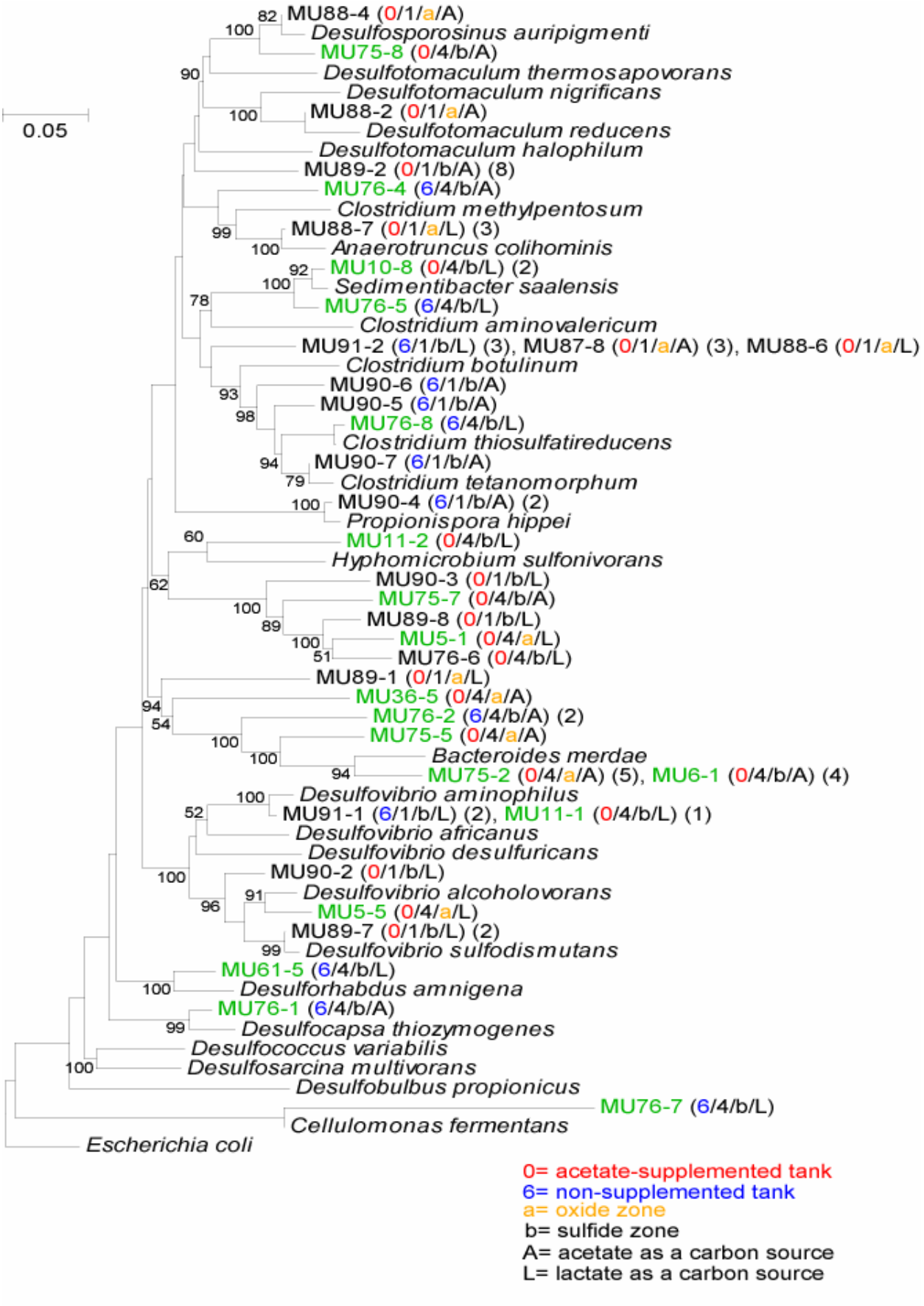

0.05
MU88-4 (0/1/a/A)
Desulfosporosinus auripigmenti
MU75-8 (0/4/b/A)
Desulfotomaculum thermosapovorans
Desulfotomaculum nigrificans
MU88-2 (0/1/a/A)
Desulfotomaculum reducens
Desulfotomaculum halophilum
MU89-2 (0/1/b/A) (8)
MU76-4 (6/4/b/A)
Clostridium methylpentosum
MU88-7 (0/1/a/L) (3)
Anaerotruncus colihominis
MU10-8 (0/4/b/L) (2)
Sedimentibacter saalensis
MU76-5 (6/4/b/L)
Clostridium aminovalericum
MU91-2 (6/1/b/L) (3), MU87-8 (0/1/a/A) (3), MU88-6 (0/1/a/L)
Clostridium botulinum
MU90-6 (6/1/b/A)
MU90-5 (6/1/b/A)
MU76-8 (6/4/b/L)
Clostridium thiosulfatireducens
MU90-7 (6/1/b/A)
Clostridium tetanomorphum
MU90-4 (6/1/b/A) (2)
Propionispora hippei
MU11-2 (0/4/b/L)
Hyphomicrobium sulfonivorans
MU90-3 (0/1/b/L)
MU75-7 (0/4/b/A)
MU89-8 (0/1/b/L)
MU5-1 (0/4/a/L)
MU76-6 (0/4/b/L)
MU89-1 (0/1/a/L)
MU36-5 (0/4/a/A)
MU76-2 (6/4/b/A) (2)
MU75-5 (0/4/a/A)
Bacteroides merdae
MU75-2 (0/4/a/A) (5), MU6-1 (0/4/b/A) (4)
Desulfovibrio aminophilus
MU91-1 (6/1/b/L) (2), MU11-1 (0/4/b/L) (1)
Desulfovibrio africanus
Desulfovibrio desulfuricans
MU90-2 (0/1/b/L)
Desulfovibrio alcoholovorans
MU5-5 (0/4/a/L)
MU89-7 (0/1/b/L) (2)
Desulfovibrio sulfodismutans
MU61-5 (6/4/b/L)
Desulforhabdus amnigena
MU76-1 (6/4/b/A)
Desulfocapsa thiozymogenes
Desulfococcus variabilis
Desulfosarcina multivorans
Desulfobulbus propionicus
MU76-7 (6/4/b/L)
Cellulomonas fermentans
Escherichia coli
0= acetate-supplemented tank
6= non-supplemented tank
a= oxide zone
b= sulfide zone
A= acetate as a carbon source
L= lactate as a carbon source

Figure 3.3. Distance dendrogram based on partial 16S rDNA sequences of clones, which retrieved using *Desulfovibrio-Desulfomicrobium* group-specific primers. The tree was constructed using Neighbor-joining method with Jukes and Cantor distance corrections. The numbers at nodes represent >50% bootstrap support (100 replicates). The scale bar indicates 10 nucleotides substitutions per 100 nucleotides. The name of sequences recovered after acetate supplementation into the acetate-fed tank was marked as green. The designation of clone sequences in the first parentheses represents (tank/core/reaction zone/substrate for culture). The number in the second parentheses indicates the frequency of occurrence of an OTU.

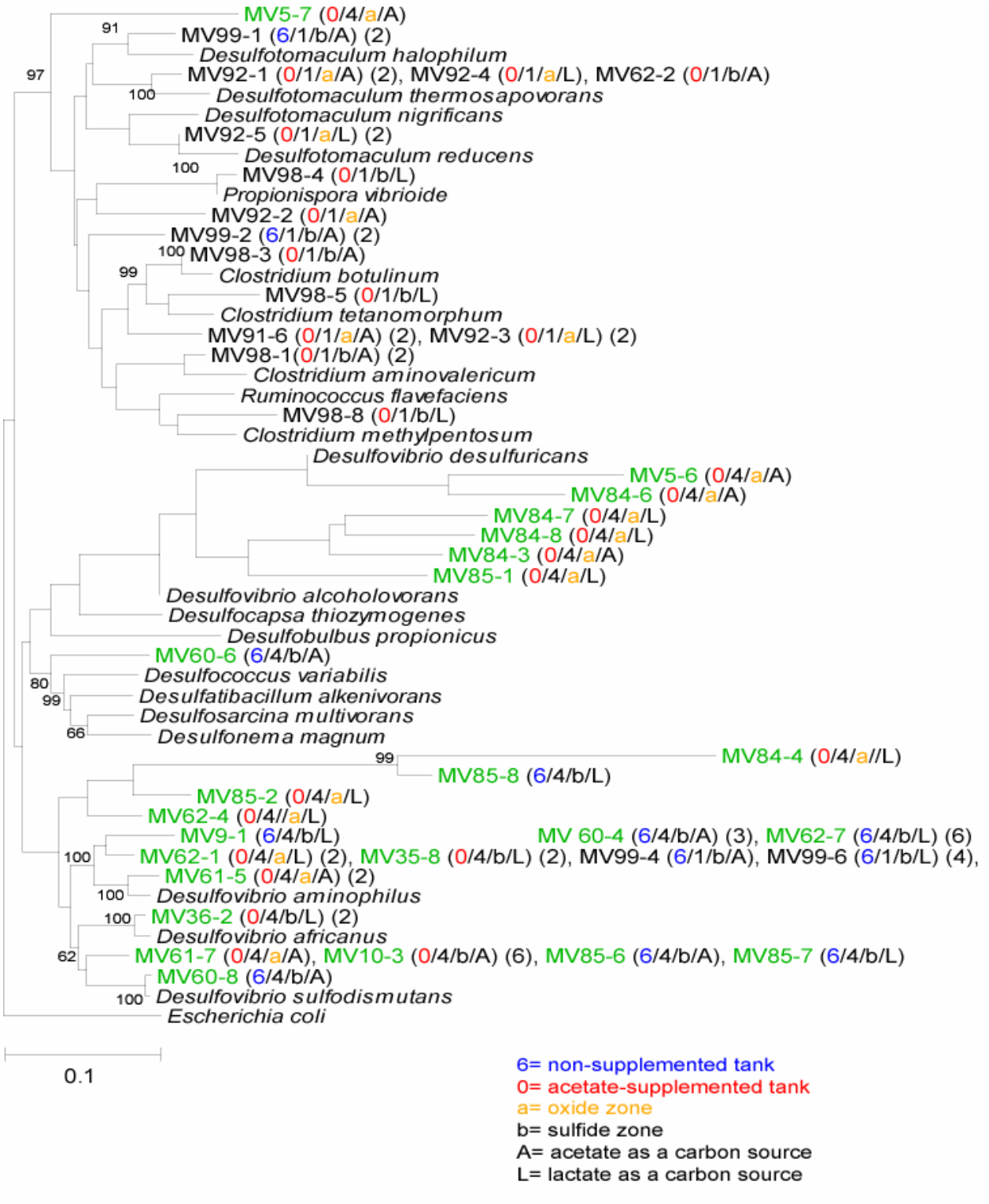
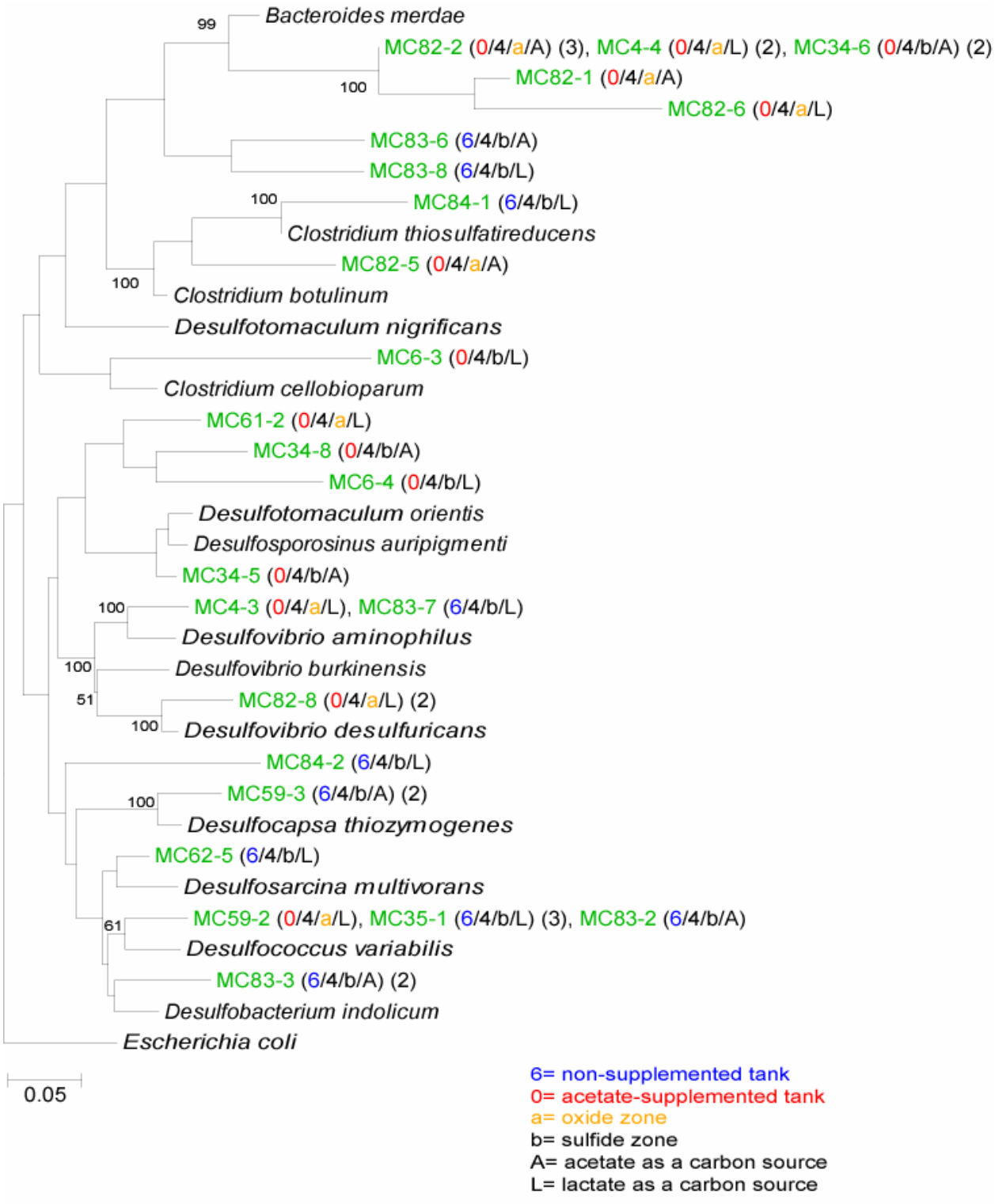

MV5-7 (0/4/a/A)
91
MV99-1 (6/1/b/A) (2)
Desulfotomaculum halophilum
97
MV92-1 (0/1/a/A) (2), MV92-4 (0/1/a/L), MV62-2 (0/1/b/A)
100
Desulfotomaculum thermosapovorans
Desulfotomaculum nigrificans
MV92-5 (0/1/a/L) (2)
Desulfotomaculum reducens
100
MV98-4 (0/1/b/L)
Propionispora vibrioide
MV92-2 (0/1/a/A)
MV99-2 (6/1/b/A) (2)
100
MV98-3 (0/1/b/A)
99
Clostridium botulinum
MV98-5 (0/1/b/L)
Clostridium tetanomorphum
MV91-6 (0/1/a/A) (2), MV92-3 (0/1/a/L) (2)
MV98-1(0/1/b/A) (2)
Clostridium aminovalericum
Ruminococcus flavefaciens
MV98-8 (0/1/b/L)
Clostridium methylpentosum
Desulfovibrio desulfuricans
MV5-6 (0/4/a/A)
MV84-6 (0/4/a/A)
MV84-7 (0/4/a/L)
MV84-8 (0/4/a/L)
MV84-3 (0/4/a/A)
MV85-1 (0/4/a/L)
Desulfovibrio alcoholovorans
Desulfocapsa thiozymogenes
Desulfobulbus propionicus
MV60-6 (6/4/b/A)
80
Desulfococcus variabilis
99
Desulfatibacillum alkenivorans
Desulfosarcina multivorans
66
Desulfonema magnum
99
MV84-4 (0/4/a//L)
MV85-8 (6/4/b/L)
MV85-2 (0/4/a/L)
MV62-4 (0/4//a/L)
MV9-1 (6/4/b/L)
MV 60-4 (6/4/b/A) (3), MV62-7 (6/4/b/L) (6)
100
MV62-1 (0/4/a/L) (2), MV35-8 (0/4/b/L) (2), MV99-4 (6/1/b/A), MV99-6 (6/1/b/L) (4),
MV61-5 (0/4/a/A) (2)
100
Desulfovibrio aminophilus
100
MV36-2 (0/4/b/L) (2)
Desulfovibrio africanus
62
MV61-7 (0/4/a/A), MV10-3 (0/4/b/A) (6), MV85-6 (6/4/b/A), MV85-7 (6/4/b/L)
MV60-8 (6/4/b/A)
100
Desulfovibrio sulfodismutans
Escherichia coli
0.1
6= non-supplemented tank
0= acetate-supplemented tank
a= oxide zone
b= sulfide zone
A= acetate as a carbon source
L= lactate as a carbon source

Figure 3.4. Phylogenetic tree constructed with partial 16S rDNA sequences of clones, which retrieved using *Desulfococcus-Desulfonema-Desulfosarcina* group-specific primers. The tree was inferred using Neighbor-joining method with Jukes and Cantor distance corrections. Bootstrap values above 50% are shown at nodes. The scale bar indicates 5 nucleotides substitutions per 100 nucleotides. The name of sequences recovered after acetate supplementation into the acetate-fed tank was marked as green. The designation of clone sequences in the first parentheses represents (tank/core/reaction zone/substrate for culture). The number in the second parentheses indicates the frequency of occurrence of a OTU.


Bacteroides merdae
MC82-2 (0/4/a/A) (3), MC4-4 (0/4/a/L) (2), MC34-6 (0/4/b/A) (2)
MC82-1 (0/4/a/A)
MC82-6 (0/4/a/L)
MC83-6 (6/4/b/A)
MC83-8 (6/4/b/L)
MC84-1 (6/4/b/L)
Clostridium thiosulfatireducens
MC82-5 (0/4/a/A)
Clostridium botulinum
Desulfotomaculum nigrificans
MC6-3 (0/4/b/L)
Clostridium cellobioparum
MC61-2 (0/4/a/L)
MC34-8 (0/4/b/A)
MC6-4 (0/4/b/L)
Desulfotomaculum orientis
Desulfosporosinus auripigmenti
MC34-5 (0/4/b/A)
MC4-3 (0/4/a/L), MC83-7 (6/4/b/L)
Desulfovibrio aminophilus
Desulfovibrio burkinensis
MC82-8 (0/4/a/L) (2)
Desulfovibrio desulfuricans
MC84-2 (6/4/b/L)
MC59-3 (6/4/b/A) (2)
Desulfocapsa thiozymogenes
MC62-5 (6/4/b/L)
Desulfosarcina multivorans
MC59-2 (0/4/a/L), MC35-1 (6/4/b/L) (3), MC83-2 (6/4/b/A)
Desulfococcus variabilis
MC83-3 (6/4/b/A) (2)
Desulfobacterium indolicum
Escherichia coli
99
100
100
100
100
100
51
100
100
61
0.05
6= non-supplemented tank
0= acetate-supplemented tank
a= oxide zone
b= sulfide zone
A= acetate as a carbon source
L= lactate as a carbon source

CHAPTER 4

GRACILIBACTER THERMOTOLERANS, A NOVEL ANAEROBIC THERMOTOLERANT BACTERIUM FROM A CONSTRUCTED WETLAND RECEIVING ACID SULFATE WATER[1]

[1]Lee, Y.-J., C. S. Romanek, G. L. Mills, and J. Wiegel. 2005. To be submitted to *International Journal of Systematic and Evolutionary Microbiology*.

ABSTRACT

An obligatory anaerobic, thermotolerant, asporogenic bacterium, strain JW/YJL-S1^T, was isolated from a sediment sample of the oxide zone (pH 2.3-3.8) in a constructed wetland system receiving acid sulfate water (pH 1.6-3.0). Cells of strain JW/YJL-S1^T were straight to curved rods 0.2-0.4 μm in diameter and 2.0-7.0 μm in length. Strain JW/YJL-S1^T grew optimally at 42.5-46.5°C, pH^{25C} 6.8-7.75 and 0–0.5 % (w/v) NaCl. The isolate produced acetate, lactate, and ethanol as fermentation end products from glucose. The G+C content of the genomic DNA of the isolate was 47 mol% (HPLC). Strain JW/YJL-S1 has polymorphism of 16S rRNA gene (GenBank accession numbers DQ117465 through DQ117469). The closest relative to strain JW/YJL-S1^T using BLASTN search against GenBank was *Clostridium pascui* (cluster I) with 92.7 % similarity, however, the inferred phylogenetic trees placed strain JW/YJL-S1^T distantly between the *Clostridium* cluster I, II and III. Based on the evidence from morphological and phylogenetic properties and the high G+C content of the genomic DNA, it is proposed that strain JW/YJL-S1^T is placed into a novel taxon, *Gracilibacter thermotolerans* gen. nov., sp. nov. (DSM 17427^T =ATCC BAA-1219^T).

It is well known that various microbial communities are involved not only in the generation but in the remediation of acid mine drainage. Most of the microorganisms isolated from mining environments are iron-, sulfur-oxidizing bacteria and sulfate-reducing bacteria. Although heterotrophic fermentative bacteria are tightly associated with other microbial communities in acid mine drainage, little is known about their diversity and functions in such environments. For instance, heterotrophic fermentative bacteria can remove organic acids that can inhibit chemolithotrophic bacteria such as *Leptospirillum ferrooxidans* and *Acidithiobacillus*

ferrooxidans (Johnson, 1998). Therefore, the generation of acid mine drainage can be facilitated by indigenous heterotrophic bacteria. Conversely, they also support sulfate- or metal-reducing bacteria by degrading biopolymers into monomers and fermentation products, which serve them as substrates, thus, contributing to the bioremediation of acid mine drainage.

Here we report on a novel isolate recovered from a constructed treatment wetland system receiving acid sulfate water (pH around 2). On the basis of physiological and phylogenetic evidence, we propose to place the novel isolate into the new taxon *Gracilibacter thermotolerans* gen. nov., sp. nov.

Strain JW/YJL-S1 was isolated from a MPN (most probable number) tube that was inoculated with surficial sediment from a constructed treatment wetland system receiving acid sulfate runoff from a coal pile located at Savannah River Site (SC., U.S.) (Lee, 2005). The acid runoff has an acidic pH around 2.3-3.8 and was characterized by high sulfate and ferric iron concentrations. The uppermost sediment in the constructed wetland was dominated by iron oxyhydroxide precipitates coating and placing an inorganic substrate amended with limestone. The organic substrates used for the constructed treatment wetland were composed of composted stable wastes, spent brewing grains and composted cricket manure. Thus no defined habitat can be given. The isolate was cultured in a carbonate-buffered basal medium (Widdle & Bak, 1994) supplemented with 20 mM acetate and 0.1 mM ferric citrate at pH^{25C} 6.8 and 37^{o}C under anaerobic condition (N_2, 100%) using a modified Hungate technique (Ljungdahl & Wiegel, 1986). The morphology of the isolate was observed using light microscopy (Olympus VANOX phase-contrast microscope). In agar-roll-tube cultures incubated at 37^{o}C, the surface colonies of strain JW/YJL-S1 appeared after 2-3 days and were less than 1 mm in diameter, circular to

irregular, mostly translucent and filamentous. Vegetative cells in liquid culture were straight to curved with 0.2-0.4 μm in diameter and 2.0-7.0 μm in length (Fig. 4.1a). Cells were either single or formed chains. Infrequently, elongated cells of up to 45 μm were detected. No active motility was observed (phase contrast microscopy), however, retarded peritrichous flagella were detected (Fig. 4.1b; negative stain) using electron microscopy (JEM-1210 Transmission Electron Microscope, JEOL, Inc.). The occurrence of spores was not detected by microscopy nor by heat treatment (10 min. at 80^{o}C). Cells staining was performed according to standard procedures (Doetsch, 1981) and showed that cells stained Gram-negative. The detailed results of morphological and physiological characteristics are summarized in Table 4.1 and in the genus and the species description.

Using a temperature-gradient incubator (Scientific Industries, Inc.), the temperature range for growth was determined to be from 25 to 54^{o}C with an optimum at 42.5-46.5^{o}C. No growth was detected below 20^{o}C or above 58^{o}C. The pH range for growth was determined at 37^{o}C and performed in the basal medium supplemented with 10 mM of each MES, HEPES and TAPS. The pH^{25C} ranges for growth was 6.0-8.25 with an optimum at pH^{25C} 6.8-7.75. No growth was detected at pH below 5.7 or above 8.5. Salinity range for growth was 0-1.5% (w/v) with an optimum at 0.5% of NaCl plus KCl at the ratio of 9:1; no growth was detected above 2% salt. The utilization of possible substrates (0.2%, w/v) was tested in the presence of 0.02% yeast extract (Table 4.1). Strain JW/YJL-S1 used casamino acids, tryptone, peptone, maltose, sucrose, arabinose, fructose, galactose, glucose, mannose, xylose, mannitol and sorbitol as carbon and energy sources. No growth was observed with cellobiose, lactose, raffinose, ribose, trehalose, inositol, mannitol, xylitol, acetate, lactate, pyruvate or methanol as carbon and energy sources. The isolate required yeast extract for growth. The culture was incubated for up to 20 days, with

growth judged positive if the optical density (at 600nm) of the culture was twice above the value of control culture containing only yeast extract (0.02%). None of the following electron acceptors, tested in media containing 20 mM lactate or 0.1% yeast extracts were utilized: Fe(III), AQDS, fumarate, nitrate, sulfate, sulfite, thiosulfate, elemental sulfur, and Mn(IV) at the concentration of 20 mM except sulfite (2 mM). Fermentation end products of 20 mM glucose were analyzed by HPLC equipped with Aminex-H87 column (Bio-Rad) and Beckmann detector. The main fermentation end products from 20 mM glucose were acetate, lactate, ethanol, CO_2 and H_2. Antibiotic susceptibility was tested with ampicillin, chloramphenicol, erythromycin, rifampicin, streptomycin, tetracycline at 10 and 100 μM. Strain JW/YJL-S1 was resistant to ampicillin and streptomycin at the concentration of 10 μM.

For phylogenetic and G+C content analyses, DNA was extracted by using DNeasy genomic DNA purification kit (Qiagen). The DNA G+C content was measured by HPLC as described previously (Mesbah *et al*., 1989), with the modification of using S1 nuclease (Invitrogen) and 0.3 M sodium acetate (pH 5.0). The G+C content of the genomic DNA was 47 mol% (HPLC), which is the average of 4 replicates. The 16S rRNA gene sequence analysis of strain JW/YJL-S1 was carried out with a bacterial domain-specific primer set, 27 forward and 1492 reverse (Lane, 1991). The PCR amplification was performed using the Easy-A High-Fidelity PCR Cloning Enzyme (Stratagene) with 30 cycles of denaturation (94^{o}C, 30 sec), annealing (58^{o}C, 30 sec), and extension (72^{o}C, 1 min) after initial denaturation at 94^{o}C for 3 min. Final extension was for 7 min at 72^{o}C. To confirm strain JW/YJL-S1 was a pure isolate, the PCR product of the 16S rRNA gene was cloned and transformed using the TOPO TA cloning kit (Invitrogen). Plasmid DNA was extracted using Eppendorf FastPlasmid Mini Kit (Brinkmann), amplified as described above, purified using QIAquick PCR Purification Kit (Qiagen), and

sequenced by Macrogen Inc. (Seoul, Korea). The similarities of partial sequences were determined using the Sequencher^{TM} v4.1.4 (Gene Codes). Retrieved 16S rDNA sequences were analyzed using BLAST (basic local alignment search tool) and then aligned manually using ClustalX v1.81 (Thompson *et al*., 1997) to create a multiple sequence alignment. A phylogenetic tree (Fig 4.2) was inferred by the neighbor-joining method (Saitou & Nei, 1987) using Jukes and Cantor distance corrections (Jukes & Cantor, 1969) with the phylogenetic analysis package PHYLIP v3.6a2.1 (Felsenstein, 2001). A total of five clones of the 16S rRNA gene were sequenced and revealed the presence of polymorphism of the 16S rRNA gene in strain JW/YJL-S1. Four of the five sequenced 16S clones were grouped together with above 99% similarity. The other sequenced clone showed ~2% divergence from the rest. When these 16S rDNA sequences, containing 1,550 bp (approx. positions -107–1,472 according to *E. coli* (X80725) numbering), were compared using a BLASTN search against the sequences in the GenBank, they yielded the same correlations that strain JW/YJL-S1 was closely related to uncultured strains, with *Clostridium pascui* as the closest cultured relative (*Clostridium* cluster I based on the classification of Collins *et al*., 1994) with 93% for the first 160 bp, 92% for 1013 bp, and 96% for 53 bp. However, in an inferred phylogenetic tree strain JW/YJL-S1 placed distantly between Collins' *Clostridium* cluster I/II and III (Fig. 4.2). Further phylogenetic analyses were performed with all type species of known genera in the family *Clostridiaceae* (Bergey's manual, 2004), and inferred phylogenetic tree indicated strain JW/YJL-S1 may not fall within the family *Clostridiaceae*. Note that in a newly defined genus *Clostridium* (Wiegel *et al*., in press), the cluster II species such as *Clostridium proteolyticum* fall within cluster I and were classified as members of cluster I (the genus *Clostridium* sensu stricto), while cluster III represents a new family. Based on polyphasic evidence including nonsporeforming property, 16S rDNA

sequence-based analysis and high G+C content of the genomic DNA, we propose to place the strain into the novel taxon, *Gracilibacter thermotolerans* tentatively belonging to the family *Clostridiaceae* within the order *Clostridiales* (Wiegel *et al*., in press).

Description of *Gracilibacter* gen. nov.

Gracilibacter (Gra.ci.li.bac'ter. L. Adj. *gracilis* slender; N.L. n. *bacter* masc. equivalent of Gr. neut. n. *baktron* rod or staff; N.L. masc. n. *Gracilibacter* slender rod, referring to its shape)

The genus *Gracilibacter* belongs to the low G+C, Gram-type positive Bacillus-Clostridium subphylum. Specific habitat unknown. Obligately anaerobic and thermotolerant chemoorganotrophs. No growth indication on H_2/CO_2 (80:20, v/v). No spores observed. The type species is *Gracilibacter thermotolerans*.

Description of *Gracilibacter thermotolerans* sp. nov.

Gracilibacter thermotolerans (ther.mo.to.le'rans. Gr. n. *thermê* heat; L. pres. part. *tolerans* tolerating; N.L. part. adj. *thermotolerans* heat-tolerating)

The cells are straight to curved rods, 0.2-0.4 μm in diameter and 2.0-7.0 μm in length. Autoplasts (L-shaped cells) occur infrequently during the late-stationary growth phase. No spores observed. Motility was not detected, while retarded flagella (1-5) were identified. The temperature range for growth is 25-54^{o}C, with an optimum at 42.5-46.5^{o}C. The pH^{25C} range for growth is from 6.0 to 8.25, with an optimum at 6.8-7.75. The salinity range for growth is from 0 to 1.5% (w/v), with an optimum at 0.5%. In the presence of 0.02% yeast extract, casamino acids, tryptone, peptone, maltose, sucrose, arabinose, fructose, galactose, glucose, mannose, xylose, mannitol, sorbitol serve as carbon and energy sources. The main fermentation end products from glucose are

acetate, lactate, ethanol. No indication of using Fe(III), nitrate, thiosulfate, elemental surfur, sulfate, sulfite, MnO_4, and fumarate as electron acceptors. Resistant to ampicillin and streptomycin (10 μM each). The G+C content of the genomic DNA is 47 mol% (HPLC). The type strain is JW/YJL-S1^T (DSM 17427^T, ATCC BAA-1219^T).

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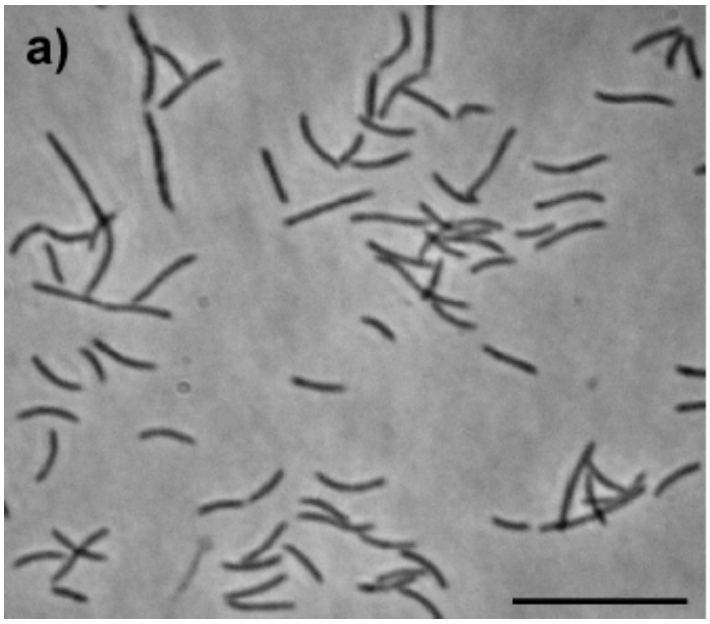
Table 4.1. Morphological and physiological characteristics of strain JW/YJL-S1 and its closest relative: 1, *Gracilibacter thermotolerans* JW/YJL-S1^T, this study; 2, *Clostridium pascui* Cm19^T, Wilde *et al*. (1997).

Character	1	2
Source	Soil mixed with horse manure	Soil, donkey pasture
Cell size (μm)	0.2-0.4 x 2.0-7.0	0.75-1.0 x 3.2-8.0
Temperature range (^{o}C)	25-54	10-43
Optimum temperature (^{o}C)	42-47	37-40
pH range	6.0-8.25	5.5-9.0
Optimum pH	6.8-7.75	6.4-7.8
Salinity (NaCl)	0-1.5	NR
Optimum salinity	0.5	NR
G+C content (mol%)	47	27
Gram stain	-	-
Spores observed	-	+
Motility	+	NR
Utilization of		
arabinose	+	-
fructose	+	-
glucose	+	-
galactose	+	-
ribose	-	-
xylose	+	-
lactose	-	-
maltose	+	-
cellobiose	-	-
raffinose	-	-
casamino acids	+	-
mannitol	+	-
sorbitol	+	-
inulin	-	-
glutamate	-	+

NR, Not reported

Figure 4.1. Phase-contrast micrograph (a, Bar: 10μm) and electron micrograph of retarded peritrichous flagella of strain JW/YJL-S1 (b, Bar: 0.5μm). Phase-contrast micrographs of bacteria were taken using agar-coated slides (2%, w/v). Periodicity of the retarded peritrichously inserted flagella is 2.4-2.5 μm (arrow).


a)

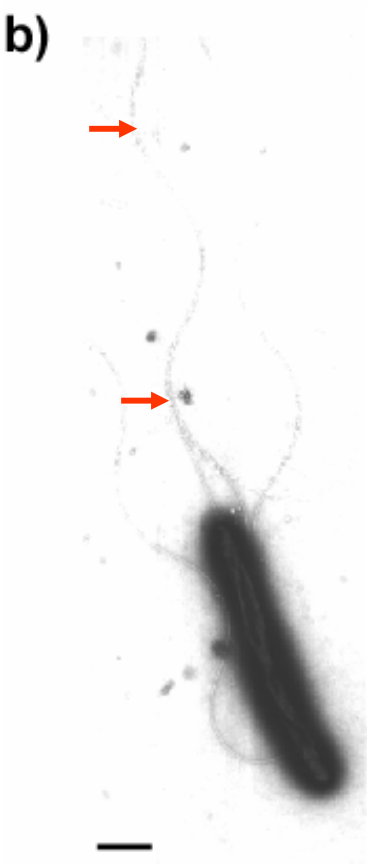
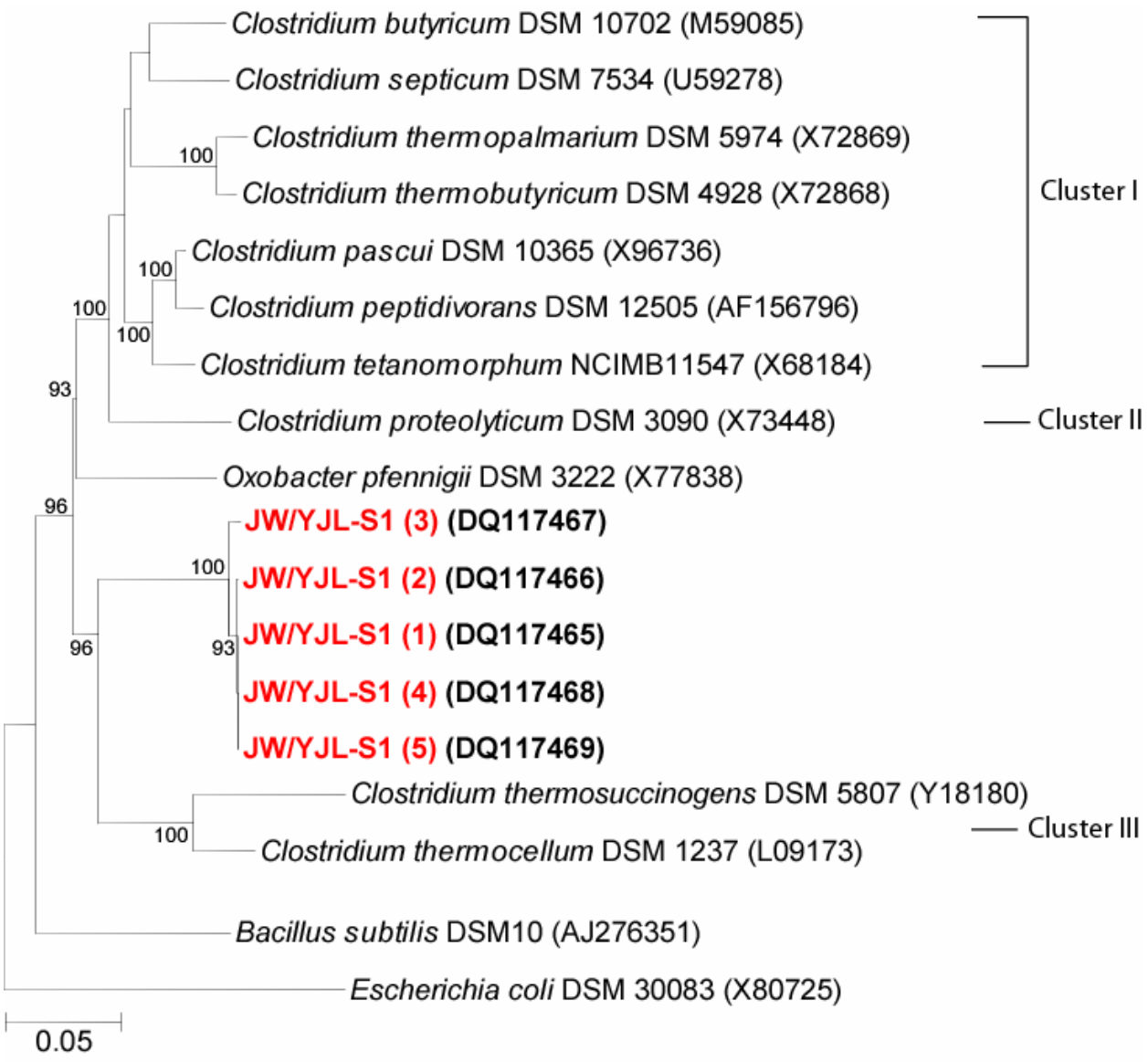

b)

Figure 4.2. A phylogenetic dendrogram based on 16S rRNA gene sequence showing the positions of strain JW/YJL-S1 (boldface text) among members of the family *Clostridiaceae*. The 16S rRNA data used represent *Escherichia coli* DSM 30083^{T} nucleotide positions 1-1,499. The tree was constructed using the neighbor-joining method with Jukes and Cantor distance corrections. Numbers at the nodes represent the bootstrap values (100 replicates); values above 90% were considered significant. Numbers in parentheses indicate numbering of different 16S rDNA sequences obtained from strain JW/YJL-S1. The scale bar indicates 5 nucleotides substitutions per 100 nucleotides.


Clostridium butyricum DSM 10702 (M59085)
Clostridium septicum DSM 7534 (U59278)
Clostridium thermopalmarium DSM 5974 (X72869)
Clostridium thermobutyricum DSM 4928 (X72868)
Clostridium pascui DSM 10365 (X96736)
Clostridium peptidivorans DSM 12505 (AF156796)
Clostridium tetanomorphum NCIMB11547 (X68184)
Clostridium proteolyticum DSM 3090 (X73448)
Oxobacter pfennigii DSM 3222 (X77838)
JW/YJL-S1 (3) (DQ117467)
JW/YJL-S1 (2) (DQ117466)
JW/YJL-S1 (1) (DQ117465)
JW/YJL-S1 (4) (DQ117468)
JW/YJL-S1 (5) (DQ117469)
Clostridium thermosuccinogens DSM 5807 (Y18180)
Clostridium thermocellum DSM 1237 (L09173)
Bacillus subtilis DSM10 (AJ276351)
Escherichia coli DSM 30083 (X80725)
100
100
100
100
93
96
96
100
93
100
Cluster I
Cluster II
Cluster III
0.05

CHAPTER 5

CONCLUSIONS

Microorganisms play a fundamental role in various biogeochemical processes occurring in environment. In this study, we focused on these microorganisms associated with a constructed wetland system for treatment of acid sulfate water. Their diversity and ecology were identified, characterized and inferred using culture-independent molecular techniques and culture-based method.

Acid sulfate water drained from coal piles has features of low pH, high concentration of sulfate and toxic metals. A constructed treatment wetland system built for treatment of this acidic water successfully improved water quality by raising pH and removing metal contaminants. In the previous study, isotopic analysis indicated that half of the alkalinity generated came from biological reactions, mainly microbial sulfate reduction. Therefore, in the bioremediation process indigenous microbial communities including sulfate reducing bacteria played a significant role.

Four distinct reaction zones were developed in constructed treatment wetlands: oxide, transitional, sulfide and unreacted zones. Each reaction zone possessed unique chemical features and affiliated microbial processes. We focused on the sulfide zone in which microbial sulfate reduction took place, thus pH increased, and metals precipitated as sulfides. Acetate was also supplemented in a tank system to enhance bacterial sulfate reduction and to evaluate the effects of the substrate on microbial communities and their function. Microbial profiles from the sulfide zone of acetate supplemented tank was surveyed by using 16S rDNA sequencing and showed variety of microbial diversity. Major groups of bacteria were the same over time, but the richness

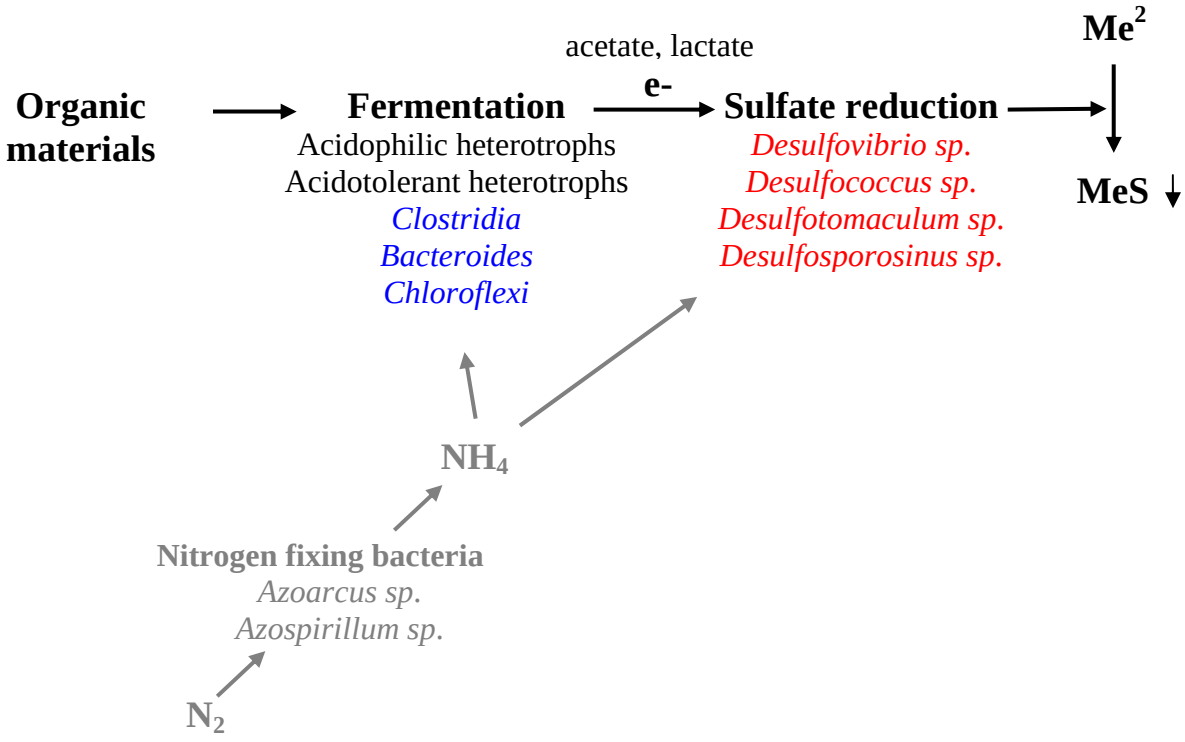
of the major groups sequenced showed a shift, and only *Proteobacteria* increased in numbers. LIBSHUFF analysis indicated that microbial compositions were significantly different in both tanks and even in the same tank. Sequences retrieved with SRB group specific primers showed increased number of SRB groups after acetate supplementation although there may be biases caused during DNA extraction and PCR amplification. Horizontal and vertical distributions of microbial communities were investigated using DGGE. The results from DGGE also showed spatial changes of microbial diversity even in the same reaction zone of the same tank.

SRB enumeration obtained from most probable number method showed only difference in the size of communities detected in the oxide zone indicating acetate might enhance the sulfate reduction in this zone. Acetate can enhance either acidophilic or acid-tolerant sulfate-reducing bacteria or heterotrophic fermentative bacteria. The latter group can degrade biopolymers and produce primary carbon sources, which then support the growth of sulfate-reducing bacteria. Therefore, heterotrophic fermentative bacteria and sulfate-reducing bacteria are syntrophically associated together and play a major role in the bioremediation process occurring in the wetland system (Fig. 5.1).

Isolation and subsequent physiological characterization of bacteria in an environment are quite useful to understand the bacteria-mediate biogeochemical processes. So a total of 101 pure strains were isolated from the highest positive MPN cultures for sulfate-reducing bacteria. Based on 16S rDNA analysis, the isolates belonged mainly to two phyla, the low G+C Gram-type positive bacteria and the *δ-Proteobacteria*. Clostridia outnumbered sulfate-reducing bacteria among isolates indicating heterotrophic fermenters are more abundant and versatile. Among isolates, only *Desulfosporosinus* and *Desulfovibrio* species was recovered as SRB. Four novel species identified were isolated from the oxide zone except one from coal pile runoff basin, and

all belonged to the low G+C Gram-type positive bacteria including a *Desulfosporosinus* species. Their true habitat is in question. SRB are sensitive to acidity, so Gram-type positive SRB might be more responsible for sulfate reduction in acidic environment.

Figure 5.1. A proposed scheme of metal removal in a constructed wetland system receiving acid sulfate water.


Organic materials
Fermentation
Acidophilic heterotrophs
Acidotolerant heterotrophs
Clostridia
Bacteroides
Chloroflexi
acetate, lactate
e-
Sulfate reduction
Desulfovibrio sp.
Desulfococcus sp.
Desulfotomaculum sp.
Desulfosporosinus sp.
Me2
MeS
NH4
Nitrogen fixing bacteria
Azoarcus sp.
Azospirillum sp.
N2