MOLECULAR ANALYSIS OF BACTERIAL COMMUNITIES ASSOCIATED WITH A FORESTED WETLAND IMPACTED BY REJECT COAL

by

JENNIFER ELLEN BROFFT

(Under the direction of Lawrence J. Shimkets and J Vaun McArthur)

ABSTRACT

The Savannah River Site's D-area harbors an exposed reject coal pile from which acidic, metal-rich saline run-off has impacted an adjacent wetland. Culture-independent 16S rDNA analysis was utilized to assess spatial and temporal bacterial community changes. This environment possesses features unique to acid mine drainage (AMD) systems examined previously. The identification and characterization of the associated bacterial constituents should aid in the understanding of the ecology of AMD systems. The 16S rDNA types recovered via PCR from sites having relatively high and low contamination (RCP1 and RCP2, respectively) were distinct from those recovered from an adjacent unimpacted forested wetland (FW). An interesting trend was the predominant recovery of as yet uncultivated bacteria phylogenetically associated with isolates or environmentally-derived sequences retrieved from habitats having similar chemical characteristics. An exciting discovery was the recovery of several particularly divergent lineages. One of these groups (termed WSRCP) was recovered via a specific PCR assay only from acidic habitats and phylogenetic analysis placed this clade mostly consistently as a novel subdivision of the candidate division WS6. The bacterial

communities associated with the RCP1 and RCP2 sites were monitored in five-week increments over a year by both *Cfo*I and *Msp*I T-RFLP analysis and the relative abundance of peak areas between sites were compared. Previously isolated environmental clones related to TRA5-3, TRB3, RCP1-48, as well as *Actinomycete, Acidobacterium*, and *Leptospirillum*-related lineages contributed to the predominant peaks. The samples were spatially and temporally differentiated based on principal component analysis of measured environmental parameters and of T-RFLP data. T-RF's corresponding to *Acidobacterium* and *Actinomycete* sequence types were negatively and positively correlated with pH; accordingly these groups were relatively more abundant in the RCP2 and RCP1 samples respectively. In either sample site, TRA5-3, TRB3 and *Leptospirillium* groups exhibited a strong fluctuation that appeared cyclic in nature. The *Leptospirillium* group was predominant during the time of year (July-December) when percent moisture values were lower; conversely, the TRA5-3 and TRB3 groups were relatively less abundant in these same samples. In sum, we were able to relate environmental parameters to as yet uncultivated bacteria associated with acid mine drainage-type systems.

INDEX WORDS: Bacterial communities, 16S rRNA analysis, T-RFLP, Reject coal, Acid mine drainage

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CHAPTER 1

INTRODUCTION

Sulfide mineral dissolution: environmental hazards and industrial promise:

Sulfide mineral dissolution generates sulfuric acid as part of the global sulfur cycle. When mining practices increase the rate of this process, acid mine drainage (AMD) is inevitably produced by a complex suite of reactions that involve the participation of bacterially and chemically controlled steps. Mining increases the surface area of the ore body and subsequently exposes it to oxygen and water when coal waste or tailings are deposited on land surfaces or when water percolates underground mines (Nordstrom and Alpers, 1999b). Documented environmental hazards associated with AMD date to the ancient Greeks and Romans (Nordstrom and Alpers, 1999b) and our society continues to pay for the consequences of improper mining practices. The U.S. mining industry spends approximately one million dollars a day to treat the AMD-associated pollution (Evangelou and Zhang, 1995). As of 1985, the United States had generated 50 billion tons of mining waste, with current production rates of approximately one billion tons per year (EPA, 1995). Consequently, approximately 17,000 km of streams and 180,000 acres of lakes and reservoirs have been impacted over the past century (Evangelou and Zhang, 1995), killing billions of fish (Nordstrom and Alpers, 1999b). The effects on the health of humans have on occasion been dramatic; the oxidation of mine wastes in Thailand poisoned citizens due to groundwater contamination with arsenic (Nordstrom and Alpers, 1999).

Acid sulfate soils can produce an environmental disturbance similar to AMD. This circumstance occurs upon drainage of sulfide-rich soils, found mostly in tropical coastal regions. Approximately 24-28 million hectares of sulfide-rich soil are estimated to exist in coastal plain and tidal swamps worldwide (Dent and Pons, 1995). Aeration of these soils has occurred naturally (by sea level lowering or by uplifting and subsequent erosion) and as a result of human impact (drainage for development or agriculture) (Dent and Pons, 1995). As a consequence, pyrite-rich soils can become acidified and coated with insoluble ferric iron precipitates, diminishing soil quality and producing a run-off that causes downstream damage (Pronk and Johnson, 1992).

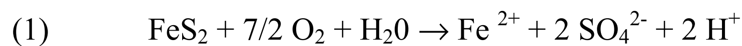
Despite the environmental consequences associated with the accelerated dissolution of sulfide minerals, this process has been successfully exploited for industrial purposes. Commercially viable metals can be solubilized from ores and mining wastes by a process referred to as bioleaching or biohydrometallurgy (Bosecker, 1997). In this process, a large pile of ore/waste material is treated with a leach solution (usually ferric iron and hydrogen sulfide). The solution along with the solubilized metal exits the pile, the metal is precipitated and the leach solution is reapplied to the pile (Hutchins *et al.* 1986). Bioleaching is an economically advantageous means to recover metals from low-grade ores (this process is too slow to be efficient compared to smelting for high grade ores); this technology may be carried out in underground mines, circumventing the expensive transportation of waste rock (Rawlings and Silver, 1995). Bioleaching has been applied primarily to recover copper and to a lesser degree uranium. Additionally, bioleaching represents a proven means to pretreat gold bound in sulfide minerals prior to recovery via cyanidation (Rawlings and Silver, 1995). Bioleaching processes may also

be exploited to desulphurize coal. Coal combustion produces sulfur dioxide, an atmospheric pollutant that has serious consequences on ecosystem health due to the production of acid rain and ozone. The reduction of sulfur emissions from coal combustion represents a significant challenge given the anticipated increase of high sulfur coal reserves (Gomez *et al.,* 1999). Sulfur dioxide is typically removed after combustion by flue gas desulfurization, an expensive process which has not been universally adopted in developing countries (Kargi, 1982). As a result, there exists a market for the development of inexpensive precombustion sulfur removal methods; the removal of inorganic sulfur from coal by bioleaching potentially has economic advantages over chemical and physical pretreatment methods (Kargi, 1982).

<u>Mechanism of sulfide mineral dissolution: a chemical and bacterially mediated process</u>

The environmental disturbances occurring with AMD and acid sulfate soils along with bioleaching result from the same general process; as sulfide minerals are exposed to oxygen, water, and bacteria, a complex series of reactions ensue, producing a toxic acidic run-off enriched with soluble salts and heavy metals. A simplified summary of the most widely accepted mechanism for AMD generation (Edwards *et al.,* 1998) is presented below. It is important to note that sulfide mineral oxidation may also produce intermediates not shown here such as elemental sulfur and sulfoxyanions (thiosulfate, polysulfonates, and sulfite) (Nordstrom and Southam, 1997). Some of these compounds may act as substrates for sulfur oxidizing bacteria, which would contribute to acid production. However, the significance of such reactions is not clear since these sulfur substrates are often undetectable at pH values less than 3.0, particularly when ferric iron concentrations are high (Nordstrom and Alpers, 1999b).

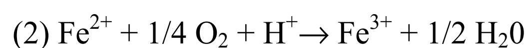
The most common type of sulfide minerals are iron sulfides (notably pyrite, FeS_2) which can represent significant constituents of coal deposits (1-20%) and can frequently co-exist with other metals (Johnson, 1998). Many commercially viable metals themselves occur as sulfides, including copper, gold, lead, silver and zinc. Excavation of sulfide minerals during coal and metal mining results in sulfuric acid production mediated either biologically by sulfur oxidizing bacteria attached to the mineral surface or chemically during the following initiation reaction (using pyrite as the example):



(1) $$FeS_2 + 7/2\ O_2 + H_20 \rightarrow Fe^{2+} + 2\ SO_4^{2-} + 2\ H^+$$

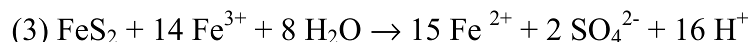
AMD isolates capable of oxidizing reduced sulfur compounds include members of the genus *Thiobacillus* and *Sulfobacillus* (Table 1.1), though it is not clear whether either one of these bacterial groups and/or an as yet uncharacterized organism are primarily responsible for this process. It is also not clear whether this reaction is catalyzed to a significant extent by bacteria (explained below).

The ferrous iron (Fe^{2+}) formed in the above reaction can serve as an electron donor for lithotrophic bacteria such as *Thiobacillus ferrooxidans, Leptospirillum ferrooxidans,* and members of the *Archaea* genus *Ferroplasma* (Table 1.1). In neutral conditions, ferrous iron is spontaneously oxidized in the presence of oxygen. However, the rate of this chemical reaction is comparatively slow in acidic conditions, allowing bacteria to compete for the substrate in aerobic regions. At pH values less than 3.0, the bacterially catalyzed oxidation rate of ferrous iron is approximately 5 orders of magnitude greater than the chemical rate (Nordstrom and Southam, 1997):



(2) $$Fe^{2+} + 1/4\ O_2 + H^+ \rightarrow Fe^{3+} + 1/2\ H_20$$

In acidic conditions, it has been determined that ferric iron (Fe^{3+}) is a more efficient oxidant of pyrite than oxygen, increasing the reaction rate of pyrite oxidation by 3-100 fold (McKibben and Barnes, 1986). Since ferric iron is soluble only under acidic conditions, this reaction is likely only significant at low pH valuesd.



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

This reaction will proceed only as fast as ferric iron is supplied and does not depend on the diffusion of oxygen to the mineral surface, though it is indirectly dependent on oxygenated conditions (Nordstrom and Southam, 1997). Therefore, the fundamental importance of acidophilic iron oxidizing bacteria in bioleaching systems lies in their ability to regenerate ferric iron, the rate-limiting step of this process. The same general reaction mechanism can occur with metal sulfides other than pyrite (eg: chalcopyrite, $CuFeS_2$ or arsenopyrite $AsFeS_2$), though distinctions in this process exist among various minerals.

Whether bacteria contribute more towards sulfide dissolution by a direct or indirect mechanism remains a controversial subject. The direct mechanism refers to the hypothesis that sulfide minerals can be directly oxidized via an extracellular enzyme system (which has yet to be identified) of surface-attached bacteria (Silverman and Ehrlich, 1964). The proposed indirect mechanism involves the chemical oxidation of pyrite by ferric iron generated as a result of lithotrophic bacterial growth, described above. Support for the direct model mainly relies on observations from several experiments conducted with the sulfur and iron oxidizing bacterium *T. ferrooxidans*. Dissolution patterns having the approximate size and shape of a rod-shaped bacterium were observed on pyrite surfaces containing attached *T. ferrooxidans* (Bennett and

Tributsch, 1978). It has also been demonstrated that *T. ferrooxidans* forms a monolayer on the pyrite surface (Sand *et al*., 1995) and preferentially colonizes metal sulfides possessing a higher reactivity (Lawrence *et al*., 1997), suggesting that direct contact is required for growth (Arredondo *et al*., 1994, Edwards *et al*., 2001). Perhaps the most compelling evidence supporting the direct mechanism came from experiments conducted with washed *T. ferrooxidans* and synthetically prepared metal sulfides free of iron (cobalt sulfide [CoS], millerite [NiS], sphalerite [ZnS]) (Duncan et al., 1967; Rickard and Vanselow, 1978). Greater oxygen consumption and metal solubilization rates were observed with this treatment then with incubations in the absence of *T. ferrooxidans*. However, Sand *et al*. (1995) observed that *T. ferrooxidans* possessed extracellular polysaccharides that contain significant amounts of ferric iron not removed by washing. Therefore, previous experiments conducted with iron-free sulfide minerals were presumably influenced by this iron contamination. No significant dissolution was observed when *T. ferrooxidans* repeatedly subcultured in an iron-free containing media was added to iron-free synthetic metal sulfides (Sand *et al*., 1995). Further evidence casting doubt on the significance of the direct mechanism was provided recently by Edwards *et al.* (2001). This group demonstrated that sterile sulfide minerals (pyrite [FeS_2], marcasite [FeS_2], and arsenopyrite [$AsFeS_2$]) incubated with only ferric iron formed cell sized/shaped dissolution patterns similar to those observed when these same minerals were incubated with either *T. ferrooxidans* or the iron-oxidizer *Ferroplasma acidarmanus.* In summary, the relevancy of the direct mechanism remains questionable while the indirect mechanism is likely always occurring due to the high concentrations of ferric iron in bioleaching habitats (Nordstrom and Southam, 1997).

<u>Rationale for researching the bacterial constituents associated with bioleaching habitats.</u>

Microbiological research of acid leaching environments has a long history, due in part to bacterial participation in the occurrence of AMD. Understanding the microbiology and geochemistry associated with sulfide dissolution may ultimately lead to the ability to limit acid production in leaching environments (Nordstrom and Southam, 1997). Therefore, identifying and elucidating the roles of the bacteria inhabiting AMD systems is an important goal. Identification of AMD bacterial constituents is also necessary in order to assess the long-term effectiveness of remediation strategies. For instance, indigenous anaerobes may potentially alter the solubility and thus mobility of toxic substances. Arsenic and other heavy metals can adsorb onto iron and maganese hydroxides, forming insoluble precipitates. The reduction of these compounds by iron and manganese reducing bacteria would solubilize these elements, potentially releasing them into the groundwater (Lovely, 1991). Conversely, hydrogen sulfide generated from bacterial sulfate reduction can reduce soluble forms of metals, forming insoluble metal sulfides that persist within the system (Wielinga *et al.,* 1999).

Bacteria inhabiting bioleaching environments have an industrial interest. Currently, some bioleaching operations use an inoculum containing an undefined bacterial community, usually enrichments from acidic environments or samples associated with metal ore bodies (Yates *et al.,* 1986); an optimized consortia or the alteration of pile parameters that select for different bacterial groups might increase the efficiency of this process. Bioleaching conditions are often based on parameters extrapolated from the properties of a few well-studied bacteria, primarily *Thiobacillus ferrooxidans* (Goebel and Stackebrandt 1994a). However, bioleaching communities are

known to harbor bacteria besides *T. ferrooxidans* (Johnson 1998), many of which likely contribute to the leaching process. The enzymes synthesized by acidophiles occupying leaching environments may also be of commercial interest. Such catalytic agents could be useful in the synthesis of compounds requiring acidic conditions (Johnson 1998), such as enzymes added to animal feeds to improve digestion efficiencies.

Similar to other extremeophiles, acidophilic bacteria are found at high densities in isolated regions (Edwards *et al.,* 2000). Despite their localized distribution, AMD bacterial communities have a great impact on global sulfur cycling; approximately one half of the total sulfate deposited by rivers into the ocean comes as the result of anthropogenic sources, including mining practices (Brimblecome *et al.,* 1989). Finally, due to the selective conditions found in acid leaching environments, the associated bacterial biodiversity appears to be relatively low compared to pristine systems. This inference is based the limited recovery of cultivatable heterotrophs and unique 16S rDNA sequence types (Wassel and Mills, 1983; Goebel and Stackebrandt, 1994b; Bond 2000b). Therefore, investigation of AMD habitats provides a chance to investigate the bacterial community composition, structure, and dynamics occurring in a relatively simple system (Bond *et al*., 2001).

<u>Bacterial constituents of bioleaching environments: results from culture-based analysis.</u>

Results from culture-based analysis suggest that the biological diversity in bioleaching environments is low compared to pristine habitats (Wassel and Mills, 1983; Goebel and Stackebrandt, 1994) and is generally prokaryotic, although green algae, fungi, protozoans, and rotifers have been recovered (Johnson 1998). Many different types of bacteria have been isolated from bioleaching environments (Johnson, 1998;

Johnson *et al*., 2001) (Table 1.1). Nevertheless, research has focused primarily on the lithoautotrophs *Thiobacillus ferrooxidans (*now referred to as *Acidithiobacillus ferrooxidans*) (Kelly and Wood, 2000), *Leptospirillum ferrooxidans* and *Thiobacillus thiooxidans* in addition to members of the heterotrophic genus *Acidiphilium.* *Acidithiobacillus ferrooxidans* (*At. ferrooxidans)* was the first bacterium cultivated from an AMD type system (Colmer and Hinkle 1947) and its addition to pyrite has been demonstrated to increase sulfuric acid production rates (Leathen *et al.,* 1953). *At. ferrooxidans* is an acidophilic, aerobic lithoautotroph that uses either ferrous iron or reduced sulfur compounds as energy sources. Members of this species have been considered the most significant participants in sulfide mineral oxidation because they are readily cultivated from bioleaching habitats (Goebel and Stackebrandt, 1994b; Wakao *et al.,* 1991; Walton and Johnson, 1992). Correspondingly, the physiology and genetics of *At. ferrooxidans* has been extensively studied to the exclusion of other acidophilic iron and sulfur oxidizers (Rawlings, 2001) and its use in pure culture laboratory experiments has been the basis for determining the rate and the variables influencing sulfide mineral oxidation (Nordstrom and Southam, 1997). However, other bacteria previously isolated and yet to be cultivated may also participate significantly in acid generation. The environmental conditions under which these organisms function optimally may be distinct from *At. ferrooxidans*, which would therefore alter acid generation models based on the growth parameters of the latter. For instance, the iron oxidizing bacterium *Leptospirillum ferrooxidans* is also suspected to play an important role in sulfide mineral oxidation. This organism appears to have a distinct mechanism for iron oxidation as it lacks rusticyanin (an acid stable redox protein of *At. ferrooxidans*) and contains a novel

soluble red cytochrome (Rawlings, 2001). Furthermore, this organism is comparatively more tolerant of low pH, high temperatures, and elevated metal concentrations (Hallman *et al*., 1992).

L. ferrooxidans is distinct from *At. ferrooxidans* both phylogenetically (member of the division *Nitrospira* versus the gamma *Proteobacteria* respectively) and in its inability to oxidize reduced sulfur species (Johnson, 1998). Based on cultivation analysis, the relative importance of these two iron oxidizers in bioleaching habitats is unclear. Historically, *At. ferrooxidans* has been shown to predominate (McGuiness and Johnson, 1993; Schippers *et al.,* 1995; Berthelot *et al.,* 1997; Johnson 2001), yet other cultivation surveys have suggested that *L. ferrooxidans* is, at the very least, as abundant (Sand 1992; Walton and Johnson 1992). *At. ferrooxidans* appears to dominate at low temperatures (Sand 1992, Johnson 2001), but few other clear patterns have emerged from these studies to explain the predominance of either. Shortcomings associated with cultivation approaches exacerbate the difficulty of interpreting and comparing these studies. For instance, the lower growth rate of *L. ferrooxidans* on energy sources such as ferrous sulfate (Pronk and Johnson, 1992) along with the difficulty associated with growing it on solid media (Johnson, 1995) might lead to the underepresentation of *L. ferrooxidans* in cultivation studies.

Sulfur oxidizing bacteria are also capable of acid generation, though their relative significance compared to iron oxidizing bacteria is unclear. Members of the genus *Thiobacillus* are capable of oxidizing various sulfur compounds (S^0, H_2S, $S_2O_3{}^{2-}$,$S_4O_6{}^{2-}$). *T. thiooxidans* and *T. acidophilus* represent two of the most commonly isolated sulfur oxidizers, with neither able to utilize ferrous iron as an energy source (Johnson, 1998).

Less frequently recovered are members of the genus *Sulfobacillus*, which are also capable of sulfur oxidation. These organisms possibly oxidize sulfide minerals directly; reduced sulfur compounds produced as byproducts of sulfide mineral oxidation might also represent the energy source for these organisms.

Lithoautotrophy represents the base of the food web in bioleaching environments since they are often devoid of sunlight (Johnson, 1998). However, heterotrophic microorganisms (*Archaea*, *Bacteria*, fungi, yeasts, and protozoa) represent a numerically significant proportion of the community (Sand *et al.,* 1992; Walton and Johnson, 1992; Johnson, 2001) despite the fact that these environments typically contain low concentrations of dissolved organic carbon (<20 ppm) (Johnson, 1998). Members of the genus *Acidiphilium* represent the most commonly isolated heterotroph, although *Acidobacterium capsulatum*, and *Acidocella* spp. have also been retrieved (Johnson 1998). Sulfur (ex: *Acidophilium acidophilium*) and iron oxidizers (ex: *Ferromicrobium acidophilium*) that require organic carbon represent a distinct type of acidophilic heterotroph. The relative importance of these organisms compared to autotrophic sulfur and iron oxidizers is unclear, but they appear to be ubiquitous among bioleaching systems (Bacelar-Nicolau and Johnson, 1999). *F. acidophilium* is able to increase pyrite oxidation rates in the presence of organic carbon or even in the absence of exogenously supplied organic carbon when a sulfur oxidizer such as *T. thiooxidans* is present (Bacelar-Nicolau and Johnson, 1999). Heterotrophy may also affect sulfide mineral oxidation by enhancing the growth rate of autotrophic, iron oxidizing bacteria (Johnson 1998). Increased pyrite oxidation rates have been observed with co-cultures of iron oxidizers and heterotrophs as compared to pure cultures of the former (Hallman 1993). Acidophilic

heterotrophs and lithoautotrophs are thought to form a mutualisic relationship as the heterotrophs obtain organic carbon from exudates and lysis products of the latter and, in turn, consume low molecular weight organic acids toxic to many iron-oxidizing bacteria (Johnson, 1998). In fact, early reports of *At. ferrooxidans* exhibiting mixotrophic growth on glucose and ferrous iron are now attributed to contamination by heterotrophic bacteria (Harrison, 1984).

Bioleaching environments may also harbor anaerobic organisms. Ferric iron and sulfate represent the most likely terminal electron acceptors due to their high concentrations in AMD systems whereas nitrate and manganese concentrations are relatively low (Johnson, 1998). The high redox potential of Fe^{+2}/Fe^{+3} (+770 mv at pH 2.0) suggests that for lithoautotrophs that utilize ferrous iron as an energy source, sulfate represents a more likely alternative terminal electron acceptor than ferric iron, whereas sulfur oxidizers can hypothetically use ferric iron (Johnson, 1998). Heterotrophic and sulfur-oxidizing acidophiles capable of anaerobic growth utilizing ferric iron include *At. ferrooxidans, Sulfobacillus* spp (coupling oxidation of tetrathionite), and the heterotrophs *Acidimicrobium ferrooxidans,* some *Alicyclobacillus* isolates and one strain of *Acidiphilium*. While sulfate reduction has been demonstrated in acidic environments (pH <4.0) by incubation of sediments with $^{35}SO_4^{2-}$ (Satake 1977, Gyure *et al.,* 1990), no acidophilic or acid tolerant sulfate reducer has been isolated in pure culture (Bond *et al.,* 2000).

<u>Culture-independent bacterial community analysis of AMD environments</u>

Culturing approaches are necessary to discern the metabolic capabilities of bacterial populations inhabiting a given environment, yet they may not representatively

sample organisms for a given system or process (Amann *et al*., 1995). Specific groups of bacteria are selected for depending on the cultivation conditions employed; for instance, under certain environmental conditions (i.e. pH < 1.8 and/or low ferrous iron concentrations), *L. ferrooxidans* outcompetes *At. ferrooxidans* (Rawlings 1995; Sand et al., 1995; Pizarro et al., 1996; Schrenk *et al.,* 1988). Cultivation of acidophiles is also prone to bias due to the sensitivity of some to the acid hydrolysis products of agar-like agents (Johnson, 1995). The majority of research associated with sulfide mineral dissolution has focused on a few organisms based upon the relative ease with which they are cultivated. However, bioleaching environments could also possess a significant bacterial diversity that has not yet been cultivated. The most common cultivation-independent means for exploring bacterial diversity is the rRNA approach. Application of this methodology to a variety of habitats has revealed that bacterial diversity is much greater than expected, and for the most part does not correspond to that described previously by traditional cultivation approaches (Hugenholtz *et al.,* 1998).

Similar to the culture-based research described above, the majority of molecular studies conducted in acid leaching environments possessed relatively less extreme chemical and physical features (pH 2-3, temperature < 30°C) (Lane *et al.,* 1985; Goebel and Stackerandt, 1994; Pizarro *et al.,* 1996; Vasquez and Moore, 1999; Espejo and Romero, 1997; Edwards *et al.,* 1998). Generally speaking, these surveys recovered 16S rDNA types that were identical to bacteria commonly isolated from these same samples. This was a surprising finding considering analogous studies conducted in non-acid leaching environments universally isolated sequences quite distinct from previously cultivated organisms (Hugenholtz *et al.,* 1998). Results from 16S rDNA-based molecular

surveys also suggested that the bacterial diversity present within acid-leaching environments is low. In an early study, Lane *et al.,* (1985) isolated two 5S rRNA molecules from a copper leaching pond sediment; one of which was identical to the sequence of *At. ferrooxidans* (ATCC 19859) while the other represented an unidentified sequence associated within the *Proteobacteria*. Direct 16S rDNA analysis of bacteria inhabiting a copper leaching bioreactor (pH 2.4) retrieved three distinct 16S rDNA types (from 80 screened clones), two of which had sequences identical to isolates cultivated from these same samples (*Leptospirillum*-related isolate MIM Lf30 and *At. ferrooxidans* strain MIM SH12) and one of which (A70) represented a organism yet to be cultivated but which was affiliated with the sulfur-oxidizing genus *Sulfobacillus* (Goebel and Stackebrandt, 1994). Similar results were obtained from a study conducted on bioleached copper ore. PCR analysis recovered the amplified 16S-23S spacer regions having identical length to those of *At. ferrooxidans*, *T. thiooxidans*, and *L. ferrooxidans* (Pizarro *et al.,* 1996; Espejo and Romero, 1997). Approximately one half of the clones retrieved from a survey of a pH 2.4 runoff stream within Iron Mountain, CA (see below) were less than 98% similar to members of the genus *Acidiphilium* and cultivated representatives of the genus *Leptospirillum* (Edwards *et al.,* 1998). The majority of the remaining sequences were highly related to other cultivated bacteria, however a few novel clones within the gamma *Proteobacteria* (clones TRA5-3 and TRA2-7) and within the *Acidimicrobidae* group of the Actinomycetes were isolated.

A series of molecular-based studies were recently conducted in Iron Mountain, an AMD-affected system having relatively extreme characteristics that distinguish it from bioleaching environments investigated previously. Iron Mountain located near Redding,

California contains a large sulfide deposit (up to 95% pyrite) which has been extensively mined for gold, silver, copper and zinc (Nordstrom and Alpers, 1999b). Tunnels directly in contact with the ore body typically display pH values in the 0-1.0 range, temperatures from 30-50°C, metal ion concentrations in the decagram per liter range, and sulfate concentrations among the highest measured (hundreds grams/liter) (Edwards *et al*., 2000). In a pivotal study applying fluorescent *in situ* hybridization (FISH), Schrenk *et al.,* (1998) found *At. ferrooxidans* was associated (up to 30% total bacterial cells) only with sampling sites draining the ore body (pH 2-4; temp < 30°C) and was virtually absent within the chemically more extreme tunnels mentioned above. This finding coincides with the observation that conditions within this extreme niche lie outside the organism's growth range. The distribution of *At. ferrooxidans* within this system led the researchers to speculate that this bacterium may not significantly contribute to acid generation as previously assumed, as the oxidant this organism regenerates would not likely come into contact with the pyrite ore. The actions of this organism may actually be beneficial in that at pH values greater than 3.0, ferric iron is insoluble and may co-precipitate toxic metals present in the system (Schrenk *et al.,* 1998). Subsequent temporal analysis confirmed these trends as *At. ferrooxidans* consistently represented a minor bacterial component (0-5% total bacterial cells) within sites associated directly with the ore (Edwards *et al.,* 1999). Molecular surveys using PCR amplification also failed to retrieve sequences closely related to *At. ferrooxidans* from a subareal biofilm (pH 0-1.0) (Bond *et al.,* 2000b), a stream in contact with the pyrite ore (pH 0.5) (Edwards *et al.,* 1998), and even a runoff stream (pH 2.4) (Edwards *et al.,* 1998), although the latter libraries were not extensively screened. It was therefore speculated that *At. ferrooxidans*

may only be significant in AMD environments having a relatively higher pH (>2.0), which is consistent with findings from 16S rDNA surveys of relatively moderate bioleaching habitats, described above. This was not the first research group to provide molecular evidence that the proportion of *At. ferrooxidans* in acid leaching habitats has been overestimated . Muyzer *et al*., (1987) using fluorescent antibodies specific to *At. ferrooxidans* found that the majority of the bacteria present in coal leaching samples (30°C, pH 2.0) consisted of bacteria other than *At. ferrooxidans*

Temporal analysis indicated *L. ferrooxidans* was a significant component (up to 20% of the bacterial cells using FISH) within Iron Mountain's extreme niches (Edwards *et al.,* 1999), suggesting that this organism is involved in acid perpetuation as previously believed. A novel cluster of *Leptospirillum*-related sequences retrieved from a biofilm (pH 0-1.0) form what has been termed *Leptospirillum* group III (Bond *et al.,* 2000b). Previous phylogenetic analysis placed the cultured representatives of this genus into two groups, I and II, which interestingly coincide with their optimum temperature growth ranges (group I: 26-30°C; group II: 30-40°C) (Goebel and Stackebrandt, 1994b). The 16S rRNA genes of three groups are approximately 90% similar to one another. Goebel and Stackebrandt (1994b) suggested that bioleaching conditions favor members of group I. However, both FISH and 16S rDNA sequence analysis suggests that types II and particularly type III represented the dominant *Leptospirillum* within the extreme regions of Iron Mountain (Bond and Banfield, 2001). The environmental factors that control the relative distributions of these groups remain unclear. Organisms representing type III sequences have so far defied cultivation and the significance of this group within relatively less extreme bioleaching systems remaining unexplored. Probes specifically

targeting this group have not been applied within such habitats and the few relevant molecular surveys have so far failed to retrieve type III sequences.

Contrary to less extreme bioleaching environments, molecular analysis indicated that the microbial community of Iron Mountain was not dominated by traditionally isolated *Leptospirillum* and *Thiobacillus* spp. Based on FISH analysis, no more than 25% of the bacterial populations within extreme Iron Mountain niches consisted of *At. ferrooxidans* along with *Leptospirillum* types I and II (Edwards *et al.,* 1999, Schrenk *et al.,* 1998). 16S rDNA surveys were conducted for stream, biofilm, and sediment samples directly in contact with the ore in an attempt to characterize the bacterial types that actually inhabit this system. Retrieved sequences were generally affiliated with but not closely (<97% similar) related to cultivated acidophiles, including the *Acidimicrobium* group of the Actinomycetes and the archaeal order *Thermoplasmales* along with sequences comprising *Leptospirillum* group III (Bond and Banfield, 2001; Bond *et al.,* 2000a). This finding contrasted with previous research applying molecular strategies that suggested bioleaching habitats consisted primarily of organisms amendable to cultivation. Only a small proportion of clones retrieved from the Iron Mountain surveys were highly similar (>97%) to cultivated acidophiles (including *Ferromicrobium acidophilius* and members of the genus *Acidiphilium)*. Still other 16S rDNAs, such as delta *Proteobacteria* sequences represented by BA18 and BA71 were not closely related (78-82%) to any GenBank sequence, environmental clone, or isolate. The retrieval of so many novel sequences from this habitat was considered a consequence of the extreme nature of the system (Bond *et al.,* 2000). Yet as with the molecular-based studies of less extreme AMD environments, the diversity associated with these bacterial communities

appeared low compared to that of non-polluted environments. The various extreme niches of Iron Mountain were typically dominated by 1-3 types of organisms, with the most dominant being members of the order *Thermoplasmales* (*Ferroplasma* spp. in particular), *Leptospirillum* groups II and III, and *Sulfobacillus* spp (Bond *et al.,* 2000a; Bond and Banfield, 2001).

The significance of *Archaea* within bioleaching environments remained largely unexplored prior to the studies conducted at Iron Mountain. Edwards *et al.* (1999) found that the *Archaea* abundance was correlated with dissolved solid levels and represented over 50% of the total bacterial population in certain sites as monitored by FISH. A novel archaeal iron oxidizer, *Ferromonas acidarmanus* has since been isolated (Edwards *et al.,* 2000). This bacterium is situated within the order *Thermoplasmales*, a *Euryarchaea* group that contains hyperacidophiles possessing only a cytoplasmic membrane. *F. acidarmanus* dominates certain sediments and slimes, comprising up to 85% of the bacterial community when the conductivity of the sample was high (100-160 mS/cm) (Edwards *et al.,* 2000). The relative abundance of the *Thermoplasmales* among distinct niches within Iron Mountain suggests that members of this group may be more competitive at relatively moderate temperatures and low pH values, which is consistent with the physiology of this group (Bond *et al.,* 2000). Additional isolates and environmental clones affiliated with the *Thermoplasmales* subsequently have been retrieved from low pH environments. *F. acidiphilium* was isolated from a bioleaching reactor (Golyshina *et al.,* 2000) and Vasquez *et al.,* (1999) retrieved a related archaeal 16S rDNA sequence type from a copper leaching solution. While the *Thermoplasmales* have not traditionally been associated with bioleaching environments, it appears that

members of this group may represent iron oxidizers significant to these systems. Interestingly, *Archaea* appear to dominate within acidic waters present within a South African gold mine (pH 3.0; 1.8 km depth) as the proportion of archaeal rDNA within the total community rDNA was determined to be 74.4% (Takai *et al.,* 2001). However, the two 16S rDNA types acquired were not members of the *Thermoplasmales*, but instead affiliated within the *Crenarchaeota*. One sequence type (11% clones) was highly related to the environmental clones of the soil crenarchaeotic group (SCG) while other (89% clones) was related to a cluster of sequences isolated from various sites (including non-acidic ones) within this particular mining system. Clearly there remains much to be learned about the types and distribution of *Archaea* within bioleaching systems.

<u>The purpose of this work</u>

There exists great interest in the types of bacteria inhabiting acid leaching systems due to their role in sulfide mineral oxidation. Because of the inherent bias associated with traditional culture-based methodologies, the application of cultivation-independent 16S rRNA-based approaches represents a desirable means to simultaneously survey different types of bacteria inhabiting a given system. To date, aside from industrial bioleaching settings, few comparatively less extreme acid-leaching habitats (pH 2-3) have been inventoried for their rRNA diversity. The biodiversity of these industrial systems might be low compared to natural ones since the environmental parameters of the former are somewhat controlled, leading to systems possessing relatively constant features. Therefore, we chose to investigate the bacterial diversity associated with an ecosystem affected by AMD-type run-off. The chemical and physical parameters of our system would be considered less extreme compared to the conditions found within Iron

Mountain. Compared to industrial bioleaching systems, our system is comparatively more complex, possessing distinguishing features such as fluctuating water levels, exposure to sunlight, and potential organic input from healthy adjacent areas. These features may be more representatives of features found in other AMD-impacted environments rather those associated with a copper bioleaching pile. These characteristics likely affect the types of bacteria present within the system and, as such, novel ecotypes important to the ecology of acid-generating environments may be retrieved.

The first study was conducted to describe the bacterial diversity associated with a forested wetland impacted by coal storage run-off. This was accomplished by applying a culture-independent 16S rRNA cataloging approach. 16S rDNA sequences were retrieved from samples taken along a contamination gradient so that those representing bacteria endemic to the polluted region could be identified by comparison with an unaffected area. This study also sought to identify commonalties between 16S rDNA types retrieved here and previously studied AMD-like systems.

The second study sought to monitor the sequence types retrieved from impacted sampling sites over time. With the exception of Iron Mountain, the majority of bacterial diversity studies conducted in bioleaching habitats have been based on only one sample. The molecular profiling technique known as terminal restriction fragment length polymorphism (T-RFLP) was utilized to compare the bacterial community structure of samples taken over the course of the year at two chemically distinct sites within the affected wetland. The proportion of the profile accounted for by the 16S rDNA sequence types retrieved from the first study was determined as was the relative

abundance of these types. Chemical and physical parameters measured with each sampling event were used to determine whether any of these variables might be linked to spatial or temporal community shifts.

The third chapter relates to an interesting 16S rDNA sequence group retrieved from the most contaminated region of the study site. We recovered three sequences (~ 90% similar to one another) encompassing a divergent cluster that was approximately 75% related to any previously characterized. Phylogenetic analysis determined that this group may represent a novel subdivision of the candidate division WS6. Primers targeting these sequences were designed and tested to determine their specificity. These primers were then applied to various environmental samples using a PCR assay to determine whether the distribution of this group was restricted to acidic habitats and whether additional members could be retrieved to aid in determining its appropriate phylogenetic placement.

The final chapter represents the phylogenetic characterization of 16S rDNA sequences obtained from the unaffected forested wetland sampled in the first study. Southeastern riparian wetlands represent the most extensive wetland category in the U.S. and to our knowledge have not been investigated for their 16S rRNA diversity. The high productivity coupled with the oxygen gradients formed under inundated conditions should enable these systems to harbor a diverse bacterial community. The phylogeny of the sequence types is described and compared to those recovered in other similar habitats.

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TABLE 1.1. Mesophilic bacteria isolated from acidic environments (pH <3.0) (adapted from Bond and Banfield, 2001).

Organism	Taxonomic affiliation	Physiology
Bacteria		
Thiobacillus ferrooxidans	γ-*Proteobacteri*a	Iron and sulfur oxidizer (autotroph)
T. thiooxidans	γ-*Proteobacteria*	Sulfur oxidizer (autotroph)
T. caldus	γ-*Proteobacteria*	Sulfur oxidizer (autotroph)
Leptospirillum ferrooxidans	*Nitrospira/Leptospirillum* group I and II	Iron oxidizer (autotroph)
Acidimicrobium ferrooxidans	Actinomycetes	Iron oxidizer (autotrophic/heterotroph)
Ferromicrobium acidophilus	Actinomycetes	Iron oxidizer (heterotroph)
Sulfobacillus acidophilus	High G + C gram positive division	Iron and sulfur oxidizer (autotroph/heterotroph)
S. thermosulfidooxidans	High G + C gram positive division	Iron and sulfur oxidizer (autotroph/heterotroph)
Alicyclobacillus spp.	High G + C gram positive division	Heterotroph
Acidiphilium spp.	α-*Proteobacteria*	Heterotroph
Acidocella spp.	α-*Proteobacteria*	Heterotroph
Acidomonas methanolicola	α-*Proteobacteria*	Heterotroph
Archaea		
Thermoplasma acidophilum	*Thermoplasmales*	Heterotroph
Picrophilus oshimae	*Thermoplasmales*	Heterotroph
Ferroplasma acidiphilum	*Thermoplasmales*	Iron oxidizer (autotroph)
Ferroplasma acidarmanus	*Thermoplasmales*	Iron oxidizer (autotroph/heterotroph)

CHAPTER 2

16S rDNA SURVEY OF BACTERIAL COMMUNITY COMPONENTS ASSOCIATED WITH A FORESTED WETLAND IMPACTED BY REJECT COAL EFFLUENT.

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[1]Brofft, J.B., McArthur, J.V. and L.J. Shimkets (accepted with revision) *Environmental Microbiology.*

Abstract

Sulfide mineral mining together with improperly contained sulfur-rich coal represents a significant environmental problem due to leaching of toxic material. The Savannah River Site's D-area harbors a 22 year old exposed reject coal pile (RCP) from which acidic, metal rich, saline runoff has impacted an adjacent forested wetland. In order to assess the bacterial community composition of this region, composite sediment samples were collected at three points along a contamination gradient (high, middle and no contamination) and processed for generation of bacterial and archaeal 16S rDNA clone libraries. Little sequence overlap occurred between the contaminated (RCP samples) and unimpacted sites, indicating that the majority of 16S rDNAs retrieved from the former represent organisms selected for by the disturbance. Bacterial diversity tended to increase with samples taken further from the coal pile, suggesting that this disturbance reduced species diversity. Archaeal diversity within the RCP samples was considerably lower than that of the *Bacteria* and consisted mainly of sequences related to the genus *Thermoplasma* and to sequences of a novel type. Bacterial RCP libraries contained 16S rRNA genes related to isolates (*Acidiphilium* sp*, Acidobacterium capsulatum, Ferromicrobium acidophilium*, and *Leptospirillum ferrooxidans*) and environmental clones previously retrieved from acidic habitats, including ones phylogenetically associated with organisms capable of sulfur and iron metabolism. These libraries also exhibited novel 16S rDNA types not retrieved from other acid mine drainage habitats, indicating that significant diversity remains to be detected in acid mine drainage-type systems.

Introduction

United States coal use is rising to meet the demand for domestically produced energy. While power plants typically maintain a 90-day coal supply (Swift, 1985), increased coal production is expected to expand storage at power plants and mines, including sites that formerly did not store coal, and thus lack sufficient containment areas and treatment technologies (Davis and Boegly; 1981, Swift, 1985). Improper containment of coal pile waste has already seriously impacted numerous ecosystems (Davis and Boegly, 1981). Leachate from high sulfur coal piles is similar to acid mine drainage (AMD) in that each represents an acidic, saline runoff enriched with sulfate, iron, and heavy metals.

The microbial ecology of aerobic AMD environments has been extensively studied due to the importance of acidophilic iron and sulfur oxidizing bacteria in perpetuating acid production in these systems. Until recently, these studies have relied on traditional culture-based methodology (Johnson, 1988). AMD bacterial communities have been thought to consist mainly of the chemolithoautotrophs *Acidithiobacillus ferrooxidans* (formerly known as *Thiobacillus ferrooxidans*) (Kelly and Wood, 2000) and *Leptospirillum ferrooxidans* along with members of the heterotrophic genus *Acidiphilium*. Subsequent AMD research focused on elucidating the distribution and controlling factors of these bacteria despite the fact that the relative importance of as yet uncultivated acidophiles had not been explored. A recent collection of 16S rDNA-based studies conducted within Iron Mountain, CA, an extreme AMD system (pH ~ 0-1, temperature 30 to 50°C), revealed the associated bacterial communities to consist primarily of uncultivated organisms that are phylogenetically related to described

acidophilic bacteria (Bond and Banfield, 2001). Conversely, studies that have applied 16S rDNA methodology towards less extreme metal bioleaching waters (pH~2-3, temperature less than 30°C) have recovered 16S rDNA sequences which were generally identical to those of bacteria commonly cultured from these same samples (Goebel and Stackebrandt 1994a, Goebel and Stackebrandt 1994b, Pizarro *et al*., 1996). The goal of our study was to conduct a 16S rDNA molecular survey of the bacterial diversity present in a forested wetland (FW) impacted by coal storage runoff. We chose to sample over a chemical gradient so that sequences representing bacteria unique to the contaminated system might be revealed by comparison with an unaffected area.

The U.S. Department of Energy's Savannah River Site (SRS) D-area (400D) contains an exposed 22-year old reject coal pile that has chronically impacted an adjacent wetland (Carlson, 1990). Coal that has a low British Thermal Unit or high sulfur content is termed "reject" and is deposited separately from the active coal pile (Carlson, 1990). Leachate emanating from the SRS pile has polluted part of a nearby wetland to the extent that it no longer supports vegetation; yet the chemical parameters of this site (pH 2-4, temperature ~ 30°C) are more moderate than the extreme characteristics at Iron Mountain. This wetland possesses features distinguishing it from AMD environments sampled in other molecular based studies, including fluctuating water levels, exposure to sunlight, and potential organic input from healthy adjacent areas, in addition to differences in the underlying geology of this site and of the coal source. This study therefore sought to identify commonalities between 16S rDNA types retrieved here and previously studied AMD-like systems, in addition to retrieving novel ecotypes which may be important to the ecology of acid generating environments.

Material and Methods

Sample site and collection. On May 9, 2000, surface sediment samples were collected from a seasonally flooded forested wetland located in the SRS D-area (Fig. 2.1, 2.2). This region contains a coal-fired power plant and its associated wastes, including an uncontained reject coal pile from which both coal and its leachate has been transported downhill into a forested wetland. The affected wetland contains two unvegetated regions and the westernmost of these two was chosen for this study. Additional description of this site can be found in Carlson (1990) and Carlson and Carlson (1992). Three sites were sampled that differed in their proximity to the reject coal pile (RCP): a site within the contaminated region close to the coal pile (RCP1), a site in this same region at the farthest edge away from the pile (RCP2), and an adjacent unaffected site located southwest of the contaminated region (FW) that harbors vegetation such as loblolly pine (*Pinus taeda* L.), sweetgum (*Liquidambar styraciflua* L.), wax myrtle (*Myrica cerifera* L.) and broom sedge (*Andropogon virginicus* L.) (Carlson and Carlson, 1992). Both the unaffected and contaminated areas were similarly inundated at the time of sampling. Aerial photographs show that the contaminated region was vegetated prior to reject coal deposition in 1978. Loblolly pine and sweetgum seedlings are found at the periphery of the contaminated region; the few loblolly pine seedlings that temporarily establish within do not survive past the first growing season (Carlson and Carlson, 1992).

A compound sampling scheme was employed for this study. At each of the three sites, 25 sediment cores (1.5 cm diameter, 2.0 cm depth) were taken with a sterile 12 ml syringe with the bottom removed. The cores were taken several centimeters apart along a straight line parallel to the coal pile, placed in a sterile Whirl-Pak bag (Nasco), mixed and

immediately frozen on dry ice. The frozen sediment samples were maintained at -80°C until processing. Twenty-five additional sediment cores were taken in a similar manner and stored on regular ice for chemical analysis. The surface sediment temperature of the three sites ranged from 29-31°C. Organic matter content in the RCP2 and FW samples were 3.4 and 9.7 percent respectively. Organic matter content was not measured for the RCP1 sediments since these samples contained a large number of coal grains which confounded this measurement. Total nitrogen was similar between the two contaminated sites (552 ppm [RCP1] and 474 ppm [RCP2]) and less than that of the FW site (1446 ppm). Nitrate levels were similar among all three sites.

DNA extraction and 16S rDNA library construction. From each composite sediment sample, DNA was directly extracted using the soil DNA Mega extraction kit (MoBio Laboratories) according to instruction except that the 65°C heating step was extended to 45 minutes. This kit relies on mechanical cell lysis by vortexing with beads along with prolonged high temperature incubation. DAPI stained cell lysates were observed via fluorescent microscopy and few intact cells were observed. Coupled with the abundant recovery of gram positive 16S rDNA sequences (Table 2.1), we believe that the cell lysis protocol was sufficiently robust for our samples.

Bacterial and archaeal 16S rDNA sequences were selectively amplified in independent reactions. Bacterial 16S rRNA gene amplification was performed using primers 27f (5' AGAGTTTGATCCTGGCTCAG 3') (Lane, 1991) and 1392R (5' GACGGGCGGTGTGTAC 3') (Lane, 1991) whereas selection of archaeal 16S rDNA was obtained using primers 25f (5' CYGGTTGATCCTGCCRG 3') (Dojka *et al.*, 1998) and 1492r (5' GGTTACCTTGTTACGACTT 3') (Dojka *et al.*, 2000). Bacterial 16S

rDNAs were amplified from 20-30 ng DNA in reactions containing 1.5 U of *Taq* polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM of each dNTP, and 0.2 μM of each primer (Ready-To-Go PCR beads, Amersham Pharmacia). Conditions were as follows: initial denaturation at 94°C for 3 min followed by 15 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min and ending with a final extension at 72°C for 7.5 min. Archaeal 16S rDNA amplicons were generated from 20-30 ng DNA in reactions containing 2.5 U of HotStar *Taq* polymerase, 1X PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP and 0.2 μM of each primer (Qiagen Inc.). An initial denaturation at 12 minutes (for enzyme activation) was followed by 25 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min and ending with a final extension at 72°C for 7.5 min. In order to minimize variation in 16S rDNA representation within the libraries, 5 individual PCR reactions were pooled for cloning into PCR 2.1 TA cloning vector (Invitrogen). Archaeal 16S rDNA PCR products were gel purified using UltraClean 15 Kit (Mo Bio Laboratories) prior to cloning.

Screening and sequencing of rDNA clones. Different strategies were taken to screen the bacterial and archaeal libraries. Clones from the bacterial library were partially sequenced using the 27f primer and clones >97% similar within 90-620 nt (*E. coli* numbering) were considered a unique operational taxonomic unit (OTU). This segment contains variable regions 2 and 3, which are located near the 5' end of the 16S rRNA molecule. A 97% sequence similarity criterion has previously been utilized to group 16S rDNA genes into OTUs (McCaig *et al*., 1999) and this cut-off was based on the observation that this similarity sometimes correlates with a bacterial species as determined by DNA-DNA reassociation (Stackebrandt and Goebel, 1994). The degree

of sequence similarity was determined using BestFit analysis contained within the Accelrys package. Archaeal clones were grouped instead based on restriction fragment length polymorphism (RFLP) analysis; sequences with identical restriction patterns were considered as belonging to one OTU. Archaeal 16S rDNA was reamplified from clones using PCR reaction conditions as described except that approximately 500 pg of plasmid DNA was used as a template. PCR products were then digested with 1U of either *Bst*UI or *Rsa*I (Moyer et al. 1996) in separate reactions and the restriction patterns were resolved using a 2.0 % TreviGel 500 (Trevigen) gel. For both the bacterial and archaeal libraries, one representative from each OTU was extensively sequenced (>1150 bp) using the amplification primers mentioned above in addition to 533f (5'GTGCCAGC MGCCGCGGTAA 3') (Lane, 1991) and the M13 forward and reverse primers recommended for use with our vector (Invitrogen).

OTU diversity indices were calculated for the bacterial libraries using the Shannon-Weaver index (*H*), Simpson's dominance (*c*), Pielou's evenness (*e*) and equitability (*J*) (Odum, 1983). As a reference point, these indices were also calculated from data generated in two independently published bacterial 16S rDNA surveys conducted from samples harboring either a high (McCaig *et al*., 1999) or low (Bond *et al*., 2000b) diversity of sequences. The indices were not calculated for the RCP archaeal libraries due to the low number of sequence types present and to the small library size. OTU richness, or the total number of OTUs, was calculated for an equal sample size of 70 clones by rarefaction (Brewer and Williamson, 1994) with the Rarefaction Calculator [located online at http://www.uga.edu/~strata/software (Holland, 2001)]. Collector's curves (Odum, 1983) (the number of distinct OTUs recovered versus the number of

clones screened) were generated for the RCP and FW libraries based on the same rarefaction analysis. LIBSHUFF was used to determine whether the bacterial RCP1 and RCP2 libraries significantly differed from one another (Singleton, 2001) by calculating the differences between homologous and heterologous coverage curves using a Camer-von Mises test statistic. If two libraries are suggested to be significantly different from one another, additional parameters calculated by this program indicate which sequence types (i.e. species-level vs. deeper taxa) might be responsible for such differences.

Phylogenetic assessment. 16S rDNA sequences were compared to those available in the GenEMBL using the FastA search program of the Accelrys package to infer an approximate phylogenetic placement. Sequences were then aligned using the Accelrys program PILE_UP. Programs contained in the PHYLIP package were used for phylogenetic tree construction. Evolutionary distances were calculated using the Jukes-Cantor model from DNADIST to determine sequence dissimilarities. Dendrograms were determined using the Fitch-Margoliash least squares algorithm of the FITCH program. Sequences were checked as possible chimeric molecules using the Ribosomal Database Project program CHECK_CHIMERA. Suspected chimeric 16S rDNA sequences were split into two fragments based on the most likely junction point predicted by CHECK_CHIMERA and independent phylogenetic trees were constructed for each fragment to further test for artifact formation. Several trees were based on less than 1000 nucleotide positions due to the inclusion of related database 16S rDNAs that were only partially sequenced.

Nucleotide sequence accession numbers. The sequences of the rDNA clones have been assigned GenBank accession numbers (AF523872-AF524029)

Results

Chemical analysis. We sampled sediments at three sites over a gradient of reject coal effluent. With increasing distance from the coal pile, the chemical characteristics of the sediments were generally less extreme; total iron, sodium, sulfate, hydrogen ion, cadmium and soluble salt concentrations decreased (Fig. 2.2) while total aluminum, calcium, manganese, and magnesium levels increased (data not shown). The pH of the FW wetland sediment was mildly acidic which is typical for a pine-dominated forested wetland.

Bacterial libraries. At least 70 clones per sample were screened from each bacterial library and grouped into OTUs based on 97% sequence similarity (Table 2.1). Two possible chimeric molecules were identified and excluded from the analysis. The diversity of OTUs was compared between samples by considering the number and relative abundance of each OTU within a library (Table 2.1). Diversity indices have previously been applied to 16S rDNA data in order to estimate bacterial richness and evenness within a sample (McCaig *et al.*; 1999, Nübel *et al.*; 1999, Cho and Kim, 2000). Table 2.1 shows the calculated indices along with the theoretical maximum and minimum values normalized to 70 clones. The OTU richness was higher for the FW sample than the RCP samples. The diversity of the FW sequences approached the hypothetical maximum and was similar to that calculated for a grassland soil; comparatively, the RCP libraries were less diverse, having both a lower Shannon value and a higher Simpson's dominance. The Simpson's dominance along with equitability and evenness indices suggest that the RCP libraries are dominated by fewer members as compared to the FW library, although Pielou's evenness did not suggest there to be as great a difference. In

general, the indices indicate that the diversity within the RCP2 library is slightly higher than the RCP1 library; however, the differences were small. Among the bacterial libraries, the slope of the rarefaction curve was much higher for the FW versus the contaminated samples, indicating the same screening effort obtained a higher coverage of the later (Fig. 2.3). Collectively, these data suggest that the diversity of bacterial 16S rDNAs increases with distance from the coal pile. The diversity of both RCP samples was considerably higher than that conducted in a ~1.0 pH AMD biofilm, with the latter having a much less even distribution of 16S rDNA sequences (the Iron Mountain BA library was dominated by the *Leptospirillium* type III clone BA29) (Bond *et al*., 2000b).

The two RCP samples shared 16 % of their OTUs and many of these overlapping sequences represented 16S rDNAs which were nearly identical (>99%) to one another. Nevertheless, LIBSHUFF showed that the libraries differed significantly ($P = 0.001$ and 0.034 for $\Delta C_{RCP1/RCP2}$ and $\Delta C_{RCP2/RCP1}$ respectively). The distribution of $(C_{RCP1} - C_{RCP1/RCP2})^2$ with D (evolutionary distance) suggests that the differences between the two libraries consists mainly of species and genus level differences ($D < 0.1$ or 16S rDNA sequence similarity of >90%) and to a lesser extent taxa representing deeper phylogenetic groups ($0.2 < D < 0.3$).

Overlap of sequence types between the contaminated samples versus the FW site was rare. The Actinomycet*e* sequences FW-103 and RCP1-34 were 98.0% identical (over 1262 nt) and represented the only such example of OTU repetition. However, there were instances of FW and RCP sequences that were phylogenetically associated with one another. The lack of overlap between the bacterial RCP and FW libraries indicates that the majority of RCP sequences represents organisms endemic to this system.

Archaeal libraries. We chose to screen the archaeal libraries by RFLP analysis rather than by partial sequencing since preliminary restriction analysis suggested a low 16S rDNA diversity. Approximately 30 clones were screened from each RCP library; no archaeal library was generated for the FW sample (Table 2.2). Sequence analysis revealed RFLP grouped sequences which were at least 98% similar (over > 1150 nt) within an OTU. One chimeric molecule was identified, leaving 64 archaeal 16S rDNAs for analysis. The RCP libraries shared 23% of their OTUs and 67% of their sequences, implying that the archaeal libraries were very similar to one another; LIBSHUFF was not performed to test whether they were significantly different due to the small number of clones screened. Coverage curves for these libraries overlapped with one another and plateaued, further suggesting that *Archaea* diversity within the contaminated zone is low compared to the *Bacteria* (Fig 3).

Distribution of 16S rDNA clones among taxa. While we attempted to keep PCR-associated bias to a minimum, we cannot be sure that the frequency of a particular 16S rDNA clone can be correlated to its abundance in the original sample (Polz and Cavanaugh, 1998; Suzuki *et al*., 1998). Therefore, clone distribution among taxa is presented as the percentage of unique OTUs retrieved from each library (Table 2.2). In this way, the presence or absence rather than the percent abundance of a particular 16S rDNA sequence was considered. In actuality, the relative clone abundance versus OTU abundance among divisions within any one library was fairly similar. This was especially evident for the FW library, as the majority of OTUs were comprised of only one sequence.

Clones associated with the alpha, delta, and gamma *Proteobacteria* along with the *Acidobacteria* and Actinomycet*e* divisions were among the most abundantly recovered in the RCP libraries (Table 2.2). The majority of RCP *Archaea* sequences consisted of *Euryarchaeota* members. While the Green non-sulfur members were abundant in the FW library, sequences from this group were not retrieved from the RCP sites, indicating that the corresponding organisms may not be adapted for survival in AMD systems. The proportion of High G + C gram positive sequences retrieved increased with proximity to the coal pile (Table 2.2). The vast majority of these sequences represented members of the *Acidimicrobidae* group, which contains members isolated from acidic environments.

Phylogenetic distribution of notable RCP 16S rDNA sequences. Due to the large number of clones analyzed for this study (319 sequences, 174 OTUs), we chose to simplify the presentation of phylogenetic data by focusing on RCP sequences obtained from taxa that were either frequently retrieved or phylogenetically associated with environmental clones or cultured organisms isolated from acidic habitats. Only in instances when FW sequences were associated with these specific RCP sequences are they included in the analysis.

***Acidimicrobidae*-associated clones.** The Actinomycetes were the most abundant bacterial division derived from RCP1, with the majority of these clones associating specifically with the *Acidimicrobidae* group (Fig. 2.4). This group contains environmental clones obtained from various acidic environments including the TM clones (Rheims *et al*., 1996) isolated from a peat bog (pH 2.7) along with clones obtained from Iron Mountain niches (~1.0 pH biofilm and 2.4 pH stream) (Edwards *et al*., 1999; Bond *et al*., 2000). The *Acidimicrobidae* contains two cultured representatives,

Acidimicrobium ferrooxidans and *Ferromicrobium acidophilus,* both of which represent acidophilic, heterotrophic iron oxidizers (Bridge and Johnson, 1998; Bacelar-Nicolau and Johnson, 1999). One OTU isolated from RCP1 was highly related to *F. acidophilus* (>99% sequence similarity), yet collectively the RCP sequences greatly expand the diversity within this group as approximately 50% of the OTUs retrieved were only 90-94% similar to described *Acidimicrobidae* 16S rDNA sequences.

Alpha *Proteobacteria*. Two OTUs (16 clones) from the RCP wetland were associated with the *Acidiphilium*-related Iron Mountain clone TRB3 (isolated from 0.5 and 2.4 pH samples) (Edwards *et al*., 1998) (Fig. 2.5). One additional OTU (2 clones) was essentially identical to the 16S rDNA of *Acidiphilium multivorum*.

Three other OTUs (7 clones) were only 92.8-94.8% similar to 16S rDNAs present in the database. These OTUs cluster with *Rhodophila globiformis* and *Acidisphaera rubrifaciens*, the latter of which was isolated from an acid mine drainage site (Hiraishi *et al*., 2000). Environmental clones isolated from an acidic geothermal spring and a polychlorobiphenyl-contaminated soil (Nogales *et al*., 2000) were also associated with this group.

Gamma *Proteobacteria*. Three abundantly retrieved RCP groups fell within the gamma *Proteobacteria* (Fig. 2.6). One OTU containing nine clones clustered (97% similarity) with Iron Mountain clone TRA5-3 (isolated from a pH 2.4 stream). Another OTU (8 clones) clustered with the Monserrat clone MS11, which was obtained from an acidic spring (97.2% similarity). A third group consisted of 2 OTUs (7 clones) which share 89% similarity to *Thiobacillus prosperus* and *Nitrosococcus halophilus*, though it clusters with the latter.

Clones closely related to *Acidothiobacillus ferrooxidans* were not isolated from the 144 RCP sequences. A 16S rDNA sequence related to this organism (>97% sequence similarity) was isolated from a different RCP1 library (generated from a sample taken in March 2000) indicating, at the very least, that *A. ferrooxidans*-like sequences are amplifiable with our selected amplification conditions.

Delta *Proteobacteria*. Two groups of RCP clones were phylogenetically situated within the delta *Proteobacteria* (Fig. 2.7). One group (3 OTUs, 5 clones) is related (>97% sequence similarity) to Iron Mountain clone BA71 (from a ~1.0 pH biofilm) (Bond et *al*., 2000b). A second cluster comprised of RCP sequences along with FW-136 displayed 85-86% similarity to a uranium waste pile clone.

Candidate division WS6. 3 OTUs (5 sequences) retrieved from the RCP1 site were distantly related (73.6-74.1 % sequence similarity) to rDNAs present in the database and grouped as a distinct lineage within the candidate division WS6 (Fig. 2.8), which contains no cultured representatives. We do not believe these sequences represent chimeric molecules as they have been subsequently reamplified from the RCP1 sample using specific primer sets. Many WS6 sequences display extensive sequence divergence in the 515F region of the 16S rRNA gene whereas this segment is considered universally conserved (Dojka *et al*., 2000). However, our WS6 sequences do not contain such divergences and are similar in this respect to the BMS clones associated with this candidate division. As far as we are aware, members of the WS6 division have not been previously retrieved from highly acidic habitats.

***Leptospirillum*-related sequences.** *L. ferrooxidans* is a chemolithoautotroph that oxidizes ferrous iron but not reduced sulfur for energy (Hippe, 2000). *Leptospirillum*-

related 16S rDNA clones were recovered from both RCP samples (Fig. 2.9) with the vast majority (3 OTUs, 9 clones) associating with *Leptospirillum* group III. This group contains no cultured representatives and consists of clones with roughly 89-93% similarity to group I and II *Leptospirillum* lineages (Bond *et al*., 2000b). One additional *Leptospirillum*-related clone was affiliated with group II (99.3% similarity) sequences.

Sulfobacillus-related sequences. Four RCP OTUs were affiliated with members of the genus *Sulfobacillus* (Fig. 2.10) yet display low sequence similarity (80-86.7%) to 16S rDNAs comprising this group. One of these OTUs, RCP1-20b was retrieved from RCP1 at a different sampling time (March 2000). Members of this genus are acidophilic facultative autotrophs that have the ability to oxidize sulfur, iron, and metal sulfides (Clark and Norris, 1996). *S. thermosulfidooxidans* and *S. acidophilus* also possess the ability to reduce ferric iron under oxygen limiting conditions. *Sulfobacillus*-like 16S rDNAs and isolates have been isolated from Iron Mountain (Bond and Banfield, 2001), metal bioleaching samples (Goebel and Stackebrandt , 1994b), and an acidic geothermal spring (Atkinson *et al*., 2000).

Acidobacterium. The *Acidobacterium* division was abundantly represented in all three samples (Table 2.1). However, the diversity of RCP sequences was limited to subdivision one (Hugenholtz *et al*., 1998) (Fig. 2.11) whereas the FW sequences were affiliated throughout the division (data not shown). Sequences represented by RCP2-3 were ~97% similar to the moderately acidophilic heterotroph *Acidobacterium capsulatum* (Kishimoto *et al*., 1991). The majority of 16S rDNAs possessed 93-97% sequence similarity to environmental clones retrieved from both acidic and nonacidic habitats; the OTU represented by RCP2-90 was only ~88.5% similar to database sequences.

Archaea. The majority of *Archaea* sequences obtained from the RCP wetland consist of euryarchaeal members (Fig. 2.12). Four OTUs (35 clones) contained 16S rDNA sequences almost identical (>99% similarity) to clones isolated from an Iron Mountain ~1.0 pH biofilm and are associated with the genus *Thermoplasma*. This genus is known to be associated with AMD systems as *Thermoplasma acidophila* was first isolated from a coal reject pile (Segerer and Stetter, 1992). Members are thermoacidophilic heterotrophs capable of growth as facultative anaerobes on molecular sulfur (Segerer and Stetter, 1992). The next most abundant group contained sequences (2 OTUs, 18 clones) only ~85% related to the *Euryarcheaeal* clone WCHD3-16, a rDNA isolated from an aquifer contaminated with chlorinated solvents (Dojka *et al*., 1998).

Chrenarchaeal clones included 1 OTU (2 sequences) which displayed 83% sequence similarity to sulfur reducers *Thermofilum pendens* and *Pyrodictium occulatum,* These organisms are both capable of dissimilatory reduction of sulfur compounds (Stetter *et al*., 1983; Zillig *et al*., 1983). An additional OTU clustered with a sequence retrieved from an acidic hot spring (Takai, and Sako, 1999).

Discussion

The aim of this study was to conduct a molecular survey to characterize the bacterial constituents of the SRS reject coal impacted wetland. In order to elucidate which sequence types may represent organisms significant to this system, we chose to compare data from multiple samples. We consider these samples to be as representative as possible based on a rigorous lysis protocol, use of amplification conditions which should limit bias (avoidance of primer degeneracies [Polz and Cavanaugh, 1998], pooled PCR products, and low [15 cycle] amplification [Polz and Cavanaugh, 1998; Suzuki *et*

al., 1998]), along with a composite sampling scheme. The sampling sites examined included two contaminated regions that differed in their proximity to the coal pile and an unaffected region adjacent to the contaminated area. There was little overlap between the RCP and FW sequences. The phylogenetic association of RCP sequences with those obtained from acidic habitats, including heterotrophic and autotrophic bacteria capable of iron and sulfur cycling, supports the contention that the majority of RCP sequences retrieved represent organisms selected for by the disturbance of the acidic run-off. The diversity of the RCP samples appears to be low enough that our screening extent generated a sufficient coverage of the associated libraries. Though a significant number of bacterial taxa were shared (16% of OTUs) between RCP libraries, the LIBSHUFF statistic considered them to be significantly different from one another, which is consistent with the distinct chemical makeup of the sites from which they were derived.

A variety of indices collectively indicated that the bacterial diversity tended to decrease with proximity to the coal pile (Table 2.2), with the most notable difference occurring between the contaminated and unaffected sites. Other studies have also noted this trend in response to certain types of pollution (Atlas, 1983; Woodwell 1970). Relevant to this study, Wassel and Mills (1983) determined that the diversity of cultured heterotrophic bacteria was lower at sites receiving AMD. The harsh chemical composition of the RCP wetland likely restricts the bacterial community composition as it also restricts the reestablishment of native vegetation. Along these lines, the bacterial diversity of the RCP wetland was higher than that found within an Iron Mountain ~1.0 pH biofim, perhaps because the environmental parameters of the latter are even more extreme.

A. ferrooxidans has traditionally been regarded as the most important organism associated with sulfide mineral oxidation. Molecular approaches have determined this organism is not particularly abundant within environments having very low pH (~1.0) and high temperature (>30°C) and is thought to be more significant in conditions (pH 2-3, temperature <30°C) similar to those found here (Schrenk *et al*., 1998; Edwards *et al*., 1999). Interestingly, a 16S rDNA survey of an Iron Mountain stream (pH 2.4, temperature 20°C) also failed to retrieve clones related to *A. ferrooxidans* (Edwards *et al*., 1998). Clearly factors other than pH and temperature also determine the distribution of this organism (Norris, 1990). Because the RCP wetland was inundated at the time of sampling, the sediment cores likely contained a significant anaerobic zone, which could have led to dilution of strictly aerobic organisms. However, this may not completely explain the absence of *A. ferrooxidans* in our libraries since several clones likely representing aerobic organisms were successfully isolated.

In contrast to molecular studies conducted in other less extreme AMD habitats, novel sequence groups in addition to those corresponding to commonly cultivated AMD isolates were retrieved. An attractive feature of 16S rDNA based methodology lies in the possibility of inferring the potential physiological capacities of the organisms representing the retrieved sequences. If a 16S rDNA sequence is determined to cluster phylogenetically with organisms that share common physiological traits, it is not unreasonable to hypothesize that the corresponding organism may display the same characteristics (Hugenholtz *et al*., 1998) and we make such inferences about several RCP 16S rDNAs below.

We retrieved several clone groups which were closely related (>97% sequence similarity) to those obtained from Iron Mountain (Bond *et al*., 2000b; Edwards *et al*., 1998). This includes members of the uncultivated group *Leptospirillum* III, which may represent acidophilic organisms capable of iron oxidation (Bond *et al*., 2000b). The relative abundance of group III *Leptospirillum* 16S rDNA sequences over those of isolates contained within groups I and II has also been noted at Iron Mountain (Bond and Banfield, 2001). Sequences nearly identical to the *Thermoplasma*-related SC clones (Bond *et al*., 2000b) were by far the major RCP archaeal sequence type isolated. This group has been determined to be numerically significant within Iron Mountain (Bond *et al*., 2000a) and may also constitute an abundant heterotroph within the RCP wetland. Additional acidophilic heterotrophs closely related to the genus *Acidiphilium* (97-99.6% sequence similarity) and *A. capsulatum* (97.8% sequence similarity) were also detected. Heterotrophic organisms are thought to play an important role in AMD systems by consuming organic acids toxic to lithotrophs, such as *L. ferrooxidans* (Johnson, 1998). Clones associated with the Iron Mountain clone BA71 were also retrieved. Because of their placement within the delta *Proteobacteria*, it has been postulated that these bacteria may represent sulfate or iron reducers (Bond *et al*., 2000b). The recovery of novel sequences previously retrieved only from extreme niches within Iron Mountain suggests that the corresponding organisms have a less restrictive distribution than previously believed.

We identified several novel sequence groups within our RCP samples not retrieved in other AMD molecular studies (Goebel and Stackebrandt, 1994a; Goebel and Stackebrandt, 1994b; Pizarro *et al*., 1996, Edwards *et al*., 1998, Bond *et al*., 2000b) that

were phylogenetically associated with acidophilic isolates. OTUs associated with the genus *Sulfobacillus* (80.0-86.7% sequence similarity) were particularly divergent and may represent novel iron and/or sulfur oxidizers. RCP2-8 clustered (90.0-94.8% sequence similarity) with acidophilic alpha *Proteobacteria* that synthesize bacteriochlorophyll (bchl). *Acidisphaera rubrifaciens,* an AMD isolate, is an aerobic heterotroph whose growth is stimulated by light (Hiraishi *et al*., 1998). Also present within this cluster is *Rhodophila globiformis*, a sulfur utilizing anoxygenic phototroph which grows optimally in mildly acidic conditions (pH 4.5-5.5) (Pfennig, 1974). We infer that these sequences may correspond to acidophilic organisms capable of utilizing light energy for growth. The absence of vegetation within the RCP sampling sites results in exposure of the sediment surface to the sun, supporting the idea that light may play a role in the microbial ecology of this system. However, since the level of similarity is low, it may be presumptuous to conclude that the corresponding organism produces photopigments. Johnson *et al*. (2001) recently isolated an acidophilic heterotroph (93% similar to RCP2-8) having the same level of 16S rDNA sequence similarity to *A. rubrifaciens*, yet it does not form red colonies indicative of carotenoids. Several additional novel OTUs clustered with the heterotrophic iron oxidizers *F. acidophilum* and *A. ferrooxidans* within the *Actinomycetes*. Bacteria corresponding to these organisms may possess similar traits, though it is important to note strains of *F. acidophilum* and *A. ferrooxidans* display additional metabolic diversity in that they are also capable of iron reduction and autotrophy, respectively (Clark and Norris, 1996).

Several novel RCP groups represent lineages for which we cannot infer the metabolic potential of the corresponding organisms. One such group includes the

euryarchaeal group consisting of RCP2-10 and RCP1-30. Additional clusters include those contained within the candidate division WS6 along with the abundantly retrieved gamma lineages represented by RCP1-48 and RCP2-96. Two clones represented by RCP2-18 (~ 82.5% similar to 16S rDNAs present in the database) formed no stable association with any described division (data not shown). We do not believe that these sequences represent a chimeric molecule due to their independent isolation from both RCP libraries. These sequences represent ones which have not been detected in any other environment, acidic or otherwise. Both the sample type taken (inundated sediment cores) in addition to distinguishing features of this contaminated wetland likely contribute to the retrieval of sequence groups not isolated by molecular surveys of other AMD environments. This finding argues that there remains more to be learned about the bacterial community composition of these systems.

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TABLE 2.1. Comparison of diversity indices determined from 16S rDNA libraries generated from reject coal impacted and unaffected sampling sites to those constructed in independent studies.

Parameter[a]	Clone library					Index range (n=70)	
	RCP1	RCP2	FW	Unimproved grassland soil[b]	Iron Mountain Biofilm[c]	theoretical minimum	theoretical maximum
Total no. of clones analyzed	70	74	100				
No. distinct OTUs	35	41	86				
OTU abundance[d]	32.2	36.6	58.5	62.2	5.7	1	70.0
Shannon (H)[e]	3.323	3.408	4.146	4.088	0.796	0	4.250
Equitability (J)[f]	0.782	0.802	0.976	0.962	0.187	0	1.000
Evenness (e)[g]	0.935	0.930	0.997	0.926	0.444	0	1.000
Simpson's dominance (c)[h]	0.053	0.044	0.018	0.018	0.633	1	0.014

[a]For all indices except OTU abundance, the clone number in each library was reduced to 70 by randomly removing sequences from the RCP2, FW, unimproved grassland soil and Iron Mountain lithotrophic biofilm 16S rDNA libraries.
[b] SAF clones from McCraig et al. 1999
[c]BA clones from Bond et al. 2000b
[d]OTU abundance was determined by rarefaction where the observed number of OTUs was calculated for 70 sequences screened.
[e]Calculated by $H = - \sum P_i (ln P_i)$, where P_i is the proportion of each OTU within a library.
[f]Calculated by H/H_{max}, where H_{max} is the theoretical maximum Shannon diversity index for a clone library of 70.
[g]Calculated as $e = H/ lnS$, where S is the total number of OTUs in each library once the clone number was normalized to 70.
[h]Calculated as $c = \sum (P_i)^2$, where P_i is the proportion of each OTU within a library.

TABLE 2.2. Relative abundance of Operational Taxonomic Units (OTUs) among bacterial and archaeal taxons within RCP and FW libraries.

Taxon	% OTU distribution[a]		
	RCP1	RCP2	FW
Bacteria			
No. clones analyzed	70	74	100
No. distinct OTUs	35	41	86
Proteobacteria			
α subdivision	11.4 (20.0)	19.5 (21.6)	9.3 (9.0)
β subdivision	0.0	0.0	4.7 (4.0)
δ subdivision	8.6 (5.7)	7.3 (5.4)	19.7 (21.0)
γ subdivision	8.6 (20.0)	9.8 (14.9)	2.3 (2.0)
Acidobacterium	17.1 (10.0)	19.5 (23.0)	20.7 (23.0)
Cyannobacteria and chloroplasts	0.0	4.9 (2.7)	2.3 (2.0)
Verrucomicrobium	0.0	2.4 (1.4)	2.3 (2.0)
Planctomyces	0.0	4.9 (4.1)	3.5 (3.0)
Actinomycetes	25.7 (18.6)	12.2 (8.1)	3.5 (4.0)
Bacillus and relatives	2.9 (2.9)	7.3 (5.4)	0.0
Cytophaga-Flexibacter-Bacteroides	0.0	2.4 (1.4)	0.0
Nitrospira	2.9 (4.3)	4.9 (9.4)	7.0 (9.0)
Spirochaeta	5.7 (2.9)	0.0	2.3 (2.0)
Green non-sulfur	0.0	0.0	10.5 (9.0)
Deinococcus-Thermus	2.9 (5.7)	0.0	0.0
Candidate division BD	0.0	0.0	1.2 (1.0)
Candidate division TM7	0.0	0.0	1.2 (1.0)
Candidate division OP10	0.0	2.4 (1.4)	1.2 (1.0)
Candidate division OP11	2.9 (1.4)	0.0	4.7 (4.0)
Candidate division WS5	0.0	0.0	1.2 (1.0)
Candidate division WS6	8.6 (7.1)	0.0	0.0
Candidate division PBS	0.0	0.0	1.2 (1.0)
Unaffiliated	2.9 (1.4)	2.4 (1.4)	1.2 (1.0)
Archaea			
No. clones analyzed	35	30	0
No. distinct OTUs	7	5	0
Crenarchaeota	28.6 (8.3)	20.0 (6.7)	ND[b]
Euryarchaeota	71.4 (91.7)	80.0 (93.3)	ND

[a]OTUs were defined based on 97% sequence similarity (*Bacteria*) or on RFLP analysis (*Archaea*) of 16S rDNA clones. For comparison, the numbers in parentheses indicate the percent clone abundance distributed among the taxons within each library.

[b] ND, not determined.

FIG. 2.1 Map relating sample sites to one another.

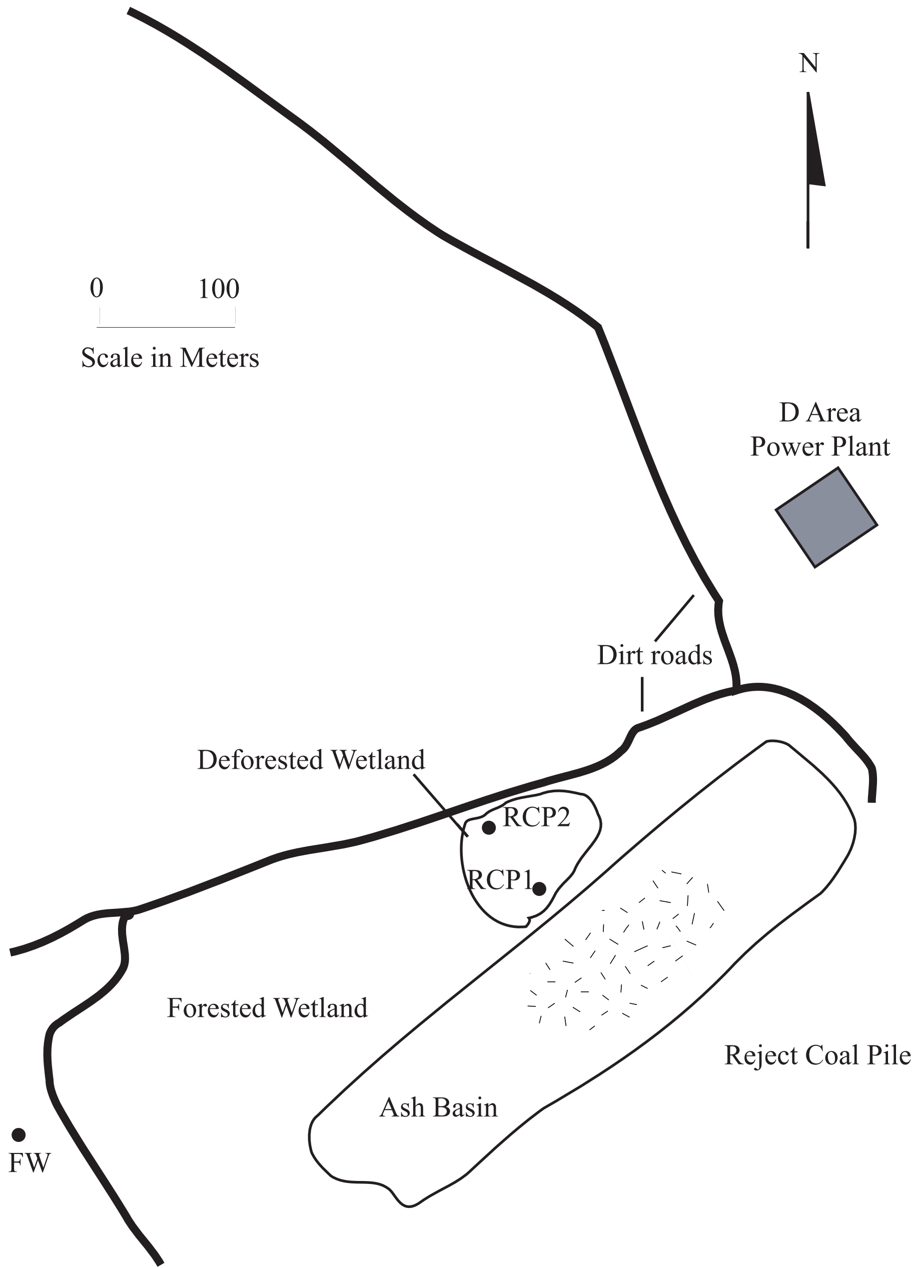
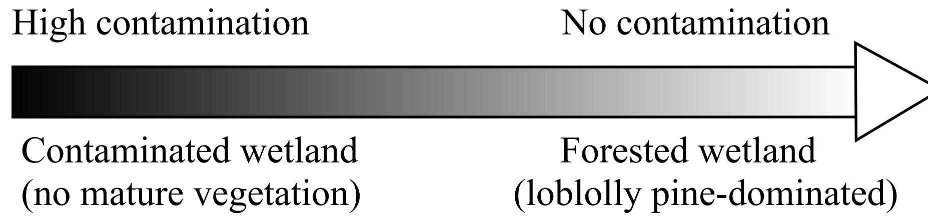
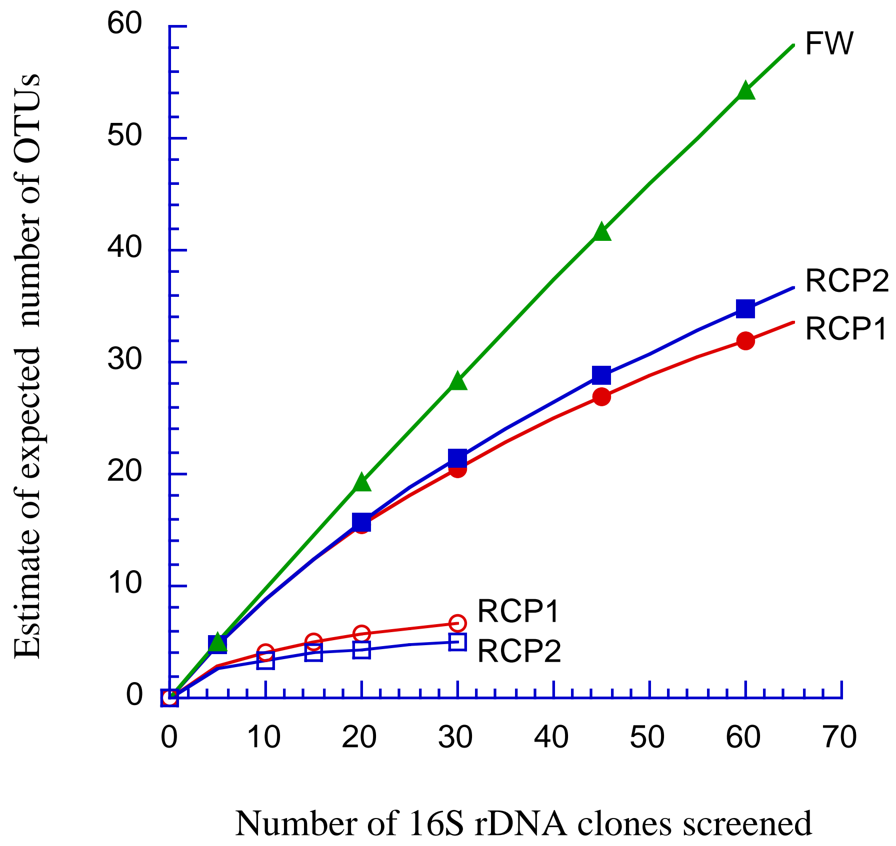

N
0
100
Scale in Meters
D Area
Power Plant
Dirt roads
Deforested Wetland
RCP2
RCP1
Forested Wetland
Reject Coal Pile
Ash Basin
FW

FIG. 2.2 Chemical characteristics of wetland sediment samples.



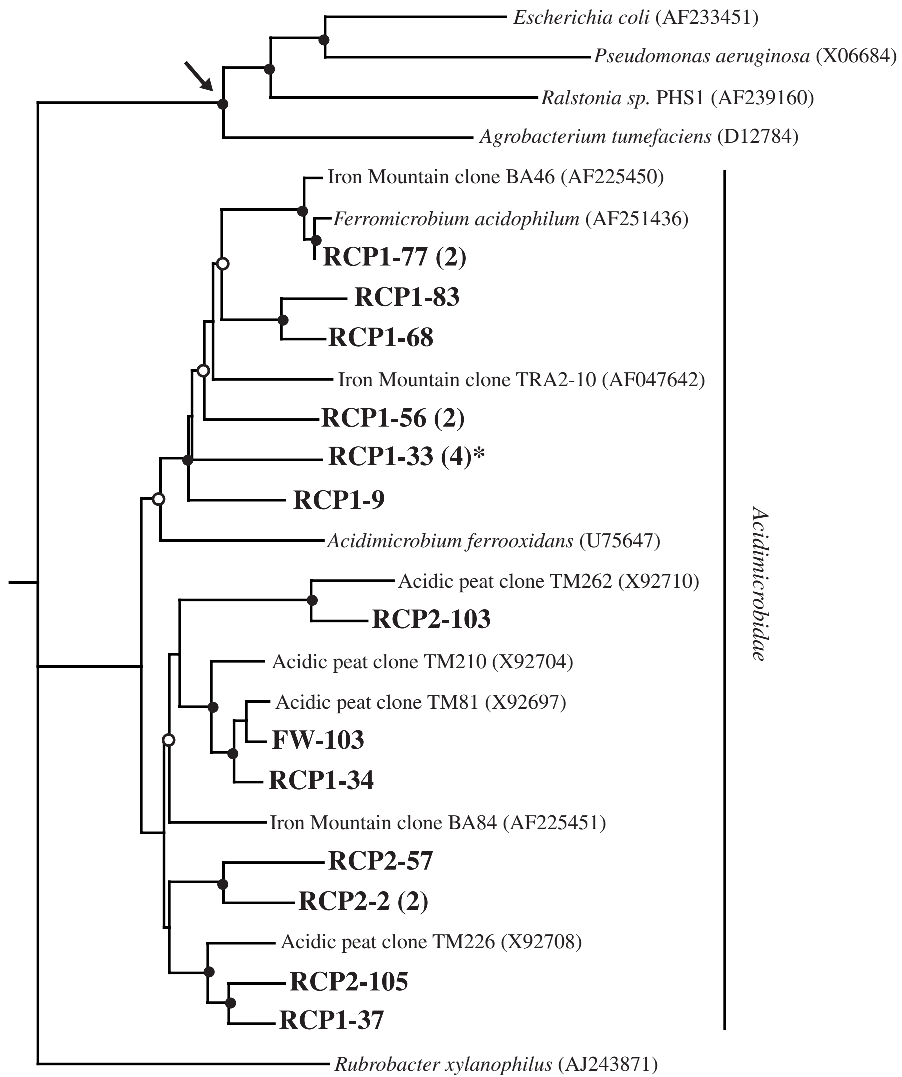
Sampling site name:	RCP1	RCP2	FW
pH:	2.5	3.0	5.2
Soluable salts (mmhos):	1.4	0.62	0.45
Total Fe (ppm):	171,620	50,000	15,000
Total Na (ppm):	1,525	94	< 1.0
Total Cd (ppm):	3.7	1.5	< 0.08
Total Sulfate (ppm):	78,332	28,654	16,630

FIG. 2.3. Rarefaction curves for 16S rDNA clones obtained from RCP and FW wetlands. Sequences were grouped into OTUs based on sequence similarity for *Bacteria* clones or on RFLP analysis for *Archaea* clones. Closed and open symbols refer to *Bacteria* and *Archaea* libraries respectively.



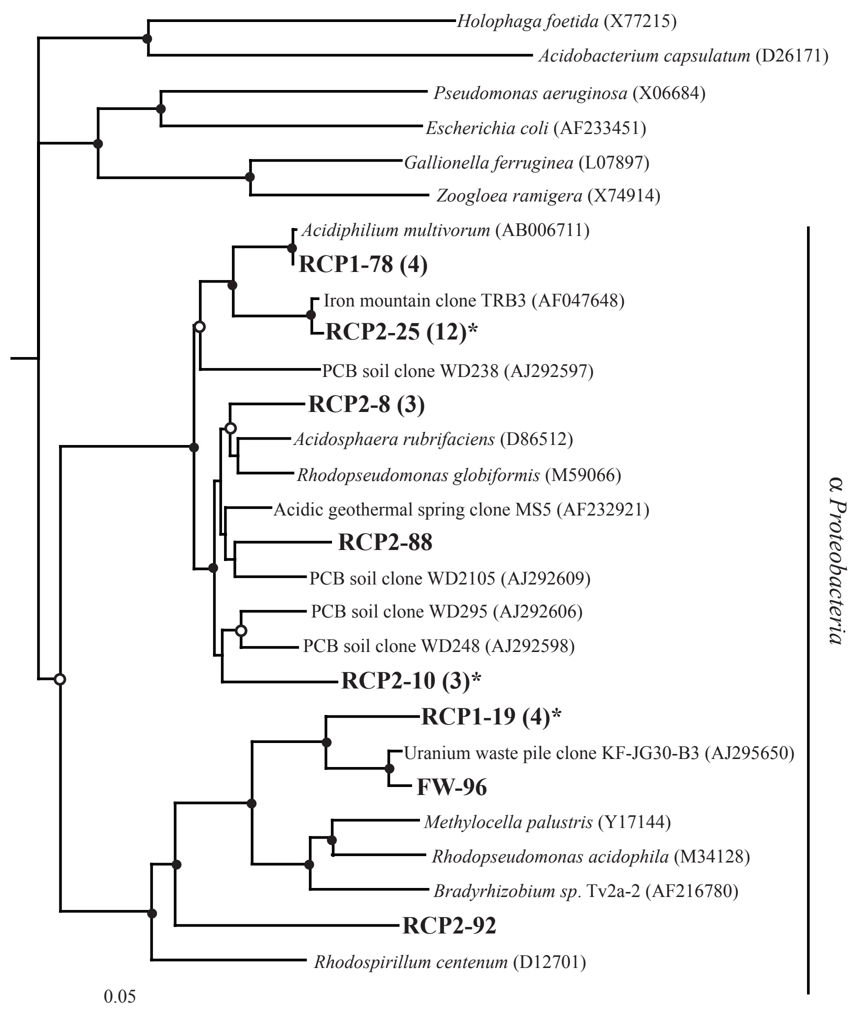
Estimate of expected number of OTUs
Number of 16S rDNA clones screened
FW
RCP2
RCP1
RCP1
RCP2
0
10
20
30
40
50
60
70

FIG. 2.4. Dendrogram based on 719 aligned nucleotides showing phylogenetic placement of RCP and FW clones affiliated with the Actinomycetes. Asterisks designate an OTU containing sequences retrieved from both RCP1 and RCP2 samples. Numbers within the parentheses denote the number of clones contained within the OTU. Symbols at the nodes represent the bootstrap values out of 100 resamplings (closed circles >75%, open circles 50 to 74%). Bootstrap values not statistically significant (<50%) are not shown. The scale bar represents .5 nucleotide substitutions per 100 nucleotides. The *Proteobacteria* members, *E. coli*, *P. aeruginosa*, *Ralsonia sp*. PHS1, and *A. tumefaciens* were used as the outgroup.



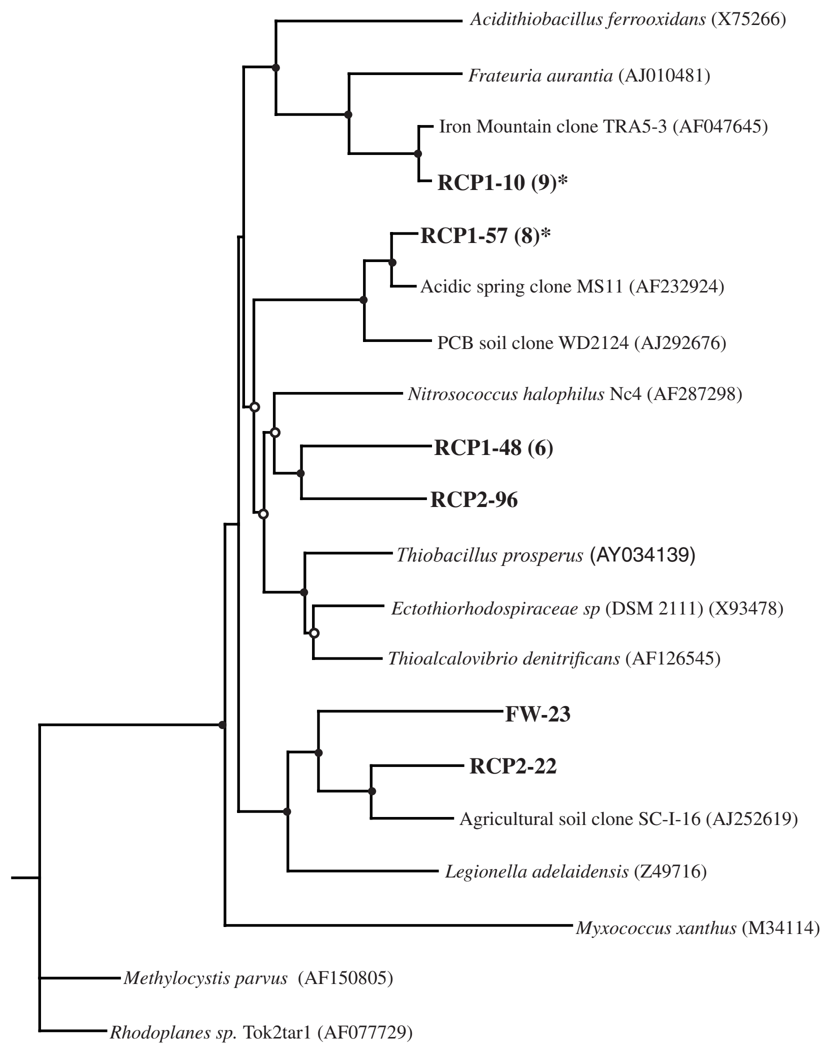
Escherichia coli (AF233451)
Pseudomonas aeruginosa (X06684)
Ralstonia sp. PHS1 (AF239160)
Agrobacterium tumefaciens (D12784)
Iron Mountain clone BA46 (AF225450)
Ferromicrobium acidophilum (AF251436)
RCP1-77 (2)
RCP1-83
RCP1-68
Iron Mountain clone TRA2-10 (AF047642)
RCP1-56 (2)
RCP1-33 (4)*
RCP1-9
Acidimicrobium ferrooxidans (U75647)
Acidic peat clone TM262 (X92710)
RCP2-103
Acidic peat clone TM210 (X92704)
Acidic peat clone TM81 (X92697)
FW-103
RCP1-34
Iron Mountain clone BA84 (AF225451)
RCP2-57
RCP2-2 (2)
Acidic peat clone TM226 (X92708)
RCP2-105
RCP1-37
Rubrobacter xylanophilus (AJ243871)
Acidimicrobidae
0.05

FIG. 2.5. Dendrogram based on 814 aligned nucleotides showing phylogenetic placement of RCP and FW clones affiliated with the alpha *Proteobacteria*. Asterisks designate an OTU containing sequences retrieved from both RCP1 and RCP2 samples. Numbers within the parentheses denote the number of clones contained within the OTU. Symbols at the nodes represent the bootstrap values out of 100 resamplings (closed circles >75%, open circles 50-74%). Bootstrap values not statistically significant (<50%) are not shown. The scale bar represents 5 nucleotide substitutions per 100 nucleotides. The *Acidobacterium* members *A. capsulatum* and *H. foetida* were used as the outgroup.



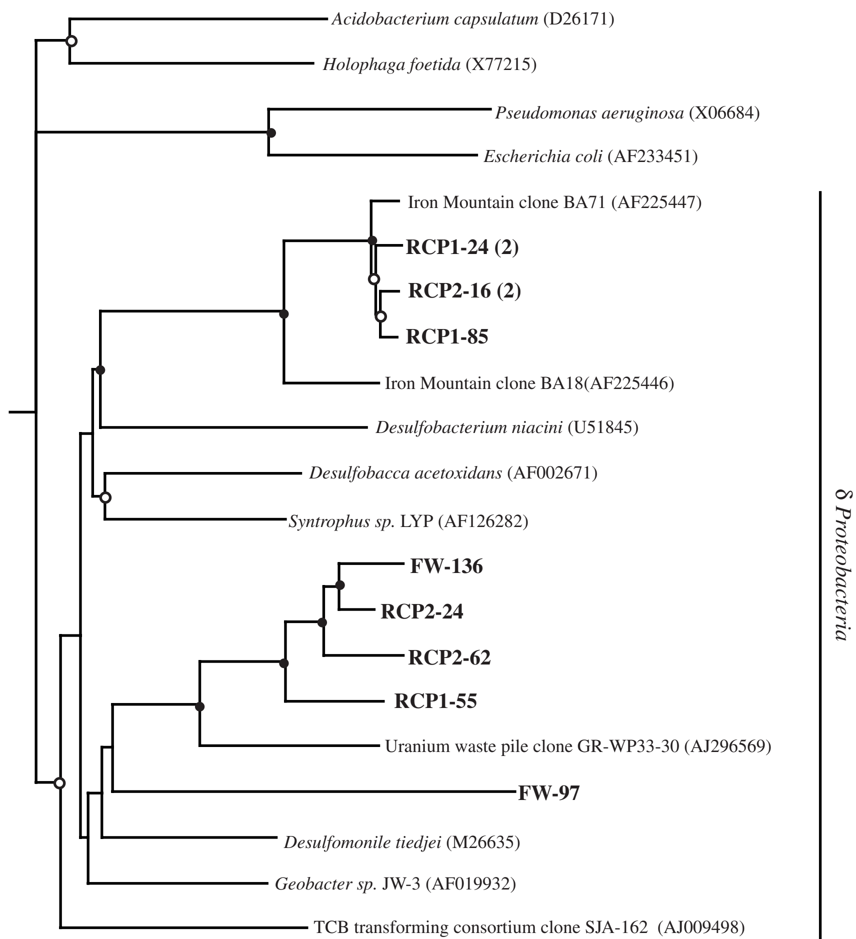
Holophaga foetida (X77215)
Acidobacterium capsulatum (D26171)
Pseudomonas aeruginosa (X06684)
Escherichia coli (AF233451)
Gallionella ferruginea (L07897)
Zoogloea ramigera (X74914)
Acidiphilium multivorum (AB006711)
RCP1-78 (4)
Iron mountain clone TRB3 (AF047648)
RCP2-25 (12)*
PCB soil clone WD238 (AJ292597)
RCP2-8 (3)
Acidosphaera rubrifaciens (D86512)
Rhodopseudomonas globiformis (M59066)
Acidic geothermal spring clone MS5 (AF232921)
RCP2-88
PCB soil clone WD2105 (AJ292609)
PCB soil clone WD295 (AJ292606)
PCB soil clone WD248 (AJ292598)
RCP2-10 (3)*
RCP1-19 (4)*
Uranium waste pile clone KF-JG30-B3 (AJ295650)
FW-96
Methylocella palustris (Y17144)
Rhodopseudomonas acidophila (M34128)
Bradyrhizobium sp. Tv2a-2 (AF216780)
RCP2-92
Rhodospirillum centenum (D12701)
α Proteobacteria
0.05

FIG. 2.6. Dendrogram based on 1003 aligned nucleotides showing phylogenetic placement of RCP and FW clones affiliated with the gamma *Proteobacteria*. Asterisks designate an OTU containing sequences retrieved from both RCP1 and RCP2 samples. The number within the parentheses denotes the number of clones contained within the OTU. Symbols at the nodes represent the bootstrap values out of 100 resamplings (closed circles >75%, open circles 50 to 74%). Bootstrap values not statistically significant (<50%) are not shown. The scale bar represents 5 nucleotide substitutions per 100 nucleotides. The *Proteobacteria* members, *M. xanthus*, *M. parvus*, and *Rhodoplanes sp*. Tok2tar1 were used as the outgroup.



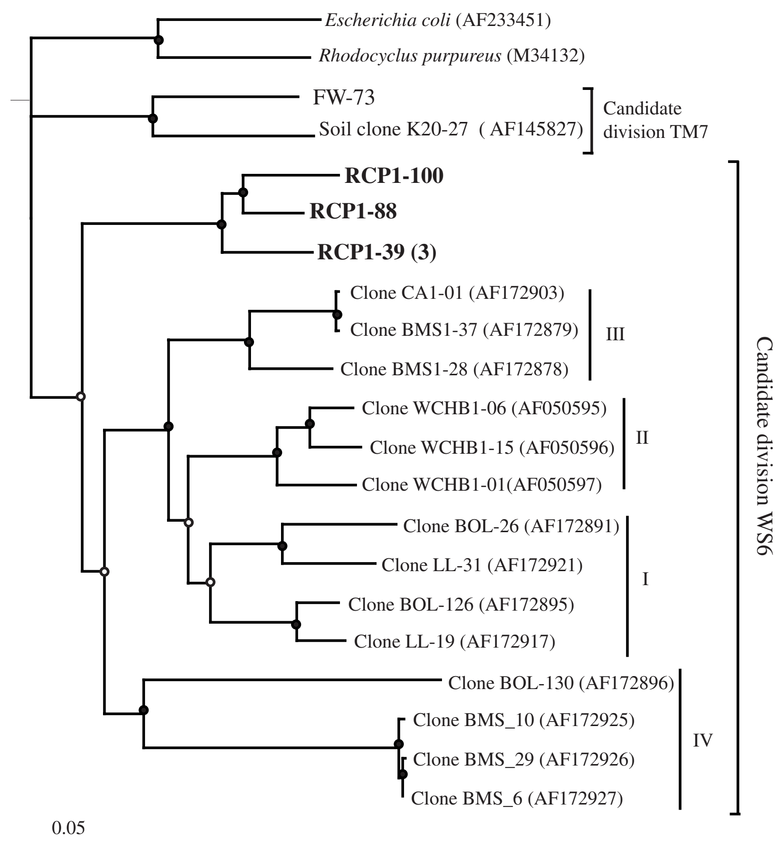
Acidithiobacillus ferrooxidans (X75266)
Frateuria aurantia (AJ010481)
Iron Mountain clone TRA5-3 (AF047645)
RCP1-10 (9)*
RCP1-57 (8)*
Acidic spring clone MS11 (AF232924)
PCB soil clone WD2124 (AJ292676)
Nitrosococcus halophilus Nc4 (AF287298)
RCP1-48 (6)
RCP2-96
Thiobacillus prosperus (AY034139)
Ectothiorhodospiraceae sp (DSM 2111) (X93478)
Thioalcalovibrio denitrificans (AF126545)
FW-23
RCP2-22
Agricultural soil clone SC-I-16 (AJ252619)
Legionella adelaidensis (Z49716)
Myxococcus xanthus (M34114)
Methylocystis parvus (AF150805)
Rhodoplanes sp. Tok2tar1 (AF077729)

FIG 2.7. Dendrogram based on 1240 aligned nucleotides showing phylogenetic placement of RCP and FW clones affiliated with the delta *Proteobacteria*. The number within the parentheses denotes the number of clones contained within the OTU. Symbols at the nodes represent the bootstrap values out of 100 resamplings (closed circles >75%, open circles 50 to 74%). Bootstrap values statistically not statistically significant (<50%) are not shown. The scale bar represents 5 nucleotide substitutions per 100 nucleotides. The *Acidobacterium* members *H. foetida* and *A. capsulatum* were used as the outgroup.



Acidobacterium capsulatum (D26171)
Holophaga foetida (X77215)
Pseudomonas aeruginosa (X06684)
Escherichia coli (AF233451)
Iron Mountain clone BA71 (AF225447)
RCP1-24 (2)
RCP2-16 (2)
RCP1-85
Iron Mountain clone BA18(AF225446)
Desulfobacterium niacini (U51845)
Desulfobacca acetoxidans (AF002671)
Syntrophus sp. LYP (AF126282)
FW-136
RCP2-24
RCP2-62
RCP1-55
Uranium waste pile clone GR-WP33-30 (AJ296569)
FW-97
Desulfomonile tiedjei (M26635)
Geobacter sp. JW-3 (AF019932)
TCB transforming consortium clone SJA-162 (AJ009498)
δ *Proteobacteria*
0.05

FIG. 2.8. Dendrogram based on 589 aligned nucleotides showing phylogenetic placement of RCP and FW clones affiliated with the candidate division WS6. Roman numerals are used to denote subdivisions determined previously (15). The number within the parentheses along side an OTU signifies the number of clones contained within the OTU. Symbols at the nodes represent the bootstrap values out of 100 resamplings (closed circles >75%, open circles 50 to 74%). Bootstrap values not statistically significant are not shown. The scale bar represents 5 nucleotide substitutions per 100 nucleotides. The *Proteobacteria E. coli* and *R. purpureus*, along with members of the candidate division TM7 were used as the outgroup.



Escherichia coli (AF233451)
Rhodocyclus purpureus (M34132)
FW-73
Soil clone K20-27 (AF145827)
Candidate division TM7
RCP1-100
RCP1-88
RCP1-39 (3)
Clone CA1-01 (AF172903)
Clone BMS1-37 (AF172879)
Clone BMS1-28 (AF172878)
III
Clone WCHB1-06 (AF050595)
Clone WCHB1-15 (AF050596)
Clone WCHB1-01(AF050597)
II
Clone BOL-26 (AF172891)
Clone LL-31 (AF172921)
Clone BOL-126 (AF172895)
Clone LL-19 (AF172917)
I
Clone BOL-130 (AF172896)
Clone BMS_10 (AF172925)
Clone BMS_29 (AF172926)
Clone BMS_6 (AF172927)
IV
Candidate division WS6
0.05

FIG. 2.9. Dendrogram based on 663 aligned nucleotides showing phylogenetic placement of RCP clones affiliated with the *Nitrospira*. Roman numerals to the left of the tree denote *Leptospirillum* subgroups determined by Bond *et al*. (7). The number within the parentheses indicates the number of clones contained within the OTU. Symbols at the nodes represent the bootstrap values out of 100 resamplings (closed circles >75%, open circles 50 to 74%). Bootstrap values not supported statistically (<50%) are not shown. The scale bar represents 5 nucleotide substitutions per100 nucleotides. *T. thermophilus* and *D. radiodurans* were used as the outgroup.

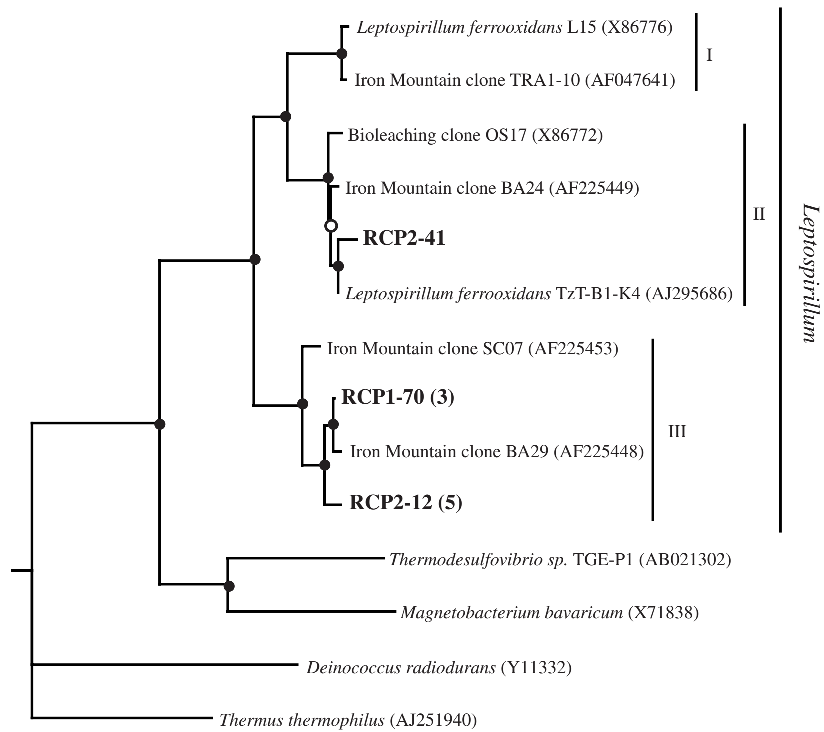

Leptospirillum ferrooxidans L15 (X86776)
Iron Mountain clone TRA1-10 (AF047641)
I
Bioleaching clone OS17 (X86772)
Iron Mountain clone BA24 (AF225449)
RCP2-41
Leptospirillum ferrooxidans TzT-B1-K4 (AJ295686)
II
Iron Mountain clone SC07 (AF225453)
RCP1-70 (3)
Iron Mountain clone BA29 (AF225448)
RCP2-12 (5)
III
Leptospirillum
Thermodesulfovibrio sp. TGE-P1 (AB021302)
Magnetobacterium bavaricum (X71838)
Deinococcus radiodurans (Y11332)
Thermus thermophilus (AJ251940)
0.05

FIG. 2.10. Dendrogram based on 1059 aligned nucleotides showing phylogenetic placement of RCP clones affiliated with the genus *Sulfobacillus*. RCP1-20 was retrieved from a library generated from a RCP1 sample taken on March, 2000. The number within the parentheses along side an OTU denotes the number of clones contained within the OTU. Symbols at the nodes represent the bootstrap values out of 100 resamplings (closed circles >75%, open circles 50 to 74%). Bootstrap values not supported statistically(<50%) are not shown. The scale bar represents 5 nucleotide substitutions per 100 nucleotides. *S. lactis* and *S. pneumoniae* were used as the outgroup.

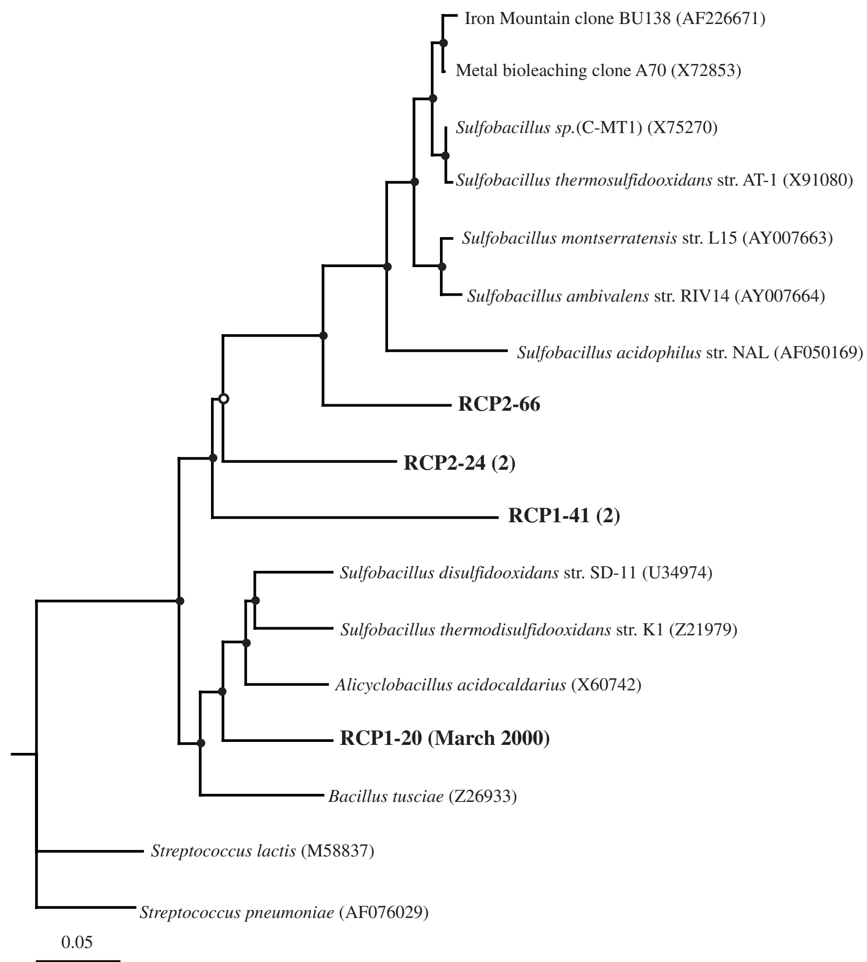
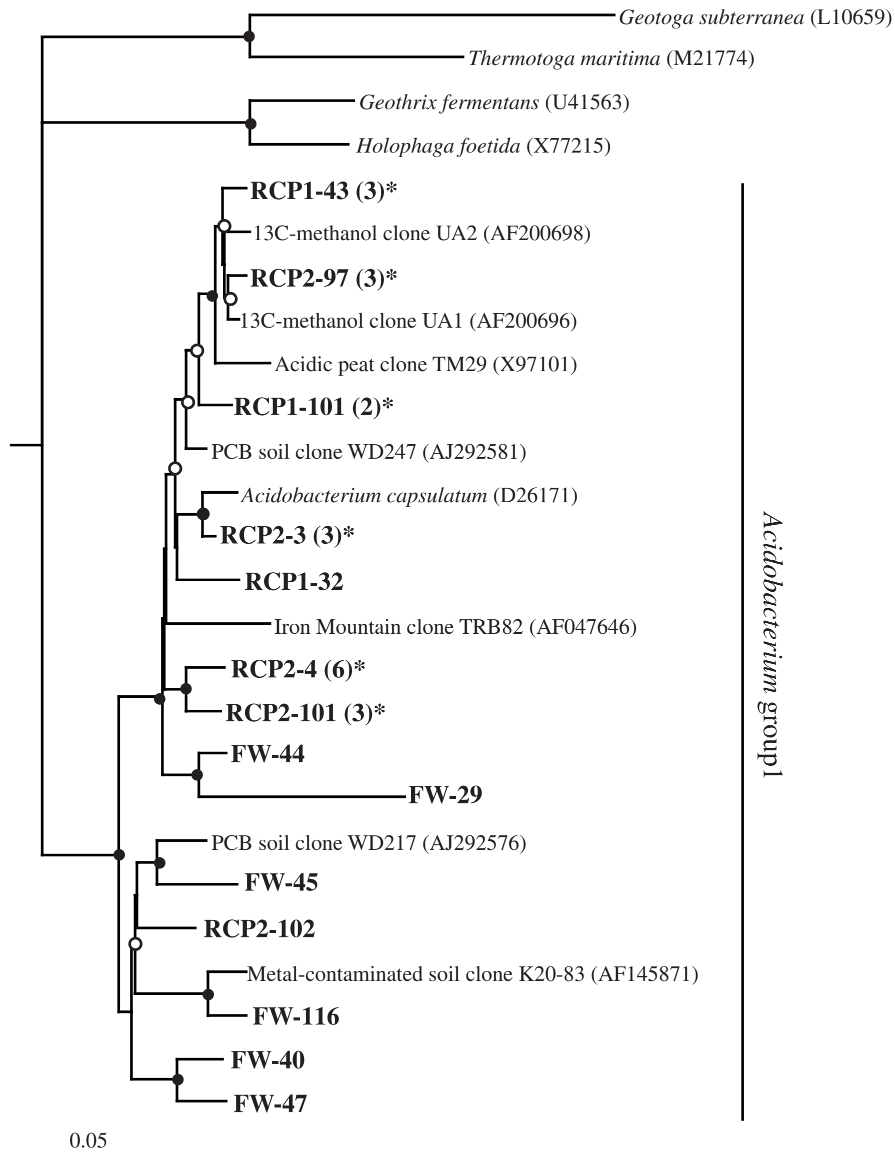
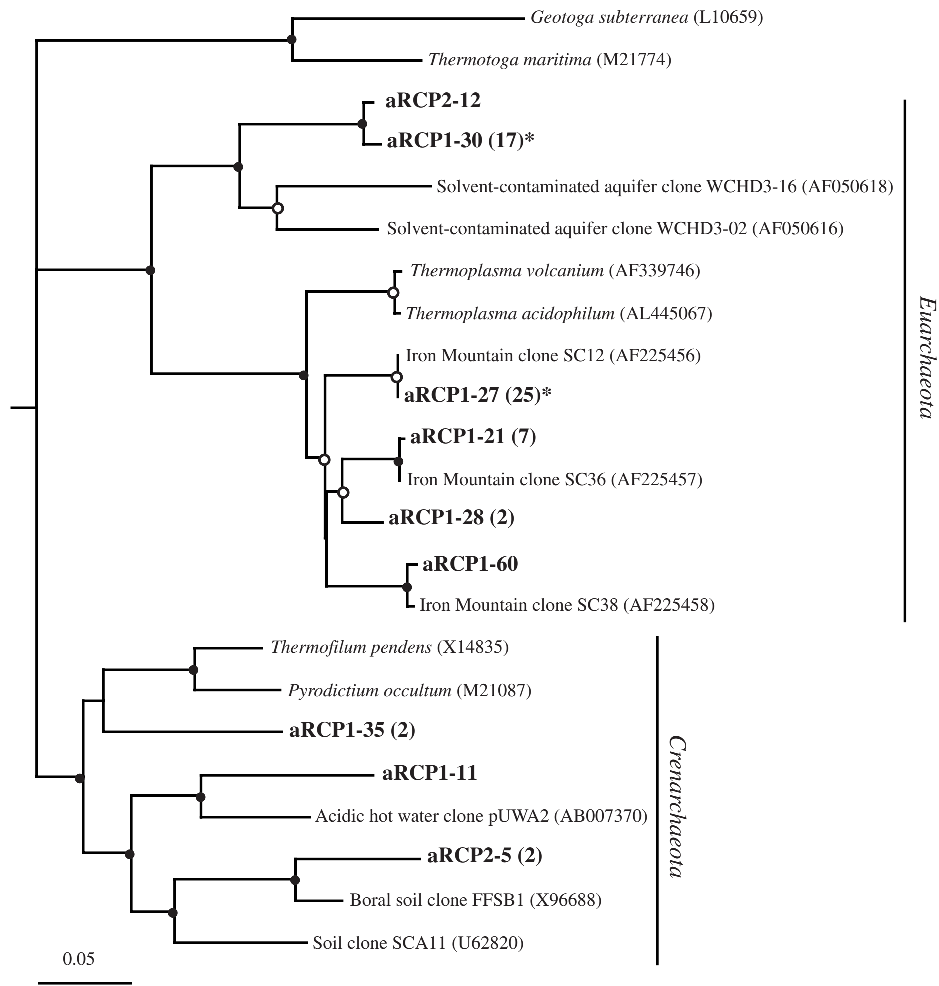

Iron Mountain clone BU138 (AF226671)
Metal bioleaching clone A70 (X72853)
Sulfobacillus sp.(C-MT1) (X75270)
Sulfobacillus thermosulfidooxidans str. AT-1 (X91080)
Sulfobacillus montserratensis str. L15 (AY007663)
Sulfobacillus ambivalens str. RIV14 (AY007664)
Sulfobacillus acidophilus str. NAL (AF050169)
RCP2-66
RCP2-24 (2)
RCP1-41 (2)
Sulfobacillus disulfidooxidans str. SD-11 (U34974)
Sulfobacillus thermodisulfidooxidans str. K1 (Z21979)
Alicyclobacillus acidocaldarius (X60742)
RCP1-20 (March 2000)
Bacillus tusciae (Z26933)
Streptococcus lactis (M58837)
Streptococcus pneumoniae (AF076029)
0.05

FIG. 2.11. Dendrogram based on 650 aligned nucleotides showing phylogenetic placement of RCP and FW clones affiliated within subdivision 1 of the *Acidobacterium* (26). Asterisks designate an OTU containing sequences retrieved from both RCP1 and RCP2 samples. Numbers within the parentheses denote the number of clones contained within the OTU. Symbols at the nodes represent the bootstrap values out of 100 resamplings (closed circles >75%, open circles 50 to 74%). Bootstrap values not statistically significant (<50%) are not shown. The scale bar represents 5 nucleotide substitutions per 100 nucleotides. *G. subterreanea* and *T. maritima* were used as the outgroup.



Geotoga subterranea (L10659)
Thermotoga maritima (M21774)
Geothrix fermentans (U41563)
Holophaga foetida (X77215)
RCP1-43 (3)*
13C-methanol clone UA2 (AF200698)
RCP2-97 (3)*
13C-methanol clone UA1 (AF200696)
Acidic peat clone TM29 (X97101)
RCP1-101 (2)*
PCB soil clone WD247 (AJ292581)
Acidobacterium capsulatum (D26171)
RCP2-3 (3)*
RCP1-32
Iron Mountain clone TRB82 (AF047646)
RCP2-4 (6)*
RCP2-101 (3)*
FW-44
FW-29
PCB soil clone WD217 (AJ292576)
FW-45
RCP2-102
Metal-contaminated soil clone K20-83 (AF145871)
FW-116
FW-40
FW-47
0.05
Acidobacterium group1

FIG. 2.12. Dendrogram based on 331 aligned nucleotides showing phylogenetic placement of RCP clones affiliated within the *Crenarchaea* and *Euryarchaea* subdomains of the *Archaea*. Asterisks designate an OTU containing sequences retrieved from both RCP1 and RCP2 samples. The number within the parentheses along side an OTU denotes the number of clones contained within the OTU. Symbols at the nodes represent the bootstrap values out of 100 resamplings (closed circles >75%, open circles 50 to 74%). Bootstraps not supported statistically (<50%) are not shown. The scale bar represents 5 nucleotide substitutions per 100 nucleotides. Members of the bacteria division *Thermotoga*, *T. maritima* and *G. subterranea*, were used as the outgroup.


Geotoga subterranea (L10659)
Thermotoga maritima (M21774)
aRCP2-12
aRCP1-30 (17)*
Solvent-contaminated aquifer clone WCHD3-16 (AF050618)
Solvent-contaminated aquifer clone WCHD3-02 (AF050616)
Thermoplasma volcanium (AF339746)
Thermoplasma acidophilum (AL445067)
Iron Mountain clone SC12 (AF225456)
aRCP1-27 (25)*
aRCP1-21 (7)
Iron Mountain clone SC36 (AF225457)
aRCP1-28 (2)
aRCP1-60
Iron Mountain clone SC38 (AF225458)
Thermofilum pendens (X14835)
Pyrodictium occultum (M21087)
aRCP1-35 (2)
aRCP1-11
Acidic hot water clone pUWA2 (AB007370)
aRCP2-5 (2)
Boral soil clone FFSB1 (X96688)
Soil clone SCA11 (U62820)
0.05
Euarchaeota
Crenarchaeota

CHAPTER 3

TEMPORAL INVESTIGATION OF BACTERIAL COMMUNITIES AFFECTED BY REJECT COAL USING TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM

Brofft, J.B., McArthur, J.V. and L.J. Shimkets. To be submitted to Applied and Environmental Microbiology.

Abstract

Bacteria associated with bioleaching systems are known to participate in acid generation, yet little is known about their temporal dynamics. In order to expand upon previously conducted 16S rDNA survey analysis, the bacterial communities inhabiting a southeastern wetland sediment contaminated by coal pile run-off were analyzed using terminal restriction fragment length polymorphism (T-RFLP). The bacterial community structure of two chemically distinct sites (RCP1 and RCP2) was monitored in five-week increments over a year by both *Cfo*I and *Msp*I T-RFLP analysis and the relative abundance of peak areas were compared between sites. The community profiles were well-represented by 16S rDNA sequences previously collected from this system, with sequences related to the environmental clones TRA5-3, TRB3, RCP1-48, as well as Actinomycete, *Acidobacterium,* and *Leptospirillum*-related lineages contributing to the predominant peaks. Differences among RCP bacterial communities were attributable more to changes in the relative abundance rather than the presence or absence of terminal restriction fragments (TRFs). The samples were spatially and temporally differentiated based on principal component analysis of measured environmental parameters and of T-RFLP data. A positive correlation existed between TRFs corresponding to the Actinomycete-related sequences and pH; accordingly this group was relatively more abundant in the RCP1 samples. TRFs representing *Acidobacterium* lineages were inversely related to the Actinomycete group and were relatively more abundant in the less acidic RCP2 samples. In either sample site, TRA5-3, TRB3 and *Leptospirillum* groups exhibited a strong temporal fluctuation that appeared cyclic in nature. The *Leptospirillum* group was predominant at time points T3-T7 (July-December) when

percent moisture values were lower and was less predominant in the remaining samples. Conversely, the TRA5-3 and TRB3 groups were relatively abundant in samples having higher percent moisture values, T9-T1 (February-May), including two sample collected at the same time approximately one year apart.

Introduction

The chemical and biological dissolution of sulfide-bearing minerals is a naturally occurring weathering process that leads to the small-scale production of sulfuric acid (22). However, when mining practices expose large sulfide deposits to the air and water, the rate of acid production is accelerated drastically leading to acid mine drainage (AMD) conditions. AMD produces an acidic leachate enriched with heavy metals and salts that has dramatically altered the chemistry of many ecosystems. Acidophilic iron oxidizing bacteria catalyze the rate-limiting step of acid production via the regeneration of ferric iron, an efficient oxidant of sulfide minerals at low pH (21). The industrial process known as bioleaching takes advantage of this potential by exposing ores and mine wastes to dilute acid and oxygen in order to encourage the growth of these bacteria (3). The acidic conditions that ensue lead to the solubilization of commercially viable metals for recovery. Thus, the primary motivation for examining the microbial ecology of AMD affected systems stems from the consequences of acid production for the environment and for industry.

Despite the well-established observation that traditional cultivation approaches do not adequately sample environmental bacteria, the consistent isolation of the iron-oxidizing, chemolithotrophs *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*

has led to the notion that these organisms represent the primary constituents in bioleaching systems. Results from cultivation-independent studies have differed in support of this view. Commercial bioleaching habitats were among the first subjected to cultivation-independent 5S and 16S rRNA analysis (13,17). Contrary to molecular surveys applied to other environments, the rRNA sequence types recovered were generally similar to organisms commonly isolated from these systems, suggesting that the majority of bacteria inhabiting AMD environments were uniquely cultivatable. However, molecular investigation of an extreme AMD system (pH <1.0) located within Iron Mountain, CA revealed the majority of associated bacteria consisted instead of as yet uncultivated organisms (2, 11). This contrasting result was attributed to the unique chemistry of the Iron Mountain system (2). We recently surveyed the 16S rDNA types associated with a southeastern wetland sediment disturbed by acidic coal leachate. Though the site conditions are relatively benign by Iron Mountain standards, the majority of 16S rDNA sequences also represented uncultivated bacteria (6). Several recovered sequence types were related to those obtained from Iron Mountain or were phylogenetically associated with isolates and environmental sequences originating in acidic habitats. Still others were not highly related to any database sequence, refuting the idea that less extreme bioleaching environments consist primarily of cultivated bacteria.

In order to expand upon the 16S rDNA survey analysis conducted previously, the temporal dynamics of the bacterial community associated with this contaminated wetland was investigated. We applied terminal restriction fragment length polymorphism (T-RFLP) to monitor bacterial community structure shifts. T-RFLP has become a commonly used method to profile 16S rDNA types recovered by PCR amplification

(4,14,18,25). Incorporating a fluorescent molecule at the 5' end of one PCR primer generates a labeled fragment during PCR amplification that is subsequently cut with one or more restriction enzymes. The labeled, polymorphic terminal 16S rDNA fragments are then resolved using an automated sequencer. We were interested in applying this approach to establish whether temporal changes in community structure could be observed and, if so, to determine which taxa contribute to seasonal groupings.

Material and Methods

Site description and sample collection. The Savannah River Site's (SRS) D-area near Aiken, S.C. houses a coal-fired power plant and its associated wastes. In 1978, reject coal was deposited onto an unlined area and the establishment of vegetation was never attempted. As a result, acidic leachate and coal grains have been washed into a seasonally flooded forested wetland, producing a region of vegetation die-off. The affected region contains two unvegetated zones, the westernmost of which was chosen for this study. Yellow precipitates, indicative of ferric sulfates such as jarosite, were observed in drier conditions. Additional description of this site can be found in Carlson (1990) and Carlson and Carlson (1992).

Beginning on March 24, 2000, surface sediment samples (sandy loam) were collected at two chemically distinct sites within the contaminated region approximately every 5 weeks until April 2, 2001 (11 samples total). Of the two sampling sites, RCP1 was situated within the contaminated region in closest proximity to the coal pile whereas RCP2 represented a site located at a further distance from the pile relative to RCP1. Twenty five composited surface sediment cores (upper 2 cm) were collected and

combined as described for DNA extraction and chemical analysis (6). For each site, the eleven samples collected are referred to in the text as T0-T10 and correspond to the following dates: T0 (3/24/00); T1 (5/9/00); T2 (6/16/00); T3 (7/21/00); T4 (8/29/00); T5 (9/28/00); T6 (11/2/00); T7 (12/6/00); T8 (1/11/01); T9 (2/27/01); T10 (4/2/01).

Chemical and physical parameters.

For each sample, the following chemical and physical parameters were determined. Total metal concentrations and sulfate levels were measured by the University of Georgia's Chemical Analysis Laboratory. These measurements were determined with an Inductively Coupled Plasma (ICP) Emission Spectrometer of sediment prepared by the EPA method 3050 and with a Braun+Luebbe Auto Analyzer II Continuous Flow System respectively. Organic matter content (determined by the loss on ignition method for 3 hrs at 360°C), total nitrogen, and soluble salts were determined by the University of Georgia's Agricultural and Environmental Services Laboratory. pH was determined by measurement of dried sediment mixed 1:1 with distilled deionized water. The percent moisture content was determined by measuring the weight loss by 40 g of sediment after drying at 60°C. The temperature of the wetland sediment surface was determined in the field.

DNA extraction. DNA was directly extracted using the soil DNA Mega extraction kit (MoBio Laboratories) as described (6). DNA extracts were further purified by binding a portion to a small-scale MoBio column (combined with 2 volumes of solution 3); the sample was then washed with solution 4, dried, and eluted with 50 µl of solution 5.

T-RFLP analysis. The bacterial community structure of the RCP samples was analyzed by T-RFLP. From 15 ng of community DNA, PCR products were amplified with the

bacterial-specific 27f primer (5' AGAGTTTGATCCTGGCTCAG 3') (16) labeled at the 5' end with 6-carboxyfluorescein (FAM) (Life Technologies) and the 1392r primer (5' GACGGGCGGTGTGTAC 3') (16). DNA concentrations were determined by fluorometry. PCR conditions were identical to those described previously to generate 16S rDNA clone libraries for sequence analysis (6) except the amplification cycle was increased from 15 to 20 to ensure sufficient product yield for analysis. The amplification cycle number was kept low on order to minimize potential bias associated with PCR-related approaches (23). It has been observed that the relative proportion of peaks in a T-RFLP profile does not change significantly with amplification cycle when this number is less than 27 (24). For each sample, ten PCR reactions were pooled and gel excised using the Qiaquick gel extraction kit (Qiagen). PCR products (100 ng) were digested for 5 hours with 10 U of either *Cfo*I or *Msp*I (NEB) in a 25 µl volume. Additional restriction enzymes having four base pair recognition sequences (*Sau*3A1, *Rsa*I, and *Hae*III) were avoided since they either produced profiles in which the major taxa formed peaks of a similar length or profiles having a low total fluorescence. The restriction enzyme was heat denatured at 65°C for 20 min and the reaction was ethanol precipitated with 0.75 M ammonium acetate, dilute glycogen, and 3 volumes of cold 100% ethanol for 30 min at -70°C. Precipitated DNA was pelleted (15,000 X g, 30 min, 4°C) and vacuum dried. The dried reactions were resuspended (12.5 µl deionized formamide and 0.75 µl of internal lane standard Tamara 2500 [Applied Biosystems Instruments (ABI)]), denatured for 5 min at 95°C and immediately placed on ice for 2 min. This size standard consists of fluorescently labeled DNA fragments of known size that are co-injected with the sample labeled with a different fluorescent molecule. The lengths of the TRFs (terminal

restriction fragments) were determined with an ABI Prism 310 automated sequencer using the GeneScan software (version 3.1); the software estimates the size of the sample DNA fragments by generation of a standard curve based on the run time of the known DNA fragments.

We were interested in determining whether bacterial 16S rDNA clones previously recovered and analyzed from the RCP samples (6) contributed to peaks comprising the community profiles. The anticipated size of the 16S rDNA inserts was estimated using the program MAP (Accelrys). However, since these libraries were sequenced using the 27f primer, the actual lengths of the TRFs could not be accurately predicted due to the lack of sequence information at the 5' end of the 16S rDNA. The fragment sizes were therefore determined directly by T-RFLP analysis. 16S rDNA inserts were reamplified from representative clones comprising the 76 operational taxonomic units (OTUs) recovered from the RCP bacterial libraries. The TRF sizes of OTU members having less than 99% sequence similarity were also assessed. Amplification conditions were the same as described above except 400 pg of plasmid was used as the template and a different fluorescent 5' label was used for the 27f primer {either 5-tetrachlorofluorescein (TET) or 5-hexachlorofluorescein (HEX); [Life Technologies]}. Ten nanograms of gel purified amplification product was added to 100 ng of FAM-labeled community PCR product which was digested and analyzed as described above. By coinjection of this mixture, it could be determined whether the TRF of the 16S rDNAs recovered from this environment cointegrated with the plasmid PCR products.

Data analysis. The integrated area under the peaks was used as a means to compare the relative abundance of taxa between environmental samples. Area rather than peak height

was used since TRFs greater than 400 bp often appeared flatter. For each sample, two replicate profiles were manually aligned and TRFs between 50 and 650 bp in length that represented greater than 0.5% of the total peak area were included in the analysis. Peaks less than 0.5 bp apart from a larger peak were combined. A program in Visual Basic for MS Excel developed by Ramunas Stepanasukas (University of Georgia) was used to facilitate this analysis.

For each sample, a profile was generated that included only TRFs present in each of two replicate profiles. Representative profiles generated with a given enzyme were then compared among samples. Peaks were omitted from the analysis if they were only observed in one sample. Due to a slight shift in peak length between profiles, peaks were assumed to be the same between samples when they were within the size variation observed between replicates of the same sample. This number usually represented a 1 bp difference, but sometimes consisted of up to 2 bp, especially with peaks that were < 50 bp or > 400 bp.

The variable reduction method, principal component analysis (PCA), was used to determine which samples were more similar to one another and which TRFs were most correlated with these groupings. PCA was carried out by a full cross validation of unweighted data (i.e. relative peak areas for each variable [TRF]) using the program Unscrambler 6.11 (Camo Inc.). A PCA was also conducted based on the environmental parameters measured; because these variables were measured in different units, the data was standardized by dividing the relative abundance values by the variable's standard deviation. A multiple regression model was developed using a partial least squares algorithm using weighted data (Unscrambler 6.11). Linear regression models based on a

single independent variable were developed using Sigma Plot (Jandel Scientific). This program uses the Marquardt-Levenburg algorithm to determine the correlation coefficients that give the best fit between the regression equation and the data.

Results

We examined two chemically distinct regions of a reject coal impacted wetland (RCP1 and 2) to determine the temporal dynamics of the associated bacterial communities, with time points 0 and 10 collected at roughly the same time one year apart (3/24/00 and 4/2/01 respectively). Because the sediments are seasonally flooded, the degree of saturation along with the concentration of chemical species varies. Thus a major aim of this research was to determine whether changes associated with the predominant bacterial taxa could be linked to shifts in various environmental parameters.

Representation of TRF profiles by sequencing data. Previously, 16S rDNA clone libraries were constructed from RCP samples collected on 5/9/00 (T1) and screened to the extent that a fairly high coverage (i.e., [1- the number of unique sequence types]/total number of sequences; 71.4% [RCP1] and 64.0% [RCP2]) was obtained compared to other soil and sediment habitats surveyed in a similar manner. *Msp*I digestion produced a greater number of TRFs, or peaks, [45] than *Cfo*I [35]. Correspondingly, a greater proportion of the *Msp*I profile was unexplained by the sequencing data. At T1, 33 % [RCP1] and 38 % [RCP2] of the *Msp*I peaks could not be attributed to 16S rDNA sequencing data collected from the same time point (Fig. 3.1). The majority of these unknown TRFs represented minor components of the profile, individually contributing no more than 4% and collectively representing 25 % [RCP1] and 16.8 % [RCP2] of the total peak area. At other times of the year, some of these unexplained TRFs (notably m86,

c75, and m505) became prominent, individually comprising 9-14% of the total peak area. [A TRF abbreviation such as m86 refers to a peak that was formed by *Msp*I digestion (accordingly, "c" denotes a TRF generated by *Cfo*I) that has a size of 86 bp]. The proportion of the profiles that was unexplained was similar between sampling sites and varied up to two-fold among different time points with no observed seasonal trend.

The dominant peaks in the T-RFLP profiles could generally be attributed to abundantly recovered 16S rDNA sequences from the RCP1 and RCP2 libraries (Table 3.1, Fig. 3.1). Moreover, *Msp*I and *Cfo*I TRFs corresponding to these particular taxa generally displayed similar relative peak areas within the corresponding profiles (Table 3.1); this agreement is also illustrated by the close proximity of these TRFs along the principal component axes (Fig. 3.1). The environmentally derived sequences related to MS11 (γ *Proteobacteria*), RCP1-75 (*Deinococcus*-related) and to members of the genus *Acidiphilium* were abundant at T1, but significantly less so in the majority of the remaining samples. Consistently predominant groups included two gamma *Proteobacteria* lineages, one of which was highly related to an environmentally derived sequence, TRA5-3, obtained from an AMD habitat (pH 2.5 stream sediment, Iron Mountain, CA) (11). This group fluctuated between 2-30% (proportion of peak area) depending on the time of year. A second lineage, represented by RCP1-48 (0-25%) is unique to the RCP site and has not been isolated from any other habitat. The *Cfo*I and *Msp*I TRFs representing these taxa exhibited similar relative abundance values among the samples. The relative abundance of *Leptospirillum* group III TRFs (c404 and m73) fluctuated between 2-30% depending on the time of year and was similar among samples, with the BA71 sequences (δ *Proteobacteria*) contributing ~ 0-3 % to c404 (as determined

by their contribution to the *Msp*I profile). This analysis did not distinguish between *Leptospirillum* group III, which contains no cultured representatives, and at least some of the cultivated *Leptospirillum*. Because a sequence highly related to *L. ferrooxidans* (RCP2-41 of the *Leptospirillum* group II) also formed the same TRF size, the relative contribution of these two groups is unknown. The higher recovery of *Leptospirillum* group III (6.25% versus 0.7% sequences) in the clone library analysis suggests that it may be proportionally more significant, but this idea needs to be further tested. RCP sequences related to the Iron Mountain clone TRB3 (recovered from pH 0.5 pyritic sediment) (11) correspond to the abundant TRFs m455 (0-30%) and c384 (0-12%), though the former TRF was often significantly higher due, in part, to the contribution of additional α *Proteobacteria* RCP sequences. Other significant groups include RCP sequences affiliated with subdivision 1 of the *Acidobacterium* along with the *Acidimicrobium* group of the Actinomycetes. These groups encompassed many RCP operational taxonomic units (OTUs) and accordingly were more divergent than the taxa described above (Table 3.1). Therefore, these OTUs could not be simultaneously tracked with each enzyme. For instance, Actinomycete sequences that formed 2 TRFs upon *Cfo*I digestion were separated into 7 *Msp*I TRFs, some of which were shared with unrelated taxa, confounding comparisons between the two profile types.

Sample site similarity based on environmental parameters. Principal component analysis (PCA) was utilized to compare the samples based on either the environmental or the bacterial T-RFLP variables. When samples are compared based on a large number of variables, the data set may be too complex to reveal relationships (26). PCA is a variable reduction method that finds variables that contribute similarly to making the samples

different from one another (i.e., that have a high variance among samples). The two combinations of variables (i.e., principal components [PC's]) that explain the most variance within the data form a new set of axes (PC1 and PC2) on which the samples are graphed (26). Thus, the data set is reduced so that as much of the original information (variance) in the data set is retained as possible. The spatial closeness of the samples along the axes reflects their similarity. The manner in which they are similar can be inferred from the graphical depiction of the initial variables along the PCs. The farther away these variables are positioned from the center, the greater their influence on the sample groupings. PCA has previously been applied to interpret T-RFLP data (4, 9).

Based on measured chemical and physical variables, PCA grouped the sediment samples into five clusters (Fig. 3.2a), with the first two components explaining 80% of the variance in the data set. Among these clusters, the samples were first separated from one another based on site, with RCP1 samples being more similar to one another than to RCP2 samples (Fig. 3.2a). Environmental parameters heavily influencing principal component 1 (PC1) (pH, metals, salts, and sulfate) (Fig. 3.2b) were primarily responsible for these groupings. The RCP1 sediments possess higher metal (other than aluminum), sulfate, and salt levels and lower pH values. Within each site, the samples formed groups based on percent moisture and, to a lesser extent, temperature, both of which exerted a high influence on PC2. Samples positioned towards the upper portion of the graph tended to be collected at higher temperatures when the percent moisture content of the sediments was lower and vice versa. Percent organic matter was not included in this analysis because the presence of coal within the RCP1 sediments confounded this measurement. However, since organic matter was correlated with temperature within the

RCP2 samples, it likely also influences the clustering of these samples. For the RCP2 site, two distinct sample groups formed; those taken at time points 3 through 7 (i.e. T3-T7) and those collected from T9, T10, T0, T1 and T2 (i.e. T9-T2). Within the RCP1 site, the same general trend can be observed, with samples collected at T3-T7 collected towards the upper half of the PC graph and the remaining samples positioned within the lower half. However, samples collected at T4, T5, T7-T10 and T2 were positioned comparatively close to one another and therefore form a distinct cluster within the RCP1 site, with T3/T6 and T0/T1 samples forming two additional groups. The RCP2 samples formed more coherent clusters than those of the RCP1 site (Fig. 3.2a).

Sample site similarity based on TRF data. PCA based only on the TRF data (i.e., the presence and relative abundance of TRFs) temporally clustered the samples. Samples collected from T3-T7 were clearly separate from samples T9-T2 (Fig. 3.3a). An exception occurred with the T2 sample of the RCP1 site. The TRFs m73, c404, and m455, the most influential variables associated with PC1 (Fig. 3.3b), were primarily responsible for this separation. The TRFs m73 and c404, corresponding to *Leptospirillum*, were more prominent in samples T3-T7, particularly RCP1/T4-T7 (Fig. 3.3b, Fig. 3.4). The abundance of TRF m455, which is partially comprised of TRB3-related sequences, was inversely related to the *Leptospirillum* TRFs (Fig. 3.3b, Fig. 3.4) and therefore was generally less abundant in the T3-T7 samples. While the TRB3 TRF c384 exhibited a similar trend, it did not exert as great an influence on the sample groupings.

PCA also separated the samples based on spatial considerations, with RCP2 samples more similar to one another than those obtained from the RCP1 site. An

exception to this trend was the placement of RCP2/T9 with the RCP1 T9-T1 cluster. A repeated run yielded a similar result, indicating either that an unknown factor(s) is driving the T9 samples to be similar. Similar to the analysis based on environmental parameters, the RCP2 samples formed tighter clusters. The TRFs c90, c92, and m263, which correspond to the *Acidobacterium* 16S rDNAs, influence PC2 and are generally more abundant in the RCP2 samples, particularly in the T8 and T10-T2 samples. The RCP1-48 group (γ *Proteobacteria*), represented by TRFs c201 and m137, also influenced PC2 and have especially higher values in RCP1/T0-T5 (9-25%) and in RCP2/T3-T8 (4-9%; excluding T9); in the majority of other time points, they were virtually absent from the profile (0-2%).

Other numerically abundant TRFs heavily influenced both PCs, meaning they were particularly abundant in certain samples of a particular site. Gamma *Proteobacteria* sequences related to TRA5-3, represented by c203 and m495, are particularly abundant in the RCP1/T9-T0 samples, along with RCP2/T9 (Fig. 3.3b, Fig. 3.4). The Actinomycete TRFs (c400 and m165) also contribute to both PCs and are particularly abundant in RCP1/T5-T8.

Because the PCA was conducted on unweighted data, the contribution of numerically less abundant TRFs is not emphasized due to their lower variance among samples. The raw data were therefore utilized due to the greater interest in the dynamics of the predominant TRFs. PCA performed on weighted data gives the less abundant TRFs an equal opportunity to influence the sample groupings by giving all the TRFs the same variance. The use of weighted data for the analysis did not significantly alter the findings described above. The same samples clusters formed except that RCP1/T2

grouped with RCP1/T3-T7 samples as opposed to behaving as an outlier (data not shown). Additionally, the T8 samples from each RCP site were distinctly clustered in between the T3-T7 and the remaining samples.

Correlation of major TRF groups to chemical and physical parameters. The distribution of independent variables (environmental parameters) relative to dependent variables (TRFs) within a partial least squares regression suggested the possibility that correlations existed between the two variable sets (Fig. 3.5). pH was found to be correlated positively with the *Acidobacterium* TRFs c90/m263 (r = 0.76, P<0.001; r = 0.79, P<0.001) and negatively with the Actinomycete TRFs c400/m165 (r = 0 .73, P<0.001; r = 0.66, P<0.001) (Fig. 3.6a,b). These TRFs could also be correlated with salts, heavy metals and sulfate concentrations to various degrees as these variables themselves were inversely related to pH (Fig. 3.5). However, the highest correlation coefficients for these TRFs were found with pH. Other TRFs corresponding to these groups were not significantly correlated with pH. A weaker correlation was found between percent moisture and several other predominant TRFs. Percent moisture was positively correlated with the TRFs corresponding to the sequences TRA5-3 (c203 [r = 0.53, P<0.01]; m495 [r = 0.54, P<0.008]) (Fig. 3.5, Fig. 3.6c) and TRB3 (c384 [r = 0.63, P<0.005]; m455 [r = 0.58, P>0.005]) and negatively correlated with the *Leptospirillum* TRFs c404/m73 (r = 0.63, P<0.005, r = 0.57, P<0.01) (Fig. 3.5, Fig. 3.6d). These taxa were correlated to a lesser extent with magnesium. The RCP1-48 TRFs were weakly correlated with temperature (c201 [r = 0.52, P<0.02]; m136 [r=0.53, P<0.02]).

Discussion

The most frequently recovered sequence types from the clone library analysis (6) appeared to contribute to the largest peaks in the T-RFLP profiles. These groups included sequences that have been previously linked to bioleaching habitats (*Acidobacterium* division, the *Acidimicrobium* group of the Actinomycetes, and *Leptospirillum* groups III and II) as well as groups not previously known to represent major groups in these habitats (sequences related to RCP1-48, TRA5-3 and TRB3). While the environmental sequences TRA5-3 and TRB3 were originally recovered from Iron Mountain (11), subsequent bacterial community analysis of this system has not focused on these groups. Based on the work conducted here, they appear to represent relatively abundant components of the RCP system.

Culture-based and molecular examinations of bioleaching bacterial communities are generally based on one sampling event and do not incorporate chemical measurements (19). A notable exception includes the temporal analysis of the low pH habitats (pH ~1.0) within Iron Mountain, in which the abundance of particular taxa was related to environmental parameters using *in situ* hybridization analysis (10). This study found that *L. ferrooxidans* was comparatively abundant in lower pH niches relative to *T. ferrooxidans*; additionally, *Archaea* cell abundance was positively correlated with conductivity. Bacterial communities associated with relatively less extreme AMD habitats have not been monitored over time using a non-cultivation approach.

Previous 16S rDNA analysis of the bacterial communities inhabiting this coal impacted wetland revealed the presence of many novel sequence types (6). To more thoroughly investigate these groups, we applied T-RFLP to profile community shifts in

this system over time. Because this approach relies on PCR amplification, T-RFLP is subject to the same known biases and at best can only be considered semiquantitative (27). However, because we kept the cycle number at 20 to minimize bias, it is reasonable to assume that the abundantly amplified 16S rDNAs represent the predominant types within a sample. Direct probing of bulk nucleic acids with specific oligonucleotides has been used to confirm the predominance of abundantly amplified 16S rDNA types (12,14). Furthermore, assuming the protocol steps are uniformly applied, the relative abundance of TRFs can be compared between samples since profile differences can be attributed to community shifts rather than to methological variance (18).

In order to compare bacterial communities by T-RFLP, ideally the predominant peaks in a profile should be represented almost exclusively by 16S rDNA sequences known to exist within the system. In this situation, shifts in peak abundance can then be related to a particular taxon. A shortcoming of T-RFLP occurs when unrelated taxa contribute to the same peak, a situation that appears to be exacerbated with complex samples such as pristine soil or sediments (4). Previous sequence analysis of T1 revealed the bacterial diversity within the RCP system to be relatively low, suggesting that T-RFLP might be conducive for temporally monitoring the associated bacterial communities. Indeed, in this study we found that *Msp*I and *Cfo*I TRFs corresponding to the same taxa generally displayed similar relative abundance values, as seen by their close proximity along the PCA axes. TRF data of bacterial 16S rDNA types generated from two enzymes has not been previously demonstrated to be so congruent, providing evidence that single taxa are being identified in many of the major peaks.

Two enzymes were utilized to confirm that the relative abundance of a taxon in one profile (i.e. a *CfoI*I or *Msp*I profile) was not significantly overestimated by the contribution of additional sequence types. This did not appear to be the case with TRFs representing the RCP1-48, *Leptospirillum sp.*, and TRA5-3 groups (Table 3.1, Fig. 3.3b). However, comparisons of *Cfo*I/*Msp*I TRFs for a given taxa were not always well correlated with one another. This situation could result from multiple sequence types comprising one peak. For instance, the summed abundance values of the *Acidobacterium* TRFs c90 and c92 were generally higher (at times greater than 2 fold) when compared to those for m263. While c92 (RCP1-43, 101, Table 3.1) was also comparatively abundant in the RCP2 samples, it was not correlated with m263 and c90, which themselves possessed similar abundance values as seen by their close association along the principal component axes (Fig. 3.3b). It is therefore possible that additional sequences significantly contribute to c92.

The majority of the *Msp*I profiles could be at least partially accounted for by RCP sequence data (up to 74% peak number and 83% peak area among all samples). While this proportion may represent an overestimate, it is nevertheless high considering 16S rDNA sequence information was obtained from only one portion of the year. Nevertheless, this study would benefit from additional clone library analysis. For instance, only one known RCP sequence (RCP2-105 of the Actinomycete*s*) potentially contributes to the TRF m165. This peak, minor at T1 (RCP1 [2%], RCP1 [1%]), eventually becomes a dominant (up to 35% in RCP1/T6) component of the *Msp*I profile. Because m165 is correlated with c400 (to which RCP2-105 also contributes), we suspect that this sequence and possibly additional unknown Actinomycete sequences are the

significant contributors to this peak. However, 16S rDNA analysis of those time points is needed to confirm this idea.

Differences among RCP bacterial communities were attributable more to changes in the relative abundance rather than the presence or absence of TRFs. Limited screening of T0-16S rDNA clone libraries suggested that sequence types in this system may not change drastically over time. From twenty-five clones, only four sequence types were not >99% identical to those recovered from the T1 library (data not shown). A higher proportion of the variance in the TRF data (40%) was explained by PC1 which temporally divides the samples, indicating the relative abundance of the dominant taxa changed more with time than between sampling sites. Yu and Mohn (2001) also observed more significant differences in the bacterial community structure over time (as determined by 16S-23S rDNA ribosomal intergenic spacer length polyporphism [RIS-LP]) than among sampling sites in an aerated lagoon receiving pulp and paper run-off. Macalady *et al.* (2000) instead noted greater spatial among bacterial communities inhabiting a mercury-polluted lake. The distance between sampling sites in the later study were more extensive (>km) than the RCP sites. The relative strength of spatial or temporal bacterial community patterns will likely depend on the scale of study and the magnitude of physical and chemical forces on the system in question.

Within a site, the proportion of TRFs corresponding to *Leptospirillum*, TRA5-3 and TRB-related sequences changed as much as 30%, with the most dramatic shifts occurring between sampling periods T3-T7 and T9-T1. Within T3-T7, the *Leptospirillum* TRFs were proportionally higher and the TRB3 and TRA5-3 TRFs were proportionally lower (Fig. 3.4). The temporal variation of these constituents may be

cyclical as relative abundance values were similar between samples taken at roughly the same time one year later (T0/T1 and T10). The similar dynamic of the taxa in the two different sampling sites is consistent with the idea that a common controlling factor(s) is affecting their temporal variation. Percent moisture exerted a strong influence on grouping samples T3-T7 as distinct from T9-T1. It is tempting to speculate a lower moisture content might coincide with higher oxygen levels, however, oxygen levels were not measured. Interestingly, the *Leptospirillum* TRFs, (which based on sequence data we suspect were represented significantly by *Leptospirillum* type III sequences), were higher in these samples, and cultured members of this group are obligate aerobes (2). However, it is important to recognize that correlation does not denote causation; though groups may fluctuate with percent moisture, other parameters correlated with percent moisture may exert a more direct influence on the dynamics of these groups.

Samples from each RCP site were more similar to one another, with the extent of similarity appearing greater among the RCP2 samples based on their tighter clustering in the PCA plots. The comparatively high variance among the RCP1 samples might be due, in part, to the coal fragments interspersed in the near-surface sediments of this site. This source material may correspond to hot spots for potential sulfur/iron transformations, acid production, and salt/metal leaching, the magnitude of which will depend on optimal environmental conditions. The distinct chemistry of each RCP site might be expected to translate into differences in bacterial community composition. However, only four TRFs (c60, c183, m162, and m179) were found to be restricted to either sampling site, each of which corresponded to minor peaks in the TRF profiles. This suggests that, at least based on the T-RFLP data, the majority of the bacterial groups were present in both sites. The

major differences between the RCP1 and RCP2 samples were attributable instead to differences in the relative abundance of various TRFs. The comparative abundance of the *Leptospirillum* group (in T3-T7 samples, Fig. 3.4) and the Actinomycete groups in the RCP1 samples suggests the possibility of a more direct role in acid generation than the organisms corresponding to the *Acidobacterium* taxa, as bacteria present in the RCP1 site are in closer contact to the coal source. This is consistent with the phylogenetic placement of these as yet uncultivated bacteria with isolates capable of iron oxidation.

T-RFLP is a relatively fast, sensitive and reproducible means to fingerprint bacterial communities (18). Shortcomings of the approach include a lack of resolution due to the potential for multiple taxa, related and unrelated, to contribute to one peak. We have found that clone library analysis can provide useful information to flush out such occurrences, as can comparisons of distinct profiles generated with different restriction enzymes. As a result, T-RFLP can successfully be applied to determine the identification of dominant 16S rDNA types and to monitor shifts in their relative distribution. Emerging trends can be further explored using higher resolution approaches such as T-RFLP using group-specific primers or those involving oligonucleotide hybridization. Virtually every bacterial community examined by molecular approaches is revealed to consist primarily of as yet uncultivated organisms (15). In the absence of actually culturing the corresponding organisms, the application of molecular methods provides a promising opportunity for learning about the characteristics of these groups by relating their abundance to chemical and physical gradients in order to reveal possible forces driving their selection. We were able to utilize this approach to detect seasonal and spatial trends in several groups of as yet uncultured bacteria that appear to be

numerically dominant in an acid leaching habitat and to correlate the abundance of some of these groups to pH or percent moisture.

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TABLE 3.1. Percent relative abundance of major taxa based on 16S rDNA clone libraries and T-RFLP analysis from RCP samples collected on 5/9/200 (T1).

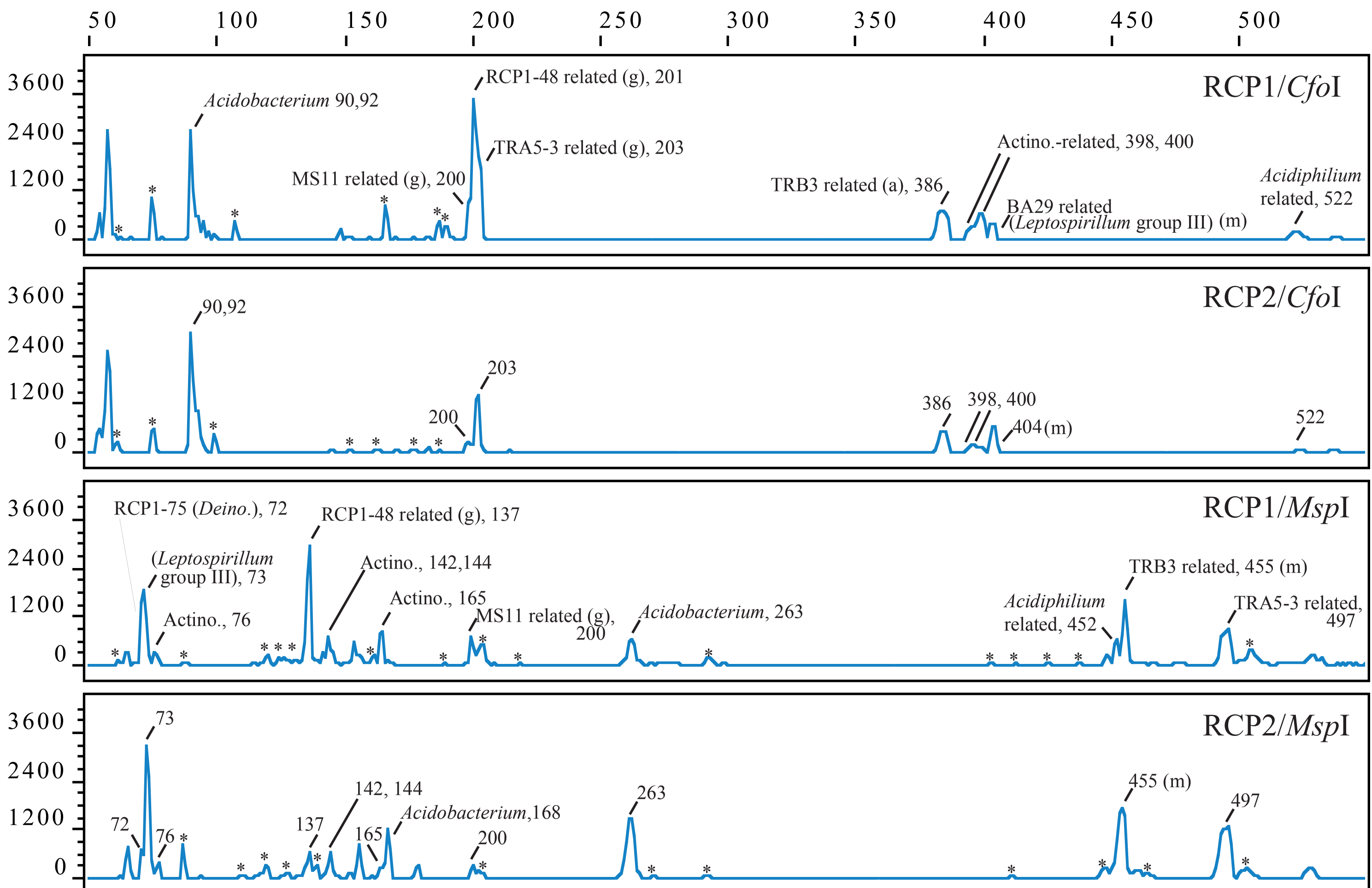
	TRF sizes(bp)[a]		Percent relative abundance[b]					
			RCP1			RCP2		
Taxa	*Cfo*I	*Msp*I	CL	*Cfo*I	*Msp*I	CL	*Cfo*I	*Msp*I
α-*Proteobacteria*								
TRB3-related	386	455*	8.5	9.0	10.0*	6.7	6.0	21.0*
Acidiphilium	522	452	5.7	4.0	3.5	0	0	0
γ-*Proteobacteria*								
MS11-related	200	202	8.6	4.0	5.0	2.7	2.7	2.0
RCP1-48-related	201	137	8.6	15.3	10.0	1.4	0	3.0
TRA5-3-related	203	497	2.9	10.3	11.0	9.5	10.3	12.7
Acidobacterium								
RCP1-43,101	92	263	2.8	1.7	5.0	4.1	6.7	13.3
RCP1-32, RCP2-3	90	263	7.0	7.7		15.0	14.7	
RCP2-4,90,97,101								
RCP2-90	54*	168	0	1.7*	0	2.7	3.7*	4.0
Actinomycete[c]								
RCP1-68,77,83	398	142	18.6	17.9	12.0*	8.7	4.3	8.7*
RCP1-56	398	76						
RCP1-9	398	144						
RCP2-105	400	165						
RCP1-33	400	144						
RCP2-57,RCP2-2	400	66*						
RCP2-103	395	144						
RCP1-37	395	162						
RCP2-68	149	176						
RCP1-34	>DR	66*						
Nitrospira								
Leptospirillum-III	404*	73	4.3	5.3*	4.0	8.1	9.3*	5.7
Deinococcus								
RCP1-75	<DR	72	5.7	<DR	6.0	0	<DR	2.7

[a](*) designates a TRF for which multiple unrelated taxa are known to contribute.

[b]Percent relative abundance of the 16S rDNA sequences based on either their representation in the clone library (CL) analysis or on the area of peaks within T-RFLP profiles generated by *Cfo*I or *Msp*I digestion. DR refers to TRFs with sizes outside the detection range (i.e. < 50bp or > 550 bp). The corresponding taxa are therefore not included in the analysis of these profiles.

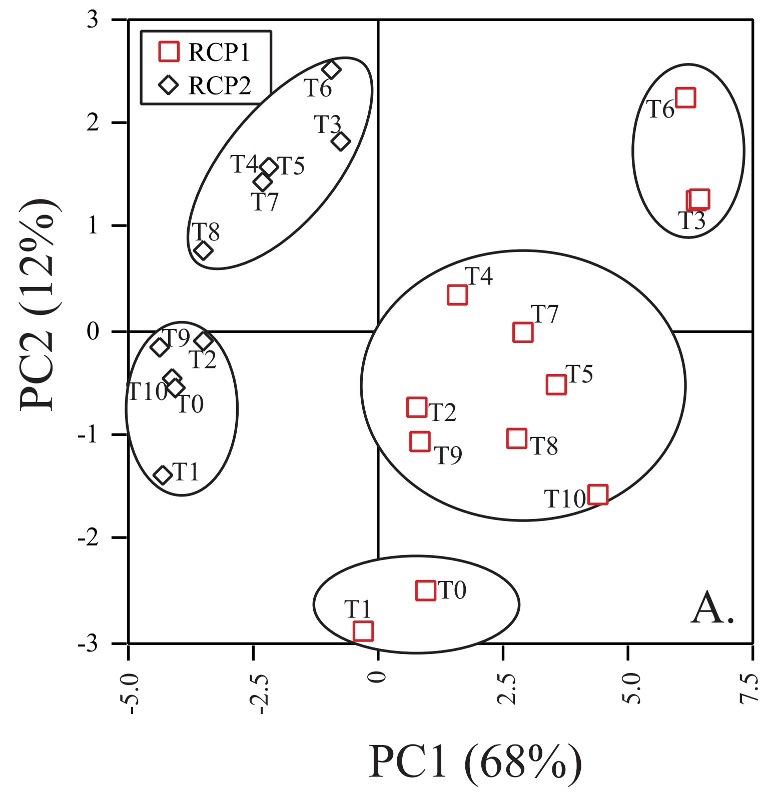
[c]Because the Actinomycete-related sequences form many distinct TRFs upon *Msp*I digestion, the percent relative abundance of these individual sequences as determined by clone library analysis anby T-RFLP profiles are summed for simplicity.

FIG. 3.1. Bacterial community structure of RCP1 and RCP2 samples at time point 1 (5/9/00) as determined by T-RFLP with separate *Cfo*I and *Msp*I digestions. Predominant peaks corresponding to taxa discussed in the text are labeled by name and their TRF length. The abbreviations *Deino*. and *Actino.* refer to *Deinococcus* and Actinomycete respectfully, (m) denotes a peak consisting of more than one unrelated taxa and (*) signifies a peak of unknown affiliation.



RCP1/*Cfo*I
Acidobacterium 90,92
RCP1-48 related (g), 201
TRA5-3 related (g), 203
MS11 related (g), 200
TRB3 related (a), 386
Actino.-related, 398, 400
BA29 related
(*Leptospirillum* group III) (m)
Acidiphilium related, 522
RCP2/*Cfo*I
90,92
203
200
386
398, 400
404 (m)
522
RCP1/*Msp*I
RCP1-75 (*Deino.*), 72
(*Leptospirillum* group III), 73
Actino., 76
RCP1-48 related (g), 137
Actino., 142,144
Actino., 165
MS11 related (g), 200
Acidobacterium, 263
Acidiphilium related, 452
TRB3 related, 455 (m)
TRA5-3 related, 497
RCP2/*Msp*I
72
73
76
137
142, 144
165
Acidobacterium,168
200
263
455 (m)
497

FIG. 3.2. Principal component analysis (PCA) based on measured environmental parameters. The plots correspond to the positions of samples (A) and environmental variables (B) along the PC axes. Sampling times and environmental parameters are labeled next to each data point, with SS referring to soluble salts. The amount of variance in the original data set explained by PC1 and PC2 is shown in parentheses.


RCP1
RCP2
T6
T3
T4
T5
T7
T8
T9
T2
T10
T0
T1
T4
T7
T5
T2
T9
T8
T10
T6
T3
T0
T1
A.
PC2 (12%)
PC1 (68%)
3
2
1
0
-1
-2
-3
-5.0
-2.5
0
2.5
5.0
7.5

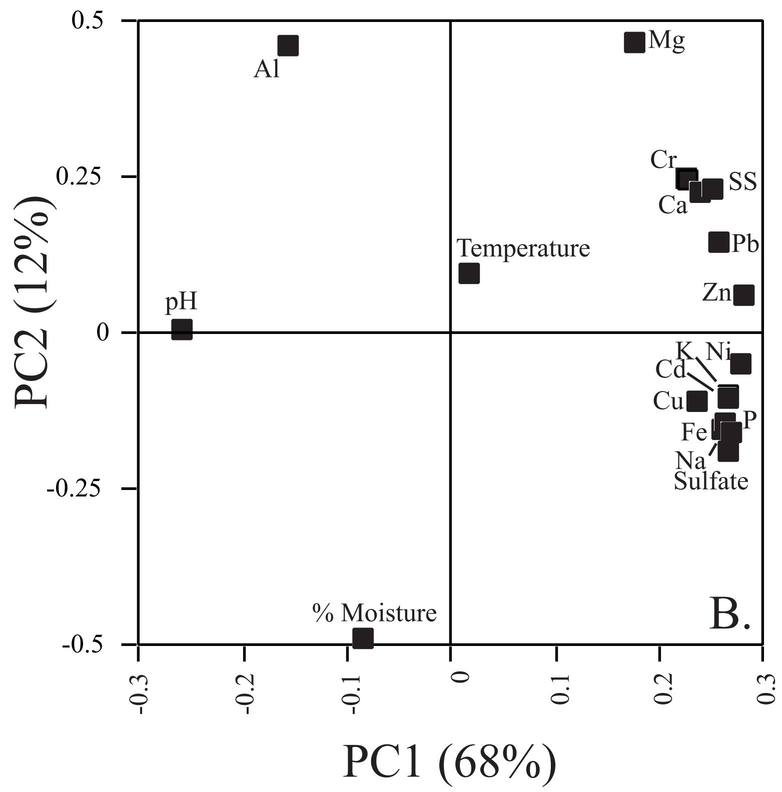
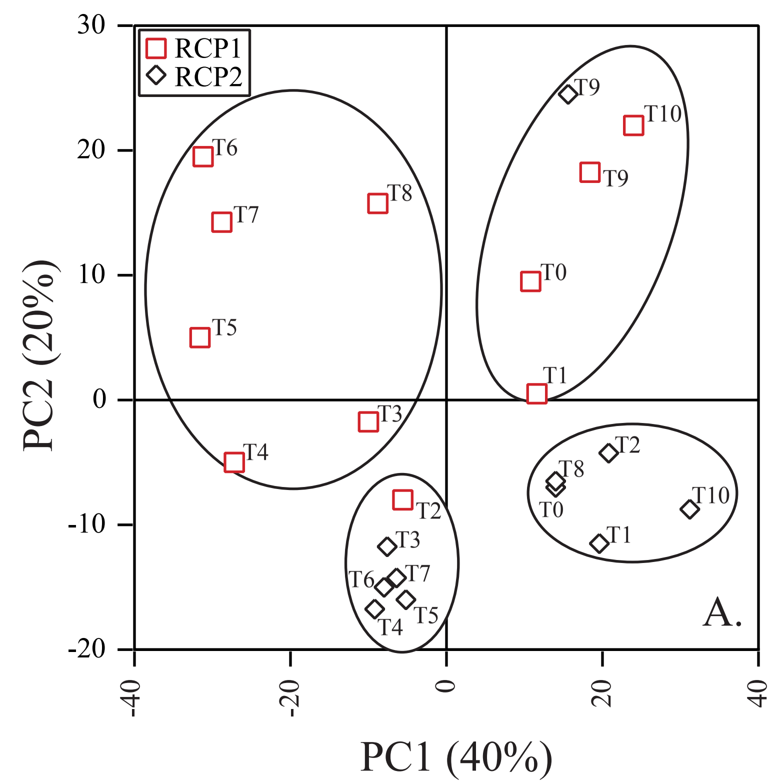

Al
Mg
Cr
SS
Ca
Pb
Temperature
Zn
pH
K
Ni
Cd
Cu
P
Fe
Na
Sulfate
% Moisture
B.
PC2 (12%)
PC1 (68%)
0.5
0.25
0
-0.25
-0.5
-0.3
-0.2
-0.1
0
0.1
0.2
0.3

FIG. 3.3. PCA based on measured T-RFLP data. The plots correspond to the positions of the samples (A) and TRFs (B) along the PC axes. Sampling times and TRFs are labeled next to each data point. The amount of variance in the original data set explained by PC1 and PC2 is shown in parentheses. Squares of the same color indicate TRFs representing the same taxa (clockwise from top: Actinomycete*,* green; TRA5-3, blue; TRB3, yellow; *Acidobacterium,* red; RCP1-48, black; *Leptospirillum,* purple.


RCP1
RCP2
T6
T7
T8
T5
T3
T4
T2
T9
T10
T0
T1
A.
PC1 (40%)
PC2 (20%)

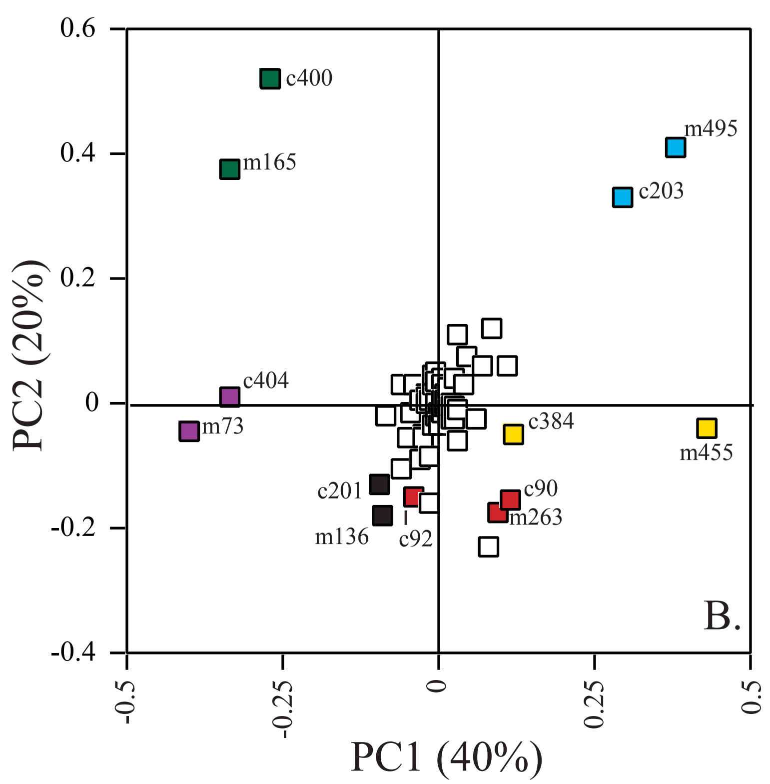
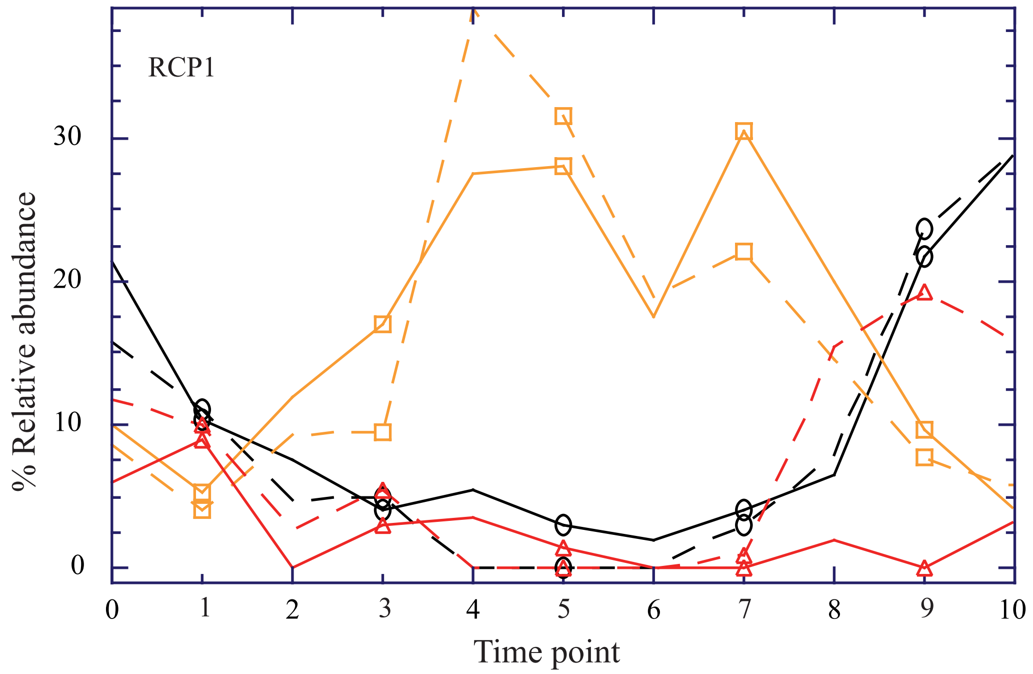

c400
m165
m495
c203
c404
m73
c384
m455
c201
m136
c92
c90
m263
B.
PC1 (40%)
PC2 (20%)

Fig 4. Temporal changes of the *Leptospirillum*, TRA5-3 and TRB3 groups in the RCP1 and RCP1 samples expressed as the percent relative abundance of the corresponding TRF peak areas. Orange lines: *Leptospirillum* TRFs (c73, m404); black lines: TRA5-3 (c203, m495); red lines: TRB (c384, m455). The straight and hatched lines represent *Cfo*I and *Msp*I data respectively.


RCP1
% Relative abundance
Time point
0
10
20
30
0
1
2
3
4
5
6
7
8
9
10

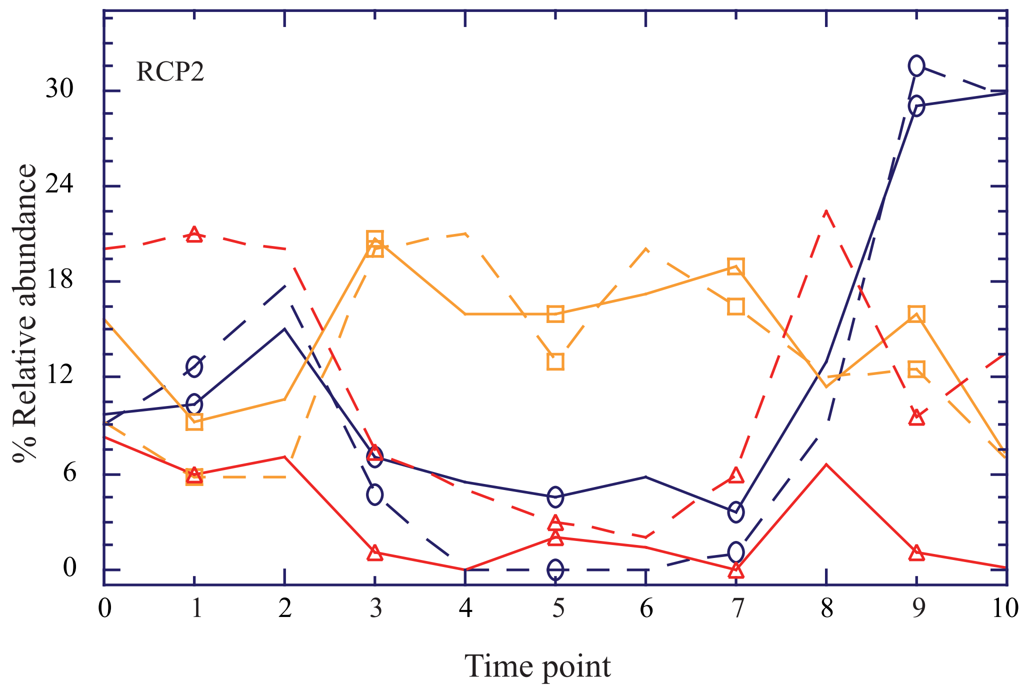
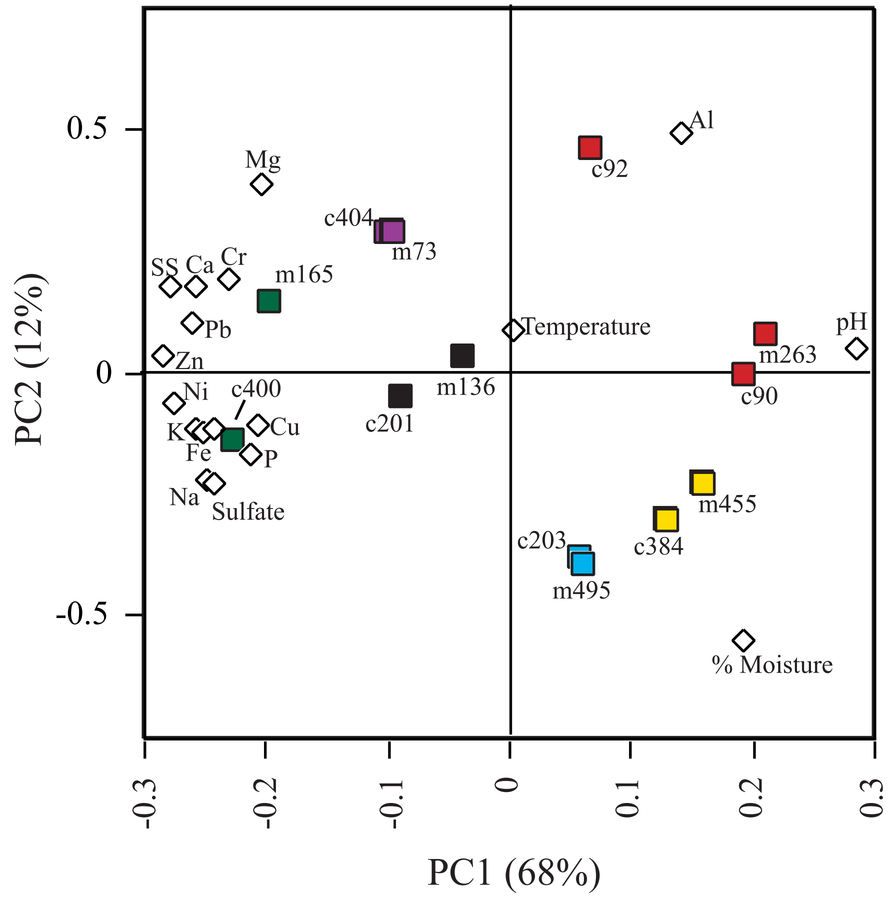
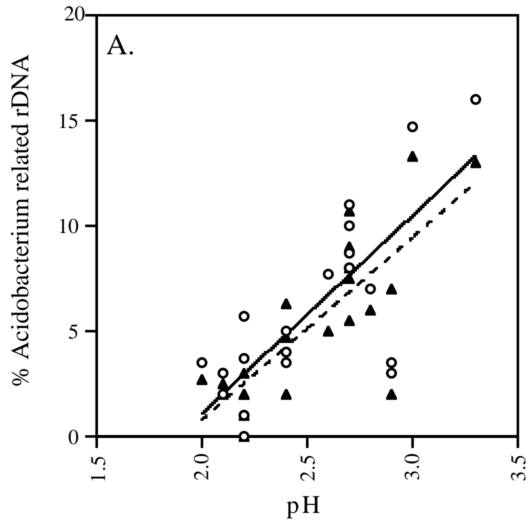

RCP2
% Relative abundance
Time point
0
6
12
18
24
30
0
1
2
3
4
5
6
7
8
9
10

FIG. 3.5. Loading plot of a partial least squares multiple regression. ■, dependent variables (environmental variables); ◇, independent variables (TRFs). The amount of variance in the data set explained by each PC is shown in parentheses.

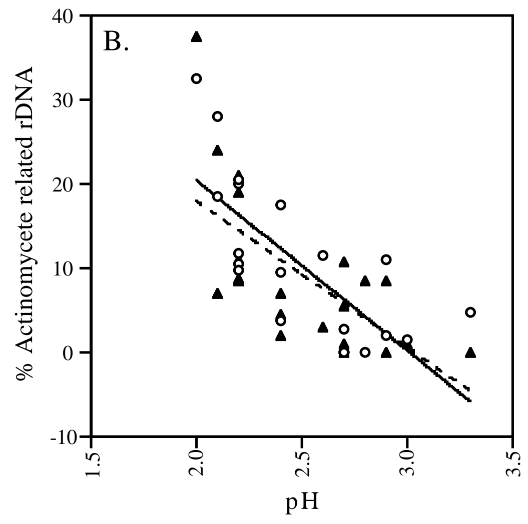


0.5
0
-0.5
PC2 (12%)
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
PC1 (68%)
Mg
c404
m73
SS
Ca
Cr
m165
Pb
Zn
Ni
c400
K
Cu
Fe
P
Na
Sulfate
m136
c201
Temperature
Al
c92
pH
m263
c90
m455
c384
c203
m495
% Moisture

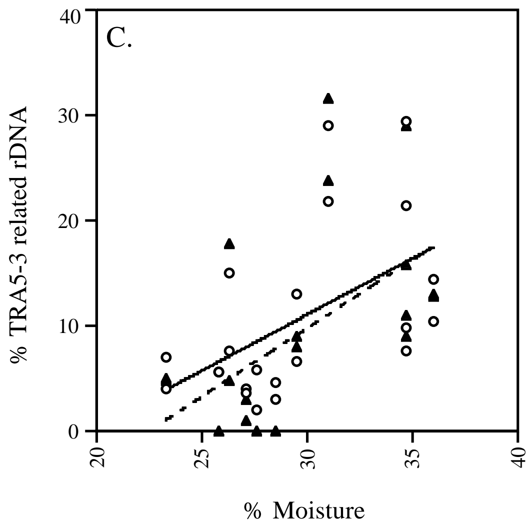
FIG. 3.6. Linear regression plots relating the percent relative abundance of specific rDNAs (as determined by the corresponding TRF peak area) to either pH or percent moisture. (A.) *Acidobacterium* TRFs c90 and m263; (B.) Actinomycete*s* TRFs c400 and M165; (C.) TRA5-3 TRFs c203 and m495; (D*.) Leptospirillum* TRFs c404 and m73. ❍, denotes *Cfo*I TRFs and ▲, signifies *Msp*I TRFs The straight and hatched lines represent the regression lines for the *Cfo*I and *Msp*I data respectively.



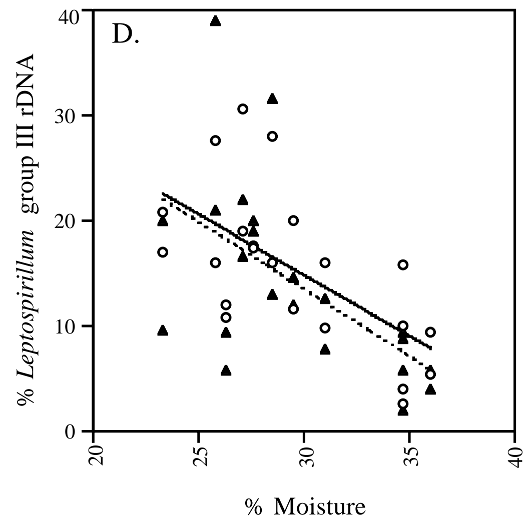
A.
% Acidobacterium related rDNA
pH



B.
% Actinomycete related rDNA
pH



C.
% TRA5-3 related rDNA
% Moisture



D.
% Leptospirillum group III rDNA
% Moisture

CHAPTER 4

DISTRIBUTION AND DIVERGENCE OF A NOVEL BACTERIAL SUBDIVISION RECOVERED FROM AN ACID LEACHING HABITAT.

Brofft, J.E., McArthur, J.V., and L.J. Shimkets. To be submitted to *Extremeophile*

Abstract

A PCR assay was used to examine the distribution of a novel 16S rDNA sequence group (~74-76% similarity to database sequences) recovered from an acidic wetland polluted by coal pile run-off (RCP1). PCR primers designed to target this group (termed WSRCP) were used to screen a variety of environmental samples in order to determine its distribution. Three additional sequence types were recovered that are affiliated with this group. WSRCP sequences were only recovered from acidic habitats and these sequences were nearly identical (>99% sequence similarity) to those previously isolated from RCP1. Phylogenetic analysis determined that the WSRCP group represents a novel subdivision of the candidate division WS6 which as yet harbors 16S rDNA sequences recovered only from acidic habitats.

A common finding of environmental molecular surveys is the recovery of 16S rDNA sequences representing as yet uncultivated bacteria (1). Occasionally, such sequences form deeply divergent lineages that expand the breadth of the domain *Bacteria*. Since the advent of 16S rDNA based culture independent studies, the number of bacterial divisions has increased from Woese's (1987) initial estimation of 12 to approximately 40 (9). However, thirteen of these are referred to as "candidate" divisions because they consist only of environmentally derived 16S rDNA sequences (9,14). Consequently, little may be learned about the characteristics of the corresponding organisms until their cultivation. A complementary culture-independent approach involves the design of specific 16S rDNA primers and probes to explore the dynamics and distribution of the group of interest. Hugenhholtz *et al*., (2001) applied *in situ* hybridization and transmission electron microscopy to determine that at least some members of the candidate division TM7 present in an activated sludge sample possess a gram positive type cell wall. Dojka *et al*., (2000) utilized a PCR primer targeting the candidate division WS6 to delineate that this group is commonly detected in anaerobic environments.

A recent bacterial diversity study of a wetland sediment impacted by coal storage run-off recovered 16S rDNA types that were distantly related (74-76% similarity) to those of any known bacterium or environmental clone sequence (Brofft et al., 2002). Phylogenetic analysis tentatively placed these sequences within the candidate division WS6 as a novel subdivision. A subdivision has been defined by Hugenholtz *et al* (1998) as a clade composed of at least two 16S rDNA sequences that is reproducibly monophyletic (i.e. robust to different phylogenetic algorithims) and unassociated with

other sequences contained in the division. This group comprised a significant proportion of the sequences analyzed (~7%) from the most acidic site examined (pH 2.5), yet was not observed in libraries generated from a less polluted sample (pH 3.0) or an unaffected sample (pH 5.2). The recovery of sequences this divergent from an extreme habitat warranted further analysis of this group. Therefore, specific primers were designed to test for its occurrence in a variety of environmental samples to determine if it is confined to similar habitats or rather has a cosmopolitan distribution. We also sought to recover related members as this might aid in confirming the group's correct phylogenetic placement.

The novel group of interest (termed WSRCP) was initially comprised of three 16S rDNA sequences retrieved with primers considered inclusive for the majority of bacteria, 27f (5'AGAGTTTGATCCTGGCTCAG 3') and 1392R (5' GACGGGCG GTGTGTAC 3') (11). Putative primers were identified by alignment of these sequences with representatives from the candidate divisions WS6, OP11, TM6, TM7, and OP5 using the program PILE_UP (Accelrys). Regions of the 16S rRNA gene unique to the WSRCP group were identified using BOX_SHADE (Accelrys). The specificity of the potential primers was tested empirically against 16S rDNA sequences present in the Ribosomal Database Project (RDP) using the program Probe Match (12) which lists RDP sequences that will most likely bind to the probe and displays the mismatches between the probe/target pair. The program OLIGO (Molecular Biology Insights) was used to analyze the primers for potential secondary structural formation.

Based on these analyses, the specificity of three primers was experimentally tested in a PCR assay. Each WSRCP primer was paired with either 27f or 1392r. The

primer sets were applied to positive controls consisting of WSRCP 16S rDNA sequences (RCP1-39,-88, and –100) contained within the pCR 2.1 vector (Invitrogen). Negative controls consisted of cloned 16S rDNAs having the same or a similar number of mismatches in the specific primer target region as the highest hit revealed by Probe Match. In one instance, the positions of the missmatches within the highest hit and our negative control were the same. Amplification conditions are described as follows: 2.5 U of HotStar *Taq* polymerase, 1X PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP and 0.2 μM of each primer and Q buffer (Qiagen Inc.). An initial denaturation at 15 minutes (for enzyme activation) was followed by 30 cycles at 94°C for 1 min, variable annealing temperature for 1 min, 72°C for 2 min and ending with a final extension at 72°C for 7.5 min. The optimal annealing temperature was determined to be the one at which PCR products of the correct size were obtained for the positive but not the negative controls (Table 4.1). The primer sets were applied to the environmental genomic DNA sample from which the WSRCP sequences were initially derived (RCP1). PCR products were cloned by TA cloning into the pCR 2.1 or pCR 4-TOPO vector and sequenced using the M13 forward and reverse primers recommended for use (Invitrogen). Every clone sequenced from libraries generated with the primer pairs 650f/1392r and 848r/27f was related to the WSRCP group, whereas the primer pair 1107r/27f recovered a significant number of non-specifically amplified 16S rDNA types. In subsequent experiments, we utilized 650f paired with the universally conserved primer 1492r (5' GGTTACCTT GTTACGACTT 3') (6) since this combination produced an amplification product having a larger region of homology with the majority of WS6 sequences. The replacement of

1392r with 1492r did not appear to alter the specificity; 10/10 clones screened from a RCP1 library created with the primer pair 650f/1492r represented WSRCP sequences.

The initial recovery of this group from an acid leaching system suggested that it might be enriched in other acidic systems. Therefore, a variety of environmental samples, acidic and non-acidic, were screened for the presence of the WSRCP group. Two wetland sediment samples (upper 2 cm; located in Savannah River Site's D-area, Aiken, SC) impacted by coal pile run-off were analyzed. Both samples were acidic and contained elevated salt, iron, sulfate and metal concentrations, though one (RCP1) was more contaminated than the other (RCP2). Sediment from an unaffected region of the same wetland system was also examined (FW). An additional acid mine drainage (AMD) impacted sample was also analyzed; acid water samples obtained from a dribbling hole (pH 2.8; 2 km depth) within shaft 2 of the Evander mine located in South Africa were provided by Duane Moser (Princeton University). An agricultural soil sample was collected from the upper 4 cm. of a tilled plot located in Horseshoe Bend, Athens, GA. A mildly acidic soil sample (upper 10 cm, pH 4.9) collected from the Athens Municipal Landfill, Athens, GA was provided by Mark Wise (University of Georgia). The samples described above were maintained at –80°C prior to DNA extraction by either the large or small scale soil DNA extraction kit (MoBio). Cell lysis is based on vortexing with beads and extended heating at 70°C (large-scale) or on bead beating (small scale).

Salt marsh water and sediment DNA obtained from the Sapelo Island coast along with DNA extracted from an enrichment utilizing *Spartina alterniflora* as the sole carbon source were provided by Alison Buchan and Wendy Ye (University of Georgia).

A nucleic acid sample obtained from a freshwater tributary flowing into San Francisco Bay was provided by Ramunus Stepanasukas (University of Georgia). Nasreen Bano (University of Georgia) provided DNA recovered from samples collected at depths of 2m, 19m, 25m, 26m, and 28m from Mono Lake, CA. Mono Lake is alkaline (pH~10) and saline (~80g/l) and at the time of sampling harbored anoxic bottom waters (>25m) due to meromixis.

These environmental samples were screened for the WSRCP group with the primers pair 650f /1492r using PCR conditions described above, except the annealing temperature was lowered to 50°C and the Q buffer was omitted in order to lower the reaction stringency. The conditions were initially relaxed to generate sufficient FW product for cloning. Because sequence analysis did not reveal evidence of non-specific amplification, these conditions were adopted for other environmental samples to increase the chance of amplifying WSRCP sequences present in low abundance. Because a low DNA yield was obtained from the acid water sample, the bacterial 16S rDNA amplification product (using the 27f /1492r primer pair) was used as a template for amplification with 650f /1492r. A range of DNA template concentrations were utilized (1-20 ng) to account for the possibility that a negative result might be due to low biomass or to the presence of coextracted inhibitory substances. Within this range, 16S rRNA genes were amplified with the 27f /1392r primer pair. Positive and negative controls consisted of RCP1 DNA and of no added template, respectively. PCR products of the correct size were cloned and sequenced as described above. The RCP1 sample was also screened by restriction fragment length polymorphism (RFLP) in an attempt to identify novel sequence types. Using the 650f/1492r primer pair, inserts were reamplified from

plasmids (~400 pg) using the conditions described above with 20 cycles and an annealing temperature of 52°C. PCR products were digested with 1.0 U *Mse*I and the restriction patterns were resolved on a 2.0 % TreviGel 500 (Trevigen) gel. The program MAP (Accelrys) was used to determine that upon *Mse*I digestion, each WSRCP sequence type yielded a distinct restriction pattern. Phylogenetic trees were constructed as described (3), with Parsimony (DNAPARS) being used as an alternative algorithm with the same alignment (4).

In this report, we applied specific primers to evaluate the distribution of a novel group of 16S rRNA sequences (termed WSRCP) originally recovered from an acid leaching habitat. Under optimal conditions, two of the designed primers appeared to be specific for the WSRCP group yet inclusive enough to recover additional affiliated sequences. Only when the stringency was relaxed did we observe nonspecific amplification (Mono Lake 25,26,28m samples and the acid water sample), demonstrating that the identity of PCR products obtained with specific primers from different environmental samples should be confirmed by sequence analysis.

Three novel operational taxonomic units (OTUs) related to the WSRCP group were recovered from the RCP1 sample using a 97% sequence similarity criterion. Two OTUs represented by WSRCP1-7 and WSRCP1-9 (Fig. 4.1) were approximately 95.6-95.9% similar to RCP1-88. A third sequence type, WSRCP1-2 (recovered using the 27f/848r primer pair), was approximately 95.9% similar to RCP1-100 and not included in the phylogenetic analysis shown in this report because only 280 nt overlapped with the aligned sequences. Extensive RFLP screening revealed no evidence (i.e. distinct restriction pattern) of additional WSRCP OTUs present in the RCP1 samples.

The WSRCP sequences were recovered only from acidic habitats, but were not retrieved from every acidic sample tested. Using the 650f/1492r primer pair, WSRCP sequences were recovered from every acidic environmental sample with the exception of the landfill soil (Table 4.2). The WSRCP group appears to persist in the contaminated wetland as PCR products were also produced from samples taken at a later date (1/11/01); a PCR product was not attained with the FW sample taken from this same date. No novel WSRCP OTUs were recovered from the RCP2, FW and acidic water samples that were not also recovered from the RCP1 sample; sequences from these libraries were practically identical (>99% sequence similarity) to the WSRCP OTUs retrieved previously. Sequences highly related to WSRCP1-7 (10) and WSRCP1-9 (1) were recovered from the RCP2 sample. Every sequence examined from the FW library (10 clones) was nearly identical to RCP1-100 and one clone recovered from the acid water sample was 99% similar to WSRCP-7. The soil, sediment and water samples analyzed encompassed a variety of different pH and salinity values and included ones derived from aerobic and anaerobic sources. The environmental samples testing positive for the WSRCP group were distinct in their nature and geographic location, yet a unifying characteristic appears to be low pH.

WSRCP sequences have not been isolated from other published 16S rDNA bacterial surveys of acidic habitats (2, 7,8,13,15). It is possible that these sequences may have been present, but not at levels high enough to be revealed by screening 16S rDNA libraries constructed with bacterial or universal primers. For instance, WSRCP sequences were not detected from the analysis of RCP2 and FW bacterial 16S rDNA libraries (74 and 100 clones respectively), yet they were subsequently recovered using a

WSRCP specific primer. Likewise, the WSRCP group may be present in the samples testing negative, but not above the detection limit of our approach. Related but divergent sequences endemic to these systems might not be amplifiable with the primers utilized in this study. The 650f primer was designed from alignment of only three WSRCP sequences and as such may not be sufficiently inclusive to recover related sequences that may exist.

It is interesting that 16S rDNA sequences nearly identical (>99%) to one another were recovered from such geographically distant sources i.e. surface sediments in South Carolina and acid water 2 km deep in South Africa. There have been other examples of this phenomenon occurring in bioleaching environments. Sequences more than 99% similar to the Euryarchaeal clones SC12, SC36, and SC38, isolated from an extreme AMD system in Iron Mountain, CA (2), were recovered from the contaminated wetland system studied here (3). The phylogenetic association of the SC clones with the acidophilic heterotroph *Thermoplasma acidophila* supports the idea that they may represent acidophiles (2). In contrast, the phylogentic isolation of the WSRCP group offers no reasonable inference about the potential physiology of corresponding organisms. Therefore, the retrieval of nearly identical WSRCP sequences exclusively from low pH environments supports the hypothesis that they may represent acidophilic or, at the very least, acidotolerant organisms. A correlation between 16S rDNA sequences representing uncultivated organisms and a specific niche has also been demonstrated previously (15). Based on three independent studies, four clusters associated with the *Proteobacteria*, Green non-sulfur and the Low G+C gram positive

divisions contain only 16S rDNA sequences recovered from freshwater anaerobic dechlorinating consortia.

The WSRCP sequences appear to form a novel subdivision within the WS6 candidate division (Fig. 4.1). The bootstrap analysis supported this placement regardless of whether distance or parsimony algorithms were used. The phylogenetic position of this group was also stable upon altering the types and amount of sequences in the alignment. To our knowledge, sequences affiliated with this division have not been recovered previously from acidic environments (5). Prior to phylogenetic analysis, it was not obvious that these sequences would be associated with the candidate division WS6 due to a low sequence similarity and to the lack of a signature sequence deviation in the otherwise universally conserved 515F region of the 16S rRNA gene (5). However, the BMS clones contained within the WS6 division also do not possess this signature sequence and the percent 16S rDNA sequence dissimilarity between the WSRCP group and WS6 division is lower than the divergence contained within the later (26%) (5). We anticipate that additional WSRCP sequences eventually will be recovered which may consequently alter the group's phylogenetic position. However, based on the sequence information available at this time, we believe this group comprises a fifth subdivision of the WS6 and is perhaps limited in scope to acidic habitats.

We thank Alison Buchan, Wendy Ye, Duane Moser, Raunus Strepanasukas, and Nasreen Bano (in association with the Mono Lake Microbial Observatory) for donating DNA and environmental samples. We also appreciate Dave Singleton's assistance in sample collection at Horseshoe Bend. This research was supported by the Financial Assistance Award DE-FC09-96SR18546 from the U.S. Department of Energy.

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TABLE 4.1. WSRCP specific primers

Primer[a]	Sequence (5’ – 3’)	Optimal annealing temperature (°C)
650f	GATGGAACTTCTGGTGGAGTA	52
848r	TGCGTTAGCTGCGACACTCAT	57

[a] The number denotes the position of the 3’ end according to *E. coli* numbering.

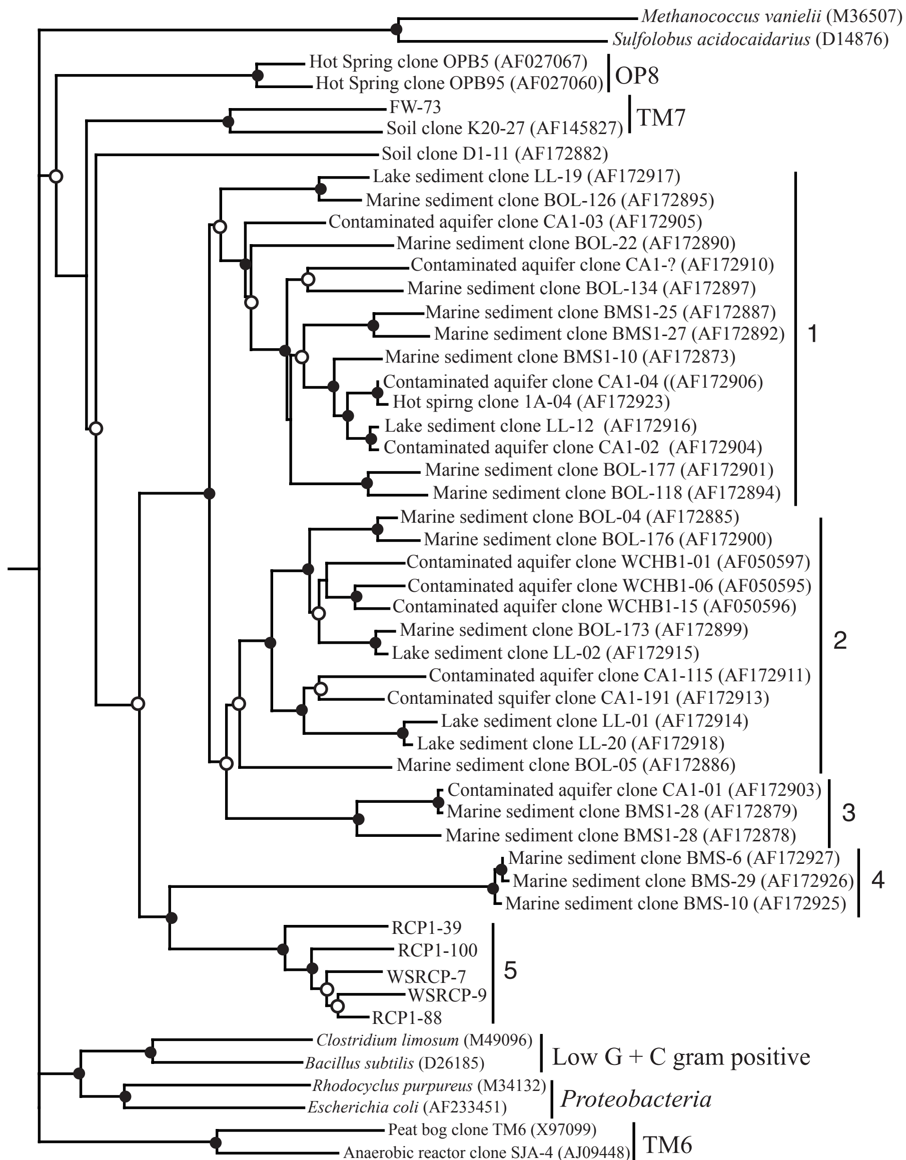
TABLE 4.2. Environmental samples analyzed for presence of the WSRCP group.

Sample type	WSRCP group detected	No. of clones screened[a]	OTU number[b]
AMD-contaminated wetland sediment (RCP1) (pH 2.5)			
5/9/00	yes	78	5
1/11/01	yes	63	5
AMD-contaminated wetland sediment (RCP2)(pH 3.0)			
5/9/00	yes	11	2
Uncontaminated forested wetland sediment (FW) (pH 5.2)	yes	10	1
Acid water, deep South African Mine (pH 2.8)	yes	3	1
Landfill top soil (pH 4.9)	no		
Salt marsh sediment, Sapelo Island, GA	no		
Salt marsh water column, Sapelo Island, GA	no		
Spartina alterniflora enrichment	no		
Tilled agricultural soil, Athens, GA	no		
Mono Lake, CA (2,19, 25, 26, 28 m) (pH ~10.0)	no		
Sacramento River, CA	no		

[a] For the RCP1 sample, clones were screened by direct sequencing and by RFLP, while the remaining samples were screened by the former.

[b] Sequences were grouped into OTUs based on sequence similarity of 97%.

FIG. 4.1 Dendrogram based on 457 aligned nucleotides showing phylogenetic placement of the WSRCP group among the four subdivisions of the candidate division WS6. Symbols at the nodes represent the bootstrap values out of 100 resamplings (closed circles >75%, open circles 50 to 74%). Values not supported statistically (<50%) are not shown. The scale bar represents 5 nucleotide substitutions per 100 nucleotides. Archaeal members *M. vanielii* and *S. acidocaidarius* were used as the outgroup



Methanococcus vanielii (M36507)
Sulfolobus acidocaidarius (D14876)
Hot Spring clone OPB5 (AF027067)
Hot Spring clone OPB95 (AF027060)
OP8
FW-73
Soil clone K20-27 (AF145827)
TM7
Soil clone D1-11 (AF172882)
Lake sediment clone LL-19 (AF172917)
Marine sediment clone BOL-126 (AF172895)
Contaminated aquifer clone CA1-03 (AF172905)
Marine sediment clone BOL-22 (AF172890)
Contaminated aquifer clone CA1-? (AF172910)
Marine sediment clone BOL-134 (AF172897)
Marine sediment clone BMS1-25 (AF172887)
Marine sediment clone BMS1-27 (AF172892)
Marine sediment clone BMS1-10 (AF172873)
Contaminated aquifer clone CA1-04 ((AF172906)
Hot spirng clone 1A-04 (AF172923)
Lake sediment clone LL-12 (AF172916)
Contaminated aquifer clone CA1-02 (AF172904)
Marine sediment clone BOL-177 (AF172901)
Marine sediment clone BOL-118 (AF172894)
1
Marine sediment clone BOL-04 (AF172885)
Marine sediment clone BOL-176 (AF172900)
Contaminated aquifer clone WCHB1-01 (AF050597)
Contaminated aquifer clone WCHB1-06 (AF050595)
Contaminated aquifer clone WCHB1-15 (AF050596)
Marine sediment clone BOL-173 (AF172899)
Lake sediment clone LL-02 (AF172915)
Contaminated aquifer clone CA1-115 (AF172911)
Contaminated squifer clone CA1-191 (AF172913)
Lake sediment clone LL-01 (AF172914)
Lake sediment clone LL-20 (AF172918)
Marine sediment clone BOL-05 (AF172886)
2
Contaminated aquifer clone CA1-01 (AF172903)
Marine sediment clone BMS1-28 (AF172879)
Marine sediment clone BMS1-28 (AF172878)
3
Marine sediment clone BMS-6 (AF172927)
Marine sediment clone BMS-29 (AF172926)
Marine sediment clone BMS-10 (AF172925)
4
RCP1-39
RCP1-100
WSRCP-7
WSRCP-9
RCP1-88
5
Clostridium limosum (M49096)
Bacillus subtilis (D26185)
Low G + C gram positive
Rhodocyclus purpureus (M34132)
Escherichia coli (AF233451)
Proteobacteria
Peat bog clone TM6 (X97099)
Anaerobic reactor clone SJA-4 (AJ09448)
TM6
0.05

CHAPTER 5

MOLECULAR ANALYSIS OF BACTERIAL COMMUNITIES ASSOCIATED WITH THE SEDIMENT OF A BOTTOMLAND HARDWOOD FOREST.

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Abstract

The phylogenetic diversity of bacteria inhabiting a South Carolina bottomland hardwood forest sediment was determined by the analysis of PCR amplified 16S rDNA sequences. Genomic DNA extracted from composite sediment samples was used to construct 100 clones which were grouped into 86 operational taxonomic units (OTUs) each with at least 97% 16S rRNA gene sequence similarity over about 500 nt. The majority of OTUs recovered (67%) were affiliated with the *Acidobacterium*, delta *Proteobacteria*, alpha *Proteobacteria*, Green-non sulfur, and the *Nitrospira* taxa. The phylogenetic placement of several sequences suggested the corresponding organisms may possess an anaerobic metabolism or tolerance to acidic habitats, consistent with features of the system at the time of sampling. While the division and subdivision-level groups detected were similar to those obtained from other freshwater systems, the specific taxa comprising these groups were generally distinct. Conspicuously novel groups included a possible subdivision within the *Acidobacterium* division along with a divergent *Nitrospira* clade (collectively comprising 12% of recovered sequences). Roughly one quarter of the recovered sequences were less than 90% similar to any recovered 16S rDNA, indicating that this site harbors bacterial diversity not yet encountered in other habitats.

Introduction

Wetlands represent ecotones between terrestrial and aquatic habitats that have been traditionally difficult to delineate since they possess features of both systems [1]. While a wide variety of wetland types exist, they collectively share the distinguishing characteristics of hydric soils, saturating conditions and the establishment of plants adapted to withstand periods of anoxia [2]. Wetland soil is often referred to as hydric due to the anaerobic conditions that inevitably develop within the upper horizons upon prolonged inundation. The decreased diffusion rate of oxygen in water coupled with bacterial respiration of surface deposited organic matter result in the development of anaerobic conditions. Yet even during saturating conditions, wetland sediments do not become completely anoxic; along with a shallow oxygenated zone at the sediment surface, oxygen leakage from hydrophyte aerenchyma is known to aerate the rhizosphere [2]. The resulting oxygen and redox gradients presumably contribute to niche complexity in these dynamic systems and as such, wetland sediments should harbor one of the more diverse bacterial community compositions.

The majority of wetland area in North America is located inland and includes freshwater marshes, northern peatlands, southern deepwater swamps, and riparian forested wetlands [2]. Riparian wetlands located in the southeast (also referred to as bottomland hardwood forests) represent one of the most extensive wetland classes in the U.S., estimated to consist of approximately 6.6 – 13 million hectares [3]. This region includes the Mississippi floodplain and Atlantic coast rivers and is characterized by the presence of woody vegetation. These systems are also significant due to their ability to remove and transform substances (i.e. nutrients, toxins, suspended sediments) originating

upland, thus limiting deposition in downstream systems [4]. For instance, bottomland hardwood forests are estimated to be capable of removing up to 80-90% of the non-point source total nitrogen and phosphorous respectively [4]. While the filtering function of wetlands can be attributed to several of their components (i.e., plant uptake and sediment trapping), the indigenous microbial communities are thought to be responsible for the majority of this processing.

The 16S rRNA approach is a culture-independent means to describe the bacterial types present in an environmental sample [5]. The inventorying of various habitats has confirmed that traditional cultivation techniques do not representatively sample environmental bacteria. The types of environmentally derived sequences and bacterial isolates recovered from the same habitat generally differ from one another. Several of these environmentally derived 16S rDNA sequences correspond to deeply divergent lineages that form novel divisions and subdivisions within the bacterial domain [5]. The diversity of bacteria as revealed by 16S rDNA-based analysis has been examined in freshwater environments [6], yet few such surveys have been directed toward wetland habitats. These studies have been limited to northern peat [7], a Carolina Bay [8] and a flooded rice paddy [9], but to our knowledge have not included a riparian forested wetland.

In a complementary study, the bacterial community composition of a southeastern forested wetland impacted by runoff from an exposed reject coal pile was determined over a contamination gradient [10]. The diversity and composition of 16S rDNA sequences directly extracted from composite sediments of two contaminated areas were compared to an unaffected region of the wetland. From this latter sample, 100 16S rDNA

clones were sorted into operational taxonomic units (OTUs) based on 97% sequence similarity within the region 90-620 nt (*E. coli* numbering). The OTU richness along with the relative distribution of OTUs among the major bacterial taxa was compared to those retrieved from the contamination areas of the wetland system. In the present study, we describe the phylogenetic diversity of the bacterial community present in the forested wetland sediment by 16S rDNA sequence analysis, focusing on the relatively abundant taxa recovered.

Material and methods

The forested wetland sampled for this study, located within the U.S. Department of Energy's Savannah River Site (SRS) D-area (400D), is in close proximity to the Savannah River. This area harbors loblolly pine (*Pinus taeda* L.), sweetgum (*Liquidambar styraciflua* L.), wax myrtle (*Myrica cerifera* L.) and broom sedge (*Andropogon virginicus* L.) and typically floods during the winter through spring. These features are most consistent with a zone IV designation (based on plant community composition and hydroperiod) for bottomland forests [11]. The samples were collected on May 9, 2000 when the sediment surface was inundated several centimeters and the surface temperature was 29°C. The sediment was light grey in color (indicative of reduced iron) and covered with a thin humic layer. Twenty-five composite surface sediment cores (1.5 cm diameter, 2.0 cm depth) were dried for chemical analysis. A pH value of 5.2 was determined by measurement of dried sediment mixed 1:1 with distilled deionized water. Analysis by an Inductively Coupled Plasma (ICP) Emission Spectrometer of sediment prepared using EPA method 3050 revealed the following element concentrations (ppm): phosphorous (404.3), iron (15,119), calcium (1662.4),

magnesium (457.8). Sulfate levels were determined to be 16,630 ppm by analysis with a Braun+Luebbe Auto Analyzer II Continuous Flow System. The University of Georgia Chemical Analysis Laboratory conducted the ICP and sulfate analysis. Organic matter content was determined to be 9.7% by the loss on ignition method for 3 hrs at 360°C and total nitrogen (1446 ppm) was measured by the University of Georgia's Agricultural and Environmental Services Laboratory.

Details concerning sample collection, construction of the bacterial 16S rDNA PCR library, and the way in which the phylogenetic assessment of the sequences was determined are described elsewhere [10]. Briefly, bulk community DNA was extracted from 25 composite surface sediment cores (1.5 cm diameter, 2.0 cm depth) using the soil DNA Mega extraction kit (MoBio Laboratories). Five independent PCR amplification reactions (15 cycles) designed to selectively enrich for *Bacteria* 16S rRNA genes were pooled for cloning into the pCR 2.1 TA vector (Invitrogen). One hundred clones were partially sequenced and those having >97% similarity within 90-620 nt (*E. coli* numbering) were considered one operational taxonomic unit (OTU). One representative from each OTU was sequenced more completely (>1150 bp). These sequences have been assigned GenBank accession numbers (x –x) and are referred to as FW clones (abbreviation for forested wetland). Programs contained in the PHYLIP package were used to construct phylogenetic trees. The Jukes-Cantor model was used to calculate sequence dissimilarities (DNADIST) and the dendrograms were determined from the distance matrices using the Fitch-Margoliash least squares algorithm (FITCH). When necessary, phylogenetic groupings were tested further by performing parsimony (DNAPARS) and also by employing different division-level outgroups on the same

alignment [12]. To test the significance of the inferred topologies, bootstrap values (SEQBOOT and CONSENSE) were calculated from 100 resamplings.

Results and discussion

Although a few freshwater sediment types have been sampled for their bacterial 16S rDNA composition, as far as we are aware, this is the first culture-independent survey of a forested wetland sediment. Similar to other freshwater sediments, the diversity of sequences was high; the coverage curve for the library was a straight line [10] indicating that the screening extent did not sufficiently sample the bacterial diversity present. No single 16S rDNA sequence was retrieved more than once and few OTUs contained more than one member, with partial sequencing grouping 100 16S rDNA sequences into 86 unique OTUs at a similarity level of 97%. This suggests that, at least at the sampling scale utilized, this environment does not harbor numerically dominating bacterial species. We did not determine any of our FW clones to be chimeric molecules, but one of our delta *Proteobacteria* 16S rDNAs, FW-18, was truncated. We were unable to determine the phylogenetic placement of FW-61 (73.2% similar to the closest 16S rDNA database sequence) and it was unclear whether this sequence represents a novel division-level lineage.

The majority of the 86 OTUs recovered represent members of 5 bacterial taxa: *Acidobacterium*, delta *Proteobacteria*, Green-non sulfur, alpha *Proteobacteria*, and *Nitrospira* (Fig. 5.1), with approximately 36% of the OTUs affiliated with the division *Proteobacteria*. No 16S rDNA sequence obtained here was identical to any contained within the public database, yet many were closely related to other environmentally derived sequences (50% were 92-99% similar). Twenty three percent of the OTUs were

less than 90% similar to any sequence present in the database, with roughly half of these only 85% similar. This indicates that a significant portion of the dominant community members represent sequences distantly related to those recovered from the multitude of 16S rDNA surveys conducted.

***Acidobacterium* and Green non-sulfur sequences.**

The largest portion of recovered 16S rDNA sequences (21% OTUs) were affiliated with subdivisions I, II, III and VII of the *Acidobacterium* (Fig. 5.2). *Acidobacterium capsulatum* (a moderate acidophile; growth range pH 3.0-6.0) or environmentally derived sequences recovered from acidic habitats (DA,TM,UA, and WD clones) represent the closest relatives of most of these sequences. A cluster of sequences comprised of FW-32, 105, and 144 (83.6-84.7% similar to database sequences) represents either an extension of subdivision VII or a novel *Acidobacterium* subdivision.

Approximately 10.5% of the OTUs retrieved were affiliated with the Green-non sulfur (GNS) subdivisions I and II, the former of which contains no cultivated representatives [5]. All but one of the GNS-related FW clones were less than 91% similar to any 16S rDNA sequence in the database. Both the *Acidobacterium* and GNS divisions consist primarily of 16S rDNA sequences recovered in culture-independent studies, particularly those of soil and sediment habitats [5]. Consequently, little is known about the organisms comprising these groups.

Delta *Proteobacteria* sequences

Aside from the *Acidobacterium* division, the next largest group OTUs (20%) recovered were associated with the delta *Proteobacteria* (Fig. 5.3). This group contains a higher proportion of cultivated organisms, thereby allowing hypotheses concerning the

potential metabolic capacity of the organisms representing 16S rDNA sequences based on their phylogenetic context. Inference of phenotypic potential based on phylogeny alone is questionable, as organisms having a high 16S rDNA relatedness can possess distinct metabolic capabilities. However, if a sequence clusters within a monophyletic group that shares a particular phenotype, then it is reasonable to speculate the corresponding organism may also possess that property, particularly if the features of the system support this contention [14]. For instance, several FW sequences were associated with organisms having an anaerobic metabolism, including sulfate reducing bacteria (SRB). The sequence FW-133 clustered (93.0 % similarity) with the genus *Desulfomonile*, members of which are obligately anaerobic and respire sulfur compounds along with 3-chlorobenzoate [15]. Four additional OTUs clustered (~90% similarity) with the sulfate reducer *Desulfobacca acetooxidans*. FW sequences were also associated with organisms capable of iron reduction and fatty acid fermentation. FW-140 grouped (94.0% similarity) with the genus *Geobacter*, members of which have been cultivated from a wide variety of sediments and are capable of using ferric iron, metals and humic acids as terminal electron acceptors [16]. Two additional OTUs cluster with the genera *Syntrophus* and *Smithella* (92.5-93.2% similarity), bacteria capable of fatty acid fermentation in the presence of either H_2 or formate utilizers such as methanogens and SRB.

Two sequence types (FW-35 and FW-46) were associated (89.2-95.0% similarity) with the myxobacteria, members of which are heterotrophs that form multicellular structures in response to nutrient limitation [17]. Myxobacteria glide over surfaces, producing extracellular enzymes that degrade macromolecules as well as other

microorganisms, with *Polyangium* (*"Sorangium"*) *cellulosum* capable of utilizing cellulose. The sequence FW-35 was 95% similar to the recently characterized [18] *Anaeromyxobacter dehalogans*, which is capable of using chlorophenolic compounds in addition to nitrate and fumerate as terminal electron acceptors. This organism is the first facultative anaerobic representative within myxobacteria. Despite the common isolation of myxobacteria from soil and sediments, this group is rarely encountered in culture-independent 16S rDNA surveys of these same environments.

Additional delta *Proteobacteria* sequences either formed novel lineages or clustered with other environmental sequences. Two OTUs (FW-15 and FW-31) were found to be related (97.8-87.1% similar) to the clone 4-29, which was originally proposed to be a member of the division *Nitrospira*. Upon repetitive phylogenetic analysis, this sequence along with the related FW clones were determined to instead be associated with the delta *Proteobacteria* at significant bootstrap values.

Alpha *Proteobacteria* sequences.

Approximately 9.3% of the FW OTUs were affiliated with the alpha *Proteobacteria* (Fig. 5.4). The OTU represented by the clone FW-94 was nearly identical (99.0%) to the 16S rRNA gene of the nitrogen-fixing leguminous symbiont *Bradyrhizobium elkanii*. Several of the remaining sequences were associated with environmental clones and isolates recovered from mildly acidic habitats (pH 3-5), similar to the pH of our system. FW-131 was closely affiliated (97% sequence similarity) with the acidophilic methanotroph *Methylocella palustris*, isolated from a bog [19], while FW-8 was associated with anaerobic phototroph *Rhodopseudomonas acidophila* (96.3%

sequence similarity). Included within this cluster of sequences is the isolate *Beijerinckia indica* (not shown), which has a pH optimum of 5.0, along with several UP and TM 16S rDNA sequences recovered from low pH soil and sediments [7,20]. FW-91 and FW-138 cluster with the acidic grassland clone DA1222 and with the environmentally derived sequence MHP14, amplified with methanotrophic-specific primers from northern peat [21]. Collectively, this suggests that the organisms corresponding to these FW sequences may be adapted to surviving acidic niches.

***Nitropira* sequences.**

Six OTUs (9% of sequences) retrieved from the FW library formed a cluster within the division *Nitrospira* which included the salt marsh clone LCP-6 (88.3-95.1% similarity) (Fig. 5.5). Members of the genera *Magnetobacterium* and *Thermobacterium* represent the most closely related cultivated organisms, albeit distantly related. The phylogenetic placement of this group is not conducive for speculating about the phenotype of the corresponding organisms.

Additional taxa.

Four OTUs were members of the Candidate division OP11 (5%); 3 of these sequences, FW-9, 17, and 129 represented clones which are only 80-82.7% related to any known 16S rRNA sequence. Little information about the characteristics of this division exists except that it is comprised solely of environmentally derived sequences, primarily ones recovered from soil/sediment samples habitats [5]. Though not abundantly represented, the retrieval of clones associated with the candidate divisions TM7, OP10, WS5, PBS-25, and BD further substantiates that these groups represent actual bacterial lineages rather than PCR artifacts.

The phylogenic placement of several FW sequences was consistent with features of a study site that is predominantly anaerobic, mildly acidic and contains high sulfate levels. Several OTUs clustered with bacteria having an anaerobic metabolism and with bacteria and environmentally derived 16S rDNAs obtained from acidic habitats. This later group includes *Beijerinickia*-related 16S rDNAs (contained within the alpha *Proteobacteria)* and those of the division *Acidobacterium*, both of which have been noted to co-occur in 16S rDNA libraries generated from acidic sediments [7,20,22,23].

The division and subdivision-level groups encountered in the FW library were similar to those recovered from other freshwater sediment 16S rDNA surveys (Table 1), with the *Acidobacterium* division along with the beta and delta *Proteobacteria* among the most numerically abundant and the *Verrucomicrobium* division along with the alpha, beta, delta *Proteobacteria* among the most consistently represented. Beta *Proteobacteria* sequences, a major constituent in freshwater sediments, represented only 4% of the FW clones screened. This discrepancy may be due to differences in oxygen content among sampled environments. Sequences associated with this subdivision have been observed to be enriched in the water column [6] and the oxic sediment layer [24] compared to the sediment and lower anoxic layers of freshwater habitats, respectively, and as such are hypothesized to be primarily associated with aerobic niches. A notable distinction between the FW library and those of other freshwater sediments was the presence of *Nitrospira* sequences in the former. *Nitrospira* sequences were not recovered from any other freshwater sediment survey and do not represent numerically abundant constituents of soil/sediment 16S rDNA libraries [5]. Though this group comprised a significant

percentage of the FW library, the sequences were not spread out through the division, but rather formed a cluster having 12% divergence.

Aside from division and subdivision-level commonalties among analogous habitats, there were instances of smaller-scale (possible genus and species) similarities, particularly with samples having similar pH values. Of the other wetlands surveyed for their bacterial 16S rDNA diversity, Rainbow Bay is most similar to the forested wetland examined here [8]. Both systems are located within the SRS site, situated on the Atlantic Coastal Plain, and are characterized by mildly acidic pH values and fluctuating water levels. However, the vegetation of these sites differ with the FW site harboring woody vegetation and Rainbow Bay, an pond surrounded by marsh and forested wetlands. While the cell lysis and PCR protocol dramatically differed between the two studies, 18% of the FW sequences were 92-96% similar to those recovered from Rainbow Bay (RB clones). The only other environmental 16S rDNA library that possessed a similar degree of sequence similarity was also generated from an acidic sample. Twenty percent of the FW clones were 92-97% similar to sequences recovered from an acidic (pH 4-5) drained polychlorinated biphenyl polluted moorland (WD clones) [23]. The majority of these (80%) were affiliated with subdivisions I, II, and III of the *Acidobacterium*. For comparison, 9 and 4% of the FW library were similar to clones obtained from a northern peat (pH 2.7) (TM clones) [7] and a saturated rice paddy (BSV clones) [9], respectively. These numbers suggest that the majority of sequences here were distinct from those obtained from other freshwater habitats. While this may be due to methodological differences in lysis and PCR protocols, it is more likely due to distinct features of this system. Furthermore, the proportion of similar sequences shared between habitats might

be higher if either library were screened more extensively. The screening applied in all of these studies does not come close to approximating the number of distinct organisms suggested to occupy soil and sediment habitats [25].

The phylogenetic characterization of the bacteria inhabiting a given system is a necessary first step toward the elucidation of their structure, function and dynamics. While this study represents one sampling event, the FW library was generated in a manner which should increase the likelihood of obtaining a representative sample; composite sediment cores were collected and PCR reactions performed using a low amplification cycle number (15 cycles) were pooled prior to cloning. Taking the known biases associated with PCR in to consideration [26], we believe it is still reasonable to assume the FW library is fairly representative of the bacterial types present in this system during flooding periods. However, it is important to note that the bacterial communities associated with bottomland hardwood forests may differ dramatically between systems. Within a system, physical changes (including topography, soil type, and hydroperiod) contribute to differences in plant composition that occur over a transriparian gradient [27]; bacterial community composition may correspondingly also vary. The degree to which the bacterial structure changes over such gradients along with questions addressing the dynamics and major functions of the bacteria present in these systems warrants further study.

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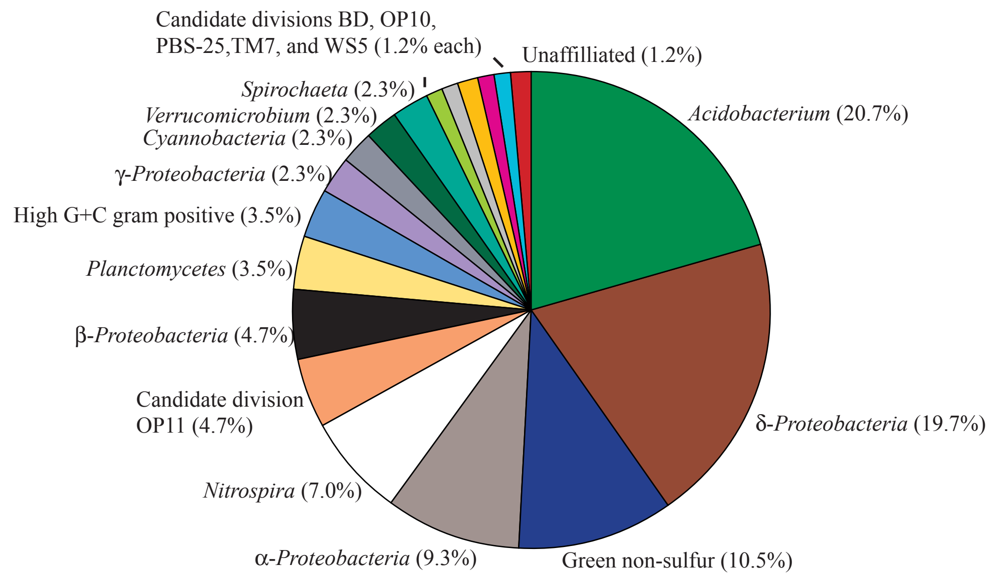
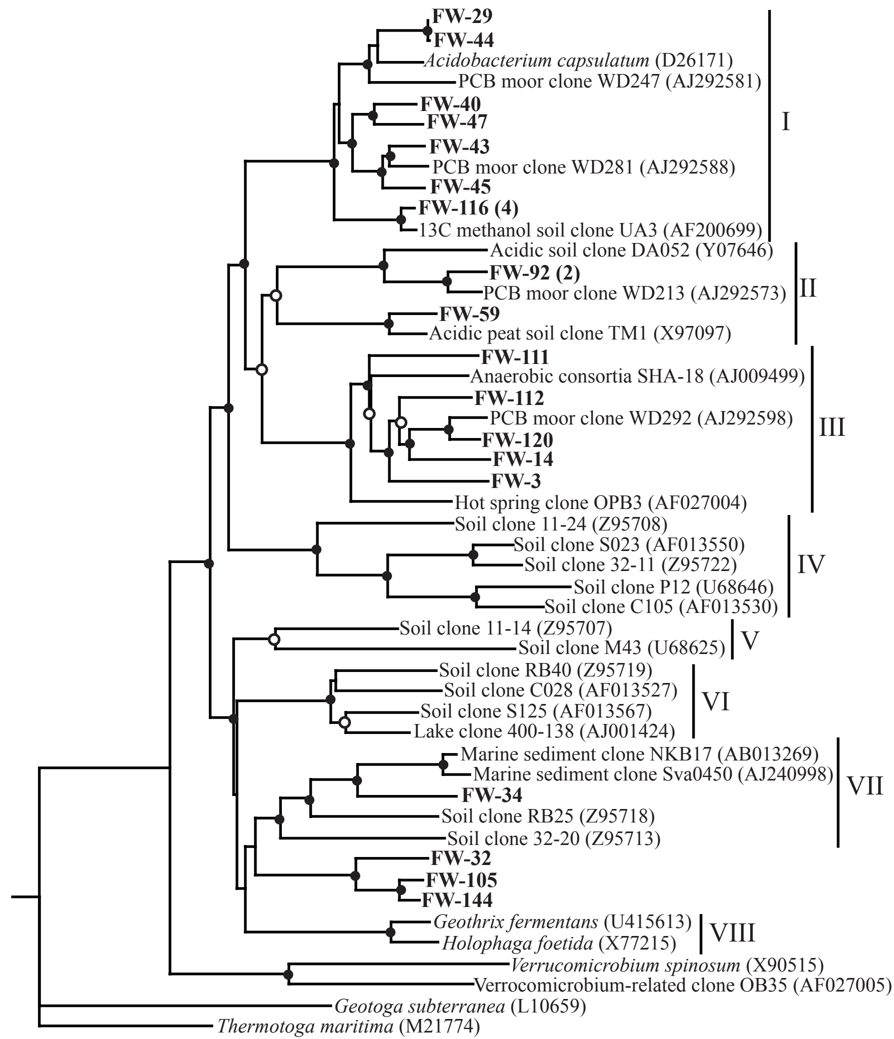
FIG. 5.1. The distribution of 16S rDNA sequences among the bacterial divisions and subdivisions detected in the FW library. Numbers in parentheses indicate the percentage of library clones affiliated with the various taxa.



Candidate divisions BD, OP10,
PBS-25,TM7, and WS5 (1.2% each)
Unaffilliated (1.2%)
Spirochaeta (2.3%)
Verrucomicrobium (2.3%)
Cyannobacteria (2.3%)
γ-*Proteobacteria* (2.3%)
High G+C gram positive (3.5%)
Planctomycetes (3.5%)
β-*Proteobacteria* (4.7%)
Candidate division
OP11 (4.7%)
Nitrospira (7.0%)
α-*Proteobacteria* (9.3%)
Green non-sulfur (10.5%)
δ-*Proteobacteria* (19.7%)
Acidobacterium (20.7%)

FIG. 5.2. Dendrogram based on 846 aligned nucleotides showing phylogenetic placement of FW clones affiliated within the 8 subdivisions of the *Acidobacteria.* The number within the parentheses denotes the clone number contained within the OTU. Symbols at the nodes represent the bootstrap values out of 100 resamplings (closed circles >75%, open circles 50 to 74%). Bootstraps not supported statistically (<50%) are not shown. The scale bar represents 5 nucleotide substitutions per 100 nucleotides. Members of the bacteria division *Thermotoga* were used as the outgroup.



FW-29
FW-44
Acidobacterium capsulatum (D26171)
PCB moor clone WD247 (AJ292581)
FW-40
FW-47
FW-43
PCB moor clone WD281 (AJ292588)
FW-45
FW-116 (4)
13C methanol soil clone UA3 (AF200699)
I
Acidic soil clone DA052 (Y07646)
FW-92 (2)
PCB moor clone WD213 (AJ292573)
FW-59
Acidic peat soil clone TM1 (X97097)
II
FW-111
Anaerobic consortia SHA-18 (AJ009499)
FW-112
PCB moor clone WD292 (AJ292598)
FW-120
FW-14
FW-3
Hot spring clone OPB3 (AF027004)
III
Soil clone 11-24 (Z95708)
Soil clone S023 (AF013550)
Soil clone 32-11 (Z95722)
Soil clone P12 (U68646)
Soil clone C105 (AF013530)
IV
Soil clone 11-14 (Z95707)
Soil clone M43 (U68625)
V
Soil clone RB40 (Z95719)
Soil clone C028 (AF013527)
Soil clone S125 (AF013567)
Lake clone 400-138 (AJ001424)
VI
Marine sediment clone NKB17 (AB013269)
Marine sediment clone Sva0450 (AJ240998)
FW-34
Soil clone RB25 (Z95718)
Soil clone 32-20 (Z95713)
VII
FW-32
FW-105
FW-144
Geothrix fermentans (U415613)
Holophaga foetida (X77215)
VIII
Verrucomicrobium spinosum (X90515)
Verrocomicrobium-related clone OB35 (AF027005)
Geotoga subterranea (L10659)
Thermotoga maritima (M21774)
0.05

FIG. 5.3. Dendrogram based on 1170 aligned nucleotides showing phylogenetic placement of FW clones affiliated within delta *Proteobacteria.* Members of the division *Verrucomicrobium* were used as the outgroup.

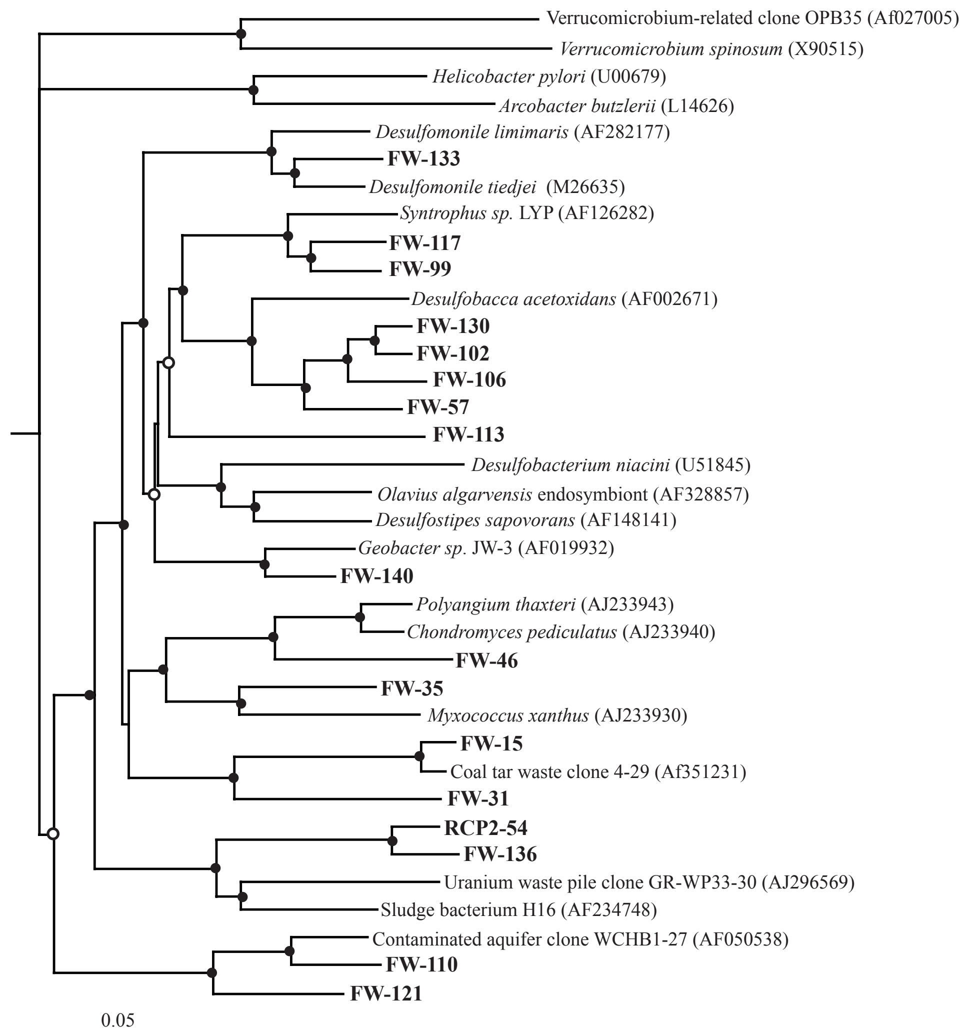
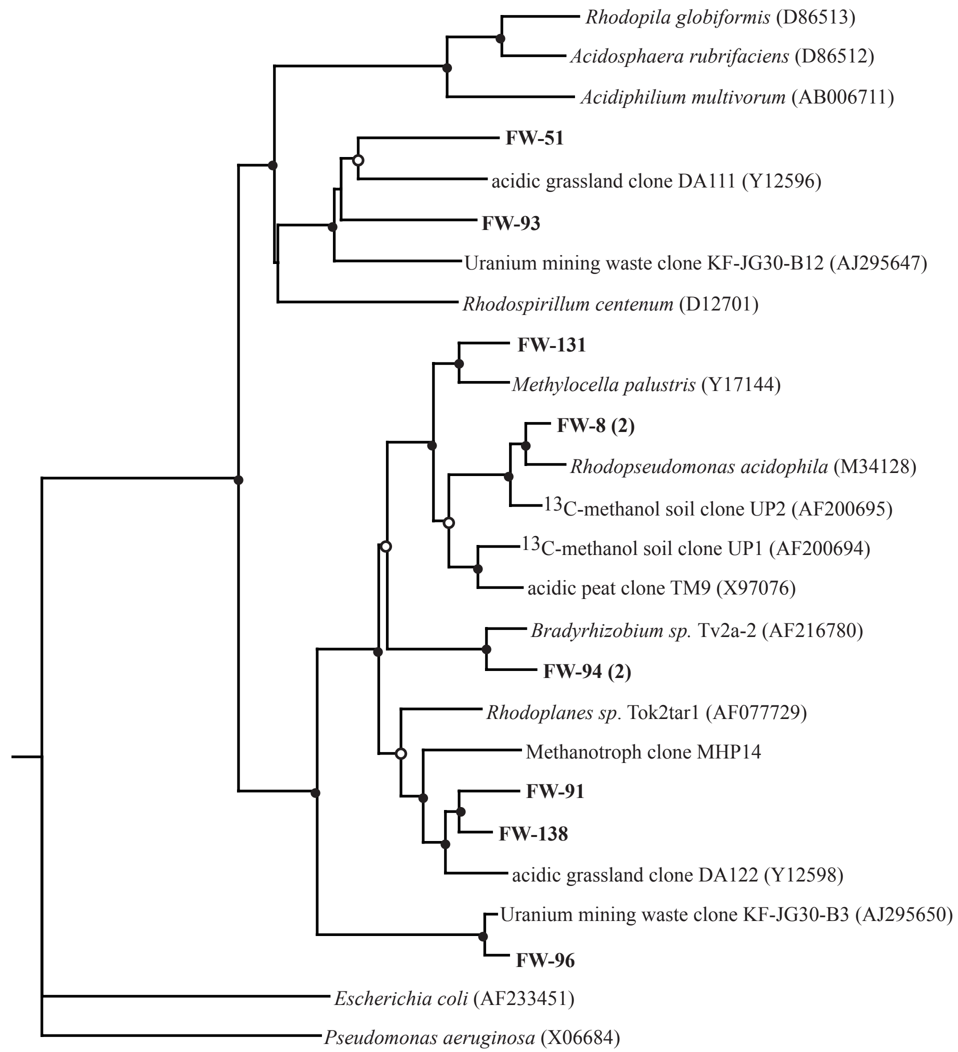
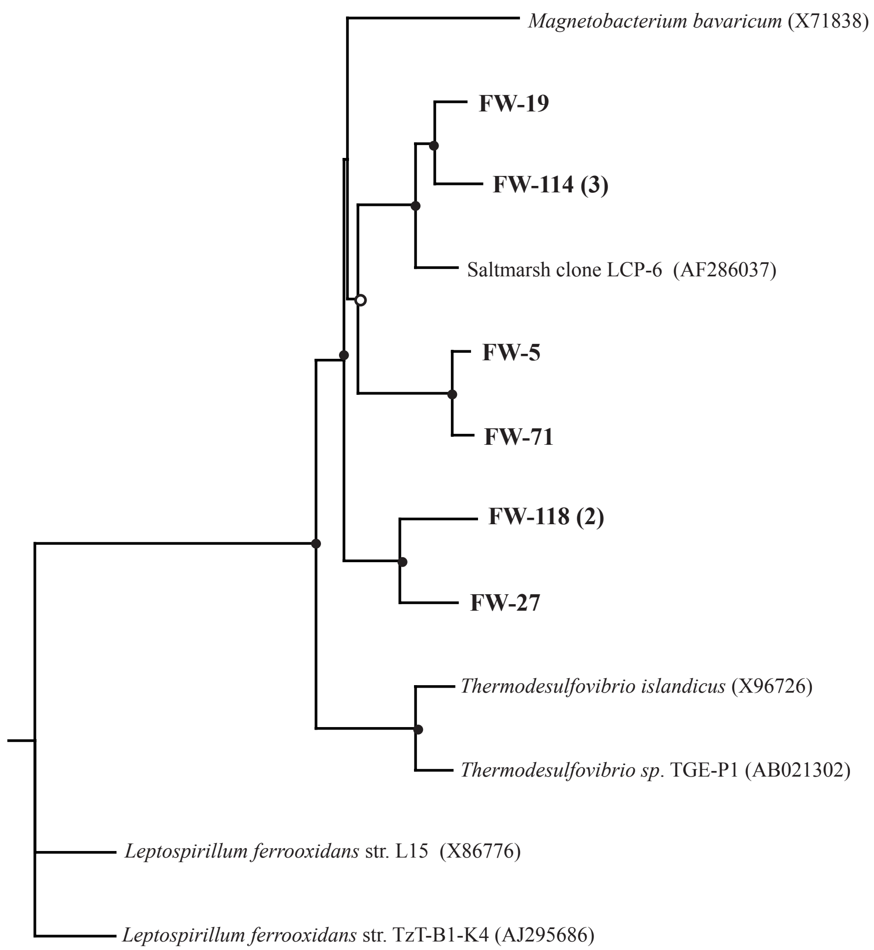

Verrucomicrobium-related clone OPB35 (Af027005)
Verrucomicrobium spinosum (X90515)
Helicobacter pylori (U00679)
Arcobacter butzlerii (L14626)
Desulfomonile limimaris (AF282177)
FW-133
Desulfomonile tiedjei (M26635)
Syntrophus sp. LYP (AF126282)
FW-117
FW-99
Desulfobacca acetoxidans (AF002671)
FW-130
FW-102
FW-106
FW-57
FW-113
Desulfobacterium niacini (U51845)
Olavius algarvensis endosymbiont (AF328857)
Desulfostipes sapovorans (AF148141)
Geobacter sp. JW-3 (AF019932)
FW-140
Polyangium thaxteri (AJ233943)
Chondromyces pediculatus (AJ233940)
FW-46
FW-35
Myxococcus xanthus (AJ233930)
FW-15
Coal tar waste clone 4-29 (Af351231)
FW-31
RCP2-54
FW-136
Uranium waste pile clone GR-WP33-30 (AJ296569)
Sludge bacterium H16 (AF234748)
Contaminated aquifer clone WCHB1-27 (AF050538)
FW-110
FW-121
0.05

FIG. 5.4. Dendrogram based on 1090 aligned nucleotides showing phylogenetic placement of FW clones affiliated within the alpha *Proteobacteria.* Members of the gamma *Proteobacteria* were used as the outgroup



Rhodopila globiformis (D86513)
Acidosphaera rubrifaciens (D86512)
Acidiphilium multivorum (AB006711)
FW-51
acidic grassland clone DA111 (Y12596)
FW-93
Uranium mining waste clone KF-JG30-B12 (AJ295647)
Rhodospirillum centenum (D12701)
FW-131
Methylocella palustris (Y17144)
FW-8 (2)
Rhodopseudomonas acidophila (M34128)
13C-methanol soil clone UP2 (AF200695)
13C-methanol soil clone UP1 (AF200694)
acidic peat clone TM9 (X97076)
Bradyrhizobium sp. Tv2a-2 (AF216780)
FW-94 (2)
Rhodoplanes sp. Tok2tar1 (AF077729)
Methanotroph clone MHP14
FW-91
FW-138
acidic grassland clone DA122 (Y12598)
Uranium mining waste clone KF-JG30-B3 (AJ295650)
FW-96
Escherichia coli (AF233451)
Pseudomonas aeruginosa (X06684)
0.05

FIG. 5.5. Dendrogram based on 1270 aligned nucleotides showing phylogenetic placement of FW clones affiliated within the division *Nitrospira. Leptospirillum ferrooxidans* was used as the outgroup.



Magnetobacterium bavaricum (X71838)
FW-19
FW-114 (3)
Saltmarsh clone LCP-6 (AF286037)
FW-5
FW-71
FW-118 (2)
FW-27
Thermodesulfovibrio islandicus (X96726)
Thermodesulfovibrio sp. TGE-P1 (AB021302)
Leptospirillum ferrooxidans str. L15 (X86776)
Leptospirillum ferrooxidans str. TzT-B1-K4 (AJ295686)
0.05

CHAPTER 6

CONCLUSIONS

Understanding the bacterial community structure of acid leaching habitats is important because of the role of bacteria in the generation of acid mine drainage (AMD) and the potential of bacteria to increase the efficiency of commercial bioleaching operations. We chose to examine the bacterial community structure of the subject system by the use of culture-independent techniques since it has been demonstrated that the traditionally cultivated AMD bacteria may not represent the dominant constituents within some of these systems (Schrenk *et al*., 1988).

Our survey analysis recovered 16S rDNAs highly related to previously cultivated organisms: *Acidiphilium* sp*, Acidobacterium capsulatum, Ferromicrobium acidophilium*, and *Leptospirillium ferrooxidans*. However*, At. ferrooxidans* was not a significant component (0.6 % of all sequences) and the majority of *Leptospirillium*-related sequences were ones comprising an uncultivated group, referred to as *Leptospirillium* group III. This finding is consistent with observations made at Iron Mountain, an extreme AMD habitat (Schrenk *et al*., 1988; Bond *et al*. 2000), rather than with observations made in comparatively less extreme bioleaching systems, similar to the one studied here (Lane *et al*. 1985; Goebel and Stakebrandt, 1994). Several recovered sequence types were related to those obtained from Iron Mountain or were phylogenetically associated with isolates and environmental sequences originating in acidic habitats. Still others were not highly related to any database sequence, refuting the idea that ambient AMD-type systems consist primarily of cultivated bacteria (Lane *et al*. 1985; Goebel and Stakebrandt, 1994). One group, termed WSRCP, appears to form a novel subdivision within the candidate division WS6. This phylogenetic placement may be premature since the number of sequences associated with this group is small; recovery of additional sequences may

reveal this group to instead represent a novel division-level lineage. Using specific primers, we recovered additional related sequences and determined that the distribution of this group appears to be limited to acidic habitats. Additional testing of other environments, particularly bioleaching habitats, should be carried out to substantiate this trend.

While 16S rDNA sequence cataloguing only generates descriptive data, the resulting information provides a foundation to address more complicated questions concerning a bacterial community. We monitored the bacterial communities associated with two chemically distinct sites (RCP1 and RCP2) within the contaminated wetland over the course of one year by terminal restriction fragment polymorphism (T-RFLP). The predominantly recovered 16S rDNA sequences corresponded to the abundant types in the system throughout the year as determined by T-RFLP. These groups included sequences that have been previously linked to bioleaching habitats (members of the *Acidobacterium* division and the *Acidimicrobida*e group of the *Actinomycetes,* as well as *Leptospirillium* groups) as well as groups not previously known to represent major types in these systems (sequences related to RCP1-48, TRA5-3 and TRB3). Future work conducted in this habitat, and perhaps similar ones, should focus on these groups and work to cultivate them. This project sought to determine whether spatial or temporal patterns could be observed in the distribution of these groups and, if so, whether environmental parameters be correlated to these changes. Identification of such relationships should provide ecological insight on these as yet uncultivated organisms.

Both the library analysis and the T-RFLP analysis characterized the bacterial communities associated with RCP1 and RCP2 sites as distinct. Only 15% of the

sequences recovered from these sites were >97% similar to one another and application of the statistic LIBSHUFF determined that the sequences recovered from each site were derived from distinct populations. The T-RFLP data suggested that the two sites differed more in terms of the percent relative abundance rather than the presence or absence of particular taxa. Comparison of communities by T-RFLP may underestimate distinctions in composition since it does not provide the same degree of resolution as sequencing (i.e. multiple taxa, related and unrelated, can form the same peak). Both analyses found that sequences related to the *Acidobacterium* were relatively more abundant in the less acidic RCP2 site whereas the *Actinomycete* sequences were proportionally more important in the more acidic RCP1 site. The *Leprospirillium* sequences (when percent moisture is low) and the *Actinomycete* group were proportionally higher in the RCP1 site. This observation, along with the fact that these sequence groups are phylogenetically associated with bacteria capable of iron oxidation, suggests that these groups may play a role in pyrite oxidation.

We were able to observe strong temporal patterns with some of the predominant sequence groups. Within a site, *Leptospirillium*, TRA5-3 and TRB3 groups exhibited large shifts that coincided with changes in percent moisture. The temporal variation of these constituents may be cyclical since the relative abundances were similar between samples taken at roughly the same time one year later (T0/T1 and T10).

These studies raise some interesting questions. First, it appears that *Leptospirillium* sequences are predominant in this system, however the relative abundance of the *Leptospirillium* groups II and III is not clear. The latter group is particularly interesting as it composed exclusively of as yet uncultivated bacteria and has

been demonstrated to represent the predominant *Leptospirillium* type in the Iron Mountain system (Bond 2000). Sequence analysis conducted at two different times (T0 and T1) also suggests that may be the case in the RCP system. Unfortunately, our T-RFLP approach did not discriminate between the two types. Application of a higher resolution technique would be needed to determine whether *Leptospirillium* group III is the predominant type in the system. The temporal study also did not address the relative contribution of the archaeal groups compared to the bacterial ones. The diversity of Archaea sequences was limited to two dominant groups: those associated with the *Thermoplasmales* or a novel *Euarchaea* group. In addition to determining their contribution compared to the bacterial groups, it would be interesting to determine whether they also exhibited a temporal trend.

Although the focus of this project centered on the bacteria associated with an extreme system, we also generated new information about the bacterial types associated with the unaffected forested wetland sediment. In general, bacterial 16S rDNAs recovered represented unique types, many of which were phylogenetically associated with environmentally derived sequences or organisms isolated from mildly acidic or anaerobic habitats, consistent with the predominant features of this systems. We recovered a novel lineage within the *Nitrospira*, a division not commonly retrieved from 16S rDNA analysis of sediments or soil, as well as a novel *Acidobacterium* group that possibly represents a novel subdivision. This information should provide a foundation for future examination of the bacterial communities associated with this system, the focus of which should be limited to specific groups that appear abundant. Examination of the

entire community by a process such as T-RFLP would be a daunting task due to the high diversity present in this system.

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