## MICROBIAL DIVERSITY ASSOCIATATED WITH METAL- AND RADIONUCLIDE- CONTAMINATION AT THE DOE SAVANNAH RIVER SITE (SRS), SOUTH CAROLINA, USA

by

#### QI YE

(Under the Direction of Chuanlun Zhang)

#### ABSTRACT

The goal of this dissertation is to utilize an integrated approach of molecular microbiology (cloning, sequencing, and real-time PCR) and microbial lipid biomarker to understand microbial diversity, and the factors and mechanisms shaping microbial community structure in the heavily contaminated soils at the Savannah River Site of the US Department of Energy in South Carolina, USA. The first task of this dissertaton was to determine the effect of heavy metals (As, Co, Cr, Cu, Pb, and Ni) on the microbial diversity in the effluent channel of the waste water from a coal-generated power plant. The predominance of antibiotic resistant species near the source water indicated that heavy metal concentrations are likely to serve as selective pressure for antibiotic and metal tolerant bacteria and genes. Members of heterotrophic clostridia in D-area may directly participate in metal reduction or provide electron donors for the metal-reducing microorganisms. Another task was undertaken to examine the microbial community structures in heavy metal (eg. nickel) and radionuclides (eg., uranium) contaminated soils in the M-area. Members of sulfate-reducing and iron-reducing bacteria, especially Geobacter species may play an important role in immobilizing the metals and radionuclides in the uranium-contaminated sites. The third task was to determine the diversity and abundance of ammonium-oxidizing archaea in metal- and organic-contaminated soils at the Savannah River Site. To the best of my knowledge, this dissertation marks the first demonstration of microbial community changes in the heavily contaminated soils at the Savannah River Site, and demonstrated that the microbial community structure and abundance changed along with heavy metal and radionuclide gradients. This implies that it is feasible to bio-stimulate the indigenous microbial populations for bioremediation of contaminants at the Savannah River Site.

INDEX WORDS: Savannah River Site, Bioremediation, 16 rRNA, Functional genes, Iron-reducing Bacteria, Sulfate-reducing Bacteria, GDGT, Ammonium-Oxidizing Crenarchaeota, Heavy metal, Radionuclide

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DOCTOR OF PHYLOSOPHY

ATHENS, GEORGIA

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#### **CHAPTER 1**

#### INTRODUCTION AND CHAPTER OVERVIEW

The U.S. Department of Energy's Savannah River Site (SRS) is an approximately 800-km<sup>2</sup> former nuclear weapons production facility situated in the Upper Atlantic Coastal Plain of South Carolina along the Savannah River near Aiken, SC. The SRS processed nuclear materials for national defense and other governmental and civil industrial purposes. SRS is contaminated with both organic and inorganic pollutants. A contaminated site may be relatively stable but can pose a future threat if not remediated. Bioremediation, as an alternative to conventional physical methods, has been developed as a rapidly growing technology to clean up the SRS. Studies at the Savannah River Ecology Laboratory (SREL) focus on the fundamental mechanisms of bioremediation of heavy metals and radionuclides (HMR) because environmental contamination by HMR cause serious problems, such as loss of ecosystem and agricultural productivity, decreased food chain quality, contaminated water resources and human illness. However, bioremediations of inorganic contaminants (e.g. heavy metals and radionuclides) are more difficult than that of organic contaminants (e.g., hydrocarbon). The reason is partly due to the fact that microorganisms can degrade organic contaminants by oxidizing them to carbon dioxide, whereas microorganisms can only change the redox of metal contaminants.

In recent years, SREL has been developing in situ immobilization methods, which reduce the potential mobility and bioavailability of toxic elements. Recent studies demonstrate that microorganisms play an important role in immobilizing heavy metals and radionuclides. Yet, the microbial communities in contaminated environments at SRS are poorly explored. The objective of this study is to determine changes in the composition and abundance of the bacterial and archaeal communities in relation to different contaminants and environmental conditions.

#### **Background and Significance**

#### **Description of the Savannah River Site**

The Savannah River Site is an approximately 800-km<sup>2</sup> former nuclear weapons production facility situated in the Upper Atlantic Coastal Plain of South Carolina along the Savannah River near Aiken, SC (Sowder et al., 2003). SRS lies at 33°00'N and 81°41 'W and has a climate characterized by humid and subtropical weather (Garten et al., 2000). Clay-rich units separate SRS aquifers, which drain into the Savannah River, its tributaries, and the Savannah River swamp. Groundwater beneath SRS flows at rates ranging from approximately 0.1 to 100 m per year (Garten et al., 2000). SRS is contaminated by heavy metals and radionuclides from the past DOE weapon production.

The waste water from metallurgical processes contains uranium, nickel and other metals or radionuclides, which contaminate groundwater and riparian sediments, particularly in the vicinity of the M-Area (Sowder et al., 2003). The Lower Tims Branch (LTB) is a second-order stream that receives contamination from an eroding former radiological setting pond called Steed Pond. LTB drains the upland region around the M-Area into Savannah River via Upper Three Runs Creek. Steed Pond received up to 44000

kg of depleted and natural uranium (U), and similar quantities of nickel (Ni) from aluminum-clad nuclear reactor targets from 1954 to 1985 (Punshon et al., 2004). The decreasing slopes and topography of the Steed Pond-Tims Branch (SP-TB) system provide an ideal depositional environment for accumulation of contaminated sediment. Thus, Beaver Ponds, Wetlands, and former farm ponds essentially function as settling basins for heavy metals and radionuclides (Punshon et al., 2003).

Besides nuclear material contamination, the 488-D-area Ash Basin contains a coal-burning electric power and steam generation facility with associated coal pile, coal pile runoff basin, ash basins, and reject coal pile (Carlson, 1990). Coal combustion products constitute a major category of solid waste in the D-area. Fly ash traditionally forms the bulk of coal combustion products in the D-area Ash Basin (Paramasivam et al., 2003). The trace-element contents of fly ash are highly variable depending on the origin of coal, combustion conditions, and ash handling practices (Jackson et al., 2003). The coal fly ash usually contains heavy metals, including cadmium, arsenic, chromium, copper, zinc, nickel, and selenium (Albelts et al., 1995).

#### **Advances in Studies of Microbial Diversity**

Microbial diversity describes complexity and variability at different levels of biological organization. Species (genetic variability within taxons), richness (the number of taxons), evenness (relative abundance of taxons) and guilds (microbial groups performing a common function in communities) are four important indexes to describe the microbial diversity in a particular environment (Torsvik and Ovreas, 2002). Culture-independent molecular methods provide a view of microbial diversity without cultivation; they often reveal that diverse uncultured organisms are predominant in the environment.

Cloning of 16S rRNA genes or functional genes encoding key enzyme for specific transforming processes is becoming a routine approach to investigate microbial communities and identify unknown microorganisms in natural settings (Dahllof, 2002). Pattern analysis (fingerprinting), such as terminal-restriction fragment length polymorphism (T-RFLP) (Horz et al., 2001), denaturing gradient gel electrophoresis (DGGE) (Muyzer and Smalla. 1998), amplified rDNA restriction analysis (ARDRA) (Brim et al., 1999), and ribosomal intergenic spacer analysis (RISA) (Fisher et al., 1999) provides information about changes in the whole community structure (Torsvik and Ovreas, 2002). However, these PCR amplification-based techniques are affected by biases introduced doing PCR. Therefore, advanced techniques are developed to avoid PCR. Some of these methods include fluorescence in situ hybridization (FISH) (Frischer et al., 1998), *in situ* polymerase chain reaction (in situ PCR) (Long and Komminoth, 1997), and microarray (Schena et al, 1995).

The PCR represents a sensitive molecular detection method due to its ability to exponentially amplify a target gene; however, this traditional PCR is not quantitative. Development of the real-time PCR technique allows the rapid quantification of the amount of template present at the start of the amplification process (Brunk et al., 2002; Ginzinger, 2002). The application of real-time PCR for the quantification of particular microbial populations in environmental samples, such as soil, water, sediments, or activated sludge, has been reported extensively (Grüntzig et al., 2001; Harms et al., 2003; Hermansson and Lindgren, 2001; Stults et al., 2001).

#### Significant gaps in studies of the SRS microbial community

Microorganisms are recognized to play an important role in bioremediation. However, there is little information on microbial community structures at SRS in relation to different HMR contaminants and environmental parameters. Understanding the structure, composition, and abundance of microbial community in indigenous environments will be important to fill the gaps in our understanding of microorganism in processes of bioremediation.

#### Hypothesis

The goal of this dissertation is to determine changes in the structure, composition and abundance of bacterial and archaeal communities in relation to different contaminants and environmental conditions. I will focus on iron- and sulfate- reducing bacteria because members of these two groups have the ability to reduce heavy metals and radionuclide. Mounting evidence suggested that crenarchaeota were prevalent in many contaminated environments. Metagenomic studies revealed that nonthermophilic crenarchaeota may participate in ammonium oxidation. However, the ecological functions of crenarachaeota in the contaminated soil are not known. The objective of this study will be achieved by testing the following hypotheses in context of the environmental conditions:

**General hypothesis:** Microbial community dynamics respond to changes in chemical conditions in the heavy metal and radionuclide contaminated soils at Savannah River Site. **Specific Hypothesis 1:** Metal-reducing and metal-resistant microorganisms exist in the SRS soils contaminated by toxic metals. Specifically, sulfate-reducing bacteria and

*Geobacter spp.* comprise a substantial portion of the microbial community and are of primary importance in in-situ bioremediation.

**Specific Hypothesis 2:** The diversity and abundance of crenarchaeota responsible for ammonium oxidation will be reduced in high metal and radionuclide environments.

These hypotheses will be tested by applying lipid analysis and cultureindependent approaches including 16S rDNA clone libraries, functional gene sequences, and quantitative real-time PCR. This research will build a strong link between microbial community with the indigenous and controlled environmental conditions. These results will provide valuable information for developing bioremediation techniques at SRS.

#### **Overview of Chapters**

In chapter 2, I reviewed recent developments on the microbial bioremediation of contaminated soils and groundwater, which provided background information for my research project in Chapters 3-5. In chapter 3, I examined microbial community changes along the overflow basin for a fly-ash-collecting pond, which was affected by heavy metals from a coal-generated power plant in the D-area. Three 16S rRNA clone libraries were constructed from both the source and downstream location that had different heavy metal concentrations. The results of lipid biomarkers-isotope signatures provided further information for understanding microbial community structures and ecological functions in these environments.

In chapter 4, I established 16S rDNA clone libraries from contaminated locations in the M-area, Lower Tims Branch, Stead Pond, and Beaver Pond. Microbial community structures in varying heavy metal and radionuclide soils were examined, and microorganisms potentially suitable for in-situ bioremediation were identified in this chapter.

In chapter 5, I described the archaeal communities and the potential ecological functions in the nitrogen cycle by members from the crenarchaeal phylum observed in heavymetal polluted soils. Comparisons of total archaeal community structures in different heavy-metal contaminated samples were examined by16S rRNA gene sequence analyses in combination with archaeal glycerol dibiphytanyl glycerol tetraethers (GDGT) profiles. The composition of the crenarchaeal ammonium oxidizers was investigated by construction of function gene (archaeal *amoA*) libraries.

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## CHAPTER 2

### MICROBIAL BIOREMDIATION OF METAL- AND RADIONUCLIDE-

CONTAMINATED SOILS AND GROUNDWATER

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#### ABSTRACT

Contamination of environments by heavy metals and radionuclides has become a serious problem worldwide. The treatment of heavy metals and radionuclides in environments by specific microorganisms such as metal- reducing and metal- resistant bacteria has become promising technology. The successful application of in-situ bioremediation will provide potential ways to clean up heavy metals and radionuclides in contaminated environments. Recent studies also focus on understanding the effect of heavy metals and radionuclides on the microorganisms in the microbial community. Microbial mats and biofilms are two representative microbial communities functioning in bioremediation. Metal speciation and valence variation, transport processes, and microbial metabolism are three important ingredients for metal and radionuclide remediation. Combining these ingredients enables us to better understand the relationships between the naturally occurring microorganisms and bioremediation processes.

#### **1. Introduction**

Contamination of soil and groundwater with both toxic organic and inorganic chemicals is one of the most serious environmental problems around the globe (Roane et al., 2001; Feris et al., 2003; Rajapaksha et al., 2004; Gillan et al., 2005). A contaminated site may be relatively stable but can pose a future threat if not remediated. Bioremediation, as an alternative to conventional physical methods, has been developed as a rapidly growing technology to clean up the contaminated sites. However, bioremediations of inorganic contaminants (e.g., heavy metals and metalloids) are more difficult than that of organic contaminants (e.g., hydrocarbon). The reason is partly due to the fact that microorganisms can degrade organic contaminants by oxidizing them to carbon dioxide, whereas microorganisms can only change the speciation of metal contaminants (Lovley and Coates, 1997).

Metal pollution is a common environmental risk, especially in mining and plating areas. Metal wastes pose adverse effects on our lives. For example, during mining operations, solutions that contain high concentrations of dissolved metals can be produced by exposed sulfide ores. These leachate solutions can affect the quality of all living organisms, including plants, animals, and human beings (Singh and Cameotra, 2004). People may be exposed to metal contamination via breathing, eating, drinking, and skin contact. Exposure to these pollutants can cause birth defects, cancer, and other health problems. Therefore, rapid detoxification of metal contaminants is important for a safe environment.

In the last two decades, collaborative efforts from microbiologists, molecular biologists, environmental engineers, geochemists, and ecologists have resulted in a

detailed understanding of various mechanisms of metal bioremediation. Recent studies demonstrate that microorganisms are capable of concentrating or changing the metals into forms that are insoluble or volatile in solution, which are less toxic and easily disposable (Gadd, 2000, 2004). These findings open up a new avenue of laboratory research with fascinating applications in various contaminated environments. This review summarizes significant advances in the use of microorganisms and microbial products for the bioremediation of metal-contaminated soil and groundwater.

#### 2. An Overview of Heavy Metals

Heavy metals have a density above 5 g/cm<sup>3</sup>. These include the transitional elements from V to the half-metal As, from Zr to Sb, and from La to Po. Other heavy metals include the lanthanides and the actinides (Nies, 1999).

Of all the heavy metals, only a few have biological importance due to three reasons. First, the occurrence of any element decreases sharply with increasing atomic mass. Thus, elements with high atomic masses are rare, and high concentrations of these elements usually do not exit in ecosystems. Second, the solubility of a metal ion determines its biological functions. Some divalent and all tri- or tetravalent cations are nearly insoluble at neutral pH and are not biologically accessible. The third reason is their toxicity (Silver, 1998; Nies, 1999, Wackett et al., 2004).

Fe, Zn, Mo, Ni, Cu, Cr, Mn, and Ni are known as required nutrients and are essential to life. The others have no biological functions and are nonessential. Essential metals function as catalysts for biochemical reactions and stabilizers of protein structures and bacterial cell walls. They are also involved in redox processes and are important components of complex molecules (Nies, 1999). However, metals at high concentrations are toxic to microorganisms. Some microorganisms have successfully adapted to the presence of high levels of selected metals (Nies, 1999). Nevertheless, heavy metals pose adverse effects on the environment. A good understanding of how microorganisms resist metals is important in developing strategies for the detoxification or removal of the metals from the environment.

#### **3. Bioremediation Technology**

Although bioremediation was first used in the sewage treatment plant in Sussex, UK in 1891, the term "bioremediation" is relatively new. It first appeared in a peerreviewed scientific literature in 1987 (NABIR, 2003). Bioremediation is defined as the process of using microorganisms to degrade or to remove hazardous components of the wastes from the environments (Hazen, 1997). The factors that influence bioremediation are energy sources, electron acceptors, nutrients, pH, temperature, and inhibitory substrates or metabolites (Boopathy, 2000).

Bioremediation technologies can be broadly divided into two categories: ex situ and in situ bioremediation (Boopathy, 2000). In situ techniques are defined as the treatment of a pollutant without the removal of the contaminated site. In contrast, ex situ technologies are treatments that involve the physical removal of the contaminated material for treatment process. Some of the bioremediation technologies are listed below:

- 1. Bioaugmentation: The addition to the environment of microorganisms that can metabolize and grow on specific contaminants.
- 2. Biostimulation: A process that increases activity of indigenous microorganisms by addition of nutrients, oxygen, or other electron donors and acceptors.

- 3. Intrinsic bioremediation: Also known as natural attenuation, is a passive remediation method that can effectively reduce contaminants in soil and groundwater to levels that do not pose a risk to human health or the environment.
- 4. Bioventing: A soil clean-up method by drawing oxygen through the soil to stimulate microbial activity.
- 5. Land farming: Solid-phase treatment system for contaminated soils.
- 6. Composting: Aerobic, thermophilic treatment process in which contaminated materials is mixed with a bulking agent.
- 7. Slurry-phase bioremediation: Also known as bioreactors, is a controlled treatment that involves the excavation of the contaminated soil, mixing it with water and placing it in a bioreactor.

The success of bioremediation technologies depends on having the right microorganisms in the right place with the right environmental factor. There are three basic principles in selecting the most appropriate strategy to treat a specific site: biochemistry (the amenability of the pollutant to biological transformation to less toxic products), bioavailability (the accessibility of the contaminant to microorganisms), and bioactivity (the opportunity for bioprocess optimization) (Dua et al., 2002).

#### 4. Mechanisms for Metal Remediation

Microorganisms play important roles in the environmental fate of toxic metals and radionuclides by affecting changes in metal speciation and mobility (Gadd, 2004). Microbial immobilization and mobilization are often involved in biogeochemical cycles for metals as well as other elements including carbon, nitrogen, sulfur, and phosphorus (Gadd, 2004).

#### 4.1. Immobilization

Immobilization is a technique used to reduce the mobility of contaminants by altering the physical or chemical characteristics of the contaminants. A number of processes lead to metal immobilization, including (1) bioaccumulation or biosorption (Chang et al, 1999); (2) binding by a range of metal-binding compounds such as peptides, proteins and polysaccharides (Pazrandeh et al., 1998; Chen, 1999); (3) metal precipitation by metal- and sulfate-reducing bacteria (Lovley, 2001); (4) bacterial and fungal oxidation(Eward and Hugues, 1991; Gomez and Bosecker, 1999); and (5) phosphatase-mediated metal precipitation (Brasnakova and Macaskie, 1999).

Microorganisms can physically remove heavy metals and radionuclides from solutions through association of these contaminants with microbial biomass. Bioaccumulation is the concentration of heavy metal or radionuclides within an organism, whereas biosorption performs association of soluble heavy metals or radionuclides with the cell surface (NABIR, 2003).

In bioaccumulation, metals are transported into the cell cytoplasm through specific membrane transport proteins. Once inside cells, metal species can be sequestered and are therefore immobile (NABIR, 2003).

Biosorption does not consume cellular energy. Microorganisms uptake both organic and inorganic metal species via physiochemical mechanisms. Biosorption provides nucleation sites such as carboxyl residues, phosphate residues, S-H groups or hydroxyl groups on the microbial cell surface. Positively charged metals ions can adsorb these negative ionic groups, which lead to precipitation of the metals (NABIR, 2003).

The polymers of cell surfaces are mostly negatively charged, therefore absorbing metal ions too.

Microorganisms can produce both specific and nonspecific metal-binding compounds. Nonspecific metal-binding compounds range from simple organic acids and alcohols to macromolecules such as polysaccharides (NABIR, 2003). Metallothioneins (MTs) and phytochelations are specific, low molecular weight metal-binding proteins. MTs are cysteine-rich proteins that bind metals ion (e.g., Cd, Pb, Hg, and Cu) and sequester them into immobile forms (Chen et al., 1999). Heterologous expression of phyotechelatin synthase (PCBs) dramatically enhances metal tolerance, which provides molecular evidence that PCS genes have the potential for bioremediation (Clemens et al., 1999).

Another process of immobilization is often achieved by microbial reduction of metals. Metals can be reduced by direct and indirect enzymatic reductions. Direct enzymatic reduction of soluble U(VI) (Lovley et al., 1991, 1993; Gorby et al., 1992; Fredrickson et al., 2000), Cr(VI) (Nakayasu, 1999) and Tc(VII) to insoluble forms has been well studied. The indirect enzymatic reduction can be carried out by both metal-reducing bacteria and sulfate-reducing bacteria. This can be achieved by coupling the oxidation of organic compounds or hydrogen to the reduction of ferric iron (Fe<sup>3+</sup>) to ferrous iron (Fe<sup>2+</sup>) or the reduction of sulfate (SO<sub>4</sub><sup>2-</sup>) to hydrogen sulfide (H<sub>2</sub>S). Either Fe<sup>2+</sup> or H<sub>2</sub>S can reduce toxic metals such as uranium, chromium, and technetium (Lovley, 1995; De Luca et al., 2001; Lloyd et al., 2002). The reduced forms of these metals are insoluble and can either precipitate as reduced oxide or hydroxide minerals or coprecipitate with iron oxides (NABIR, 2003).

Iron-oxidizers and fungi can oxidize metals, especially in acidic environments where those metals are stable when no microbes are involved. For example, *T. ferroxidans* is an acidophilic chemolithotroph, which plays a significant role in generating acid mine drainage (Ewart and Hugues, 1991). Recent studies indicate that Fe(II)-oxidizing photoautotrophic bacteria may have catalyzed the precipitation of Banded Iron Fromation, which is an ancient class of sedimentary deposits (Kappler and Newman, 2004).

Metals or radionuclides can also be precipitated as phosphates by accumulating in bacterial biomass. Brasnakova et al. (1999) described that a mixture of  $Zr(HPO_4)_2$  and hydrate zirconic oxide ( $ZrO_2$ ) was produced through mineralizing zirconium by a *Citrobacter* sp. When excess inorganic phosphate is present, uranium as  $HUO_2PO_4$  is repressed by zirconium. The cell-bound  $HUO_2PO_4$ , however, facilitates Ni<sup>2+</sup> removal by intercalative ion exchange into the polycrystalline lattice.

#### 4.2. Mobilization

Microorganisms can mobilize metals via autotrophic and heterotrophic leaching, chelation using metabolites and siderophores, methylation using volatile C-1 compounds, and mediating redox.

Bioleaching is a simple and effective technology for metal extraction from lowgrade ores and mineral concentrate (Bosecker, 1997). Autotrophic leaching is based on the activity of acidophilic chemolithoautotropic bacteria, mainly *Thiobacillus ferroxidans*, *T. Thioxidans*, and *Leptospirllum ferrooxidans*, which causes solublization of metal sulfides (Gadd, 2004). Heterotrophic leaching occurs when heterotrophic bacteria or fungi acidify their environment by proton efflux, thus leading to the release of metals. Fe(III) has low water solubility and cannot be acquired as free ions in aerobic soils; this problem can be solved by siderophores produced by microbes (NABIR, 2003). Siderophores are highly specific Fe(III) ligands and are able to bind to other metals such as magnesium, manganese, chromium(III), gallium(III), and nuclides such as plutonium (IV) (Gadd, 2004). Siderophore-mediated uptake and transport could be a useful pathway for environmental mobility (NABIR, 2003).

Methylation involves methyl groups that are enzymatically transferred to the metal by a given species, followed by the formation of a number of different methylated metal(loid)s differing in their solubility, volatility, and toxicity (Gadd, 2004). For example, several bacteria and fungi can methylate selenium or arsenic compound to volatile forms, resulting in the loss of Se and As from the environments (Gadd, 2004).

Redox transformations allow microorganisms to mobilize metals, metalloids, and organometallic compounds by reduction and oxidation process. A number of metal(loid)s including Fe(III), Mn(IV), Se(IV), Cr(VI), and U(VI) are used as terminal electron acceptors by a variety of metal-reducing microorganisms (Lovely, 2001). While Fe and Mn increase their solubility by reduction, the solubility of other metals such as U and Cr decrease in solubility when reduced, resulting in their immobilization (Gadd, 2004). 4.3. Metal immobilization and mobilization applied to bioremediation

Mechanisms of microbial immobilization and mobilization provide potential for both in-situ and ex-situ bioremediation. In the context of bioremediation, mobilization enables metals to be removed from solid phases, such as soil, sediments, and industrial wastes. Alternatively, immobilization of metals provides a means to transform mobile metals into insoluble forms. Immobilization processes are efficiently applied to remove metals from mobile phases such as groundwater and leachates. A number of articles have reviewed the recent applications for treating toxic metal(loid)s by microbial solubilization and immobilization mechanisms in different contaminated environments (Bosecker, 1997; White et al., 1997; Gadd, 2000).

#### 5. Microbial Metabolism Affecting the Metal(loid)s Bioremediation

Basic microbial metabolic processes include respiration, fermentation, and methanogenesis (NABIR, 2003). These processes all have potential for metal bioremediation. There are two basic forms of respiration: aerobic and anaerobic. Aerobic respiration is the use of  $O_2$  as a terminal electron acceptor. Anaerobic respiration occurs when the terminal electron acceptors are inorganic compounds other than  $O_2$ .

In aerobic respiration, compounds other than carbon dioxide such as reduced iron [Fe(II)], ammonium sulfide  $(NH_4)_2S$ , or molecular hydrogen  $(H_2)$  can act as electron donors with oxygen as the electron acceptor. These reactions hold promise for bioremediation. For example, when dissolved Fe(II) is oxidized to Fe(III), hydrous iron-oxide minerals precipitate. These precipitates are characterized by high surface activity and specific surface areas, which enhance reactions with other metals and radionuclides, allowing complexation to occur with contaminants and changing contaminant mobility. This will reduce the risk of contaminants entering groundwater (NABIR, 2003).

Nitrite reduction (denitrification), iron reduction, and sulfate reduction are major processes of anaerobic respiration. The distribution of the above mentioned microbial communities is highly correlated to the redox profile when a long-term equilibrium is reached. Denitrification is a two-step process: Nitrate is reduced to nitrite, which is then reduced to  $N_2$ . Under certain conditions, the reduction of nitrate is faster than the reduction of nitrite. This may cause the accumulation of nitrite, which may inhibit the activity of dissimilatory iron- or sulfate- reducing bacteria (NABIR, 2003).

A wide range of microorganisms are able to reduce Fe(III) to Fe(II). In the domain Bacteria, iron reducers include species of *Geobacter* (Coates et al, 1996), *Shewanella* (Nealson and Myers, 1992), *Desulfuromonas* (Krumoholz, 1997), *Pelobacter* (Lonergan et al., 1996), *Geovibrio* (Caccavo et al., 1996), *Geothrix* (Coates et al., 1999), *Bacillus* (Boone et al., 1995), *Pyrobaculum* (Kashefi and Lovley, 2000), *Thermus* (Kieft et al., 1999), and *Thermoterrabacterium* (Slobodkin et al. 1997). In the domain Archaea, iron reducers include *Archaeoglobus*, *Pyrococcus*, and *Pyrodictium* (Vargas et al. 1998). Dissimilatory iron-reducing microorganisms can reduce mineral-associated iron to produce reactive sites or to directly reduce contaminants, such as uranium and chromium (Lloyd et al., 2003).

Some sulfate-reducing bacteria, such as *Desulfovibrio desulfuricans*, can reduce a variety of metals including Fe(III), U(VI), Cr(III), Tc(VIII), Mo(VI), and Pd(II) in lieu of sulfate (Fude et al., 1994; Lloyd et al., 1998, 1999; Chang et al., 2001). However, these sulfate-reducers are unable to couple growth to metal reduction. *Desulfotomaculum reducens* can conserve energy by reduction of Cr(VI), Mn(IV), Fe(III) and U(VI) (Tebo, 1998; Lloyd et al. 2001). Metal reduction by SRB usually causes the precipitation of a low valence of metal and, therefore, fixes a variety of metal(loid)s in contaminated environments.

Fermentation is an anaerobic process in which energy generation occurs by redox reaction and in which an organic substrate serves as both an electron donor and an electron acceptor. Although fermentation does not affect the metal(loid)s, it can be an

important step in the production of substrates used by dissimilatory iron- and sulfatereducing bacteria (Lovley et al., 1993). In addition, fermentation products can also serve as metal-complexing agents to increase metal contaminant mobility in sediments (NABIR, 2003).

#### 6. Microbial Mats and Biofilms for Bioremeditation

Biofilms and microbial mats can be defined as surface-associated layers of microbial cells embedded in extracellular polymeric substance (Beer and Kühl, 2001). Biofilms and microbial mats are thus important communities in most aquatic ecosystems both today and through geological time. Recent studies demonstrate biofilms and microbial mats have potentials for heavy metal remediation.

#### 6.1. Microbial mats

Microbial mats are laminated, cohesive microbial communities, composed of a consortium of bacteria dominated by photoautotrophic cyanobacteria. Mats are ubiquitous in nature, commonly found over the sediments surfaces or as floating masses in marine waters. In nature, mats generally attach tightly to soil or submerged sediments. Microbial mats can sequester organic and metals from their environments (Bender and Phillips, 2004). There are several mechanisms that likely result in the removal of specific heavy metals.

1). Surface binding/ion exchange mechanism. Mats have highly negatively charged surfaces, which can bind an enormous amount of positively charge metals (Blanco et al., 1999).

2) Community-level mechanism. The reduction of heavy metallic ions requires multi-species communities. Microbial mats, especially in the anaerobic zone, provide ideal environments for such reactions (Bender et al., 2000).

3) Synthesis of negatively-charged bioflocculants. Bioflocculants are extracelluar micromolecules. Both soil bacteria and cyanobacteria were discovered to produce bioflocculant that clean up the surrounding environments. *Oscillatoria* sp., a type of cynaobacteria, dominates in many microbial mats and produces bioflocculants, which can effectively remove heavy metals such as Cu, Zn, Co, Cr, Fe, and Mn from water (Bender et al., 1994; Ahuja, 1999).

#### 6.2. Biofilms

A biofilm is a complex community of microorganisms that develop on surfaces in diverse environments (O'Toole et al., 2000). Microorganisms localized within biofilms have better chance of adaptation and survival than when cells are present in solution (Davey and O'Toole, 2000). Several studies demonstrate that microbial biofilms have different mechanisms to absorb the metals. For example, sulfate-reducing bacterial biofilms are highly efficient in precipitation of heavy metals such as metal sulfides from metal-contaminated water (Labrenz et al., 2000).

Canstein et al. (2001) studied the mercury retention efficiency of packed bed bioreactor under different biological conditions. They used a mercury-reducing strain and a mercury-reducing biofilm that contains six mercury-resistance isolates and one genetically modified mercury-resistant *Pseudomonas putida* strain. The mixture culture displays higher efficiency than monoculture under changing environmental conditions. Microbial diversity of biofilms provides a reservoir with complementary ecological
niches that result in a superior bioreactor performance than monospecies (Canstein et al., 2001).

Teitzel et al. (2003) studied the effect of the heavy metals Cu, Pb, and Zn on biofilms and plaktonic *Pseudomnoas aeruginosa*. They determined that biofilms were from 2 to 600 times more resistant to heavy-metal stress than free-swimming cells. This result suggests that biofilms increase resistance to heavy metal compared to the resistance of free-swimming organisms.

#### 7. Mechanisms of Metal Resistance and Implication for Bioremediation

Bacteria exist in environments that have always contained metals. Bacteria are speculated to have developed metal resistance shortly after the prokaryotic life was evolved (Bruins et al., 2000). Many microorganisms demonstrate the ability to resist metals in soil, water, and industrial waste. Bacteria have specific genes for resistance to the toxic ions of heavy metal ions including  $Ag^+$ ,  $AsO4^{3-}$ ,  $Cd^{2+}$ ,  $Co^{2+}$ ,  $CrO4^{2-}$ ,  $Cu^{2+}$ ,  $Hg^{2+}$ ,  $Ni^{2+}$ ,  $Sb^{3+}$ ,  $TeO3^{2-}$ ,  $Tl^+$ , and  $Zn^{2+}$  (Silver and Phung, 1996). Genes encoding specific resistance are located on chromosomes, plasmids, or transposons (Bruins et al., 2002). Six mechanisms are postulated to be involved in resistance system (Bruins et al., 2000): 1) metal exclusion by permeability barrier, 2) active transport of the metal away from the microorganism, 3) intracellular sequestration of the metal by protein binding, 4) extracelluar sequestration, 5) enzymatic detoxification of the metal to a less toxic form, and 6) reduction in metal sensitivity of cellular targets. The deep understanding of metal resistance may provide insight into application for their environmental decontamination.

As mentioned above, metal resistant bacteria can survive the highly contaminated environments. This feature is very attractive for decontamination of metal-polluted soil and groundwater. For example, *Ralstonis sp.* CH34 is a gram-negative bacterium with a remarkable set of resistance systems. It is able to live in an extreme environment at metal ion concentrations that inhibit other bacteria (Nies, 2000). It has the ability to simultaneously precipitate heavy metals (e.g., Zn, Cd, and Co) and degrade xenobiotics, making them future prospects for bioremediation (Nies, 2000). Another example is that enzymatic detoxification makes [Hg(II), As(V)] less toxic (Mukhopadhyay et al., 2002; Barkay et al., 2003). Mercury-resistant bacteria can reduce soluble Hg(II) to insoluble metallic Hg(0) by means of cytoplasmic enzyme mercuric reductase, which is encoded by the gene merA. The mercury remediation process offers a promising way to extract mercury from polluted wastewater because of its effectiveness and low cost (von Canstein et al., 1999).

#### **Conclusion and Perspectives**

Significant advances have been made in understanding the microbial influence on the metal immobilization and mobilization and in the applications of these mechanisms for the bioremediation of metals and radionuclides. Although diverse bacteria capable of immobilizing and mobilizing heavy metals have been isolated and characterized, the vast majority of metal-detoxifying bacteria inhabiting contaminated and bioremediation environments are uncultivable. Culture-independent rRNA approaches, including FISH, cloning, and sequencing and microarray technology, offer good tools to study the composition and dynamics of bacterial and archaeal communities surviving in metalcontaminated soils and groundwater. The development of microbial ecological techniques makes natural diversity for bioremediation *in situ* feasible.

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# **CHAPTER 3**

# MICROBIAL DIVERSITY AND GEOCHEMICAL DYNAMICS ASSOCIATED WITH METAL- CONTAMINATION AT THE DOE SAVANNAH RIVER SITE (SRS), SOUTH CAROLINA, USA

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#### ABSTRACT

The goal of this study was to determine the effect of heavy metals from a coalgenerated power plant on microbial diversity at the SRS of the US DOE in SC, USA. The study sites were the overflow basin for fly-ash-collection ponds. One sample (named as DHC) was collected from the location near its headwaters, and the other two samples (named as IRM and IRS) were taken from a downstream location which is impacted by the seepage of a holding pond, including the red microbial mat at the surface and black sediment underneath the mat. Metal analysis showed that metal concentrations of V, Ni, Cu, Cr, Co, and Pb species in soil at DHC were approximately 10-20 times higher than those at IRMS. Three 16S rDNA clone libraries were constructed by partial sequencing of randomly selected clones. Library for the source location had 96 clones, the mat had 171 clones and that for the sediment had 188 clones. In the surface mat, members of autotrophic cyanobacteria represented 35.1% of the clones; autotrophic green non-sulfur bacteria were also present appreciably (12.3%). In the underlying sediment, members of heterotrophic Firmicutes (48.9%) were predominant. These results indicate that the microbial mat functions as primary production, which supplies carbon for heterotrophs to grow below the mats. Other bacterial species in the mat and/or the sediment included  $\alpha_{-}$ ,  $\beta$ -,  $\gamma$ -, or  $\delta$ -Proteobacteria, Actinobacteria, and Planctomycetes. In particular, clones belonging to the  $\beta$ -Proteobacteria in the sediment had 99% similarity with Ralstonia sp. 13I, a heavy-metal-resistant bacterium isolated from Torch Lake, Michigan. Furthermore, most clones belonging to the  $\alpha$ - and  $\delta$ -*Proteobacteria* in the sediment were closely (93%) to 96% similarity) related to not-yet-cultured species from other contaminated environments. Interestingly, 63% of DHC cloned sequences had 97-99% similarity with

the antibiotic resistant species, *Streptococcus mitis*, which indicated that DHC location is likely to serve as sources for antibiotic and metal tolerant bacteria and genes. Results of this study provide valuable information on microbial community dynamics responding to changes in physical and chemical conditions and indicate the potential impact of coalcombustion wastes on microbial biochemistry.

#### **INTRODUCTION**

The widespread contamination of soils, sediments and groundwater with toxic metals through the combustion process of coal-fired power plants is a serious environmental problem worldwide. In the USA, coal-fired electric utilities supply more than half of the nation's electricity annually (Am. Coal Ash Assoc., 1997). Now more than 121 tons of coal combustion products are produced each year, which is expected to grow to 50% by 2010 (Goss, 2005). The increased use of coal for power generation could result in increased release of potentially toxic organic and inorganic contaminants into the environments (Sajwan et al., 2003). Fly ash is the major coal residue of coal combustion. The trace-element content of fly ash is highly variable depending on the original coal, combustion conditions and ash handling practice (Jackson et al., 2003). Coal fly ash usually contains heavy metals, including cadmium, arsenic, chromium, copper, zinc, nickel, and selenium species (Albert, 1985). The readily accumulation of these elements by organisms caused genetic damage in fishes and wildlife (Rowe, 2002). The long residence time of coal results in heavy contaminations in shallow groundwater and terrestrial vegetation of adjacent areas of contaminated location. (Carlson, 1990). A 488km<sup>2</sup>-D-area Ash Basin is located in SRS, which contains a coal burning electric power and stem generation facility with associated coal pile, coal pile runoff basin, ash basin, and reject coal pile (Carlson, 1990). Fly ash traditionally forms the bulk of coal combustion products on D-area Ash Basin (Paramasivam et al., 2003).

The treatment of toxic contaminants by specific microorganisms becomes the most promising technology to clean up such contaminated sites. However, the bioremediation of these wastes in natural settings is a complicated process because many

sites were contaminated by multiple pollutants, such as organic and inorganic matters (eg. heavy metal and radionuclides) (Fields, et al., 2006). The successful application of in situ bioremediation depends on comprehensive understanding of microbial community and activities in indigenous and controlled environments.

Recently, culture-independent molecular methods are becoming a routine approach to investigate microbial communities and identify unknown microorganisms in diverse contaminated environments (Bowman, et al., 1993, Peacock et al., 2004; Petrie et al., 2003; North et al., 2004; Nakatsu et al., 2005; Vrionis et al., 2005). Previously, Brofft et al. (2002) used cultivation-independent method to characterize bacterial and archaeal communities of an acidic (pH 2-4), metal rich, saline region in D-area. However, the microbial community is still essentially unexplored in SRS D-area. In this study, we examined bacterial communities in both source location and downstream which receive effluent from fly-ash-treatment ponds, as little is known about this niche with heavy metal gradients. The goal of this work is to determine changes of microbial community structures and ecological functions in relations to geochemical characteristic by using a combination of molecular phylogeny and lipid biomarkers-isotope signatures.

#### MATERIALS AND METHODS

**Sampling site and soil characteristics.** D-area in the Savannah River which was impacted by 22-year old reject coal pile, was located in the Upper Atlantic Coastal Plain of South Carolina along the Savannah River near Aiken, SC (Fig. 3.1). One sample was taken from source location (sample named as DHC), and the others were collected from the red microbial mat (sample named as IRC) at the surface and black sediment (samplea

named as IRS) underneath the mat of the downstream location, which received effluents from fly-ash-treatment ponds, finally drains into Savannah River (Fig.3.1).

**Metal analysis** Samples for trace metal analysis were collected on two locations (source and downstream). All samples were assayed in the analytical facility at the Savannah River Ecology Laboratory. Samples were subjected to nitric acid/hydrogen peroxide microwave digestion (CEM, Matthews, NC) followed by inductively coupled plasmamass spectrometry analysis (ICP-MS, Perkin-Elmer, Norwalk, CT). Appropriate calibration standards and blanks were used in both the digestion and analysis processes.

**DNA extraction and purification.** DNA was extracted from 5g soil samples by the freeze-thaw procedure of Zhou *et al.* (1996), followed by using Ultraclean Mega Prep Soil DNA Kit (Mo Bio Laboratory, Inc, Solana Beach, CA). The precipitated DNA was purified by gel electrophoresis plus mini-column preparation (Wizard DNA Clean-Up system, Promega, Madison, WI).

**PCR amplification and cloning.** The nearly complete 16S rRNA gene (approximately 1500 bp) were amplified in 9700 Thermal Cycler (Perkin-Elmer) with the primer pair FD1F (5' AGA GTT TGA TCC TGG CTC AG 3') and 1540R (5' AAG GAG GTG ATC CGG GC 3') (position 1 and 1540 *E. coli* designation) (Weisburg et al., 1993). The PCR reaction solution (20  $\mu$ l) contained 2  $\mu$ l 10×buffer (500 mM KCl, 100mM Tris HCl pH 9.0 and 1% Triton X-100), 1.2  $\mu$ l 25mM MgCl2, 0.2  $\mu$ l 400ng/ $\mu$ l Bovine serum albumin, 0.2  $\mu$ l 25mM 4×dNTPs (USB, Cleveland, OH), 10 pmol each primer, 2.5 U Taq polymerase, and 1  $\mu$ l purified DNA (10-20 ng). PCR-induced artifacts were minimized by using the optimal number of cycles as describe in Qiu *et al.*(2001). Briefly, each cycle gent through the following sequences: 80°C for 30 s, 94°C for 2 min; 94°C for 30 s, 58°C

for 1 min, and 72°C for 2 min. After 25 cycles, PCR products were maintained at 72°C for 7 min and then analyzed on 1.5% (wt/vol) TAE agarose gels. In order to avoid potential sample biases and to obtain enough PCR products for cloning, five replicate amplifications were carried out for each sample.

The combined PCR products (1500bp) were purified by cutting out the appropriate bands from a low-melting point agarose gel (0.8%). The PCR products were purified from the gel using the QIA quick Gel Extraction Kit (QIAGEN, Inc., Valencia, CA). Recovered DNA was resuspended in 6 $\mu$ l ddH2O, 2  $\mu$ l was ligated with pCR vector form a TA-cloning kit and competent *Escherichia coli* cells were transformed according to manufacturer's instructions (Invitrogen, San diego, CA). White colonies were randomly picked and screened for 16S rRNA gene inserts, which were amplified (25  $\mu$ l) with the vector-specific primers for the polylinker of vector pCR2.1. A total of 455 16S rRNA clones were screened from the two samples.

**Sequence and phylogenetic analysis.** The amplified inserts were purified by using the Multiscreen PCR plate (Millipore, Bedford, MA). Partial sequences were determined from the purified inserts with the 16S rRNA gene primer 529r, 5' CGC GGC TGC TGG CAC 3' (*E. coli* numbering). 5  $\mu$ l sequencing reaction contains the recovered template (1  $\mu$ l), sequence buffer (1  $\mu$ l), ABI Big Dye @ Terminator v3.1 (Applied Biosystem, Foster City, CA) (1  $\mu$ l), 3 pmol 16S rRNA gene primer 529r and water). Sequencing was determined by using a 3700 DNA analyzer (Perkin-Elmer, Wellesley, MA) according to the manufacturer's instruction.

The closest database relatives of all sequences were obtained using all available sequence information by Blast searches of Genebank. The sequences were aligned with

Clustalx 1.81 software and Bioedit (Hall, 1999). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 and phylogenetic trees were constructed with distance matrices and the neighbor-joining method with MEGA (Kumar et al., 2001). The phylogenetic trees constructed with maximum-likelihood and neighbor-joining methods were not significantly different. OTU (Operational taxonomic unit) determination is an important criteria for conducting microbial diversity studies by using environmental sequences. In this study, sequences with similarities  $\geq$ 97% were placed into the same operational taxonomic unit (OTU).

The 16S rRNA clone libraries between iron creek mat and sediment samples were compared by using computer program WebLIBSHUFF version 0.96. The communities of two different samples can be directly compared to each other based on evolutionary distance of all sequences. The DNADIST program of PHYLIP using the Jukes-Cantor model for nucleotide substitution was used to calculate the distance matrix (Singleton et al., 2001). The distance matrix was utilized to determine the significance of differences in the compositions of pairs of libraries.

Analysis and carbon isotopic composition of Lipids. 10g soil samples were extract by a modified Bligh and Dyer single-phase organic solvent system comprised of chloroform, methanol, and aqueous 50 mM phosphate buffer (pH 7.4) in a ratio of 1:2:0.8 (vol/vol/vol) as previously described (Bligh and Dye 1959; White et al. 1979). After overnight extraction, chloroform and nanopure water were added to the extract in equal volumes, which resulted in a two-phase system. The lipid confined to the lower phase was collected and fractionated on a silicic acid column into neutral lipids, glycolipids, and polar lipids. The polar phospholipid fatty acids were treated using a mild alkaline methanolysis to

produce fatty acid methyl esters. Methyl esters were analyzed with an Agilant 6890 series gas chromatograph interfaced to an Agilant 5973 mass selective detector with a 20-m nonpolar column (Zhang et al., 2002). Mass spectra were determined by electron impact at 70 eV. Methyl heneicosanoate was used as the internal standard. The fatty acid methyl esters (FAMEs) were expressed as equivalent peaks against the internal standard. Stable carbon isotopic ratios of individual lipids were determined using a GC-combustion system linked to a Finnigan MAT 252 isotope ratio mass spectrometer (IRMS) describe previously (Dai and Sun, 2007)

#### RESULTS

**Metal Analysis.** Metal concentrations of Cr, Ni, Sr and Pb species were approximately 10 times higher at source location than those at downstream one, concentrations of V, Co and Cu species were 20 times higher at source location than at downstream one, and concentrations of Zn, Cd, and Cs species were 3 to 4 times higher at source location than at downstream one. The concentrations of As and U species are similar at both sites (Table 3.1).

**Phylogenetic analysis of 16S rDNA from the source water (DHC).** A total of 96 nonchimeric 16S rRNA sequences were analyzed from the source location (DHC) in the Darea (Table 3.2). 62 DHC clones representing 64.5% of the total clones had 97-99% similarity with *Streptococcus mitis*, a well-known antibiotic-resistant species. 25 DHC clones had 99% similarity with *Veillonella parvula*.

**Phylogenetic analysis of 16S rDNA from a downstream environment.** Samples were collected from the surface mat (IRM) and from sediment (IRS) below the mat at an iron-rich creek downstream from the source of contamination. A total of 359 clones were

partially sequenced covering approximately 500 bp. The rarefaction analyses suggested that the number of sampled clones reached saturation from approximately 100 for IRM to about 150 for IRS. Therefore, size of both IRM and IRS clone libraries represent the bacterial communities in the studied sites (Fig. 3.2). Phylogenetic analyses revealed that 35.1% IRM clones were classified as cyanobacteria, followed by  $\beta$ -Proteobacteria (16.3%), Chloroflexus (12.3%),  $\delta$ -Proteobacteria (8.2%),  $\alpha$ -Proteobacteria (6.4%). In contrast, Firmicutes constitute a significant portion of the 16S rDNA clone library from the iron creek sediment, account for 48.9% of total clones. The remaining IRS clones belonged to the green non-sulfur bacteria (12.2%), Actinobacteria (8%),  $\delta$ -Proteobacteria (7.4%),  $\beta$ -Proteobacteria (4.3%),  $\alpha$ -Proteobacteria (2.7%) (Table 3.3). A total of 94 distinct OTUs were observed in the iron creek mat whereas 114 in the iron creek sediment.

**Phylogenetic analysis of creek downstream.** Partial sequences of 16S rRNA gene from both IRM and IRS samples fall into the following groups.

(1). Actinobacteria. Members of Actinobacteria are common gram-positive bacteria found in soils. 14 IRS clones and 4 IRM clones falls into Actinobacteria phylum. The majority of the Actinobacteria clone sequences displayed relationships with uncultured bacteria from various environments (Fig. 3.3). Only two sequences have 98% similarity with *Propionibacterium cenes* and 97% similarity with *Frankia. sp.* respectively.

(2). Acidobacteria. Two IRM clones cluster with GenBank member *Holophaga* and *Geothrix* 16S rRNA gene sequences. The rest of acidobacteria IRM clones were closely related to environmental sequences (Fig. 3.3). For example, two clones from Iron creek

mat exhibited 94% similarity with clones derived from 1,2- dichloropropane contaminated sediment of the River Saale, Germany (Schlotelburg, et. al., 2000).

(3). **Chloroflexi**. Phylogenetic analysis show that 22 IRM and 23 IRS clones fell into Chloroflexi phylum. It is interesting that 8 IRS and 14 IRM clones forms their own distinct OTU (Fig 3.4). IRM cluster had 90-92% similarity with uncultured chloroflexi bacterial clones from activated sludge (Juretschko, et al., 2002), microbial fuel cells (Phung et al., 2004), and sediments from trophic state (Wobus et al., 2003). Eight IRS clones had approximately 94% similarity with metal-contaminated soil clone K20-51 from super fund site.

(4). **Cyanobacteria**. Totally 60 IRM clones and only 1 IRS clone fell into cyanobacterial phylum. As shown in the phylogenetic tree in Fig. 3.5, these sequences were distributed in 7 clusters. Cluster I corresponded to the order *Microcoleus* and *Oscillatoriales*. 17 IRM clones fell in this cluster. Cluster II comprised 2 IRM sequences which had 92% similarity with *Nodularia spumigena*. Cluster III comprised the sequences of 13 IRM clones, which exhibited 95% similarity with uncultured cyanobacterial clone BGC-Fr005 from the Antarctica mat (Taton, et al., 2003). 5 clones from IRM and 1 clone from IRS samples fell in cluster IV, which has 93% similarity with *Cyanothece* sp., the microorganism with potential for N2 fixation (Mazard, et al., 2004). 4 IRM clone formed cluster V which shows 96% similarity with environmental cyanobacterial sequences from freshwater ecosystem (O'Sullivan et al., 2002). Only 1 IRM sequence was detected in cluster VI. Cluster VII comprised 16 IRM clones, these sequences exhibited 93% similarity with their closest sequence of an unclutured *Gloeothece* sp. clone. Except cluster III and V, the rest of the cluster shows at least 7% dissimilarity with the sequences

from Genbank database. These results suggested that novel cyanobacteria clusters were present in iron creek mat.

(5). Fimicutes. A significant number of Firmicutes-like sequences was observed in Iron Creek Sediment (Figs. 6 and 7). One third of them had greater than 96% identity to the Clostridium genus, including *C. butyricum* (Collins et al., 1994), *C. colicanis* (Greetham et al., 2003), *C. disporicum* (Stackebrandt et al. 1999),*C. thersulforeducens* (Hernandez-Eugenio, et al., 2002 ) *C. glyclocuim* (Chamkiha et al. 2001) and *C. lentocellum* (Collins et al., 1994), which were able to use a variety of organic matter as carbon source (Hernandez-Eugenio et al., 20020. Another one-third of the Firmicute-related clones were affiliated with uncultured clostridia clones recovered from various environments (Fig 3.6, Fig 3.7). Most of them had highly similarity to sequences from Italian rice field soils (Lueder et al., 2004).

Only four IRS sequences in the Firmicutes groups were related to *Bacillus* spp, a group of obligate or facultative aerobes. The other four clones in the Firmicutes clusters were similar to the sulfate-reducing bacteria, *Desultitobacterium metallireducens* (Finnerman et. al., 2003), and an uncultured SRB clone from Guaymas basin of Gulf of California (Dhillon et al., 2003).

#### (6). Proteobacteria

6.1. Alphaproteobacteria. 12 IRM clones, representing 110 different OTUs, were affiliated with alpha-subdivision of Proteobacteria (Fig.3. 8). Among these clones, 9 clones were affiliated with Rhizobiaceae family, related to the genera *Hyphomicrobium*, *Bradyrhizobium*, and *Methylobacterium*. These microorganisms are frequently isolated nitrogen-fixing and methane-oxidizing microorganisms. From 4 out of 6

alphaproteobacterium IRS clones were affiliated with *Acidisphaera rubrifaciens*, a bacterium isolated from acidic environment.

6.2. Betaproteobacteria . Six IRS clones, representing 3% 16S rDNA sequences of Creek sediment clones, fell into the  $\beta$ -proteobacteria. These clones have 97-99 similarity with isolate of *Ralstonia* from a copper contaminated lake sediment in Michigan lake, which are resistant to heavy metals, such as Zn, Cu, Ca, and Ni (Konstantindis, 2003) (Fig.3.9).

6.3. Deltaproteobacteria. 5 IRS clones had the 92-94% similarity with *Desulfomonile limimaris*, an anaerobic dehalogenated bacterium (Sun et al., 2001). 5 IRM and 1 IRS clones were affiliated with *Anaeromyxobacter dehalogenans*, an aryl-halorespiring facultative anaerobic myxobacterium, which can utilize nitrate, Fe(III), chlorinated hydrocarbon as electron acceptors (Sanford et al., 2001) (Fig. 3. 10).

7. Others. We also obtained 7 IRS clones representing 2 OTU and 1 IRS clones that clustered with the Planctomycetes (Fig. 3.11). 2 clones found in both IRM and IRS clone libraries cluster with Verrucomicrobia (Fig. 3.11). A few sequences related to Eukaryota, Bacterioidetes and Candidate OP10 division were also retrieved from the iron creek (Fig 3.11).

**WebLibshuff Analysis.** The SSU rRNA clone libraries were compared between Iron Creek Mat and Sediment, as they are constructed simultaneously under same conditions. The iron Creek mat had a P-value of 0.002 when it compared with Iron Creek sediment. This direct comparison of the entire sequence libraries indicated that the iron creek mat is significantly different from the underlying sediment.

**Stable Carbon Analysis of Lipid.** Phospholipid fatty acids (PLFA) in the iron creek mat sample were performed by J. Dai and M. Sun. The predominant PLFA were 16:0

(47.04%), 16:1 (18.41%), 18:1 $\omega$ 7c (13.67%), 18:1 $\omega$ 9c (6.51%), 14:0 (5.8%) and 18:0 (5.18%). In the iron creek sediment, the predominant PLFA were 16:0 (41.07%), 18:1 $\omega$ 7c (32.75%), 18:0 (6.6%), 18:1 $\omega$ 9c(6.51%), 14:0 (3.75%) and 16:1 (2.07%) (Fig 12A). A significant difference of the carbon isotopic signatures of these lipid biomarkers and total biomass (-35.77% in the mat and -32.63 % in the sediment) was observed (Fig 12B).

### DISCUSSION

Cloning of 16S rRNA gene has become a routine approach to investigate microbial communities and identify unknown microorganisms in natural settings (Dahllof., 2002). However, these PCR amplification-based techniques were affected by biases introduced by PCR artifacts (Acinas et al., 2005). In this study, we performed 5 replicate PCR amplifications to minimize PCR drift and carried out 25 PCR amplification cycles to reduce chimeras and Taq DNA polymerase errors. Various molecular techniques, such as terminal-restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophorosis (DGGE), amplified rDNA restriction analysis (ARDRA), fluorescence in situ hybridization (FISH), and 16S rRNA and functional gene cloning, have been applied to characterize microbial diversity in different natural samples. Recently, direct sequence determination of randomly selected and screened clones was employed due to a significant savings in time and effort. Also, partial sequences of informative regions of SSU rRNA gene can be used to efficiently screen libraries and thereby reduce the number of full-length sequences (Fields et al., 2006). The use of 16S rRNA partial sequences, combined with stable carbon isotope analysis of lipid biomarkers, provides a powerful tool of understanding the microbial diversity and dynamics in the iron creek mat and sediment of SRS D-area.

Previous studies have reported the relationship between antibiotic resistance and heavy metals (Alonso et al., 2001). Heavy metals have been widely reported as possible factors of selective pressure for antibiotic resistance. In the environment, bacteria in metal-contaminated areas appeared to be more tolerant to metals and antibiotics than in the control sites (Baker-Austin et al., 2006, Stepanauskas et al., 2006, Wright et al., 2006). In our study, 65% DHC cloned sequences had 97-99% similarity with *Streptococcus mitis*, which had tetracycline resistance genes (Aminov and Mackie, 2007), The predominance of antibiotic resistant bacteria in the most heavy metal contaminated samples confirmed the hypothesis that locations contaminated by high concentrations of heavy metals are likely to serve as sources for antibiotic resistant and metal tolerant bacteria and genes.

The Cyanobacteria are autotrophic producer by fixing CO2. Cyanobacteria also can convert inert atmospheric nitrogen, such as nitrate or ammonia into an organic form, which very important for healthy plant growth. Cyanobacteria are found in almost every conceivable habitat, including extreme conditions with a wide variety of pH, salinity, and temperature (Sorensen et al., 2005; Taton et al., 2003). Recently, cyanobacterial communities were detected in high-iron accumulated environments (Pierson et al., 1999, 2000; Brown et al., 2005). Several studies postulated ferrous ion may function as potential electron donor for photosythesis in Cyanobacteria (Cohen et al., 1986, Pierson et al., 1999, 2000). Ferrous may also increases the photosynthetic rate by operating at the spectrum of redox potential values of -50 to +50 mv, which is an ideal range for the photic microzone (Guerres et al., 2002). Phototrophs in microbial mats were investigated in high iron hot springs (Pierson et al., 1999, 2000, Brown, et al., 2005), little is known about diversity of Cyanobacteria inhabiting iron-rich mat in contaminated environments. In this study, we observed 3 major types of Cyanobacteria. 19 IRM clones were related to *Microcoleus* and *Oscillatoria* species, which were known to use sulfide to sustain anoxygenic photosynthesis (Cohen et al., 1986). Totally 21 IRM *cyanobacterium* clones were close to *Gleothece* sp and *Cyanothece* sp. Genus *Glothece* and *Cyanothece* were unicellular Cyanbacteria which can aerobically fix-nitrogen (Mazard et al., 2004). These results suggested that Cynabacteria in iron creek mat may potentially contribute to N2 fixation in addition to serving as the prominent primary producers. Interestingly, a group of cyanobacteria (13 clones) related to an uncultured antarctic cyanobacterial clone were also detected in iron creek mat, which temperature is higher than Antarctic lakes (Taton et al., 2003). The depletion of <sup>13</sup>C in the iron creek also reflected intense carbon cycling coupled between the autotrophic community in the surface mat and the heterotrophic community in the sediment.

The occurrence of Chloroflexus in the Iron Creek Mat is consistent with the observation that it was found in high iron mat at Chocolate Pots (Pierson et al., 2000). However, the Chloroflexus were not found in the other high iron thermal springs (Hanada et al., 1995). We assumed that organic carbon source provided by Cyanobacteria may determine the growth of Chloroflexus in different iron-accumulated environments. Phylogenetic analysis showed that most IRM Chloroflexus-like clones were affiliated with uncultured environmental sequences, the metabolic functions of Chloroflexus in SRS iron creek were still under investigation. Both IRM and IRS clone libraries contains approximately 12.2% Chloroflexus-like sequences, however, they are phylogenetically distant. Interestingly, 14 out of 23 IRS Chloroflexus fell into Cluster I in the phylogenetic

tree within Chloroflexus. The strictly anaerobic bacteria, *Dehalococcoides* spp., which were capable of oxidative degradation of a variety of dechlorinated products, were present as a groups in this cluster (Adrian et al., 2000; Maymo-Gatell et al., 1997). Previous studies detected that the "*Dehalococcoides*" groups in the various contaminated sites (Yoshida et al., 2005; Hendrickson et al. 2002). The *Dehalococcoides*-like sequences in the IRS site have high similarity with sequences recovered from a metal-contaminated soil at super fund site. This finding suggested that "*Dehalococcoides*"-related bacteria may play a role in degrading organic wastes in heavy-metal contaminated environments.

Previous studies found *Clostridium spp.* sequences dominated in different ecosystems, including rumen (Tajima et al., 2000; Whitford et. al., 1998), landfills (Burrel et al., 2004; Dyke et al., 2002) and anoxic soils (Hengstmann et al., 1999; Lueders et al., 2004). Anaerobic degradation of complex polymers, especially cellulose decomposition was considered as the main process in the landfills. Microbial diversity in soil was determined by environmental factors, such as carbon and soil water contents. In iron creek sediment, substrates were available by autotrophic product in the mat, coal pile runoff, or healthy adjacent areas. Fluctuation in the water contents were influenced by alternative seasons of flooding and dryness. IRS *Clostridium*-like sequences can be divided into 2 big groups. Clostridia clones in the first group utilized a variety of the easily metabolizable carbohydrates, whereas the stable polymers, such as cellulose serve as the carbon sources in another group. CO2 was the final products fermentation of these organic materials, in turn, provide carbon source to the autotrophic microbial communities in the above mat for CO2 fixation. The carbon isotope analysis of IRS lipid

and TOC suggested that heterotrophs may be the dominant microorganisms. The observation of several Firmicute-like clusters as abundant microorganism in the iron creek sediment appeared to be consistent with the carbon isotope results.

Transformation is a major process for bioremediation because metals are not degradable (Lovley and Coaste, 1997). In-situ bioremediation methods, which reduce the potential mobility and bioavailability of heavy metals, have been developed in recent years. Microorganisms play important roles in the environmental fate of toxic metals by changing metal speciation and mobility (Gadd, 2004). The detection of potential bioremediating bacteria in contaminated sites will greatly aid in the development of insitu heavy-metal bioremediation strategies. In this study, some clones assigned to bioremediating microorganism were observed in iron creek mat and sediment. A group of clone sequences were affiliated with Rubrobacter spp. Isolates and environmental clones clustering with the Rubrobacteria group, were previously retrieved from different heavymetal contaminated site, such as thermally polluted effluent (Carreto et al., 1996) and Znpolluted soils (Moffett et al, 2003). The group of *Rubrobacteria* is probably functionally active in heavy-metal contaminated soils (Gremion et. al., 2003). Six IRS clonal sequences highly related to *Ralstonia sp* 13I, which was isolated from Torch Lake sediment contaminated with copper, arsenic, chromium, and lead. This isolate can carry multiple resistances to several metals including Cu, Zn, Ni and Cd species. (Konstantinidis et al., 2003), which confirmed that this species could be resistant to more metals, such as high concentrations of As and V species. Another interesting result is that Anaeromyxobacter-like species were investigated in both iron creek mat and sediment samples, which had 95-97% similarity with Anaeromyxobacter dehalogenans.

*Anaeromyxobacter* species were considered as microorganisms with ability to detoxify multiple pollutants because they can utilized several electron acceptors, including nitrate, Fe(III), U(VI), and chlorinated hydrocarbons (Sanford et al., 2002). In the experiments during in-situ biostimulation of FRC subsurface sediment cocontaminated with uranium and nitrate, *Anaeromyxobacter dehalogenans*-related bacteria were predominant in biostimulating microbial community. This result indicated that these microbes may be the important metal-reducing organism in polluted environments (North et al., 2004). Effectiveness of culture-independent methods, microorganisms responsible for heavy-metal detoxification from these natural habitats must be cultivated to understand better their role in environmental processes. Future investigations on iron creek microbial communities should involve a combination of both culture-dependent and culture-independent methods to elucidate the metabolic activities in these potential bioremediating microbes.

In summary, the results of this study may provide valuable information on microbial community dynamics responding to change in the physical and chemical conditions. Also, these results suggest that novel species of heavy-metal-resistant bacterial and organic contaminant-degrading bacteria may be present in this disturbed ecosystem. These types of bacteria may be selected for potential bioremediation in this site.

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| Element | Source Location | Creek Downstream |
|---------|-----------------|------------------|
|         | (DHC), mg/kg    | (IRMS), mg/kg    |
| Mg      | 93.83           | 1568.32          |
| Al      | 2627.84         | 7894.56          |
| V       | 225.68          | 9.88             |
| Cr      | 113.70          | 11.04            |
| Mn      | 97.95           | 260.95           |
| Fe      | 44772.37        | 331265.01        |
| Со      | 61.07           | 3.29             |
| Ni      | 153.75          | 16.50            |
| Cu      | 153.16          | 6.86             |
| Zn      | 163.71          | 53.50            |
| As      | 82.74           | 60.64            |
| Sr      | 223.39          | 16.41            |
| Cd      | 0.58            | 0.14             |
| Cs      | 2.62            | 0.73             |
| Pb      | 48.39           | 3.65             |
| U       | 3.65            | 2.93             |

Table 3.1. Heavy metal data for 2 samples analyzed from the Source Location and Creek Downstream, D-area.

Representative clone	# of clones	Closest sequences in GeneBank (Accession Number)	Similarity
Total	96		
DHC-Clone43	62	Streptococcus mitis (AF393761)	97-99%
DHC-Clone11	25	Veillonella parvula (AY995767)	99%
DHC-Clone7	3	Eubacterium sp. oral clone JN088 (AY349377)	99%
DHC-Clone92	1	Neisseria sp. oral clone BP2-72 (AB121944)	99%
DHC-Clone88	3	Prevotella oris (L16474)	99%
DHC-Clone55	2	Porphyromonas gingivalis (AB035456)	99%

Table 3.2. Classification and number of 16S rRNA gene sequences in Source location (DHC).

Table 3.3. Relative abundance of operation taxonomic units (OTU) among bacterial taxon within IRM and IRS clone libraries. Sequences with similarities  $\geq$ 97% were placed into the same operational taxonomic unit (OTU).

Taxon	Iron Creek Mat	Iron Creek Sediment
Bacterial		
No. clones analyzed	171	188
(No. distinct OTUs) <sup>a</sup>	(94)	(114)
Acidobacteria	8 (7)	2 (2)
Acitonobacteria	6 (6)	15 (10)
Bacteriology	0	2 (1)
Chloroflexi	21 (13)	23 (14)
Cyanobacteria	60 (15)	2 (2)
Eukayate	7 (3)	1(1)
Firmicutes	1 (1)	92 (60)
Nitro	0	1 (1)
OP 10	0	5 (3)
Planctycemete	1 (1)	7 (2)
Proteobacteria		
Alpha subdivision	11 (10)	5 (3)
Beta subdivision	28 (17)	8 (3)
Delta subdivision	14 (12)	14 (8)
Gamma subdivision	8 (6)	0
Thermotoga	1 (1)	0
Verrimicrobial	2 (2)	2 (2)
Unclassified	3	9

Fig. 3.1. Pictures showing the headwater (A) from the coal-combustion treatment plant and downstream mat (B) in the effluent channel in the D-area at SRS. In B, both mat (red) and sediment (black) samples were collected.

Fig 3.1.





Figure 3.2. Rarefaction analysis of soil bacterial 16S rRNA gene libraries from three clone libraries (DHC, IRM, IRS), displaying number of OTUs detected versus number of sequences analyzed. Sequences with similarities  $\geq$ 97% were placed into the same operational taxonomic unit (OTU).

Fig 3.2.



Figure 3.3. Phylogenetic relationship to the Acidobacteria and Acitinobacteria of partial 16S rRNA sequences (approximately 500 bp) from the dowonstream location. The tree topology is based on Neighbor-Joining analysis using p-distance method (Mega3.1 software), and was evaluated by Maximum Parsimony analysis. One representative of sequence groups is shown; the total number of clones represented by these sequences is shown in the parenthesis. The accession numbers for the reference sequences are shown in black. 1000 bootstraps were performed and only bootstrap values of >50% are shown at the branch points.



Figure 3.4. Phylogenetic relationship to the Chloroflexi of partial 16S rRNA sequences (approximately 500 bp) from the dowonstream location. The tree topology is based on Neighbor-Joining analysis using p-distance method (Mega3.1 software), and was evaluated by Maximum Parsimony analysis. One representative of sequence groups is shown; the total number of clones represented by these sequences is shown in the parenthesis. The accession numbers for the reference sequences are shown in black. 1000 bootstraps were performed and only bootstrap values of >50% are shown at the branch points.

Fig 3.4.





Figure 3.5. Phylogenetic relationship to the cynobacteria of partial 16S rRNA sequences (approximately 500 bp) from the dowonstream location. The tree topology is based on Neighbor-Joining analysis using p-distance method (Mega3.1 software), and was evaluated by Maximum Parsimony analysis. One representative of sequence groups is shown; the total number of clones represented by these sequences is shown in the parenthesis. The accession numbers for the reference sequences are shown in black. 1000 bootstraps were performed and only bootstrap values of >50% are shown at the branch points.



0.02

Figure 3.6. Phylogenetic relationship to the Clostridia of partial 16S rRNA sequences (approximately 500 bp) from the dowonstream location. The tree topology is based on Neighbor-Joining analysis using p-distance method (Mega3.1 software), and was evaluated by Maximum Parsimony analysis. One representative of sequence groups is shown; the total number of clones represented by these sequences is shown in the parenthesis. The accession numbers for the reference sequences are shown in black. 1000 bootstraps were performed and only bootstrap values of >50% are shown at the branch points.



Figure 3.7. Phylogenetic relationship to the *Bacilius* and other groups in Firmicutes of partial 16S rRNA sequences (approximately 500 bp) from the dowonstream location. The tree topology is based on Neighbor-Joining analysis using p-distance method (Mega3.1 software), and was evaluated by Maximum Parsimony analysis. One representative of sequence groups is shown; the total number of clones represented by these sequences is shown in the parenthesis. The accession numbers for the reference sequences are shown in black. 1000 bootstraps were performed and only bootstrap values of >50% are shown at the branch points.



Figure 3.8. Phylogenetic relationship to the alphaproteobacteria of partial 16S rRNA sequences (approximately 500 bp) from the dowonstream location. The tree topology is based on Neighbor-Joining analysis using p-distance method (Mega3.1 software), and was evaluated by Maximum Parsimony analysis. One representative of sequence groups is shown; the total number of clones represented by these sequences is shown in the parenthesis. The accession numbers for the reference sequences are shown in black. 1000 bootstraps were performed and only bootstrap values of >50% are shown at the branch points.



Figure 3.9. Phylogenetic relationship to the Betaproteobacteria of partial 16S rRNA sequences (approximately 500 bp) from the dowonstream location. The tree topology is based on Neighbor-Joining analysis using p-distance method (Mega3.1 software), and was evaluated by Maximum Parsimony analysis. One representative of sequence groups is shown; the total number of clones represented by these sequences is shown in the parenthesis. The accession numbers for the reference sequences are shown in black. 1000 bootstraps were performed and only bootstrap values of >50% are shown at the branch points.



Figure 3.10. Phylogenetic relationship to the deltaproteobacteria of partial 16S rRNA sequences (approximately 500 bp) from the dowonstream location. The tree topology is based on Neighbor-Joining analysis using p-distance method (Mega3.1 software), and was evaluated by Maximum Parsimony analysis. One representative of sequence groups is shown; the total number of clones represented by these sequences is shown in the parenthesis. The accession numbers for the reference sequences are shown in black. 1000 bootstraps were performed and only bootstrap values of >50% are shown at the branch points.



Figure 3.11. Phylogenetic relationship to the Planctomycete ,Verrucomicrobia, Eukaryota, and Bacterioidete partial 16S sequences (approximately 500 bp) from the downstream location. The tree topology is based on Neighbor-Joining analysis using p-distance method (Mega3.1 software), and was evaluated by Maximum Parsimony analysis. One representative of sequence groups is shown; the total number of clones represented by these sequences is shown in the parenthesis. The accession number for the reference sequences are shown in black. 1000 bootstraps were performed and only bootstrap values of >50% are shown at the branch points.



Figure 3.12.A. Fatty Acid profiles of two samples (surface mat and sediment below) at the downstream location at the SRS D-area.

Figure 3.12.B. Variations of  $\delta^{13}$ C ratios of 14:0, 16:0, 18:1  $\omega$ 7c, 18:0, 22:0, 24:0 fatty acids, and TOC in same sample described in Figure 3.12.A.





Iron Creek Mat
Iron Creek Sediment

# **CHAPTER 4**

# MICROBIAL COMMUNITY CHANGES ALONG THREE HEAVY METAL AND RODIONUCLIEDS GRADIENTS AT M-AREA OF SAVANNAH RIVER SITES, SOUTH CAROLINA, USA

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## ABSTRACT

The Savannah River Site (SRS) is an approximately 800-km<sup>2</sup> former nuclear weapons production facility situated in the Upper Atlantic Coastal Plain of South Carolina along the Savannah River near Aiken, SC. The discharge of waste water from metallurgical process contains uranium, nickel and other metals or radionuclides, which contaminate groundwater, soils and sediments, particularly in the vicinity of the M-Area. We hypothesize that microbial community dynamics respond to changes in chemical conditions in heavy metal and radionuclide contaminated soils at SRS. To test this hypothesis, we analyzed soil samples with different levels of metal (e.g., chromium, cobalt, and nickel) and radionuclide (e.g., uranium) contaminations from the M-area, Lower Tims Branch, Stead Pond, and Beaver Pond by applying 16S rRNA gene clone libraries. The library for the Lower Tims Branch had 209 clones and those for Stead and Beaver Ponds have 242 and 200 clones, respectively. Members of Proteobacteria were predominant in all tested samples, representing 49% in Stead Pond, 53% in Beaver Pond, and 53% in Lower Tim Branch. Acidobacteria is another important group, which comprises 22.0-34.5% of clones for samples collected from the three locations. Interestingly, approximately 2% clones from Stead pond and Beaver pond had 95% similarity with Geobacter species, which were not detected in the Lower Tims Branch Sample. About 12% clones in Lower Tims Branch have 92-98% similarity with methanotrophs; only 2% clones were related to metanotrophs in the other two samples. Acidobacteria-related sequences had 95-99% similarity with those retrieved from the other DOE's uranium contaminated sites. Our results suggested that composition and structure of microbial community changed in relation to different heavy metal and radionuclide contamination and environmental conditions. Uncultured *Acidobacteria* may participate in the ecological functions in the contaminated area. Members of sulfate-reducing and iron-reducing bacteria, especially *Geobacter* species, may play an important role in immobilizing the metals and radionuclides through metabolic activities.

## **INTRODUCTION**

The widespread contamination of soils, sediments and groundwater with heavy metals and radionuclides at Department of Energy (DOE) facilities is a serous environmental problem. It has many polluted locations that need to be cleaned up. In particular, the Savannah River Site, South Carolina, has been subjected to both heavy metal and radionuclide contamination. The contaminants include the heavy metals cadmium, zinc, and nickel and radionuclides such as uranium and cesium.

Uranium is one of the most common radionuclides at SRS. A wide range of prokaryotes can reduce hexavalent uranium [U(VI)] to tetravalent uranium [U(IV)] and precipitate as a U(IV) mineral such as uraninite (UO<sub>2</sub>) (Suzuki et al., 2003). Organisms that can reduce uranium include hyperthermophilic archaea (Kashefi and Lovley, 2000), thermophilic bacteria (Liu et al., 2005), mesophilic Fe(III)- and sulfate-reducing bacteria (Coates et al., 2000; Lovley and Philips, 1992; Pietzsch et al.,1999) and fermentative bacteria (Francis et al., 1994). Recent studies revealed that sulfate-reducing microorganisms have potentials in uranium immobilization (Chang et al., 2001). Also, adsorption of uranium onto iron oxides has long been recognized as an important reaction for immobilization of uranium (Lack et al., 2002). Stimulating microbial reduction of soluble U(VI) to insoluble U(IV) shows promise as a strategy for immobilizing uranium in uranium-contaminated subsurface environments.

The effects of heavy metals and radionuclides on microbial communities have been assessed by comparing indigenous contaminated and uncontaminated communities (Gillan et al., 2005; Knight et al., 1997; Petrie, et al., 2003; Stephan, 1999) or through controlling biogeochemical processes of specific contaminant (Gillan et al., 2005; Reardon et al., 2004). The former approach provides greater realism than the latter; whereas the latter offers the greatest experimental control (Feris et al., 2004). PCR-based 16S rRNA gene clone libraries provide a powerful tool to estimate the similarity or dissimilarity between two microbial communities (Sliwinski et al., 2004). Several studies have applied cultivation-independent approaches for characterization of the DOE FRC sites (Fields et al., 2005; Petrie et al., 2003; Reardon et al., 2004). In this study I will examine the changes of microbial community changes in SRS M-area soils contaminated with heavy metals and radionuclides by construction of 16S rRNA gene clone libraries.

## **MATERIALS AND METHODS**

**Field site description.** The field site in this study is the Department of Energy's Savannah River Site (SRS), located near Aiken, SC. The discharge of waste water from metallurgical process contains uranium, nickel and other metals or radionuclides, which contaminate groundwater and riparian sediments, particularly in the vicinity of the M-Area.

Soil samples were collected from three locations in the M-area including Steed pond, Beaver pond and Low Tims Branch. Steed pond is heavily contaminated with metals and raidonuclides, which received up to 44,000 kg of depleted and natural uranium (U), and similar quantities of nickel (Ni) from aluminum-clad nuclear reactor. Lower Tims Branch is less contaminated with metals and radionuclides. It is a secondorder stream that receives contamination from an eroding former radiological setting pond. Beaver pond functions as a settling basin for heavy metals and radionuclides. **Metal analysis.** All samples were assayed in the analytical facility at the Savannah River Ecology Laboratory. Samples were subjected to nitric acid/hydrogen peroxide microwave digestion (CEM, Matthews, NC) followed by inductively coupled plasma-mass spectrometry analysis (ICP-MS, Perkin-Elmer, Norwalk, CT). Appropriate calibration standards and blanks were used in both the digestion and analysis processes.

**DNA extraction.** Environmental DNA was extracted from 10-g soil by using Ultraclean Mega Prep Soil DNA Kit (Mo Bio Laboratory, Inc, Solana Beach, CA). The precipitated DNA was purified by gel electrophoresis plus mini-column preparation (Wizard DNA Clean-Up system, Promega, Madison, WI).

**PCR amplification and cloning.** The nearly complete 16S rRNA genes (approximately 1500 bp) were amplified in 9700 Thermal Cycler (Perkin-Elmer) with the primer pair FD1F (5' AGA GTT TGA TCC TGG CTC AG 3') and 1540R (5' AAG GAG GTG ATC CGG GC 3'). In order to avoid potential sample biases and to obtain enough PCR productions for cloning, five replication amplifications were carried out for each sample. The PCR parameters were 30 s at 80°C and 2 min at 94 °C, followed by 25 cycles of amplification with each cycle consisting of 30 s at 94 °C, 30 s at 58 °C, and 90 s at 72 °C. The last cycle, however, was 7 min at 72 °C. The combined PCR products were purified by cutting out the appropriate band from a low-melting point agarose gel (0.8%) using the Wizard® SN Gel and PCR clean-up system (Promega). The Recovered DNA was ligated with pCR vector from a TA-cloning kit and competent *Escherichia coli* cells were transformed according to manufacture's instruction (Invitrogen).

**Phylogenetic analysis.** The closest database relatives of all sequences were obtained using all available sequence information by Blast searches of Genebank. The sequences were aligned with CLUSTALX and alignments compared with references sequences from the database. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 and phylogenetic trees were constructed with distance matrices and the neighbor-joining method with MEGA (Kumar et al., 2001) Trees constructed with maximum-likelihood and neighbor-joining methods were not significantly different.

**Statistical comparison of coverage.** The 16S rRNA gene clone libraries between two samples were compared by using computer program WebLIBSHUFF version 0.96. Principle-component analysis (PCA) was performed by using SYSTAT statistical computing package (version 10.0, SPSS Inc., Chicago, IL).

## **RESULTS AND DISCUSSION**

**Heavy metals and radionuclides of soil samples.** The heavy metal analysis of the soil samples provides information on important processes of contamination and for the understanding of the shift of microbial communities among different environments. The heavy metal concentrations of each of the soil samples are shown in Table 4.1. Soil collected from Lower Tims Branch had particularly high concentrations of uranium (2216 mg/kg) and nickel (715 mg/kg), followed by sample collected from Steed pond, which had medium concentrations of uranium (72 mg/kg) and nickel (54.8 mg/kg); soil taken from the Beaver pond had relative low concentration of uranium (3.9 mg/kg) and nickel

(14.6 mg/kg). Lower Tims Branch is a second-order stream that drains approximately 16 km<sup>2</sup> on the SRS, receiving substantial quantities of uranium and nickel from nuclear material production and refinement. This may cause the highest heavy metal contamination in the Lower Tims Branch soils. Effluents from Lower Tims Branch flowed into the Beaver pond and then the Steed pond. Although the Beaver pond is upstream of the Steed pond, the latter provided the longer residence time for depositing the heavy metal contaminants, which may explain the higher level of heavy metals in the Steed pond than in the Beaver pond.

**Characterization of 16S rRNA gene sequences.** Rarefaction analysis indicated that the bacterial diversity was adequately sampled within 100 clones for Lower Tims Branch, 200 for the Steed pond and the Beaver pond (Fig. 4.1).

Comparison of diversity indices suggested that the Beaver pond had the highest diversity whereas the Steed pond displayed the lowest diversity (Table 4.2.1). Pair-wise comparisons showed that 6.7-13.5% of the total OTUs recovered from each sample was shared with those of another sample (Table 4.2.2).

The 16S rRNA gene clonal libraries were compared between the sites based on LIBSHUFF analysis. The p-values between each pair of two clone libraries are 0.002, which suggested that the clone libraries are significantly different among different locations in the M-area (Table 4.2.3).

One hundred and eighty three clones were sequenced for the Lower Tims Branch, 231 clones for the Steed pond, and 201 clones for the Beaver pond (Table 4.3). The averaged length sequenced was 500 nucleotides. Full-length sequence analysis was

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performed to accurately determine the representative clones from the Steed pond. The major groups from the *Proteobacteria* and *Acidobacteria* are further described and discussed (Tables 4.4 and 4.5).

The Delta-*Proteobacteria*. Approximately 2% clones from both the Steed pond and the Beaver pond had 95% similarity with various strains of *Geobacter* species, which were not detected in Lower Tims Branch (Fig. 4.3). *Geobacter* genus has been shown to utilize ferric iron as electron acceptor to completely mineralize organic acids to carbon dioxide. Recent studies have shown that members of the *Geobacteriaceae* family are present in a variety of environments (Nogales et al., 1999; Roling et al., 2001; Rooney-Varga et al., 1999). North et al. (2004) reported that 16S rRNA sequences related to the members of the *Geobacteriaceae* family significantly increased in the FRC acidic subsurface samples by in-situ neutralization and addition of an electron donor. This can be explained by its ability to utilize the abundant electron acceptors and the presence of sufficient carbon substrate. Thus, this successful treatment may provide us an important technique to stimulate the *Geobacteriaceae* group in our SRS contaminated areas as well.

*Acidobacteria.* 16S rRNA gene-based surveys revealed that members of the *Acidobacteria* phylum were abundant in diverse settings (Hugenholtz et al., 1998; Barns et. al., 1999). Mounting evidence demonstrated that bacteria in the *Acidobacteria* phylum were ubiquitously present in soils and sediments contaminated by radionuclides (Barns et al., 2007), heavy metals (Gremion et al., 2003) and hydrocarbon contamination (Allen et al., 2007). Recent studies on microbial community structures of FRC uranium-contaminated areas has detected that *Acidobacteria* may have ability to withstand high content of uranium in acidic subsurface (Barns et al., 2007).

From the three 16S rRNA gene clone libraries generated in our study, 33.3% of clone sequences from the Beaver pond, 16% from the Steed pond, and 34.3% from the Lower Tims Branch were phylogenetically affiliation with *Acidobacteria*. Moreover, these acidobacteria-related sequences were assigned to 12 subgroups (Fig 4.4 and Table 4.6). Interestingly, 61 out of 69 sequences from the Beaver pond and 55 out of 60 sequences from the Lower Tims Branch clustered with subgroups 1, 2 and 3; whereas sequences from the Steed pond were frequently found in subgroups 17, 18, 19 and 25, which were not detected in the other two libraries.

Subgroups 1, 2, 3, 4 of the Acidobacteria are actively distributed in soil worldwide (Barns et al., 1996; Janssen, 2006; Ludwig et al., 1997; Nogales, 1999). However, no specific isolation methodologies have been developed to culture Acidobacteria, only a few acidobacterial isolates were reported. Kishimoto and Tano (1987) cultured eight isolates from Acidobacteria subgroup 1 from water, mud, and soil that were affected by acid mine runoff; one of the isolates was subsequently described as Acidobacterium capsulatum (Kuske et al., 1997). To date, Acidobacterium capsulatus is the only well described species in Acidobacteria subgroup 1. Only 4 sequences from the Beaver pond had 95-97% similarity with Acidobacterium capsulatus; the other sequences in Acidobacteria subgroup 1 had 96-99% with the sequences EF457401, EF457395, AY661979, and AY661979 retrieved from the other DOE uranium-contaminated sites (Barns et al., 2007). The presence of the similar 16S rRNA genes in our SRS samples provided further evidence that certain Acidobacteria may be widely distributed in the radionuclide- contaminated environments. Only two clones from the Beaver pond were closely related to the other taxonomically described *Geothrix Fermentans* is in

*Acidobacteria* subgroup 8. *G. Fermentans* is was an anaerobic chemo-organotroph that utilizes various organic acids as electron donors and Fe(III) as the electron acceptor.

By 2005, 11 subgroups in *Acidobacteria* phylum were obtained by using 16 rRNA gene surveys (Zimmermann et al., 2005). Analysis of sequences surveyed in subsurface sediments from uranium-contaminated sites greatly expanded the phylogeny of the Acidobacteria phylum that now consists of 26 subgroups (Barns et al., 2007). Interestingly, the sequences closely related to the new published subgroups 18, 19, 25 were only detected in Steed pond soil with intermediate uranium concentrations among three studied samples. This result may show that members of these new *Aciodobacteria* subgroups are present in the certain extent of uranium contaminated areas.

In summary, our results suggested that composition and structure of microbial community change in relation to samples with different heavy metal and radionuclide concentrations. Members of sulfate- reducing and iron-reducing bacteria, especially *Geobacter* species, may play an important role in immobilizing the metals and radionuclide through metabolic activities. Members of uncultured *Acidobacteria* may play certain ecological functions in the uranium-contaminated sites.

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Mg/KG	Lower Tims Branch	Steed pond	Beaver pond
Mg	43	38	116
Al	6353	3452	4003
V	53	52	18
Cr	82	35	12.28
Mn	540	44	191
Fe	13670	6768	4292
Со	11.96	3.95	2.63
Ni	715	54	14
Cu	41	27	10
Zn	85	101	50
As	9	8.	BDL
Sr	0.93	1.27	12
Cd	0.19	0.094	0.034
Cs	1.34	2.21	1.90
Pb	42	30	18
U	2216	72	3

Table 4.1. Heavy metal data for samples analyzed from the Lower Tims Branch, the Steed pond, and the Beaver pond in the M-area.

Samples	Lower Tims Branch	Steed pond	Beaver pond
# of clones <sup>a</sup>	231	208	200
$OTU^{b}$	120	96	135
H'°	6.61	5.82	6.98
$1/D^d$	108	36.8	207
Evennesses	0.919	0.827	0.974

Table 4.2.1. Comparison of Diversity of Soils in M-area.

a. Number of analyzed clones in each clone library.

b. Operational taxonomic units based on unique sequences detected. Sequences with

similarities  $\geq$  97% were placed into the same operational taxonomic unit (OTU).

c. Shannon index, higher number represents higher diversity.

d. Reciprocal of Simpson's index, higher number represents higher diversity.

e. Evenness, as value of evenness approaches 1, the population is more evenly distributed.

Table 4.2.2. Pair-wise comparison demonstrating the genetic overlap of 16S rRNA genes between two sites.

Site	Beaver pond	Steed pond	
Lower Tims Branch	26/231	14/216	
Beaver pond		16/255	

Each pair-wise comparison shows the number of OTUs common to both samples vs. the total number of OTUs of both samples. Sequences with similarities  $\geq$ 97% were placed into the same operational taxonomic unit (OTU).

Table 4.3. Summary of phylogenetic distribution of 16S rRNA clones from Lower Tims Branch, the Steed pond and the Beaver pond.

	Lower Tims Branch	Steed pond	Beaver pond
Proteobacteria	78 (42.65%)	96 (41.6%)	89 (44.3%)
Alpha-proteobacteria	61 (33.3%)	42 (18.2%)	49 (24.4%)
Beta-proteobacteria	3 (1.6%)	21 (9.1%)	6 (3.0%)
Delta-proteobacteria	5 (2.7%)	30 (13.0%)	24 (11.9%)
Gamma-proteobacteria	6 (3.3%)	2,(0.9%)	3 (1.5%)
Unclassfied proteobacteria	3 (1.6%)	1 (0.4%)	7 (3.5%)
Acidobacteria	61 (33.3%)	37 (16.0%)	69 (34.3%)
Actinobacteria	4 (2.2%)	9 (3.9%)	3 (1.5%)
Bacteroidetes	0	2 (0.9%)	0
Cyanobacteria	0	2 (0.9%)	0
Firmicutes	8 (4.4%)	27 (11.7%)	4 (2.0%)
Gemmatimonadetes	3 (1.6%)	0	2 (1.0%)
Lentisphaerae	0	1 (0.4%)	0
Nitrospira	1 (0.5%)	1 (0.4%)	1 (0.5%)
OP-10	0	4 (1.7%)	0
Planctomycetes	4 (2.2%)	2 (0.9%)	2 (1.0%)
Thermophilic	1 (0.5%)	0	0
Spirochaetes	0	2 (0.9%)	0
Verrucomicrobia	1 (0.5%)	20 (8.7%)	9 (4.5%)
Unclassified	22 (12.0%)	28 (12.1%)	22 (10.9%)
# of Total Clone	183	231	201

Table 4.4. Summary of the 16S rRNA gene clonal affiliation in the phylogentic tree based on the most closely related GenBank member sequences. BP = Beaver pond, SP = Steed pond, LTB = Lower Tims Branch.

Class	Order	Family	BP	SP	LTB
α-	Caulobacterales	Caulobacteraceae	4	2	1
Proteobacteria	Rhizobiales	Beijerinckiaceae	3	2	1
		Bradyrhizobiaceae	10	10	16
		Hyphomicrobiaceae	8	6	14
		Methylobacteriaceae	6	4	8
		Methylocystaceae	3	11	6
		Phyllobacteriaceae	1	0	0
		Rhizobiaceae	0	1	0
	Rhodobacterales	Rhodobacteraceae	1	0	1
	Rhodospirillales	Acetobacteraceae	8	4	3
		Rhodospirillaceae	3	2	2
	Sphingomonadales	Sphingomonadacea	3	0	9
β-	Burkholderiales	Alcaligenaceae	3	0	1
Proteobacteria		Burkholderiales	0	1	2
		Comamonadaceae	1	4	0
		Incertae sedis 5	2	9	0
		Methylophilaceae	0	3	0
		Oxalobacteraceae	0	2	0
	Rhodocyclales	Rhodocyclaceae	2	2	0
δ-	Desulfobacterales	Desulfobacteraceae	4	4	0
Proteobacteria	Desulfurellales	Desulfurellaceae	1	1	3
	Desulfuromonales	Geobacteraceae	5	5	0
	Myxococcales	Cystobacterineae	6	4	0
		Sorangineae	0	2	2
	Syntrophobacterales	Syntrophaceae	2	3	0
		Syntrophobacteraceae	3	3	0
	Chromatiales	Ectothiorhodospiraceae	0	0	5
γ-	Legionellales	Coxiellaceae	1	0	1
Proteobacteria	Methylococcales	Methylococcaceae	0	1	0
	Thiotrichales	Thiotrichaceae	2	1	0
		Total Numbers	82	87	75

Table 4.5. Diversity and composition of Acidobacteria phylum in heavy metal

contaminated	samples,	SRS.
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	Beaver pond	Steed pond	Lower Tims
			Branch
No of Clones for			
subgroups (OTUs in each			
subgroup)			
1	16 (7)	5 (4)	23 (4)
2	23 (4)	8 (4)	14 (4)
3	22 (5)	7 (4)	19 (2)
4	1	0	0
5	1	0	0
6	1	0	2 (1)
8	2 (1)	0	0
13	0	0	3 (1)
17	3 (1)	4(1)	0
18	0	7 (1)	0
19	0	4 (1)	0
25	0	2 (1)	0
Total no. of clones	69	37	60
Total no. of OTUs	18	16	12
Total no. of subgroups	8	7	5

Table 4.6. Percent sequence similarity and distribution of selected OTUs within

*Acidobacteria* phylum. BP = Beaver pond, SP = Steed pond, LTB = Lower Tims Branch.

Representative				Closest Match	Similarity
clones	חח	CD	LTD		
Cash ann ann 1	BP	SP	LIB		
Subgroup I	6	0	1.4		07.000/
11m-Clone182	5	0	14	(EF457401)	97-99%
Tim-Clone134	1	0	6	Clone BGB.0069 (EF457395)	98-99%
Tim-Clone116	0	0	2	Clone Tc129-14 (AY262647)	98%
Tim-Clone89	0	1	1	Clone 300A-B12 (AY661979)	99%
Steed-Clone108	0	2	0	Clone5V15 (DQ663803)	99%
Steed-Clone143	1	1	0	Clone BGA.0027 (EF457379)	98%
Steed-Clone80	0	1	0	Clonej29-023 (EU122635)	99%
Beaver-Clone149	2	0	0	Clone 5C64 (DQ663973)	97%
Beaver-Clone109	2	0	0	Clone EC1149 (DQ083327)	96%
Beaver-Clone39	1	0	0	Ellin6024 (AY673361)	96%
Beaver-Clone192	4	0	0	Acidobacterium capsulatum (D26171)	95-97%
Subgroup 2					
Beaver-Clone	8	3	2	CloneK20-93 (AF145877)	94-96%
Beaver-Clone160	4	2	3	Clone 5V79 (DQ663849)	98-99%
Beaver-Clone160	4	1	1	Clone5V92 (DQ663859)	97-99%
Beaver-Clone37	7	2	8	Clone KF/GS- JG36-31 (AJ295657)	94-96%

# Table 4. 6 (continued).

Representative clones				Closest Match	Similarity
	BP	SP	LTB		
Subgroup 3					
Tim-Clone94	7	4	15	Clone T15-0035	94-99%
				(EF457353)	
Steed-Clone182	6	2	0	Clone 655941	94-96%
				(DQ404813)	
Beaver-Clone53	5	0	4	Clone 5A95	96-98%
				(DQ663860)	
Beaver-Clone46	2	0	0	Ellin371	99%
				(AF498753)	
Beaver-Clone173	2	1	0	CloneN12-11WL	96-98%
				(AF431411)	
Subgroup 4					
Beaver-Clone112	1	0	0	Clonej29-023	99%
				(EU122635)	
Subgroup 5					
Beaver-Clone24	1	0	0	Clone EC1149	96%
				(DQ083327)	
Subgroup 6					
Beaver-Clone109	2	0	2	clone K20-48	96%
				(AF145843)	
Subgroup 8	-			~ ^	0.507
Beaver-Clone 27	2	0	0	G. fermentans	96%
~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~				(AF145877)	
Subgroup13				<u></u>	
Tim-Clone70	0	0	3	Clone5V92	97-99%
				(DQ663859)	
Subgroup 17					0.60/
Steed-Clone19	3	4	0	Clone RP16.0042	96%
				EF45/4/5	
Subgroup 18	0		-		0.001/
Steed-Clone18	0		0	Clone RP16.0085	99%
G 1 10				(EF45/489)	
Subgroup 19	0		0		0.00/
Steed-Clone 68	0	4	0	Clone T16.0055	99%
Garbarr 25				(EF45/369)	
Subgroup 25	0	2	-	01 (540.00	0.50/
Steed- clone 57	0	2	0	Clone 654969	95%
				(DQ404639	

Figure 4.1. Rarefaction analysis of soil bacterial 16S rRNA gene sequences from three clone libraries (Lower Tims Branch, Steed pond, and Beaver pond), displaying number of OTUs detected versus number of sequences analyzed. Sequences with similarities  $\geq$ 97% were placed into the same operational taxonomic unit (OTU).

Fig 4.1.



Figure 4.2. Percentages of 16S rRNA sequences from the Stead pond, the Beaver pond and the Lower Tim Branch in the M-area, SRS. Sequences with similarities  $\geq$ 97% were placed into the same operational taxonomic unit (OTU).



Figure 4.3. Phylogenetic tree of  $\delta$ -proteobacterial 16S rRNA gene sequences from SRS M-area soils. The tree topology is based on Neighbor-Joining analysis using p-distance method (Mega 3.1 software), and was evaluated by Maximum Parsimony analysis.. One representative of sequence groups is shown; the total number of clones represented by these sequences was shown in the parenthesis. The accession number for the reference sequences are shown in black. 1000 bootstraps were performed and only bootstrap values of >50% are shown at the branch points.



Figure 4.4. Phylogenetic tree of acidobacterial 16S rRNA gene sequences from SRS Marea soils. The tree topology is based on Neighbor-Joining analysis using p-distance method (Mega3.1 software), and was evaluated by Maximum Parsimony analysis. One representative of sequence groups is shown; the total number of clones represented by these sequences was shown in the parenthesis. The accession numbers for the reference sequences are shown in black. 1000 bootstraps were performed and only bootstrap values of >50% are shown at the branch points.



## **CHAPTER 5**

THE DIVERSITY AND ABUNDANCE OF AMMONIUM-OXIDATION ARCHAEA IN HEAVILY CONTAMINATED SOILS AT DOE SAVANNAH RIVER SITE (SRS), SOUTH CAROLINA, USA

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#### ABSTRACT

The effects of heavy metals on the soil archaeal community structures were investigated in soil samples collected from three sites with different contamination sources (former nuclear processing and coal combustion) on the DOE's Savannah River Site in South Carolina, USA. Analysis of the archaeal 16S rDNA clone library revealed that sequences retrieved from these samples were significantly different from each other. For instance, members of the nonthermophilic group 1.1a and 1.1c of Crenarchaeota constituted an important fraction in the samples obtained from the (Lower Timer Branch-Steed Pond (LTB-ST) system with M-area, which received waste water from nuclear and metallurgical processes. In contrast, the archaeal community composition was observed to be dominated by members of group 1.3b *Creanarchaeota* in soil obtained from the chemical, metal and pesticide (CMP) pits. The similar proportions of Crenarchaeota (47.5%) and the Euryachaeota (52.5%) clones were present in beaver dam creek soil within D-area, which was contaminated by fly ash from coal combustions. Glycerol dialkyl glycerol tetraethers (GDGTs) profiles showed distinct patterns among the samples from different locations. In particular, crenarchaeol, a specific lipid biomarker of pelagic Crenarchaeota within Group 1.1a, was only detected in samples from the LTH-ST system. This result was consistent with the 16S rRNA analyse. Diversity of ammoniaoxidizing archaea was examined by targeting the archaeal ammonia monooxygenase asubunit (*amoA*) gene in the same samples to better understand the ecological functions of Crenarchaeata. The total AOA sequences from all six samples 93.5% fell into the water column/soil/sediment cluster. Furthermore, quantitative real-time PCR analysis showed that ratios of copies of archaeal amoA genes (AOA) to those of bacterial amoA genes (AOB) ranged from 0.72 to 9.8 in six studied samples. The lowest ratio (0.72) of AOA to AOB in Beaver dam creek soilwas consistent with relative lower proportion of crenarchaeotal clones in this library. Our results provide evidence that Crenarchaeota may play a significant role in ammonium oxidation in radionuclide and heavy-metal contaminated environments.

#### **INTRODUCTION**

The Savannah River Site (SRS) located in Aiken, South Carolina is one of the U.S. Department of Energy's (DOE) former nuclear processing facilities. The SRS was contaminated by heavy metals and radionuclides due to different pollutant sources from the past DOE weapon production. Bacterial community structures were studied in many sites within the U.S. nuclear weapon complex managed by the Department of Energy (North et al., 2004; Petrie et al., 2003; Reardon et al, 2004; Abulencia et al., 2006). However, only few studies have examined the effect of nuclear contamination on archaeal communities impacted by heavy metals and radionuclides.

Cultivation-independent molecular methods, particularly the analysis of cloned 16S ribosomal RNA gene sequences, have proven to be powerful tools to study community composition and the relative distributions of *Archaea* in natural habitats. All of the cultured *Crenarchaeota* from freshwater and terrestrial habitats are extreme thermophiles that have optimal growth temperatures of more than 80°C (Stetter 1996). However, numerous 16S rRNA-based phylogenetic surveys have established three major groups of sequences affiliated with *Archaea* from various nonthermophic environments (Delong 1998). Members of the so called Group 1 were associated with *Crenarchaeota*, whereas Group 2 and 3 fall within the families *Methanosarcinaceae*, *Methanosaetaceae*, *Methanomicrobiaceae*, and *Methanobacteriaceae* of the *Euryarchaeota* (Delong 1998, Chin et al., 1999). Phylogenetically distinct subclusters of Group 1 *Crenarchaeota* appear related to 16S rRNA gene sequences recovered from specific marine water column, aquatic and terrestrial ecosystems (Ochsenreiter et al., 2003). A wealth of cloned sequences belonging to Group 1.1b of *Crenarchaeota* has been discovered in may soil systems (Ochsenreiter et al., 2003, Bintrim et al., 1997, Buckley et al., 1998), including forest soils (Pesaro et al., 2002), grassland soils (Nicol et al., 2003), agriculture soils (Furlong et al., 2002), and pasture soils (Borneman et al., 1997). The 16S rRNA gene from soil fosmid 54d9, a 43.3-kb metagenomic fragment which was isolated from a calcareous grassland soil, is also associated with group 1.1b Crenarchaeota (Treusch et al., 2005). Group 1.2 of *Crenarchaeota* comprises a few sequences from marine and lake sediments (Hershberger et al., 1996, Ventrini et al., 1999). A majority of cloned sequences of Group 1.1c, were recovered from forest soils (Kemnitz et al., 2007). For examples, Finnish forest soil type B (FFSB) sequences were placed within this group in phylogenetic tree (Jurgens et al, 1997). Sequences affiliated with Group1.3 of Crenarchaeota were frequently retrieved from soils, freshwater, and wastewater (Ochsenreiter et al., 2003), such as petroleum-contaminated soil (Kasai et al., 2005), rice soil (Gro Kopf et al., 1998), metal-rich freshwater reservoir (Stein et al., 2002), anaerobic digestor (Godon et al., 1997), freshwater sediment (Jurgen et al., 2000; Schleper et al., 1997). Group1.1a of Crenarachaeota consists mainly of rDNA clones from acidic forest soils (Kemnitz et al., 2007), contaminated aquifer (Dojka et al., 1998), deep subsurface (Chandler et al., 1998), marine picoplankton assemblages (Delong, 1992). Marine sponge symbiont Cenarchaeum symbiosum (Preston et al., 1996) and marine aquarium candidatus 'Nitrosopumilus maritimus' (Könneke et al., 2005) are well-known mesophilic members of *Crenarchaeota*, and these two microorganisms were associated with group 1.1a Crenarchaeota.

Although mesophilic members of *Crenarchaeota* are ubiquitous and abundant in the ocean and soils, their physiological properties and ecological functions in nature

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remain a mystery due to the absence of cultivated organisms. A number of metagenomic studies have provided multiple lines of evidences that many mosophilic *Crenarchaeota* are capable of performing ammonium-oxidation (Schleper et al., 2005; Treusch et al., 2005). Furthermore, the recently isolated pure culture *Nitrosopumilus maritimus* (Group 1.1a lineage of Crenarchaeota) containing putative *amoA*, *amoB*, and *amoC* genes of ammonia monooxygenase, can grow solely on bicarbonate and ammonia as carbon and energy sources (Könneke et al., 2005).

Nitrification is a two-step process which oxidizes ammonia  $(NH_3)$  to nitrate  $(NO_3^{-1})$ via nitrite (NO<sub>2</sub><sup>-</sup>) (Kowalchuk et al., 2001). Previous studies have shown that chemolithoautotrophic ammonia-oxidizing bacteria (AOB) carry out the oxidation of NH<sub>3</sub><sup>+</sup> to NO<sub>2</sub><sup>-</sup>, the first rate-limiting step of nitrification. All known terrestrial AOB belong to a monophyletic group within the B-subclass of the Proteobacteria, including members of the genera Nitrosomonas and Nitrosospira (Head et al., 1993). Nitrosococus oceani and Nitrosococus halophilus are the only known species in AOB within the 7subclass of the Proteobacteria which are only found in halophilic and marine environments (Ward et al., 2002). The amoA gene, which codes for the alpha subunit of ammonia monooxygenase, the protein that catalyzes the oxidation of ammonia to hydroxylamine, has been utilized as a gene target to explore AOB diversity and quantify AOB populations in a wide variety of environments (Rotthauwe et al., 1998; Okano et al., 2004). Until recently, autotrophic oxidation of ammonia was considered to be restricted to the domain *Bacteria*. In last three years, however, many studies have demonstrated the existence of ammonia-oxidizing archaea (AOA) in various mesophilic habitats, including marine and terrestrial ecosystems (Beman and Francis, 2006; Wuchter et al., 2006;

Francis et al., 2005; Ingalls et al., 2006, Schleper et al., 2005, Venter et al., 2004; Leininger et al. 2006; Weidler et al., 2007). However, no investigations have been studied AOA communities impacted by heavy metals.

The first objective of this study is to use 16S rRNA gene analyses in combination with archaeal GDGT profiles to compare the archaeal community structures in different heavy-metal contaminated samples. The second objective is to use archaeal *amoA* genes to investigate the composition of the crenarchaeal ammonium oxidizers in the same samples. Our results demonstrated that changes in archaeal communities structures associated with different contaminated sources in studied samples. Furthermore, the presence of archaeal *amoA* genes in all the studied samples indicated that *Crenarchaeota* were capable of performing ammonium oxidation in the SRS contaminated soils. This information will help us to better understand the ecological functions of *Crenarchaeota* in heavy-metal contaminated environments.

#### **MATERIALS AND METHODS**

**Site description and sample collection.** The Savannah River Site (SRS) is an approximately 800-km<sup>2</sup> former nuclear weapons production facility situated in the Upper Atlantic Coastal Plain of South Carolina along the Savannah River near Aiken, SC (Fig. 1) (Sowder et al. 2003). SRS lies at 33°00'N and 81°41'W and has a climate characterized by humid and subtropical weather (Garten et al. 2000). Former production and processing sites at the SRS are contaminated by heavy metals and radionuclides due to different pollutant sources from the past DOE weapon production (Table 1).

The discharge of waste water from metallurgical process contains uranium, nickel and other metals or radionuclides, which contaminate groundwater and riparian sediments, particularly in the LTH-ST system of M-Area (Punshon et al. 2004). Lower Tims Branch (LTB) is a second-order stream that receives contamination from an eroding former discharge settling pond called Steed Pond. Steed Pond received up to 44000 kg of depleted and natural uranium (U) and similar quantities of nickel (Ni) from aluminumclad nuclear reactor targets from 1954 and 1985.

In SRS, 488-D-area Ash Basin contains a coal-burning electric power and steam generation facility with associated coal pile, coal pile runoff basins, ash basins, and reject coal pile (Carlson, 1990). Effluents from these basins are discharged into Beaver Dam Creek, a tributary of the Savannah River Sites.

The chemical, metal and pesticide (CMP) pits are located in the central part of the SRS in the Tim Branch watershed. The chlorinated solvents, metals, pesticides, and electrical parts containing PCBs were dumped into CMP pits between 1971 and 1979. Station 3 in the Pen Branch Creek is one of the more contaminated locations in CMP pits.

Soils were sampled from five locations of the Savannah River Sites. The first sample was collected from Beaver Dam Creek (Bdam), the second from top soils from station 3 of CMP pits (CMP311). The third sample was taken from Lower branch Tim Creek (LTH), The last two samples were collected from two heavy metal-contaminated gradients, one on the shoreline of steed pond (SSh), and the other one in outer bank of the steed pond (So). A sediment core was also obtained from the outer bank of the steed pond (Ssed). The level of contamination is higher in samples from outer bank than that of shoreline of Steed pond. Abbreviations in the parentheses represent the sample name below. The samples were immediately stored at -20°C on site and stored at -80°C in the laboratory until analysis.

**DNA extraction and purification.** Genomic DNA was extracted from 7.5 g at soils or sediment measured from frozen stocks using the Ultraclean Mega Prep Soil DNA kit (MOBio Laboratories, Inc, Solana Beach, CA). The precipitated DNA was further purified by gel electrophoresis plus mini-column preparation (Wizard DNA Clean-Up System, Promega, Madison, WI).

**PCR amplification and cloning of archaeal 16S rRNA gene and** *amoA*. Six Archaeal 16S rRNA gene and *amoA* clone libraries were constructed from three areas in Savannah River sites (Table 1). Both archaeal 16S rRNA genes (approximately 930 bp) and archaeal amoA gene fragment (approximately 630 bp) fragments were amplified in a Perkin-Elmer 9700 Thermal Cycler with the primer pairs Arch-21F/Ach-958R and ArchamoAF/Arch-amoAR respectively (Table 2). To minimize sample biases and to obtain enough amplicon for cloning, the number of cycles in a PCR reaction was limited to 30, and 3 replicated amplifications were carried out for each sample. The combined PCR products were purified from a low-melting point agarose gel (1.0 %) using the QIA Quick Gel Extraction Kit (QIAGEN, Inc., Valencia, CA). The purified DNA was ligated with pCR2.1 vector, and competent *Escherichia coli* cells were transformed according to manufacturer's instructions (Topo-TA cloning kit, Invitrogen, Carlsbad, CA).

**Sequencing and Phylogenetic Analysis.** Forty to 50 colonies from each sample were randomly selected for sequencing. Plasmid DNA containing inserts were prepared using a QIAprep Spin miniprep kit (QIAGEN, Valencia, CA). Sequences were determined from the purified inserts with the vector-specific primer M13F (5'-GTAAAACGACGGCCAG-3'). Sequencing reactions were carried out using ABI BigDye Terminatior v.3.1 (Applied Biosystems. Foster City, CA) and sequenced on an ABI 3100 automated sequencer.

Sequences were edited, checked, aligned and analyzed with the following programs: Sequencher (v4.0 Gene Codes, Ann Arbor, MI), Bioedit (Hall, 1999), Belleophon (Huber et al., 2004), and RDP's CHECK\_CHIMERA (Cole et al., 2003). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1, and phylogenetic trees were constructed with distance matrices and the neighbor-joining method with MEGA (Kumar et al., 2004). Trees were constructed with maximumlikelihood and neighbor-joining methods were not significantly different. Both archaeal 16S rRNA gene and *amoA* clone libraries between paired samples were compared by using computer program WebLIBSHUFF version 0.96.

**Data Analysis.** Sequences with identities of greater than 97% were considered to represent the same optional taxonomic unit (OTU). Coverage (C) was calculated as:  $C = 1-(n_1/N)$ , where  $n_1$  is the number of phylotypes that occurred only once in the clone library and N is the total number of clones analyzed (Mullins *et al.*, 1995). Rarefaction analysis (Heck et al., 1975) and computation the Shannon diversity and the Chao and ACE Chao1 nonparametric richness estimates were performed using the software program DOTUR (Schloss and Handelsman, 2005). Principle-component analysis (PCA) was performed using the **SPSS** 15 software (**SPSS** Inc., Chicago, Ill.). Using clone library data, samples from different locations were grouped or separated based on the patterns of OTUs shared by all samples and distinct OTUs from each samples.

**Real-time PCR.** The six samples as those used for clone library construction (see above) were used to determine the abundance of certain groups of microorganisms. The 16S rRNA gene primers specific for *Crenarchaeota* (28F/457R), primers specific for bacterial and archaeal *amoA* genes (amoA-1F/amoA-2R and amoAqF/amoAqR, respecifively)

listed in Table 2 were employed for quantification of total copy numbers of crenarchaeote 16S rRNA genes and bacterial and archaeal amoA genes, respectively. Amplification conditions were 95°C for 3 min, followed by 45 cycles (15s at 94°C, 30s for annealing at the temperatures shown in Table, and 30s at 72°C) in a reaction volume of 25 µl, containing 12.5 µl of iQ<sup>TM</sup> SYBR Green Supermix (BIO-RAD, Hercules, CA) and 5 pmol of each primer. PCR products from pure culture of Sulfolobus solfataricus using the Archaea-specific primer (21F/958R) and Clone QLS4amoAB2 and Clone SobAOA-Clone12 (for AOB and AOA) were used as standards. Serial dilutions of these standard vielded standard curves for each group of organisms. All quantitative PCR reactions for the standard curves were performed in triplicate. The amplification yielded reliable exponential patterns with template amounts in the range of  $10^4$  to  $10^8$  copies of 16S rRNA gene and *amoA* genes, respectively. The data were used to create standard curves correlating the C<sub>T</sub> values with copy numbers of the 16S rRNA gene and *amoA* gene. Linear plots between the Ct value (not shown) and log (copy numbers/reaction) were obtained with correlation coefficients of R<sup>2</sup>=0.9937, 09938 and 0.9909 for Crenarchaeota, AOB and AOA, respectively.

**GDGT analysis.** Soil samples (10g) were extract by a modified Bligh and Dyer singlephase organic solvent system comprised of chloroform, methanol, and aqueous 50 mM phosphate buffer (pH 7.4) in a ratio of 1:2:0.8 (vol/vol/vol) as previously described (Bligh and Dye 1959; White et al., 1979). After overnight extraction, chloroform and nanopure water were added to the extract in equal volumes, which resulted in a two-phase system. The lipid confined to the lower phase was collected and reduced in volume under a stream of pure N<sub>2</sub> gas. Total lipids were transesterified in 2 ml of methanol and hydrochloric acid (95:5; v/v) in a heating block at 70°C for two hours, which also hydrolyzed polar side chains of glycerol dialkyl glycerol tretraethers (GDGTs). After cooling to room temperature, 1 ml of solvent-extracted nano-pure water and 2 ml of CH<sub>2</sub>Cl<sub>2</sub> were added. The transesterified lipids were passed through a C-18 solid phase extraction (SPE) column. The GDGT fraction eluted with 1:3 ethyl acetate:hexane and was dissolved in 1.4% isopropanol in hexane.

GDGTs were identified using an Agilent 1100 series high performance liquid chromatograph (HPLC) with atmospheric pressure chemical ionization-MS using a Zorbax NH<sub>2</sub> column (2.1 x 150 mm, 5  $\mu$ m particle size) and/or a Prevail CN column (2.1 x 150 mm, 3  $\mu$ m particle size) at 30°C (Pearson *et al.*, 2004). Conditions for atmospheric pressure chemical ionization-MS were nebulizer pressure of 60 lb/in<sup>2</sup>, drying gas flow of 6.0 liters/min and 350°C, vaporizer temperature of 375°C, voltage of 3 kV, and corona of 5 uA. Spectra were scanned over the *m/z* range from 1,250 to 1,350.

#### RESULTS

**Phylogenetic analyses of archaeal 16S rDNA clones.** After discarding the sequences with potential chimeric artifacts, a total of 235 clones were analyzed from six independent archaeal 16S rRNA gene clone libraries were phylogenetically analyzed. The sequences were assigned to individual OTUs based on the 97% sequence similarity criterion. Table 3 summarizes the phylogenetic distributions of archael clones in these libraries. A neighbor-joining phylogenetic tree including all of representative sequences from these libraries and reference sequences from a range of published papers of environmental archaeal clones (Fig. 3).
Among sequences from LTH, Ssed, Sout, Ssh, and CMP311 libraries, 81-100% clones were affiliated with the group 1.1a, 1.1c, 1.3b (as termed by Ochsenreiter et al. 2003) in the *Crenarchaeota*. Only in Bdam library, the proportion of *Crenarchaeota* clones (47.5%) was lower than the *Euryachaeota* ones (52.5%).

**Crenarchaeal Group 1.1a sequences.** In the four clone libraries from the LTH-ST system, the majority of sequences placed within the group 1.1a in *Crenarchaeota*. Thirty-one out of thirty-two sequences from Ssed library had 99% similarity with clone ASN7 recovered from petroleum-contaminated soil (Kasai et al., 2005). Approximately 50% sequences in both LTH and Ssh libraries fell into this group. A smaller number of sequenced clones (30%) from Sout soil belonged to this group, with 92-97% similarity with ASN7. Only three Bdam sequences were related to ANS7 clone, and no sequence was detected in the CMP311 soil within this group. The marine *Crenarchaeota N. maritimus* and *C. symbiosum* are also associated with group 1.1a *Crenarchaeota*.

**Crenarchaeal Group 1.1c sequences.** There were five distinct clusters were present in the crenarchaeal group 1.1c (Fig. 2). Cluster I consisted of the representative clones (SSHarch-Clone14, SOarch-Clone5 and LTHarch-Clone3) and clone FSSB1 retrieved from boreal forest soil in Northern Finland. Only three sequences formed cluster II, which included SOarch-Clone15, LTHarch-Clone22 and clone GSF7-9500iii from glacier foreland soil, Austria (Nicol et al., 2003). Cluster III consisted of representative clones from SSH (9 clones), SO (2 clones), CMP311 (2 clones), which were grouped with clone ARCP2-5 from forest wetland soil, USA (Brofft et al., 2002). Cluster IV contained sequences from SSH, SO, and LTH libraries, which had 91-98%

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similarity with Finnish forest soil clone FFSA2 (Jurgens and Saano, 1999). In cluster V, two CMP311 clones were similar to an austrian thermal spring clone FJQFA2 at 95% similarity.

**Crenarchaeal Group 1.3b sequences.** Cloned sequences affiliated with the Crenarachaeal 1.3b group were the dominant group in the CMP311 library. CMP311-Arch38, representing twenty clones, showed 91-95% similarity with ASC25 from petroleum-contaminated soil, Japan. Nine sequenced CMP311 clones from the second most dominant OTU, possessed a sequence similarity range of 88-95% with an other Japanese petroleum-contaminated soil clone ASC37. Thirty-two percents of the Bdam sequences were closely related (92-98% similarity) to environmental sequences, such as HTA-B10 from freshwater soil containing metal-rich particles and GSF5-9500iii from glacier foreland. These clones were also grouped with 4 clones from CMP311, 1 from SSH, and 3 from SO libraries.

*Euryarchaeota* sequences. Twenty-one out of fourty Bdam cloned sequences were related to 3 families of *Euryachaeota* including *Methanosaetaceae*, *Methanosaeta* and marine bethnic Group D (MBGD) (Fig 2 and Table 3). Ten Bdam clones were affiliated with Clone CBD-4160G previously detected in acidic peat bog (Cadillo-Quiroz et al., 2006) within the family *Methanosaetaceae*, which are strict acetotrophic methanogens. Another three Bdam *Methanosaetaceae*-like sequences were related to the clone Lrh87 from rice rhizosphere (Lu et al., 2005). Members of *Methanosaetaceae* were also observed in the hydrocarbon-contaminated sites rich in organic matter and limited electron acceptors (Dojka et al. 1998, Watanabe et al., 2006). The frequent detection of these aceticlasic methanogens within *Methanosaetaceae* suggested that they may play an

important role in degrading the organic matter in Bdam soils. Interestingly, seven sequences, accounting for 17.5% of total clones in Bdam library, had 96-98% similarity with peat soil clone CBD-305F within the MBGD group which shares a common ancestry with *Thermoplasma* groups (Vernnitizes et al., 1999). Members of MBGD were originally detected in deep-sea sediments (Vernnitize et al., 1999), but they have been subsequently reported in numerous studies of mesophilic spring (Elshahed, AEM 2004) and lake sediments (Jugen 2000, Nusslein 2001) and acidic peatland (Cadillo-Quiroz et al., 2006).

Only eight cloned sequences in the So library were placed within *Euryachaeota*. However, these clones were closely related to known methanogenic families (*Methanosaetaceae, Methanomicrobia, Methanosarcina*) as well as Rice cluster I and II. **Phylogenetic analyses of archaeal** *amoA* **clones.** A total of 264 non-chimeric archaeal *amoA* (AOA) sequences from 6 clone libraries were analyzed. Fifty-nine OTUs were found based on 97% cutoff of DNA sequence similarity. Neighbor-joining analysis revealed the presence of three phylogenetically distinct lineages, designated cluster A (soil and marine origins), cluster B (hot spring origin) and cluster C (only soil origin)

**Cluster A.** Searching for the nearest published relatives showed that these sequences were previously retrieved from the marine sediment and soils. There are two subdivisions in Cluster A. Cluster A-I contained the largest number of clones from each library. It was notable that these sequences were separated into two distinct subclusters. Of all of the Ssed, LTH and Bdam clones analyzed, the representative clones SsedAOA-Clone12, BdamAOA-Clone20 and LthAOA-Clone41 were closely related to Clone QY-

based on the origins of included reference sequences.

40 from Qiyang Red soil, China, and the levels of archaeal *amoA* sequence similarity were range from 93-97%. One the other hand, the majority of sequences from CMP311, Sout and Ssh libraries fell into Cluster A-II. However, they formed their own subcluster, which were affiliated with 87-91% similarity with Clone QY-40.

In Cluster A-II, sequences had 95-97% similarity with another Qiyang red soil clone QY-45, which accounted for 38.6% of thetotal clones in the CMP311 library, 20.5% in the So library and 25% in the Ssh library respectively.

**Cluster B.** Only a few cloned sequences from LTH and CMP311 libraries fell into this Cluster. Four CMP311 cloned sequences had 99% similarity with the Clone SV-40 recovered from a Nevada hotspring, whereas one Bdam sequence was affiliated with Austrian archaeal amoA-like clone B2.

**Cluster C** With the exception of KRO1-related sequences detected in both LTH and Bdam libraries, there was no overlap in this cluster between the communities found in 6 SRS libraries. For example, a single Bdam sequence appeared to be distantly related to clones R60-70\_248 (79% similarity) retrieved from sandy soil, Germany (Leininger et al., 2006). Another clone was related to Austrian soil Apine1 clone(77% similarity) (Leininger et al., 2006). Only one OTU, representing three Ssed sequences, which had 96% similarity with terrestrial clone 25.

**Estimated richness, coverage and rarefaction analysis.** Both archaeal 16S rRNA gene and archaeal *amoA* gene clone libraries were constructed from 6 soil/sediment samples of Savannah River Sites. To estimate the coverage and the richness of these clone libraries, we computed the nonparametric estimator Chao1 and the Abundance-based coverage estimator (ACE) for richness and the Shannon Index for both phylotype richness and evenness of community composition (Schloss and Handelsman, 2005). For these six archaeal 16S clone libraries, a total of 235 clones were sequenced and 70 OTUs were recovered, representing 71-98% of the clones analyzed. Both Chao1 and ACE estimators indicated that CMP311 soil had the highest richness, whereas Steed sediment had the lowest one. A total of 264 clones were sequenced and 59 OTUs were found in 6 archaeal amoA clone libraries, which covered 85-98% of estimated total number of phylotypes in the environments. According to the Chaol estimator, archaeal amoA richness was lower than archaeal 16S rDNA at each sample; however, the ACE estimator suggested that richness was highest in Bdam sample. The Shannon-Weiner diversity index is a good overall diversity measurement since it reflects both phylotype richness and evenness. Shannon index is positively correlated with species diversity. For example, higher value of the Shannon index means that community has more species and an even distribution of abundance. In archaeal 16S rRNA gene clone libraries, the Shannon diversity of Ssed library was significantly lower than those of other ones. The values for the Shannon diversity index were similar for all archaeal amoA clone libraries and ranged between 1.97 (CMP311 library) and 1.35 (LTH library).

Rarefaction analysis is another approach to determine the diversity coverage of clone libraries (Heck, 1975). Rarefaction curves created for each of the sampling sites are shown in Fig 4. The patterns of rarefaction plots are similar between archaeal 16S rRNA gene and AOA clone libraries. For example, according to the rarefaction plots, the expected numbers of sequence types of Ssed library are lowest in both libraries. The results from rarefaction anlysis were also consistent with the estimated diversity indices.

**PCA analysis.** PCA is a powerful tool to discriminate the spatial variations of community structures among different samples collected from SRS sites. Figure 5 (A-C) showed two-dimensional summaries of 4-dimensional data based on the relative abundance of OTU of six archaeal 16S rRNA gene clone libraries. The cumulative variation of these four principle components was account for 90% of the total variations. Figure 5 (A) showed that these samples form 3 groups including LTH/Ssed, Ssh/So and Bdam/CMP311 groups. Figure 5 (A) clearly showed that PC 1 separated LTH/Ssed libraries from the other two groups. On the other hand, Ssh/So group was a great distance from Ddam/CMP one in principle component 2 (PC 2). Figure 5 (B) displayed that CMP311and Bdam libraries were separated in PC3, but Ssh and So libraries was grouped together for PC3. Figure 5 (C) indicated that So, Ssh, CMP311 and Bdam libraries were separated from each other one in PC4, whereas LTH and Ssed libraries were cluster together in all four PCs.

Figure 5 (D) is a two-dimensional PCA ordination using the original abundance matrix of six AOA clone libraries. The cumulative variation of PC1 and PC2 accounts for 88% of the total variation. These libraries were divided into two groups: one includes libraries Ssh, So and CMP311 that loaded heavily on the first component (PC 1), and the other included the libraries LTH, Ssed and Bdam that loaded heavily on the second component (PC 2).

Quantifications of crenarchaeotal 16S rRNA, archaeal *amoA* (AOA) and bacterial *amoA* (AOB) genes in SRS samples. Real time PCR were applied to six samples targeting 3 set of genes , The crenarchaeotal 16S rRNA gene copy numbers varied dramatically over 2 orders of magnitudes, ranging from  $6.1 \times 10^5$  to  $1.5 \times 10^7$  per gram of

dry soils or sediment. The population sizes of *crenarchaeota* were more abundant in CMP311 and Sshore, followed by Ssed and LTH, and less abundant in Sout and Bdam. The smaller amounts of crenarchaeotal *amoA* gene in Bdam and Sout correspondenced with the relative lower proportion of crenarchaeotal clones in Bdam library or higher number of phylotypes within *Euryachaeota* detected in Sout library. Interestingly, AOA to AOB ratios were 0.79 in the Bdam and 0.90 in Sout samples, whereas these ratios ranged from 3.7 to 9.8 in the other samples, showing archaeal *amoA* genes dominated over bacterial *amoA* genes in Ssed, Ssh, LTH and CMP311 samples.

**Archaeal lipid analysis.** Figure 7 (A), Table 7 shows GDGT profiles as measured for samples from Steed sediment, Steed shore and Beaver Dam. The peak with a mass of 1292 corresponded to crenarchaeol, the specific biomarker for nonthermophilic crenarachaeota. The peaks of other GDGTs with masses ranging from 1,302 (no rings) through 1,294 (four rings) were equivalent to GDGT-0, GDGT-1, GDGT-2, GDGT-3, and GDGT-4 respectively. Structures of GDGT (0-4) and crenarchaeol are shown on Fig. 7 (B).

The GDGT profiles of these samples revealed the presences of GDGTs 0-3, but was not GDGT-4 detected in all samples. However, the distributions of GDGT-0 and crenarchaeol revealed three patterns for these six analyzed samples. Firstly, similar relative abundances of GDGT-0 or crenarchaeol were observed in Ssed, Ssh and Sout samples. Secondly, LTH soil possessed the highest relative abundance of crenarchaeol, whereas GDGT-0 was relatively less abundant than that in the other samples. Thirdly, both CMP311 and Bdam samples were dominated by GDGT-0, accounting for 69% of total GDGTs, however, almost no crenarchaeol was detected in both samples.

## DISCUSSION

Savannah River Sites (SRS) is one of the major US Department of Energy facilities. Approximately 90% of the Savannah River Sites is now forested with humid and subtropical climate (Garten et al., 2000). Historically, former nuclear processing facilities (M-area), coal-fired powered plants (D-area), and various waste management facilities (CMP pits) were the primary sources from which heavy metals and radionuclides entered the environments (Garten et al., 2000). In this study, we collected 4 soils/sediments from the LTH-ST system in M-area. LTH-ST samples are characteristic of the acidic soils and sediments, and displayed elevated levels of U, Ni, Cr, Cu and Pb. The levels of U and Ni are several hundred times higher than typical background soils (Seaman et al., 2003; Punshon et al., 2003). A soil sample was also obtained from the CMP pit, which was constructed to dispose of solvents, pesticides, and lighting ballast components (Baladi et al., 2003). CMP soils are polluted by chemical solvents (mainly TCE and PCB), pesticides and metals. Typical acidic soils in CMP pits consist of hardpan clay, which result in the deficient nutrient and low microbial diversity (Newsletter). Another soil was collected from Beaver Dam Creek, a tributary of Savannah River in SRS-Drea. Trace metals such as Cd, Cr, Cu, See Zn, and As, as well as chlorinated hydrocarbons are the major contaminants in this area. Currently, Beaver Dam Creek receives condenser cooling water from the coal-fired power plant, neutralization wastewater, sanitary wastewater treatment effluent, ash basin effluent waters, and various laboratory wastewaters. SRS provide a unique opportunity to study the change of archaeal communities impacted by different contamination sources.

Ten Bdam clones were affiliated with Clone CBD-4160G previously detected in acidic peat bog (Nicol et al., 2005) within the family Methanosaetaceae, which are strictly capable of utilizing acetate. Another three Bdam Methanosaetaceae-like sequences were related to the clone Lrh87 from rice rhizosphere (Lu et al., 2006). Members of Methanosaetaceae were also observed in the hydrocarbon-contaminated sites with rich organic matter and limited electron acceptors (Dojka et al., 1998, Watanabe et al., 2002). The frequent detections of these aceticlasic methanogens suggested that they may play an important role in degrading the organic matter in Bdam soils. Interestingly, seven sequences, accounting for 17.5% of total clones in Bdam library, had 96-98% similarity with peat soil clone CBD-305F within MBGD group which shares a common ancestry with *Thermoplasma* groups (Vernnititizes et al., 1999). Members of MBGD were originally detected in deep-sea sediments (Vernnitize et al., 1999), but they were subsequently reported in numerous studies of mesophilic spring (Elshahed, et al., 2004), lake sediments (Jugen 2000, Nusslein 2001) and acidic peatland (Cadillo-Quiroz et al., 2006).

Only eight cloned sequences in So library were placed within *Euryachaeota*, however, these clones were distributed among known methanogenic families (*Methanosaetaceae, Methanomicrobia, Methanosarcina*) as well as Rice cluster I and II, showing their lower frequencies in each group.

A previously described feature of archaeal communities in soils is the ubiquitous presence of group 1.1b *Crenarachaeota*. They have been considered the most abundant archaea in different types of soils (Ochsenreiter et al., 2003). In our study, no sequences affiliated with members of group 1.1b crenarcheaota were found in all the six SRS clone

libraries. The absence of group 1.1b crenarchaeota sequences was also observed in the both forest and moorland soils (Jurgens et al., 1997; Jurgens and Saano, 1999; Pesaro and Widmer, 2002; Nicol et. al., 2007). However, sequences of group 1.1b crenarchaeota were reported in the heavy-metal-contaminated soils (Sandaa et al., 1999). We reasoned that specific habitats, but not toxic heavy metals, act as a selective pressure upon the group 1.1b lineage.

Paired comparisons between the 16S rRNA gene clone libraries performed using Weblibshuff suggested that each of the libraries differed significantly from the others, except no significant difference were found between the LTH and Sed samples. These observations generally agree with results from PCA and phylogenetic analysis. Four 16S rDNA clone libraries were generated from acidic forest soils/sediments in M-area LTH-ST system. Representative clones LthArch-Clone3 (5 clones), SshArch-Clone14 (12 clones) and SoArch-Clone5 (4 clones) were 93-99% similar to FFSB1 (X96688), and representative clones LthArch-Clone40 (7 clones), SshArch-Clone19 (5 clones) and SoArch-Clone24 (3 clones) had 91-98% similarity with FFSA2 (Y08985). Both clones FFSB1 and FFSA2 were retrieved from forest soils, Finland (Jurgens et al., 1997; Jurgens and Saano, 1999). These two clones belong to different phylogenetic groups but fell into Group I.1c Crenarchaeota. The finding of FFS group of Group I.1c Crenarchaeota sequences in clone libraries from LTH-ST system, in combination with the previous studies that also frequently detected FFS group sequences in other soil clone libraries (Kemnitiz et al., 2007) suggested that these sequences may be globally distributed in forest soils.

The most striking results of our study was that members of group 1.1a crenarchaeota dominate the clone libraries derived from LTH-ST system (55% of total clones for LTH, 31% for Ssh, 43% for So, and 97% for Ssed). These sequences showed 93-99% similarity with ASN7, a clone recovered from alkaline soil in Shizouka, Japan (Kasai, et al. 2005). Considering that the 16S rRNA genes from N. maritimus and C. symbiosum are also associated with the group 1.1a Crenarchaeota, and these organisms were isolated from marine environments with average oceanic pH of 8.2. Thus, the group 1.1a-related sequence were thought to be with alkaline environments. However, the relative high abundance of this groups in these acidic samples showed that they are present in a wider range of pH value than previously thought. Interestingly, our sequences in this cluster were also closely affiliated to JG36-GR-137 (AJ535135), which was retrieved from uranium mining waste. We speculate that the dominant group 1.1a crenarchaeota described here to be tolerant to the specific extent of heavy metals and radionuclides such as uranium, and may play certain metabolic functions in the polluted areas.

The main feature of the archaeal community in the CMP surface soil, compared with other soil communities, was the abundance of a set of sequences affiliated with the group1.3b *Crenarchaeota*, mostly related to a sequence retrieved from unsaturated, petroleum-contaminated soil, Japan (Kasai, et al. 2005). Sequences fell into the lineage of group1.3b crenarchaeota were also found in freshwater (Heshberger et al., 1996), anaerobic digestor (Godon et al., 1997) and wastewater (Ochsenreiter et al., 2003). However, because no cultured representatives of group 1.3b crenarchaeota have been isolated, their ecological functions remain unknown.

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One significant observation in the community structure in Bdam was that half of cloned sequences were related to environmental *Euryachaeota* clones (CBd-4160G and CBd-305F), which were retrieved from an acidic bog, New York, USA. The close relationship of Bdam sequences (32.5% of total clones) to Clone CBd-4160G within *Methanosaetaceae* family (95 to 98% sequence similarity) allows us to assume that acetate could be a major substrate for methanogenesis in the soil of Beaver Dam Creek. The 16S rRNA sequences typical of *Methanosaetaceae* have also been retrieved from hydrocarbon-contaminated samples (Dojka et al., 1998; Kasai et al., 2005). Acetate was considered as an important intermediate in the hydrocarbon-contaminated sites. A large number of clostridia sequences in bacterial clone libraries constructed from D-areas (date not shown) indicated that the acetate may be produced by clostridial fermentation in the Beaver Dam Creek. In addition, 18% of Bdam cloned sequences had 96-98% similarity with CBd-305F, a member of marine benthic group D (MBGD). However, their ecological significance is still unknown due to no representative organisms cultured.

Besides genetic biomarkers such as 16 rRNA genes, lipid analysis provides additional information on studying distributions of archaeal community of different environments. Archaea possess a distinctive ether-linked membrane in which the major lipids are glycerol dialkyl glycerol tetraethers (GDGT). Crenarchaeota were found to contain GDGTs with zero to four cyclopentane rings (Schouten et al., 2000), which are named as GDGT-0 to GDGT-4. The unique feature of crenarchaeol is the presence of a cyclohexane ring as well as four cyclopentane rings (Schouten et al., 2000 and Damsté et al., 2002). Crenarchaeol has been proposed as a unique biomarker for the group1.1 a crenarchaeota. Crenarchaeol appears to have a wide of environmental distributions. It was

originally detected in the ocean (Schouten et al., 2000). Recent studies reported the presence of crenarchaeol in hot springs (Pearson et al. 2004), peat bogs (Weijers et al., 2004), and soils (Weijers et al. 2006). In this study, we examined six SRS soil samples from three different areas in SRS. Crenarchaeol detected from all four samples from LTH-ST system confirmed the presence of sequences affiliated with the group1.1a crenarchaeota by 16S rRNA gene analysis. A pronounced difference in GDGT profiles of SRS soils was the near absence of crenarchaeol in both CMP311 and Bdam samples, which was in agreement with predominance of Group 1.3b Crenarchaeota in clone libraries of these two samples. This result confirmed that crenchaeol was characteristic only for the GDGTs of group 1.1 Crenarchaeotes, members of group1.3b Crenarchaeotes may not produce crenarchaeol in their membrane. Previous studies showed that the presence of crenarchaeol in nearly all soils covering a large geographical range (Weijers et al. 2006). Lack of crenarchaeol in two of our soil samples indicated that group 1.1a Crenarchaeotes may not tolerate some specific contaminants. GDGT-0, a general archaeal core membrane lipid, was present in all the studied samples. Among all samples, both CMP311 and Bdam had the highest relative abundances of GDGT-0 (69%). Considering the 16S rRNA gene clone library results, GDGT-0 in Bdam soil may be derived from both methanogenic Euryarchaeotes and nonthermophilic crenarchaeota. In contrast, group 1.3b crenarchaeota were the major source of the GDGT-0 in the CMP311 soil.

Previous studies have studied the effects of toxic heavy metals on indigenous soil  $\beta$ -Proteobacterial subgroup ammonia oxidizer community structures and abundances (Stephen et al., 1999; Gremian et al., 2004). By using real-time PCR, we determined that

bacterial *amoA* gene copy numbers ranged from  $1.01 \times 10^5$  to  $4.36 \times 10^5$  per gram of dry soils in our six samples. This finding is similar to that of Stephen et al., (1999) who reported that the total numbers of bacterial ammonia oxidizers were approximately  $2.3 \times 10^5$  copies per gram of dry soil in toxic-metal-contaminated microcosms with acidic pH (5.5). However, the bacterial *amoA* copy numbers were higher, ranged from  $2.89 \times 10^6$ to  $9.34 \times 10^7$  per gram of dry soil in acidic non-contaminated soils, Qiyang, China (He et al., 2007). Our observations confirmed that the heavy metal contamination has a drastic effect on population sizes of the targeted bacterial groups. Interestingly, all the bacterial amoA sequences recovered from both contaminated (Stephen et al., 1999) and noncontaminated (He et al., 2007) samples were affiliated with Nitrosospira or Nitrosospira-like species. The dominance of Nitrosospira cluster 3 sequences in both studies suggested that contamination of heavy metals may not be a selective factor on influencing the bacterial ammonium-oxidizing community in soils. Since we did not aim to phylogenetically analyze the bacterial *amoA* community in our samples, it remains open if these Nitrosospira cluster 3 sequences are also present in SRS soils.

In the present studies, with the analysis by combining molecular methods and culture-dependent techniques, the autotrophic oxidation of ammonia was discovered not to be restricted to the domain Bacteria. Archaeal ammonium oxidizers were detected in different types of soils (Leininger et al., 2006; Lu et al., 2007). Here, we performed phylogenetic analysis of the archaeal *amoA* gene using previously established PCR primers (Francis et al., 2005) for six SRS samples. Our results demonstrate the ubiquity of archaeal *amoA* genes in the heavy-metal contaminated soils. The vast majority of our analyzed clones from LTH-ST system fell into cluster A which contains the published clones from marine, sediment and soil (Francis et al., 2005; Lu et al., 2007). Beman and Francis (2006) hypothesized

that archaeal *amoA* genes associated with *Crenarchaeota* group 1.1a are likely tofall predominantly into column/sediment cluster A, since the 16S rRNA genes from *N. maritimus* and *C. symbiosum* are associated with group 1.1a *Crenarchaeota* and that their *amoA* genes fall in the water column/sediment cluster A. Our results provided the further evidence for this hypothesis since there were relatively high abundances of group 1.1a-like *Crenarchaeota* sequences in 16S rRNA gene clone libraries from the LTH-ST system. Furthermore, approximately 90% of archaeal amoA sequence in both Bam and CMP311 also fell into Cluster A. The predominance of group 1.3b-like *Crenarchaeota* sequences in clusters A in the contaminated sites. Given the presence and abundance of archaeal ammonium oxidizers in the all SRS samples we studied, it is reasonable to speculate that they may significantly contribute to the nitrification in the heavy-metal contaminated environments.

In conclusion, this study gave us a picture of the archaeal communities which may play ecological functions in the nitrogen cycle in heavy-metal polluted soils. No cultured nonthermophilc crenarchaeota have been obtained from soils yet, this finding will help up to design strategies for enrichment and isolation for better understanding their physiological characteristics.

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Sample Name (Abbreviation Name)	Clone library Name	Location	Туре	Major contaminants	Major contaminated source
Lower Tim Branch	Lth	SP-LTB System, M area	Soil	U, Ni, Chlorinated organic solvents	Nuclear reactor
Steed Outbank	So	SP-LTB System, M area	Soil	U, Ni, Chlorinated organic solvents	Nuclear reactor
Steed Shore	Ssh	SP-LTB System, M area	Soil	U, Ni, Chlorinated organic solvents	Nuclear reactor
Steed Sediment	Ssed	SP-LTB System, M area	Sediment	U, Ni, Chlorinated organic solvents	Nuclear reactor
Beaver Dam	Ddam	D-Area	Soil	Trace Elements Chlorinated hydrocarbon	Coal Plant
CMP311	CMP311	Chemical, Metal, Pesticide (CMP) Pit	Soil	PCE and TCE	CMP Pit Wastes

Table 5.1. Site Description.

Primer Name	Primer sequence $(5' \rightarrow 3')$	Annealing Temp. (°C)	Target Group (application)	Reference
21F 958R	TTCCGGTTGATCCYGCCGGA YCCGGCGTTGAMTCCAATT	58	Archaeal 16S rRNA (Clone library)	Delong, 1992
Arch-amoAF Arch-amoAR	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	53	Archaeal amoA (Clone library)	Francis et al., 05
Cren28F Cren457R	AATCCGGTTGATCCTGCCGGACC TTGCCCCCCGCTTATTCSCCCC	60	Crenarchaeota (Q-PCR)	Schleper, et al., 1997
Arch-amoAqF Arch-amoAqR	CTG AYT GGG CYT GGA CAT C TTC TTC TTT GTT GCC CAG TA	50	Archaeal amoA (Q-PCR)	Wuchter et al., 2006
Bacβ-amoAF Bacβ-amoAR	GGG GTT TCT ACT GGT GGT CCC CTC KGS AAA GCC TTC TTC	53	β-Proteobacterial amoA (Q-PCR)	Rottauwe et al., 1997

Table 5.2. Summary of primers used in this study.

Table 5.3. 16S rRNA archaeal sequences similarity analysis of representative clones from soils and sediment of Savannah River Site, USA.

Representative Clones		I	Relative Abunda	ince			Closest Sequences	Crenarchaeatal	Ref
			Clone Numbe	r			(Accession No.)	group	
	(Seque	ence Similarit	y with the neare	st published sec	(uences)				
	CMP311	LTH	Sshore	Soutbank	Ssediment	Bdam			1
LthArch-Clone31,	0	24	12	19	31	3	ASN7	C: 1.1a	Kasai et al.,
SshArch-Clone18		(94-99%)	(92-97%)	(93-97%)	(99%)	(95-99%)	(AB161345)		05
SoArch-Clone28							(,		
SsedArch-Clone22									
BdArch-Clone10									
LthArch-Clone3	0	5	12	4	0	0	FFSB1	C: 1.1c	Jurgens et
SshArch-Clone14		(97-99%)	(93-99%)	(99%)			(X96688)		al., 1997
SoArch-Clone5									
LthArch-Clone40	0	7	5	3	0	0	FFSA2	C: 1.1c	Jurgens and
SshArch-Clone19		(95-97%)	(91-98%)	(97%)			(Y08985)		1000
SoArch-Clone24									
LthArch-Clone21	0	2 (95%)	0	0	0	0	FJQFA2 (AM039531)	C: 1.1c	Weidler et al., 2007
LthArch-Clone22	0	1	0	1	0	0	GFS7-9500iii	C: 1.1c	Nicol et al.,
SoArch-Clone15		(98%)		(98%)			(AY601300)		2005
SshArch-Clone3	2	0	9	2	0	0	ARCP2-5	C: 1.1c	Brofft et
SoArch-Clonel1	(91-94%)		(96-98%)	(89-97%)			(AF523944)		al., 2002
CMP311Arch-Clone10									
SshArch-Clone8	4	0	1	3	0	13	HTA-B10	C: 1.3b	Stein, et al.,
BdArch-Clone42	(87-96%)		(96%)	(97-99%)		(92-98%)	(AF418925)		2002
SoArch-Clone33									
CMP311Arch-Clone37									
CMP311Arch-Clone3	1 (86%)	0	0	0	0	0	SK307 (AY882855)	C: 1.3b	Gene bank
CMP311-Arch38	20 (91-96%)	0	0	0	0	0	ASC25 (AB161328)	C: 1.3b	Kasai et al., 2005
BdArch-Clone5	0	0	0	0	0	1 (98%)	ASC27 (AB161330)	C: 1.3b	Kasai et al., 2005
SoArch-Clone12	9	0	0	1	0	1	ACS37	C: 1.3b	Kasai et al.,
BdArch-Clone41	(88-95%)			(97%)		(97%)	(AB161337)		2005
CMP311-Clone45									
SsedArch-Clone5	1	0	0	1 (96%	1 (95%)	1 (97%)	ASC40 (AB161339)	C: 1.3b	Kasai et al.,
BdArch-Clonel1	(95%)								2005
SoArch-Clone19									
CMP311arch-Clone4									
CMP311Arch-Clone11	1 (94%)	0	0	0	0	0	ASC33 (AB161334)	C: 1.2	Kasai et al., 2005

## Table 5.3-Continued

Representative Clones		F	Relative Abunda Clone Number	nce s <sup>b</sup>			Closest Sequences (Accession No.)	Euryachaeotal group	Ref
	(Seque	nce Similarity	with the neares	t published sequ	uences )°				
	CMP311	LTH	LTH Sshore Soutbank Ssediment P				1	l ·	1
LthArch-Clone54	0	3	0	0	0	0	ACS10 (AB161325)	E: Methanosaeta	Kasai,
		(95-98%)							2005
CMP311Arch-Clone6	1	0	0	0	0	0	SAGMA-G	E: SAGMEG-1	Takai,
	(92%)						(AB050211)		2001
BdArch-Clone17	0	0	0	0	0	7	CBd-305F (DQ301914)	E: Marine benthic	C-Q, 06
						(96-98%)		Group D	
BdArch-Clone9	0	0	0	1 (98%)	0	10	CBd-4610G	E: Methanosaetaceae	C-Q, 06
SoArch-Clone13						(95-98%)	(DQ301881)		
BdArch-Clone4	0	0	0	0	0	1 (97%)	HTA-D5 (AF418933)	E: Methanosaeta	Stein,02
BdArch-Clone43	0	0	0	0	0	3 (98%)	LrhA87 (AJ879059)	E: Methanosaetaceae	Lu, 05
SoArch-Clone2	0	0	0	2 (99%)	0	0	CBd-367A (DQ301891)	E: Rice Cluster I	C-Q, 06
SoArch-Clone17	0	0	0	1 (97%)	0	0	CBd-b2C(DQ301895)	E: Rice Cluster II	C-Q, 06
SoArch-Clone32	0	0	0	3	0	0	WCHD3-34 (AF050620)	E: Methanomicrobia	Dojka,
				(95-98%)					98
SoArch-Clone23	0	0	0	1 (99%)	0	0	HTA-C1 (AF418929)	E: Methanosarcina	Stein,02
Total OTUs	1	2	0	6	0	4			

a. From Fig. 5.2.

b. A total of clone numbers were grouped with representative clones

c. Range of similarity within the defined groups.

Table 5.4. Archaeal *amoA* sequences similarity analysis of representative clones from soils and sediment of Savannah River Site, USA.

Representative Clones <sup>®</sup>			Relative Abundar		Closest Sequences	Ref		
		Clone Nu	mbers" (Sequence	e Similarity)"			(Accession No.)	
	CMP311	LTH	Sshore	Soutbank	Ssediment	Bdam		
CMP311AOA-Clone46	23	39	33	35	38	39	Clone QY-A40	Lu et al.,
LthAOA-Clone41	(87-99%)	(88-96%)	(87-93%)	(88-92%)	(91-99%)	(93-97%)	(EF207216)	2007
SshAOA-Clone10								
SoAOA-Clone12								
SsedAOA-Clone12								
BdAOA-Clone20								
CMP311AOA-Clone33	17	1	11	9	0	2	Clone QY-A45	Lu et al.,
LthAOA-Clone38	(95	(96%)	(97%)	(95-97%)		(91%)	(EF207221)	2007
SshArch-Clone36	-97%)							
SoAOA-Clone15								
BdAOA-Clone7								
BdAOA-Clone2	0	0	0	0	0	1	Clone B2	Genebank
						(90%)	(AM260487)	
CMP311AOA-Clone17	4	0	0	0	0	0	Clone SV-40	Gene bank
	(99%)						(EF032877)	
LthAOA-Clone34	0	2	0	0	0	2	Clone KRO1	Leininger
BdAOA-Clone15		(81-88%)				(87-93%)	(DQ534815)	et al., 2006
BdAOA-Clone21	0	0	0	0	0	1	Clone Alpine1	Leininger
						(77%)	(DQ534697)	et al., 2006
BdAOA-Clone47	0	0	0	0	0	1	CloneR60-70_245	Leininger
						(79%)	(DQ534856)	et al., 2006
SsedAOA-Clone37	0	0	0	0	3	0	Clone 25	Gene bank
					(96%)		(DQ304886)	
LthAOA-Clone29	0	2	0	0	0	0	Clone QY-A47	Lu et al.,
		(87-88%)					(EF207223)	2007

a. From Fig 5.3.

b. A total of clone numbers were grouped with representative clones

c. Range of similarity within the defined groups.

Table 5.5. Observed richness and diversity estimators based on different methods derived from both archaeal 16S rRNA and Archeal *amoA* gene clone libraries from six soil and sediment samples from Savannal River Site.

Sample	# of Sequencens	# of OTU <sup>a</sup>	Chao1 <sup>b</sup>	ACE	Shannon Index <sup>d</sup>	Coverage (%) <sup>e</sup>
Archaeal 16S rRNA						
CMP311	39	18	32	37	2.37	71.8
Bdam	40	15	20	24	2.39	82.5
Lth	44	9	9.3	9.9	1.70	95.3
Sshore	39	11	16	17	2.01	87.2
Soutank	41	15	22	23.6	2.14	82.9
Ssed	32	2	2	ND	0.13	96.8
All samples	235					
Archaeal amoA						
CMP311	44	12	17	21	1.97	86.4
Bdam	46	13	17.25	34	1.65	84.7
Lth	44	8	10	14	1.35	90.9
Sshore	44	9	11	13	1.50	90.9
Soutank	44	11	14	20	1.76	88.6
Ssed	42	6	6	6.4	1.53	97.6
All sampless	264					

a. OTUs were defined as 97% similarity in nucleic acid sequences

b. c. d. Chao1-, ACE-, and Shannon Index were calculated by DOTUR software.

e. Coverage= 1-the number of phylotypes/library size.

Table 5.6. Q-PCR for *Crenarchaeota*, archaeal *amoA* gene(AOA) and bacterial *amoA*gene (AOB) from six samples collected from three areas, SRS.

	Copie	Copies of target genes /gram (dry soils)							
Sample (Location)	Crenarchaeota	AOA	AOB	AOA/AOB					
CMP311 (CMP pit)	1.22×10	1.14×10°	1.57×10 <sup>5</sup>	7.26					
Bdam (D-area)	6.11×10 <sup>5</sup>	1.93×10 <sup>2</sup>	2.65×10 <sup>5</sup>	0.72					
LTH (M-area)	3.15×10°	9.80×10 <sup>2</sup>	1.01×10 <sup>5</sup>	9.70					
So (M-area)	6.21×10 <sup>2</sup>	2.26×10 <sup>3</sup>	2.51×10 <sup>5</sup>	0.90					
Ssh (M-area)	1.52×10 <sup>7</sup>	1.10×10°	2.15×10 <sup>5</sup>	5.11					
Ssed (M-area)	1.82×10°	1.61×10°	4.36×10 <sup>5</sup>	3.69					

Table 5.7. Relative abundance of glycerol dialkyl glycerol tetraethers (GDGTs) in soils and sediment collected from Savannah River Sites, USA,

	Relative abundance (%)							
Samples	GDGT-0	GDGT-1	GDGT-2	GDGT-3	GDGT-4	GDGT-5	GDGT-5*	GDGT-5
	(m/z 1302)	(m/z 1300)	(m/z 1298)	(m/z 1296)	(m/z 1294)	(Crenarchaeol)	Cren-150mer	/GDGT-0
CMP311soil	0.69	0.09	0.10	0.08	0.04	0	0	0
Beaver Dam	0.69	0.15	0.10	0.03	0.02	0.01	0	0.014
Lower Tim Branch	0.19	0.17	0.15	0.05	0	0.44	0	2.31
Steed Shore	0.36	0.15	0.17	0.07	0	0.22	0.03	0.61
Steed Outbank	0.37	0.19	0.22	0.09	0	0.13	0	0.32
Steed Sediment	0.49	0.13	0.11	0.04	0.02	0.21	0	0.43

Fig. 5.1. Map of CMP, Beaver Dam and LTH-Steed System in the Savannah River Site. Modified from Carroll et. al. 2006.

Fig 5.1.



Figure 5.2. Phylogenetic relationship of archaeal 16S sequences among 6 soils and sediments from Savannah River Site. The tree topology is based on Neighbor-Joining analysis using the p-distance method (Mega3.1 software) and was evaluated by Maximum Parsimony analysis. OTUs (3% cutoff) are color-coded for springs from different locations. One representative of each sequenced group is shown; the total number of clones represented by these sequences was shown in the parenthesis. The accession number for the reference sequences are shown in black. Bootstraps replicate, 1000, were performed and bootstrap values of >50% are shown at the branch points.

Fig 5.2.


Figure 5.3. Phylogenetic relationship of archaeal AOA sequences among 6 soils and sediments from Savannah River Site. The tree topology is based on Neighbor-Joining analysis using p-distance method (Mega3.1 software) and was evaluated by Maximum Parsimony analysis. OTUs (3% cutoff) are color-coded for springs from different locations. One representative of sequence groups is shown; the total number of clones represented by these sequences were shown in the parenthesis. The accession number for the reference sequences are shown in black. Bootstraps, repricates, 1000 were performed and bootstrap value of >50% are shown at branch points.



Figure 5.4. Rarefaction curves showing archaeal 16S rRNA (A) and archaeal amoA gene (B) richness within two clone libraries derived from six soil or sediment of Savannah River Sites. Clones were grouped into OTUs at a level of sequence similarity of >97% .Curves are color-coded as Fig 2 and 3.

Fig 5.4.



Figure 5.5. Principle-component analysis based on the relativeabundance of OTUs on archaeal 16S rRNA gene, A) PC1 and PC2, B) PC1 and PC3, C) PC1 and PC4, or archaeal *amoA* gene, D) PC1 and PC2. The values in parentheses are percentage of the PCA derived form the either archaeal 16S rDNA or archaeal *amoA* gene data.





Figure 5.6. Quantification of crenarchaeal 16S rRNA gene, archaealand bacterial *amoA* genes in soils and sediment from Savannah River Site by real-time PCR method.



Figure 5.7. ( A). Isoprenoidal GDGTs are attributed to archaea, numbers refer to the rings in the GDGT structures. (B) Shown GDGT series are: GDGT-0, GDGT-1, GDGT-2, GDGT-3, and crenarchaeol.

Fig 5.7.



#### **CHAPTER 6**

# SUMMARY

My focus of this dissertation is to utilize an integrated approach of molecular microbiology (cloning, sequencing, and real-time PCR) and microbial lipid biomarkers to understand microbial diversity and the factors and mechanisms shaping microbial community structure in contaminated soils. To the best of my knowledge, this study marks the first demonstration of microbial community changes in the heavily contaminated soils at Savannah River Site (SRS) and demonstrated that the microbial community structure and abundance changed in different environments contaminated with heavy metal and radionuclides.

The first study of this dissertation was to determine the effect of heavy metals (V, As, Ni, Cu, Cr, Co, and Pb) from a coal-generated power plant on the microbial diversity in a source and downstream environment. Sixty-three percents of cloned sequences from source location had 97-99% similarity with the antibiotic resistant species, Streptococcus *mitis.* Six clonal sequences from the downstream location were highly related to *Ralstonia sp* 13I, which carries multiple resistances to several metals including Cu, Zn, Ni and Cd. (Konstantinidis et al., 2003). These results indicated that heavy metal concentrations are likely to serve as selective pressure for antibiotic and metal-tolerant bacteria and genes. Another interesting result is that Anaeromyxobacter-like species were observed in both mat and sediment samples from the downstream location; these sequences 95-97% similarity Anaeromyxobacter dehalogenans. had with Anaeromyxobacter species have the ability to detoxify multiple pollutants because they can utilize several electron acceptors, including nitrate, Fe(III), U(VI), and chlorinated hydrocarbons (Sanford et al., 2002). Members of the heterotrophic *Clostridia spp* were dominant in the underlying sediment of the downstream location. They may provide electron donors for the metal-reducing microorganisms by oxidizing the complex organic matters to simple forms of carbon, or they can participate directly in metal reduction. Further investigation of *Clostridia spp*. is warranted to determine the environmental significance of this group of bacteria in the metal reduction in the contaminated areas.

The second study examined the microbial community structures in heavy metal (e.g., nickel) and radionuclides (e.g., uranium) from three M-area contaminated locations, Lower Tim Branch, Stead Pond, and Beaver Pond. One important result is that members of sulfate-reducing and iron-reducing bacteria, especially *Geobacter* species, may play an important role in immobilizing the metals and radionuclides through metabolic activities in the soils with intermediated uranium contamination. The other result provides further evidence that members of uncultured *Acidobacteria* may play certain ecological functions in the uranium-contaminated sites (Barns et al., 2007).

The third study of this dissertation gave a comprehensive picture of the diversity and abundance of ammonium-oxidizing archaea in heavily polluted soil samples collected from three sites with different contamination sources (former nuclear processing, coal combustion, and a chemical, metal, pesticide pit). Analysis of the archaeal 16S rRNA gene clone library revealed that sequences retrieved from these samples were significantly different from each other. For instance, members of the nonthermophilic group 1.1a and 1.1c *Crenarchaeota* constituted an important fraction in the samples obtained from the LTH-ST system within the M-area, which received discharge of waste water from nuclear and metallurgical processes. In contrast, the archaeal community composition was dominated by members of group 1.3b *Creanarchaeota* in soil obtained from the chemical, metal and pesticide (CMP) pits. By using archaeal ammonia monooxygenase  $\alpha$ -subunit (*amoA*) gene as a marker, ammonia-oxidizing archaea communities were detected from all the samples. Real-time PCR results showed that archaeal *amoA* genes were more abundant than bacterial *amoA* genes in most of studied samples.

The results reported in this dissertation indicate that microbial populations containing specific groups of microorganisms may be involved in contaminant degradation and immobilization but their distribution is heterogeneous. This implies that it is feasible to biostimulate the indigenous microbial populations for contaminant remediation. Further studies need to monitor the community dynamics in situ by collaborating with environmental engineers.

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# **APPENDIX 1**

# GLOBAL OCCURRENCE AND BIOGEOGRAPHIC PATTERNING OF PUTATIVE ARCHAEAL AMOA GENES IN TERRESTRIAL HOT SPRINGS

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#### SUMMARY

Despite the ubiquity of ammonia in geothermal environments and the thermodynamic favourability of aerobic ammonia oxidation, the importance of nitrification at high temperature has hardly been addressed. In this study we sampled 21 hot springs (pH 3.4-9.0, temperature 41-86°C) from the United States, Russia, and China and obtained 846 putative archaeal ammonia monooxygenase large subunit (*amoA*) gene sequences. The *amoA* gene sequences were highly diverse, and clustered with the known major clades of archaeal amoA gene sequences. Principal component analysis showed that the majority of hot spring clusters corresponded with location and not temperature, pH, or ammonia or nitrate concentration, suggesting that geography, not chemistry or temperature, might control which clades of amoA genes predominated. RT-PCR from one of the hot springs showed that *amoA* genes were transcribed and transcripts were closely related to the DNA sequences amplified from the same spring. Lastly, the majority of amoA genes were distinct from those of low temperature environments; in particular, pairwise comparisons between *amoA* gene sequences from a hot spring and the sympatric soil showed less than 85% sequence identity, demonstrating the distinctness of hot spring archaeal communities from the surrounding soil system. Our study demonstrates the global occurrence of putative archaeal *amoA* genes in terrestrial hot springs and suggests that geography may play an important role in selecting different assemblages of crenarchaeota that harbour amoA genes.

#### **INTRODUCTION**

Microorganisms carry out a variety of respiratory processes involving nitrogen species and these processes are important forces in controlling the form and fate of inorganic nitrogen in nature. Despite recent advances in our understanding of the nitrogen cycle in soils and fresh and marine waters (see reviews by Jetten et al., 2004; Geets et al., 2006; Nicol and Schleper, 2006; Francis et al., 2007), several gaps in the nitrogen cycle remain in high temperature ecosystems. In particular, although ammonia is the major source of inorganic nitrogen in the source water of most hot springs, reaching at least 34 mM in bulk water of hot springs in the Washburn area of Yellowstone National Park (YNP)(Nordstrom et al., 2005), ammonia respiration has not been known to occur above 60°C until now (de la Torre et al., 2007). The two respiratory processes involving ammonia are nitrification, the stepwise oxidation of ammonia to nitrite and then nitrite to nitrate using oxygen as the terminal electron acceptor, and anaerobic ammonia oxidation (anammox) via nitrite to produce dinitrogen gas. Thermodynamic calculations using empirical geochemical data have been reported for several springs in YNP and the Great Basin and are in the range of 43.5 kJ for the aerobic oxidation of ammonia to nitrite and 32.9 kJ for the aerobic oxidation of nitrite to nitrate (Shock et al., 2005, Inskeep et al., 2005; Costa et al., 2007), well above minimal free energy yields to support microbial life.

The recent discovery that non-thermophilic Group I crenarchaeota occurring widely in low temperature marine and terrestrial environments (see reviews by DeLong, 1998; Dawson et al., 2000; Schleper et al., 2005) are capable of the chemolithotrophic oxidation of ammonia to nitrite (Könneke et al., 2005; Francis et al., 2005) raises the possibility that thermophilic crenarchaeota might mediate the same reaction in hot

springs. Recent progress in the cultivation and study of thermophilic crenarchaeota has made a breakthrough in isolating thermophilic crenarchaeon that is known to oxidize ammonia (de la Torre et al., 2007). So far all reported cultivated thermophilic archaea belong to a single class, the Thermoprotei, which includes only three orders, Sulfolobales, Thermoproteales, and Desulfurococcales (Boone and Castenholz, 2001). On the other hand, cultivation independent studies of 16S rRNA gene sequences have revealed a much wider diversity of crenarchaeota outside the Thermoprotei in geothermal environments worldwide (Barns et al., 1996; Meyer-Dombard et al., 2005; Huang et al., 2007).

Several research groups have begun to search for thermophilic ammonia oxidizing crenarchaeota. So far, however, limited information exists about the possible occurrence of ammonia oxidizing crenarchaeota in hot springs. Several groups have reported the recovery of *amoA* genes from thermobiotic environments (Wagner and Schleper, 2006 meeting abstract; Weidjer et al., 2006; Spear et al., 2007 meeting abstract; Zhang et al., 2007 meeting abstract). And recently, crenarchaeota in a thermophilic ammonia oxidizing enrichment have been shown to fix carbon dioxide (Wagner et al. 2007 meeting abstract).

In this study, we used previously established PCR primers (Francis et al., 2005) to perform an extensive survey of putative archaeal *amoA* genes from a large number of physiochemically diverse hot springs in the United States (the Great Basin and Yellowstone National Park), China (Tengchong), and Russia (Kamchatka). Our results demonstrate that archaeal *amoA* genes are ubiquitous in geothermal systems. At least some of these genes are transcribed in situ, and *amoA* gene sequences from hot springs are different from those found in sympatric soils. Although some *amoA* gene types are found in hot springs on disparate continents, suggesting cosmopolitanism of those types, the assembly of *amoA* genes present in hot springs was strongly correlated with geography and neither chemistry nor temperature.

#### RESULTS

# Locations of hot springs and measurements of water chemistry

We analyzed archaeal *amoA* genes from 21 hot springs from the United States (the Great Basin and Yellowstone National Park), Russia (Kamchatka), and China (Tengchong). Temperature, pH, total dissolved solids (TDS), nitrate, nitrite, ammonia, sulphate, and hydrogen sulphide were determined for most of the springs (Table 1). Temperature ranged from 41°C to 86°C and pH from 3.4 to 9.0. In general, hot springs in the Great Basin (Nevada/California) and Tengchong were circum-neutral or alkaline; whereas hot springs from YNP included both bicarbonate-buffered alkaline springs and sulphuric acid-buffered acidic springs. Samples from Kamchatka were collected from two slightly acidic hot springs (BLS51 and JV73) in the East Thermal Fields and one alkaline spring (JV73) in the Geyser Valley (Table 1).

The Great Basin hot springs had low concentrations of ammonium (0.0-16.8  $\mu$ M) and hydrogen sulphide (0.0-3.9  $\mu$ M); both are electron donors for chemolithoautotrophs. Concentrations of ammonium were significantly higher in Yellowstone (68.0-5722.0  $\mu$ M), Tengchong (0.7-40.5  $\mu$ M), and Kamchatka (1105.3-1368.4  $\mu$ M), and so were concentrations of hydrogen sulfide (Table 1). Nitrite was only detected in the source water of Surprise Valley spring (0.02  $\mu$ M; Table 1) in California, but was significantly higher (0.88-2.24  $\mu$ M) in Tengchong hot springs (Table 1). Similarly high nitrite concentrations have also been observed in other Yellowstone and Kamchatka hot springs (data not show). These results suggest that the Great Basin hot springs may represent

low-potential inorganic energy systems, whereas those in YNP, Tengchong, and Kamchatka represent high-potential inorganic energy systems. Other inorganic electron donors such as Fe[II] and H<sub>2</sub> also occur variably in these systems (Spears et al., 2005; Romanek et al., unpublished data).

#### Occurrence and diversity of archaeal amoA genes in hot springs

Putative archaeal *amoA* genes were obtained from DNA extracted directly from environmental samples by PCR using primers specific for archaeal amoA genes (Francis et al., 2005). A total of 846 clones were sequenced from environmental genomic DNA/RNA from the 21 hot springs, which resulted in a total of 153 unique OTUs based on nucleic acid sequences at a 2% cutoff (Table 1). The greatest number of OTUs was 14 occurring in Yellowstone hot spring AJ41; the lowest number of OTUs was 2 occurring in the Great Basin (EV41 and GBS74) and in Kamchatka (JV73). Shannon Index (H) for amoA gene diversity ranged from 0.21 to 2.24 at 2% cutoff. In general, the Great Basin hot springs were characterized by H values less than 1.00, whereas the Yellowstone, Tengchong, and Kamchatka hot springs are characterized by H values greater than 1.00 (the highest value 2.24 occurred in AJ41; Table 1), suggesting greater diversity of the *amoA* genes in the latter. The Chao1 values represent species richness and do not always correlate with H values. For example, similar H values (at 2% cutoff) (0.95 for SV86 and 0.96 for SV42) corresponded to Chao1 values that are dramatically different (12 for SV86 and 3 for SV42), suggesting significantly greater species richness at the spring SV86. Overall, the genotypic diversity of archaeal *amoA* genes appears to be lower in the Great Basin hot springs than in Yellowstone, Tengchong, and Kamchataka hot springs. The results were generally consistent with the overall difference in ammonia concentrations at these locations. However, no significant correlation was observed between Shannon index or Chao1 and ammonia concentration or any other environmental variables ( $r^2$  values below 0.5 for linear regressions applied to H or Chao1 vs temperature, pH,  $\Sigma NH_3$ ,  $NO_2^-$ ,  $NO_3^-$ , or  $\Sigma S^{2-}$ ; data not shown).

# Phylogeny of archaeal amoA genes in hot springs

The sequences of all putative archaeal *amoA* genes from soil, freshwater, and marine habitats have been divided in two clades (Beman and Francis, 2006; Beman et al., 2007). *AmoA* genes from DNA libraries of the hot springs are diverse (up to 41% sequence divergence) within these two clades, yet they can be broken down further into four distinct groups from the Great Basin (GB Group I-IV), six from Yellowstone National Park (YS Group I-VI), five from Tengchong (TC Group I-V), and three from Kamchatka (KM Group I-III) (Fig. 1). Clade I includes GB Group I-III, YS Group I-III, TC Group I-III, and KM Group I-II, which together account for 84% of total clones. Clade II includes GB Group IV, YS Group IV-VI, TC Group IV-V, and KM Group III, which account for 15% of total clones, the remaining 1% clones are individual sequences that are not included in these groups. In each clade, the major groups rarely overlap, except for GB Group III and KM Group I, which have 99% similarities.

Clade I also includes sequences from a subsurface geothermal environment (Austrian Thermal Spring sequences) and from low-temperature marine environments as well as the pure culture *Nitrosopumilus maritimus* (Fig. 1); however, Clade I does not include any sequences from soil environments. Clade II, on the other hand, includes mostly soil sequences and a sequence from a geothermal mine adit (Fig. 1). In Clade I, sequences of TC Group I and GB Group I are 91-92% similar to the Austrian Thermal

Spring clone F2; sequences of YS Group I and TC Group II-III are 92-97% similar to the Austrian Thermal Spring clone B2; and sequences of GB Group II are 96% similar to the Austrian Thermal Spring clone OT2. TC Group IV is the only major group in Clade II that are closely related to the sequence from the geothermal mine adit (Table 2).

Major groups in Clade I were also compared with *N. maritius* and other marine sequences (Francis et al., 2005; Beman and Francis, 2006; Beman et al., 2007). These groups (GB Groups I-III, YS Groups I-III, TC Groups I-III, and KM Group I-II) are 75-85% similar to *N. maritius* and 81-85% similar to other marine sequences (Table 2). Major groups in Clade II were compared with a soil fosmid 54d9 and other soil or terrestrial sequences (Schleper et al., 2005). These groups (GB Group IV, YS Groups IV-VI, TC Groups IV-V, and KM Group III) are 72-77% similar to fosmid 54d9 (Schleper et al., 2005) but have significantly higher similarity (88-98%) to other soil sequences (Table 2).

# Cosmopolitan and endemic patterns of hot spring amoA genes

GB Group III and KM Group I are two major groups that show high similarity (99%) across the North America and Russia. There are a few other occasions when sequences from two distant locations are highly similar. These include three Yellowstone OTUs (AJ42-Clone33, WG66-Clone16, and WG66-Clone8) that are grouped within the GB Group I or II; one OTU from GB (GBS45-Clone14) that is grouped within KM Group I, and one OTU from Yellowstone (JCA82-Clone32) that is grouped within KM Group III (Fig. 1). None of these sequences, however, occurs at more than two major locations, suggesting only a small subset of putative archaeal *amoA* genes present in geographically distant terrestrial hot springs. This is clearly demonstrated in principle

component analysis (PCA) of OTUs from different locations (Fig. 2). In particular, the majority of GB sequences (GBS45 excluded) are clearly separated from Yellowstone, Tengchong, and Kamchatka on PCA 1, which accounts for 26.55% of the total variance; the latter three locations, on the other hand, are separated on PCA 2, which accounts for 21.37% of total variance (Fig. 2). On PCA 3 (19.53% total variance), Tengchong and Yellowstone sequences cluster together but are separated from Kamchatka (including GBS45); and on PCA 4 (15.66% total variance), Yellowstone, Tengchong, and Kamchatka have the least separation except for two OTUs from Yellowstone (JCA82 and WG88) (data not shown). Together these four principle components account for 83.09% of total variance.

The endemic patterning of hot spring *amoA* genes was further demonstrated by correlation matrix analysis using the abundances of OTUs, which showed positive correlation values between two springs within a geographic location (e.g., Great Basin springs) but negative or zero values between two springs from two different locations; again, the exception is GBS45 from the Great Basin, which correlates highly with all three hot springs from Kamchatka (Table 3).

#### Archaeal *amoA* gene expression in terrestrial hot springs

To evaluate the functionality of ammonia-oxidizing archaea in the hot spring environment, RT-PCR was performed on an environmental sample collected from the Eagleville hot spring (EV41) and several other springs. EV41 was chosen on the basis of prior evidence of archaeal *amoA* genes in an ammonia oxidizing enrichment culture containing Crenarchaeota (42°C, pH 9.0) established from this spring (Huang et al., unpublished data). The RT-PCR indicated expression of *amoA* in EV41 but not in any of the other springs tested using either random hexamers or archaeal-specific *amoA*-reverse primer (Fig. 3). A total of 34 clones were obtained from the cDNA library of the RT-PCR product, which resulted in four unique OTUs (based on 2% cutoff) (Table 1). Two of the expressed amoA OTUs were identical or nearly identical to genes recovered from the same sample (Fig. 4), indicating that the ammonia-oxidizing archaea may be indeed active in the natural environment.

# Comparison of hot spring amoA genes with those from sympatric soil

It has been suggested that genomic DNA from terrestrial hot springs may be prone to contamination by the surrounding soil (DeLong, 1998). The high similarity between some hot spring amoA gene sequences and some terrestrial soil sequences appear to support this hypothesis (e.g., BSB44-clone27, AJ42-clone22, WG66-clone27; Clade II; Fig. 1). To examine this possibility more directly, topsoil samples were collected from the vicinity of Eagleville (EV41) and Surprise Valley hot springs (SV86-SV42) and used as substrates for DNA extraction and *amoA* gene PCR and sequencing. The soil sequences were distinct (<85% similarity) from those of nearby hot springs (Fig. 4), suggesting that microbial communities in the Great Basin hot springs may be well isolated from surrounding soil. This does not necessarily apply to other locations and like comparisons with surrounding soil samples should be made at each site to draw a more definitive conclusion. On the other hand, it has been clearly demonstrated that archaeal 16S rRNA genes from the open ocean were highly similar to those observed from hot springs (Mincer et al., 2007), suggesting that previously recognized archaeal phylotypes from soil or marine environments may indeed have a thermophilic origin.

#### DISCUSSION

#### Nitrogen metabolism by archaea

Nitrogen metabolism is poorly understood among the archaea. A recent review on nitrogen cycle activities indicated that a limited diversity of activities are known in archaea, including mostly reductive pathways of N<sub>2</sub> fixation, nitrate assimilation, and nitrate respiration such as denitrification (Cabello et al., 2004). The detection of ammonia-oxidation by non-thermophilic crenarchaeota filled an important knowledge gap by adding an oxidative dissimilatory pathway (see Nicol and Schleper, 2006; Francis et al., 2007). So far, however, most research on archaeal ammonia oxidation has focused on low-temperature marine or soil environments (e.g., Francis et al., 2005; Leininger et al., 2006; Wuchter et al., 2006). The discovery of amoA genes in geographically and physiochemically diverse terrestrial hot springs in this study suggests that ammoniaoxidizing archaea may play an important role in the nitrogen cycle in geothermal systems. This is supported by a recent thermophilic archaeal isolate that grows optimally at 74°C and using ammonia as electron donor (de la Torre et al., 2007). Other cultureindependent genomic approaches have been used to guide the cultivation of new species (Tyson et al., 2005). For example, a novel N<sub>2</sub>-fixation hyperthermophilic archaeon was recently isolated from a deep-sea hydrothermal vent based on discovery of potentially archaeal nitrogenase genes (Mehta et al., 2005, 2006).

# Endemic and cosmopolitan patterning of ammonia-oxidizing archaea

Microbial biogeography is emerging as a cornerstone for understanding prokaryotic diversity, ecology, and evolution (Cho and Tiedje, 2000; Papke et al., 2003; Whitaker et al., 2003; Vinuesa et al., 2005; Ramette and Tiedje, 2007). Archaea have

been shown to have endemic or cosmopolitan patterns of distribution. The best example of endemic nature of archaea is the study of multilocus sequences of archaea by Whitaker et al. (2003), which identified endemic genotypes in the hyperthermophilic archaeon *Sulfolobus "islandicus"* from hot springs separated by distances of 6-15 km to >250 km. Similarly, Papke et al. (2003) identified the endemic patterning of hot spring cyanobacterial genus *Synechcoccus* from North America, Japan, New Zealand and Italy by sequencing the 16S rRNA genes and the internal transcribed spacer (ITS) regions.

Cosmopolitan distribution of archaea, on the other hand, is observed in the open ocean. For example, Massana et al. (2000) reported cosmopolitan phylotypes of planktonic crenarchaeota in widely different oceanic provinces. Francis et al. (2005) noted that the majority of water column archaeal *amoA* gene sequences from four different geographical locations fell into a single cluster; whereas sediment archaeal *amoA* gene sequences from different locations appear to have a stronger endemic patterning.

These studies demonstrate that populations of (hyper)thermophilic microorganisms are better isolated from one another by geographic barriers and geographic isolation is in part responsible for driving the recent evolutionary divergences (Papke et al., 2003; Whitaker et al., 2003) in these environments, whereas planktonic microorganisms in the open ocean lack geographic barriers and thus show the predominance of cosmopolitan patterning of distribution.

Our results support the growing evidence that species of archaea are endemic in "island-like" terrestrial hot springs (Figs. 1 and 2). We have also compared global distribution patterns of the archaeal 16S rRNA genes from hot springs, which show less

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clear endemic property than the archaeal *amoA* gene marker (Chen et al., unpublished data). The archaeal *amoA* gene may be better suited for biogeographical studies of archaea, as it is expected to have a higher resolution than the conservative 16S rRNA genes. The precise role that physical isolation plays in generation of microbial biogeography is unknown and both adaptation and geographical isolation must be considered as factors affecting microbial population speciation (Papke et al., 2003; Ramette and Tiedje, 2007).

#### Environmental factors affecting ammonia-oxidizing archaea

Currently little is known about how environmental variables may affect the diversity, abundance, and activity of archaea in low-temperature environments. Available evidence indicates that the abundance and activity of nonthermophilic crenarchaeota and particularly the ammonia-oxidizing crenarchaeotes can be correlated to environmental variables such as the concentrations of ammonia or nitrite or the rate of ammonia oxidation (Murray et al., 1999; Damste et al., 2002; Teira et al. 2006; Wuchter et al., 2006). Treusch et al. (2005) observed that addition of ammonia enhanced the expression levels of *amoA* in microcosm incubation experiments. Factors affecting the diversity of ammonia-oxidizing archaea can only be speculated. For example, Francis et al. (2005) observed that the highest diversity of the archaeal *amoA* genes was related to high ammonia concentration in water columns and sediments of the ocean.

It is much more complicated to decipher variables affecting species diversity and abundance in terrestrial hot springs because of dramatic differences in temperature and water chemistry from spring to spring. In this study, numbers of OTUs, Shannon index, and Chao1 were observed to decrease with decreasing temperature and ammonia at a single spring (SV-2) in Surprise Valley, where samples were collected along a outflow channel; however, this correlation is non-linear with the two lower-temperature samples (SV60 and SV40) having the same OTU and Chao1 values at different ammonia concentrations (Table 1). Also, Shannon Index increased again at the lowest temperature (SV40), suggesting significant increase in species diversity in this sample. No correlation was observed, however, between environmental variables and diversity parameters among different springs at a particular geographic location (data not shown). Between locations, the Great Basin has lower values of OTUs (2-6; average =  $3.7\pm1.7$ , n = 7), Shannon Index (0.21-0.95; average =  $0.71\pm0.25$ , n = 7), and Chao1 (3-12; average =  $6\pm3$ , n = 7) than either Yellowstone or Tengchong (Table 1). These lower values may be attributed to the low ammonia concentrations (0.0-16.8 QM; average =  $6.2\pm5.8$ , n =7) in Great Basin hot springs. However, the diversity indices (OTUs, Shannon Index, Chao1) are similar between Yellowstone and Tengchong hot springs even though ammonia concentrations were over tenfold higher in the former than in the latter (Table 1). Overall, analysis of water chemistry in this study shows weak or no correlation between environmental variables and species diversity, further supporting the genetic observation that the ammonia-oxidizing archaea are probably geographically isolated from each other in terrestrial hot springs.

# Comparison between non-thermophilic and (hyper)thermophilic crenarchaeota for ammonia oxidation

Archaeal ammonia oxidizers were observed to be more abundant than their bacterial counterparts in soils and thus proposed to be the most abundant ammoniaoxidizing prokaryotes in soil ecosystems on Earth (Leininger et al., 2006). It is further speculated, based on limited 16S rRNA gene diversity of crenarchaeota in marine and terrestrial environments that some groups of non-thermophilic crenarchaeota may be mostly or totally ammonia-oxidizers (Nicol and Schleper, 2006). It is worth noting that no molecular (genetic) evidence has been reported that the non-thermophilic crenarchaeota possess genes for autotrophic growth on H<sub>2</sub> or reduced sulfur species. On the other hand, hyperthermophilic archaea are known to be dominated by H<sub>2</sub> or sulfur metabolism (Stetter, 1996; Spear et al., 2005). The discovery of archaeal ammonia-oxidation adds to the metabolic diversity of hyperthermophilic autotrophic archaea; however, the relative importance of ammonia oxidation in thermophilic autotrophic archaea is unknown. Given the presence and abundance of ammonia in many hot springs, and the thermodynamic favorability of ammonia oxidation in these systems (Amend and Shock 2001; Shock et al., 2005), it is reasonable to believe that abundant ammonia-oxidizing archaea exist in the hot spring environment and potentially play an important role in nitrogen cycles.

## Evolutionary pathways of ammonia-oxidizing archaea

So far, little is known about the origin of ammonia-oxidizing archaea. Questions have been raised that this metabolism might have arisen from hot environments (Könneke et al., 2005; Francis et al., 2007; Nicol and Schelper, 2007). Our data do not exclusively address this question; however, the inclusion of low-temperature *amoA* gene sequences within the major clades of our environmental sequences from the hot spring environment does endorse the hypothesis that low-temperature ammonia-oxidizing crenarchaeota are derived from thermophilic counterparts. On the other hand, the hypothesis that the capability of ammonia oxidation by archaea may be acquired from bacterial nitrifiers

through lateral gene transfer (HGT) (Klotz et al., 2006; Francis et al., 2007) provides an alternative view of the evolutionary pathways of archaeal ammonia oxidation. Both hypotheses must be examined carefully to fully understand the mechanisms and pathways of the functional evolution of archaea between thermophilic and non-thermophilic species.

In summary, our study demonstrates the global occurrence and biogeographical isolation of previous unrecognized ammonia-oxidizing archaea in terrestrial hot springs, which may play important roles in nitrogen metabolism in the geothermal system. Future studies can take advantage of the genomic information and address evolutionary questions of ammonia-oxidizing archaea by using cultivation and comparative genomics approaches.

#### **EXPERIMENTAL PROCEDURES**

#### Site description, water chemistry, and sample collection

A total of 21 hot springs were analyzed for archaeal *amoA* genes for this study, which include seven from the Great Basin and six from Yellowstone National Park, USA, five from Tengchong, China, and three from Kamchatka, Russia. At each location, water pH, temperature, and total dissolved solids (TDS) were determined using a Hach<sup>®</sup> pHmeter equipped with a pH and temperature probe and a TDS probe. Calibration of the pH meter was performed at ambient temperature (~25°C) and measurements of pH were expressed as pH<sup>25°C</sup> in reporting. Sulfate and hydrogen sulfide were determined at the spring using Hach kits following the manufacturer's instructions. Nitrate and ammonia were preserved using HgCl<sub>2</sub> and determined colorimetrically using standard protocols in the laboratory. After chemical measurements in the field, the mat or mat-containing sediment was collected in sterile plastic tubes or plastic bags using a sterile spoon. Samples were immediately cooled on ice or frozen on dry ice when available. In the laboratory, samples were stored at -80°C until analysis of DNA.

#### DNA extraction, PCR amplification, cloning and sequencing, and phylogenetic analysis

Genomic DNA was extracted from 5-g (wet weight) mat material by using Ultraclean Mega Prep Soil DNA kit (MO Bio Laboratory, Inc, Solana Beach, CA). The precipitated DNA was purified by gel electrophoresis plus mini-column preparation (Wizard DNA Clean-Up System, Promega, Madison, WI). Archaeal amoA gene fragments (approximately 635 bp) were amplified in a 9700 Thermal Cycler (Perkin-Elmer) using the primer pair Arch-amoAF (5'STAATGGTCTGGCTTAGACG3') and Arch-amoAR (5'GCGGCCATCCATCTGTATGT3') (Francis et al., 2005). PCR cycling was performed following Francis et al. (2005) with initial denaturation at 95 °C for 15 min. In order to avoid potential sample biases and to obtain enough PCR production for cloning, 3 replicated amplifications were carried out for each sample. The combined PCR products were purified by cutting out the appropriate band from a low-melting point agarose gel (0.8%). The PCR products were purified from the gel using QIA Quick Gel Extraction Kit (QIAGEN, Inc., Valencia, CA). The purified DNA was ligated with pCR vector form TA-cloning kit and competent Escherichia coil cells were transformed according to manufacture's instructions (Invtrogen, Carsbad, CA). Forty to 50 randomly chosen colonies per sample were analyzed for insert archaeal amoA gene sequences. Plasmid DNA containing inserts of the archaeal amoA gene was prepared using a QIAprep Spin miniprep kit (QIAGEN, Valencia, CA). Sequencing reactions were carried out using primer Arch-amoAF provided in an ABI BigDye Terminatior v.3.1 kit (Applied

Biosystems, Foster City, CA). The archaeal *amoA* gene sequences were determined with an ABI 3100 automated sequencer. Sequences were typically ~600 to 700 bp long.

The sequences were aligned using CLUSTALX 1.83 and compared with reference sequences from the database. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 and phylogenetic trees were constructed with p-distance martrices and the neighbor-joining method with MEGA. Trees constructed with maximum-likelihood and neighbor-joining methods were consistent. Operational taxonomic units (OTUs) were defined as sequence groups in which sequences differed by  $\leq 2\%$  or  $\leq 5\%$ .

RNA extraction, RT-PCR analysis of archaeal amoA gene expression, and construction of cDNA tree

Samples for RT-PCR were collected from the Eagleville hot spring (California, USA) and several other springs in RNA-Free vials and saved immediately on dry ice. Total RNA was extracted using the RNeasy® Mini Kit (Qiagen) combined with ballistic cell disruption by vortexing with 0.1-mm sterile glass beads. Purified RNA (5µl) was reverse transcribed in separate reactions using random hexamers or the archaeal-specific *amoA* reverse primer (Arch-amoA-R, see below) and the Bio-Rad iScript Select cDNA Synthesis Kit. Double stranded cDNA was amplified with archaeal specific *amoA* primers (Arch-amoA-F: 5'-STAATGGTCTGGCTTAGACG, Arch-amoA-R: 5'-GCGGCCATCCATCTGTATGT) and the HotStarTaq® Plus PCR kit. PCR products (635 bp, expected *amoA* product) were analyzed by agarose gel electrophoresis (1.0%) with a 1.0 kb molecular weight marker. A clone library was constructed for the cDNA sequences from RT-PCR using the same procedure described above.

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# Statistical analysis

Rarefaction and richness including the nonparametric richness estimators Chaol and ACE and the Shannon diversity index were calculated using available programs (Schloss and Handelsman, 2005). Archaeal *amoA* clone libraries between two samples were compared by using computer program WebLIBSHUFF version 0.96 available online at http://libshuff.mib.uga.edu/. Principal-component analysis (PCA) was performed by using SYSTAT statistical computing package (Version 10.0; SPSS, Inc, Chicago, IL) using clone library data, samples from different sites were grouped or separated based on the patterns of OTUs share by all samples and the distinct OTUs from each sample. In this study, the relative amounts of the OTUs for each sample were used as variables.

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Table 1. Temperature, water chemistry and sequencing information for 21 hot springs showing positive results of archaeal amoA genes. All samples
are for DNA libraries except for Eagleville, which also contained a cDNA library. "n" = number of clones; "na" = nucleic acids; "nd" = not
determined; "BDL" = below detection limit. H = Shannon index.

determined; "BDL" = below detect	tion limit. H	= Sha	nnon inde	x.	-			-				-		
Sample	Temp	pН	ΣNH <sub>3</sub>	NO3	NO <sub>3</sub>	NO <sub>2</sub>	ΣS <sup>2</sup>		оти	l-na	,	н	Cha	o1
-	(°C)		μМ	uМ	μМ	uМ	ъM	n	2%	5%	2%	5%	2%	5%
Great Basin, USA	( 0/			part	para	pin								
Eagleville (EV41)	41.0	9.0	BDL	3.17	3.17	BDL	BDL	43	2	2	0.72	0.55	8	3
Eagleville (EV41cDNA)	41.0	9.0	BDL	3.17	3.17	BDL	BDL	34	4	4	0.76	0.76	4	4
05-GBS-04B (GBS74)	74.4	6.6	16.8	15.87	15.87	BDL	0.6	45	2	1	0.21	0.00	4	nd
05-GBS-13 (GBS45)	45.7	7.6	7.4	6.35	6.35	BDL	0.4	44	6	3	0.68	0.53	6	3
05-SV2-con-source (SV86)	86.0	6.4	8.9	3.17	3.17	0.02	3.9	29	6	4	0.95	0.64	12	5
05-SV2-con2 (SV69)	69.2	6.2	6.3	1.59	1.59	BDL	0.1	46	4	4	0.76	0.5	5	3
05-SV2-con3 (SV60)	60.4	6.0	3.7	1.59	1.59	BDL	0.1	45	3	2	0.66	0.24	3	2
05-SV2-con5 (SV42)	42.0	8.8	0.5	1.59	1.59	BDL	0.4	29	3	3	0.96	0.59	3	2
Yellowstone, USA														
Avocado Jeff's Upper Slides (AJ41)	41.0	8.4	nd	nd	nd	nd	nd	44	14	11	2.24	2.07	51	17
NGB Procerlin Basin (PB75)	75.0	3.5	68.0	<0.7	<0.7	nd	1.3	39	9	7	1.22	1.01	12	8
Josephs Coat Springs (JC3A82)	82.0	6.2	5722.0	9.60	9.60	nd	20	41	6	5	1.03	0.95	9	6
NGB Monarch Geyser (MG85)	85.0	4.4	158.0	<0.7	<0.7	nd	30	39	6	5	1.33	1.25	8	5
Calcite Spring 'B' in outflow (SSB74)	74.0	8.2	1167.0	<0.7	<0.7	nd	290	42	4	3	0.36	0.32	7	3
NBG Whirligig Geyser (WG88)	66.0	3.4	74.0	<0.7	<0.7	nd	<0.3	34	10	9	1.74	1.69	13	12
Tengchong, China														
Little Spring 1A (LS75)	75.3	7.6	8.76	BDL	BDL	0.88	nd	44	6	6	1.66	1.22	9	5
Bridge spring (BSA59)	59.3	7.5	40.53	33.02	33.02	1.13	nd	35	6	5	1.28	1.21	9	6
Bridge spring (BSB44)	43.6	7.5	8.62	27.03	27.03	2.24	nd	40	8	7	1.66	1.57	11	8
Wurning (WM77)	77.0	7.7	14.68	0.79	0.79	1.33	nd	43	6	4	0.99	0.89	5	4
Little pond 2A (LP52)	52.4	8.2	0.67	0.60	0.60	1.35	nd	33	11	9	2.21	1.94	15	10
Kamchatka, Russia														
Burliyashi D (BLS51)	50.5	6.0	1105.26	nd	nd	nd	5.88	41	8	6	1.47	0.84	12	6
Geyser Valley (GV88)	66.2	8.0	nd	nd	nd	nd	nd	35	7	6	1.89	0.91	9	6
Jenn's Vent 3 (JV73)	72.6	6.0	1368.42	nd	nd	nd	2.94	21	2	1	1.23	0.66	2	1

Table 2: Classification of archaeal amoA gene clones from DNA libraries.								
Group of clones	No. of	Percentage of	Closest published Relatives	Similarity	Similarity with	Similarity with		
	Clones	same location	(Accession Number)	(%)	N. maritimus (%)	Fosmid 54d9 (%)		
Great Basin, USA								
NV/CA Group I	182	64.53	Austrian Thermal Spring clone F2 (AM233905)	92	81%	68%		
NV/CA Group II	45	15.96	Austrian Thermal Spring clone OT2 (AM260489)	96	78%	70%		
NV/CA Group III	38	13.48	Marine Clone MX_5_OCT_12 (DQ501024)	81	80%	71%		
NV/CA Group IV	4	1.42	Terrestrial Clone25 (DQ304886)	92	66%	72%		
Yellowstone, USA								
YS Group I	12	5.15	Austrian Thermal Spring clone B2 (AM260487)	92	79%	71%		
YS Group II	13	5.57	Marine sediment Clone ES_HI_21 (DQ148788)	81	76%	68%		
YS Group III	124	53.22	Marine sediment Clone ES_HI_21 (DQ148788)	85	75%	66%		
YS Group IV	7	3.01	Soil Clone r0-10_266 (DQ534840)	98	66%	76%		
YS Group V	22	9.44	Soil Clone r0-10_239 (DQ534851)	93	63%	77%		
YS Group VI	29	12.45	Terrestrial Clone 25 (DQ304888)	97	67%	73%		
Tengchong, China								
TC Group I	47	23.04	Austrian Thermal Spring clone F2 (AM233905)	91	81%	66%		
TC Group II	12	5.88	Austrian Thermal Spring clone B2 (AM260487)	92	81%	70%		
TC Group III	96	47.06	Austrian Thermal Spring clone B2 (AM260487)	97	83%	70%		
TC Group IV	16	7.84	Geothermal Mine Clone (DQ672674)	97	67%	74%		
TC Group V	24	11.76	Terrestrial Clone 25 (DQ304886)	97	66%	73%		
Kamchatka, Russia								
KM Group I	12	12.24	Marine Clone MX_5_OCT_12 (DQ501024)	81	85%	71%		
KM Group II	71	72.45	Marine Clone MX_5_OCT_12 (DQ501024)	84	79%	71%		
KM Group III	15	15.31	Terrestrial Clone 25 (DQ304886)	88	65%	72%		

Table 3. Correlation Matrix based on abudances of OTUs from 21 hot	t springs. See Table 1 for sample IDs.
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	Nevada/California					Yellowstone						Tengchong					Kamchatka				
	GBS75	EV41	SV86	SV69	SV60	SV42	AJ41	PB75	JC3A82	MG85 3	SSB74	WG66	BSA59	BSB44	LP52	LS75	WM77	BLS51	GV66	JV73	GBS45
GBS75	1.000	0.986	0.989	0.992	0.973	0.594	-0.007	-0.034	-0.048	-0.047	-0.028	0.005	-0.046	-0.055	-0.039	-0.052	-0.037	-0.034	-0.037	-0.036	-0.030
EV41	0.986	1.000	0.998	0.973	0.992	0.719	-0.015	-0.039	-0.055	-0.055	-0.033	0.005	-0.053	-0.063	-0.045	-0.060	-0.043	-0.039	-0.043	-0.042	-0.035
SV86	0.989	0.998	1.000	0.763	0.992	0.695	-0.018	-0.042	-0.059	-0.058	-0.035	0.000	-0.056	-0.067	-0.048	-0.064	-0.045	-0.041	-0.045	-0.044	-0.037
SV69	0.922	0.973	0.963	1.000	0.973	0.858	-0.027	-0.046	-0.065	-0.064	-0.038	0.004	-0.062	-0.074	-0.053	-0.071	-0.050	-0.046	-0.050	-0.049	-0.041
SV60	0.973	0.992	0.992	0.973	1.000	0.737	-0.026	-0.046	-0.065	-0.064	-0.039	-0.004	-0.063	-0.075	-0.053	-0.071	-0.051	-0.046	-0.050	-0.049	-0.041
SV42	0.594	0.719	0.695	0.858	0.737	1.000	-0.045	-0.048	-0.068	-0.067	0.040	0.006	-0.065	-0.078	-0.056	-0.074	-0.053	-0.048	-0.052	-0.051	-0.043
AJ42	-0.007	-0.015	-0.018	-0.027	-0.026	-0.045	1.000	0.915	0.497	0.891	0.911	0.276	-0.088	-0.105	-0.075	-0.100	-0.071	-0.065	-0.071	-0.069	-0.056
BR75	-0.034	-0.039	-0.042	-0.046	-0.046	-0.048	0.915	1.000	0.411	0.851	0.989	0.307	-0.060	-0.071	-0.051	-0.068	-0.048	-0.044	-0.048	-0.047	-0.039
JCA82	-0.048	-0.055	-0.059	-0.065	-0.065	-0.068	0.497	0.411	1.000	0.475	0.417	0.772	-0.084	-0.100	-0.072	-0.095	-0.068	-0.062	-0.067	-0.066	-0.055
MG85	-0.047	-0.055	-0.058	-0.064	-0.064	-0.067	0.891	0.851	0.475	1.000	0.860	0.209	-0.083	-0.099	-0.071	-0.094	-0.067	-0.061	-0.066	-0.065	-0.055
SSB74	-0.028	-0.033	-0.035	-0.038	-0.034	-0.040	0.911	0.989	0.417	0.860	1.000	0.291	-0.050	-0.059	-0.042	-0.056	-0.040	-0.036	-0.040	-0.039	-0.033
WG66	0.005	0.005	0.000	-0.004	-0.004	0.006	0.276	0.307	0.772	0.209	0.291	1.000	-0.094	-0.112	-0.080	-0.107	-0.076	-0.069	-0.075	-0.073	-0.062
BSA59	-0.046	-0.053	-0.056	-0.062	-0.063	-0.065	-0.088	-0.060	-0.084	-0.083	-0.050	-0.094	1.000	0.965	0.711	0.649	0.839	-0.059	-0.065	-0.063	-0.053
BSB44	-0.055	-0.063	-0.067	-0.074	-0.075	-0.078	-0.105	-0.071	-0.100	-0.099	-0.059	-0.112	0.965	1.000	0.646	0.676	0.755	-0.071	-0.077	-0.075	-0.063
LP53	-0.039	-0.045	-0.048	-0.053	-0.053	-0.056	-0.075	-0.051	-0.072	-0.071	-0.042	-0.080	0.711	0.646	1.000	0.636	0.903	-0.050	-0.055	-0.054	-0.045
LP57	-0.052	-0.060	-0.064	-0.071	-0.071	-0.074	-0.100	-0.068	-0.095	-0.094	-0.056	-0.107	0.649	0.676	0.636	1.000	0.693	-0.067	-0.073	-0.072	-0.066
WM77	-0.037	-0.043	-0.045	-0.050	-0.051	-0.053	-0.071	-0.048	-0.068	-0.067	-0.040	-0.076	0.839	0.755	0.903	0.693	1.000	-0.048	-0.052	-0.051	-0.043
BLS51	-0.034	-0.039	-0.041	-0.046	-0.046	-0.048	-0.065	-0.044	-0.062	-0.061	-0.036	-0.069	-0.059	-0.071	-0.050	-0.067	-0.048	1.000	0.950	0.933	0.990
GV66	-0.037	-0.043	-0.045	-0.050	-0.050	-0.052	-0.071	-0.048	-0.067	-0.066	-0.040	-0.075	-0.065	-0.077	-0.055	-0.073	-0.052	0.950	1.000	0.864	0.965
JV73	-0.036	-0.042	-0.044	-0.049	-0.049	-0.051	-0.069	-0.047	-0.066	-0.065	-0.039	-0.073	-0.063	-0.075	-0.054	-0.072	-0.051	0.933	0.846	1.000	0.883
GBS45	-0.031	-0.035	-0.037	-0.041	-0.041	-0.043	-0.058	-0.039	-0.055	-0.055	-0.033	-0.062	-0.053	-0.063	-0.045	-0.060	-0.043	0.990	0.965	0.883	1.000

**Fig. 1.** Phylogenetic relationship of archaeal *amoA* sequences from DNA libraries among hot springs, marine settings, and soils. The tree topology is based on Neighbor-Joining analysis using p-distance method (Mega3.1 software), and was evaluated by other methods. OTUs (2% cutoff) are color-coded for springs from different locations. Numbers following location name are spring temperatures. 1000 bootstraps were performed and bootstrap value of >50% are shown at branch points.

**Fig. 2.** Principle Component analysis based on the relative abundance of OTUs on PC 1 and PC 2. The values in parentheses are percentage of the total variance of PCA derived from archaeal *amoA* gene data.

**Fig. 3.** Archaeal *amoA* gene expression analysis by RT-PCR. Reactions labeled N6 or Arch*AmoA*-R correspond to reverse transcription reactions using either random hexamers or the Archaeal-specific *amoA*-reverse primer, respectively. NT is the no template control. Samples numbered 1 through 4 correspond to samples collected from different springs. Number 4 is from the Eagleville site.

**Fig. 4.** Phylogenetic relationship of archaeal *amoA* sequences between hot springs (DNA and cDNA) and soils (DNA only) at Eagleville and Surprise Valley. The tree topology is based on Neighbor-Joining analysis using p-distance method (Mega3.1 software), and was evaluated by other methods. OTUs (2% cutoff) are color-coded for hot springs and soils. 1000 bootstraps were performed and bootstrap value of >50% are shown at branch points.

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Fig 1.



BR75 MG85 SSB74 AJ42 0.60-JCA82 ଷ WG86 0 0.30-PC 2 (21.37%) BLS51 SV42 GBS75 FV42 0.00 SV00 5 (**6** SV86 JV73 GBS45 SV69 -0.30-WM77 LP53 LS57**60** B BSA59 -0.60 BSB44 -0.20 0.20 0.40 0.60 0.80 1.00 0.00 PC 1 (26.55%)







Fig. 4

# **APPENDIX 2**

# ARCHAEAL LIPIDS AND 16S RRNA GENES CHARACTERIZING GAS HYDRATE-

# IMPACTED SEDIMENTS IN THE GULF OF MEXICO

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## ABSTRACT

The abundance and community structure of archaea may be significantly altered in marine sediments when impacted by gas hydrates or cold seeps. We studied the intact lipids and the phylogenetic compositions of archaea from marine sediments near or on top of an elevated sedimentary topography manifested in a mosaic of methane seeps, hydrate mounds, and *Beggiatoa* mats in the Mississippi Canyon Block 118 in the Gulf of Mexico. Our primary goal was to use lipid biomarkers to examine changes in community structure of archaea in normal marine versus hydrate-impacted sediments. Glycerol dialkyl glycerol tetraethers (GDGTs) showed distinct patterns between hydrate and nonhydrate samples, suggesting variation in archaeal communities caused by the presence or absence of gas hydrates. In particular, GDGT-1 to GDGT-3 having one to three cyclopentyl rings, respectively, were significantly enhanced in the hydrate or methanerich samples. Deviation of lipid profiles from normal marine sediments was verified by 16S rRNA genes to provide a phylogenetic explanation of the archaeal populations possibly causing the deviation. Clone libraries of the 16S rRNA genes from the hydrateassociated samples showed the predominance of ANME-1 subgroups, which are known to produce tetraether lipids and may be responsible for the enhanced archaeal lipids in the hydrate samples. Our study demonstrated the consistency between phenotypic and phylogenetic properties of archaea, which responded to the impact of gas hydrates in the marine environment.

#### **INTRODUCTION**

The domain *Archaea* contains the major phyla *Crenarchaeota* and *Euryarchaeota*. *Crenarchaeota* inhabit not only extreme environments such as sulfidic, hot, salty or anoxic waters, but also are widespread in other locations such as lakes, open seas and sediments (*e.g.*, DeLong, 1992; DeLong *et al.*, 1994; Fuhrman *et al.*, 1992; Hershberger, 1996; MacGregor *et al.*, 1997; Pearson *et al.*, 2004; Schleper *et al.*, 2005; Zhang *et al.*, 2006). These non-thermophilic crenarchaeota may account for one third of the planktonic prokaryotes in the open ocean (*e.g.*, Karner *et al.*, 2001). Mounting evidence indicates that many non-thermophilic crenarchaeota use ammonia as a major energy source for autotrophic growth (Francis *et al.*, 2005; Könneke *et al.*, 2005; Treusch *et al.*, 2005; Leininger *et al.*, 2006; Nicol & Schleper, 2006; Wuchter *et al.*, 2006). Other molecular and geochemical studies show that nonthermophilic crenarchaeota can take up amino acids and organic carbon, as well as inorganic carbon, indicating that some capacity for heterotrophy or mixotrophy (Ouverney & Fuhrman, 1999, 2000; Herndl *et al.*, 2005; Ingalls *et al.*, 2006; Teira *et al.*, 2006).

*Euryarchaeota* include important groups of microorganisms that mediate methane production (methanogens) and methane oxidation (ANME groups). In particular, anaerobic oxidation of methane (AOM) consumes about 90% of methane produced in marine sediments (Valentine & Reeburgh, 2000) and plays a critical role in controlling flux of methane into the atmosphere. While numerous species of methanogens have been identified (*e.g.*, Boone & Castenholz, 2001), none of the ANME groups has been brought into pure culture. However, geochemical and genomic evidence indicates that some, if not all, of the ANME groups oxidize methane using reverse methanogenesis working in

syntrophy with sulfate-reducing bacteria (*e.g.*, Hoehler *et al.*, 1994; Boetius *et al.*, 2000; Orphan *et al.*, 2001a,b; Michaelis *et al.*, 2002; Valentine, 2002; Hallam *et al.*, 2004; Nauhaus *et al.*, 2005).

*Crenarchaeota* and *Euryarchaeota* also have distinct lipids. Glycerol dialkyl glycerol tetraethers (GDGTs) are the core lipids of *Crenarchaeota* (Koga *et al.*, 1993). *Euryarchaeota* (*e.g.*, methanogens and halophiles), on the other hand, commonly are characterized by isoprenoidal diethers such as archaeol and *sn*-2-hydroxyarchaeol (Koga *et al.*, 1998; Hinrichs *et al.*, 1999; Pancost & Damsté, 2003). However, some of the *Euryarchaeota* such as *Thermococcus* and *Thermoplasmatales* also contain tetraethers as core lipids (Uda *et al.*, 2001; Macalady *et al.*, 2004).

Substantial evidence indicates that the GDGTs found in normal marine sediments predominantly originate from planktonic and/or benthic crenarchaeota (*e.g.*, DeLong *et al.*, 1998; Biddle *et al.*, 2006). The GDGTs from cold areas such as the Antarctic almost exclusively are composed of only crenarchaeol and GDGT-0; whereas in warmer areas such as the Arabian Sea, the GDGT assemblages also contain appreciable amounts of GDGT-1, GDGT-2, GDGT-3 and crenarchaeol-isomer (Schouten *et al.*, 2002). On the other hand, available information from gas hydrate or cold hydrocarbon seep environments indicates that the ANME-1 group (but not ANME-2) may also produce GDGT-0 to GDGT-3 as core lipids (Aloisi *et al.*, 2002; Blumenberg *et al.*, 2004).

The Gulf of Mexico is an important place to study biological effects on the carbon cycle, because dynamic microbial communities exist in association with gas hydrates or cold seeps/mud volcanoes in the marine sediments (Lanoil *et al.*, 2001; Mills *et al.*, 2003, 2005; Zhang *et al.*, 2002, 2003, 2005; Joye *et al.*, 2004; Orcutt *et al.*, 2004, 2005; Zhang

& Lanoil, 2004; Lloyd *et al.*, 2006; Martinez *et al.*, 2006; Reed *et al.*; 2006; Yan *et al.*, 2006). One unique feature of the Gulf of Mexico in comparison with other hydrate locations is that gas hydrates formed there contain methane not only from microbial methanogenesis but also from thermogenic break down of petroleum hydrocarbons in the deep subsurface (Sassen et al., 2001, 2004). The abundance of archaea is enhanced significantly in the hydrate environment and these organisms may play an important role governing exchange between sediments and the deep oceanic carbon cycle (Zhang *et al.*, 2003; Pancost *et al.*, 2005). Here we present an integration of lipid profiles and DNA sequence data to achieve a better understanding of archaeal community biogeochemistry, in association with gas hydrate-impacted sediments in the Gulf of Mexico.

#### **MATERIALS AND METHODS**

#### Sample collection and description

Gravity cores of 3-m or 10-m length (7 cm diameter) were collected in the Mississippi Canyon (MC) Block 118 in May 2005, onboard the R/V Pelican. MC 118 comprises a portion of the continental slope with water depths ranging from 800 to 990 m (Fig. 1) (Woolsey *et al.*, 2005). About 12 cores were taken near or on top of an elevated sedimentary topography within MC 118 (approximately 1 km<sup>2</sup> in area; Fig. 1), which is manifested in a mosaic of methane seeps, hydrate mounds, and *Beggiatoa* mats (Sassen and Roberts, 2004). These features change on a scale of a few meters as shown by submersible and ROV surveys (Sassen and Roberts, 2004). All samples for this study were from the 10-m long cores (cores 1, 8 and 9). Core 1 was in normal marine sediment and cores 8 and 9 were on top of the "hydrate mound" (Fig. 1). A core (NBP) from the

normal marine sediment at Grand Canyon Block 233 (Li *et al.*, 2007) was also included in this study.

Samples were examined visually onboard for evidence of hydrates and gas before being sub-sampled for analyses of lipids and DNA. The lithology was then characterized in detail in the laboratory at the University of Southern Mississippi. In general, all cores were dominated by mud (particle size < 0.063 mm) and contained less than 10% sand (particle size ranging from 2-0.063 mm) in most intervals (Brunner, personal communication). In core 1, the mud was olive or dark olive in color. A thin oxidized layer (~5 cm) occurred around 300 cm and 400 cm. In core 9, the mud was gray and contained carbonate nodules or pyrite shells in the top 150 cm section. Gas expansion and oil staining were noticeable in core 9 but absent in core 1, suggesting possible dispersion of hydrate in core 9. Lithological description was not available for core 8 but the lithology in this core may be similar to that in core 9 because they are both on top of the elevated topography (Fig. 1).

About 50 grams of sediments were collected at different depth intervals (5-486 cm below sediment surface). The samples were immediately stored at -20°C onboard and stored at -80°C in the laboratory until analysis.

### Porewater sulfate and sulfide

Porewater chemistry was performed on core 1 and core 9. The frozen samples were thawed to room temperature in an anaerobic chamber to minimize the oxidation of reduced species such as hydrogen sulfide. Porewater was collected by centrifugation (500 x g) using 10-15 grams of wet sample and immediately analyzed for sulfate and hydrogen

sulfide using Hach kits (Zhang et al., 2007) according to manufacturer's instructions (Hach Company, Loveland, CO).

# Lipid analysis

About five grams of a lyophilized sample were used for extraction of total lipids using a single-phase organic solvent system comprised of chloroform, methanol, and aqueous 50-mM phosphate buffer (pH 7.4) in the ratio of 1:2:0.8 (v:v:v; Zhang *et al.*, 2006). After about 12 hours, equal volumes of chloroform and nano-pure water were added, forming a two-phase system. The organic phase was collected and reduced in volume under pure  $N_2$  gas.

Total lipids were transesterified in 2 ml of methanol and hydrochloric acid (95:5; v/v) in a heating block at 70°C for two hours, which also should hydrolyze polar sidechains of GDGTs. After cooling to room temperature, 1 ml of solvent-extracted nanopure water and 2 ml of CH<sub>2</sub>Cl<sub>2</sub> were added. The transesterified lipids were passed through a C-18 solid phase extraction (SPE) column. The GDGT fraction eluted with 1:3 ethyl acetate:hexane and was dissolved in 1.4% isopropanol in hexane.

GDGTs were identified using an Agilent 1100 series high performance liquid chromatograph (HPLC) with atmospheric pressure chemical ionization-MS using a Zorbax NH<sub>2</sub> column (2.1 x 150 mm, 5 m particle size) and/or a Prevail CN column (2.1 x 150 mm, 3 m particle size) at 30°C (Pearson *et al.*, 2004). Conditions for atmospheric pressure chemical ionization-MS were nebulizer pressure of 60 lb/in<sup>2</sup>, drying gas flow of 6.0 liters/min and 350°C, vaporizer temperature of 375°C, voltage of 3 kV, and corona of 5 uA. Spectra were scanned over the m/z range from 1,250 to 1,350.

# Archaeal 16S rRNA gene analysis

Three archaeal clone libraries were constructed using samples from MC 118 core 9 (GoMT: 5-15 cm; GoMM: 15-30 cm; GoMD: 30-35 cm) to provide phylogenetic information for the possibly unusual GDGT profiles to be detected in this core. Genomic DNA was extracted from 7.5g frozen sediment using the Ultraclean Mega Prep Soil DNA kit (MOBio Laboratories, Inc, Solana Beach, CA). The precipitated DNA was purified by gel electrophoresis plus mini-column preparation (Wizard DNA Clean-Up System, Promega, Madison, WI). Archaeal 16S rRNA gene fragments (approximately 930 bp) were amplified in a Perkin-Elmer 9700 Thermal Cycler with the primer pair Arch-21F (5'-TTC CGG TTG ATC CYG CCG GA-3') and Arch-958R (5'-YCC GGC GTT GAM TCC AAT T-3') (DeLong, 1992) according to the procedure of the FailSafe PCR system reaction mix G (Epicenter Biotechnologies, Madison, WI). To minimize sample biases and to obtain enough amplicon for cloning, the number of cycles in a PCR reaction was limited to 30 and three replicated amplifications were carried out for each sample. The combined PCR products were purified from a low-melting point agarose gel (1.0%) using the QIA Quick Gel Extraction Kit (QIAGEN, Inc., Valencia, CA). The purified DNA was ligated with pCR 2.1 vector and competent Escherichia coli cells were transformed according to manufacturer's instructions (Topo-TA cloning kit, Invitrogen, Carlsbad, CA). Forty to 50 colonies from each sample were randomly chosen for sequencing. Plasmid DNA containing inserts were prepared using a QIAprep Spin miniprep kit (QIAGEN, Valencia, CA). Sequencing reactions were carried out using ABI BigDye Termination v.3.1 (Applied Biosystems. Foster City, CA) and sequenced on an ABI 3100 automated sequencer. Clone sequences were manually checked for chimeras using Ribosomal

Database Project II (http://wdcm.nig.ac.jp/RDP/html/index.html) and identified chimeric sequences were removed. Phylogenetic analysis was done according to Jiang *et al.* (2006). Sequences with identities of greater than 97% were considered to represent the same operational taxonomic unit (OTU) (Humayoun *et al.*, 2003). Coverage (C) was calculated as:  $C = 1-(n_1/N)$ , where  $n_1$  is the number of phylotypes that occurred only once in the clone library and N is the total number of clones analyzed (Mullins *et al.*, 1995). Rarefaction analyses were performed using DOTUR (Schloss & Handelsman, 2005).

#### Nucleotide sequence accession numbers

The unique sequences determined in this study have been deposited in the GenBank database under accession numbers EF116459- EF116485.

#### RESULTS

#### Concentrations of porewater sulfate and hydrogen sulfide

In core 1, sulfate concentrations ranged from 24.6 mM to 35 mM. In core 9, sulfate concentrations were below 0.3 mM. In contrast, sulfide concentrations were less than 2.5 M in core 1 but 27.8 to 979.4 M in core 9 (Table 1). However, it was possible that pore-water sulfide had undergone partial sulfide oxidation during exposure of sediment core on board the ship and during laboratory storage because of its sensitivity to oxygen in contact with the sample. Thus, measurements of sulfide may be regarded as potential underestimates of actual concentrations. Nevertheless, the results clearly indicated that extensive sulfate reduction occurred in core 9. This is consistent with the presence of carbonate and pyrite minerals are both enhanced during sulfate reduction coupled to the oxidation of methane and/or other hydrocarbons (Formolo et al., 2004).

# Archaeal lipids

Twenty-four samples were analyzed for GDGTs. Representative GDGT profiles from GC 233 NBP and MC 118 Cores 1, 8, and 9 are shown in Fig. 2. Archaeal lipids from GC 233 NBP and MC 118 core 1 are characterized by high relative abundance of crenarchaeol (peak I) and GDGT-0 (peak II) (profiles a and b, Fig. 2), which are typical of normal marine sediments (*e.g.*, Schouten et al., 2002). Archaeal lipids from the hydrate-affected samples, however, are characterized by relative decreases in crenarchaeol and GDGT-0 and increases in GDGT-1 and GDGT-2 (peaks III and IV); GDGT-3 (peak V) increases to a lesser extent in hydrate-affected samples (Table 2; profiles c and d, Fig. 2).

In all three horizons from GC233 NBP, the crenarchaeol accounted for 42-43% and the GDGT-0 30-33% of total GDGTs (Table 2). The remaining GDGTs accounted for less than 12% for each compound and decreased in abundance in the order of GDGT-2 (10-11%), GDGT-1 (8-10%), the crenarchaeol isomer (4-5%), and GDGT-3 (1-2%) (Table 2). GDGTs from MC 118 core 1 had similar distributions to those from GC 233 NBP. In particular, no significant differences were observed in the relative abundances of crenarchaeol, GDGT-0, GDGT-1 and the crenarchaeol isomer between the two locations in the 0-30 depth range (Table 2).

In contrast, GDGTs from MC 118 cores 8 and 9 had distinct patterns with depth. In core 8, crenarchaeol and GDGT-0 were relatively less abundant below 100 cm (10-17% and 26%, respectively) than above 30 cm (28-43% and 30-34%, respectively; Table 2); while GDGT-1 and GDGT-2 were significantly enriched below 100 cm (20-23% and 31-33%, respectively) compared to shallower depths (10-12% and 11-21%, respectively).

Similarly, GDGT-3 was also more abundant in the lower (below 100 cm) section (5-7%) than in the upper (above 30 cm) section (3%). Overall, in core 8, crenarchaeol and its isomer decreased with depth; whereas, GDGT-1, GDGT-2, and GDGT-3 increased gradually with depth (Table 2).

Core 9 showed a nearly opposite pattern of distributional change in GDGT composition as a function of depth. A boundary appeared to exist between 35 and 100 cm, where below 100 cm, the GDGT profiles were similar to those in GC 233 NBP and MC 118 core 1 (34-43% and 26-36%, for crenarchaeol and GDGT-0, respectively); whereas above 35 cm, crenarchaeol and GDGT-0 were relatively much less abundant (13-18% and 14-16%). The crenarchaeol isomer also is slightly less abundant in the upper (above 35 cm) section (Table 2). The most abundant GDGTs in the shallow horizons, interestingly, are GDGT-2 and GDGT-1 (39-46% and 16-18%; Table 2).

#### **Diversity of archaeal 16S rRNA genes**

A total of 42-44 clones were sequenced from each of the top three intervals (5-15 cm, 15-30 cm, 30-35 cm) of core 9 in order to provide phylogenetic information about the archaeal communities that may be responsible for the unusual profiles of GDGTs in this core. The number of clones represented 82.5-95.0% of coverage of each clone library (Table 3) and along with rarefaction analysis (data not shown) indicated that sequences in each library reached or was close to saturation. Statistical analysis of the three 16S rRNA archaeal libraries using the LIBSHUFF program (http://whitman.myweb.uga.edu/libshuff.html) showed the archaeal community structure in the bottom sample (30-35 cm) was significantly different (P = 0.001) from that in the

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top (5-15 cm) or the middle (15-30 cm) sample; whereas the community structure is similar between the top and middle samples ( $P \ge 0.05$ ).

The overall archaeal 16S rRNA gene sequences were dominated by euryarchaeota, which in turn were dominated by the ANME-1a subgroup (61-71% of the total clones at each depth; Table 3). The 15-30 cm interval also included a couple of representatives from the ANME-2a subgroup and the terrestrial miscellaneous euryarchaeotal group (TMEG); the 5-15 cm and 30-35 cm intervals had a few representatives of the ANME2-c subgroup (Table 3). On the other hand, a large proportion (23-24%) of *Thermoplasmatales* was found in the 5-15 cm and the 15-30 cm intervals but not in the 30-35 cm interval (Table 3).

The number of crenarchaeotal 16S rRNA gene sequences was limited at each interval analyzed (Table 3). The 5-15 cm interval had five clones (11%) belonging to the marine benthic group B (MBG-B); while the 30-35 cm interval had 3 clones (7%) belonging to the miscellaneous crenarchaeotal group (MCG) and 4 clones (9%) belonging to MBG-B. No crenarchaeota were detected in the 15-30 cm interval.

Figures 3 shows the phylogenetic relationships of these euryarchaeotal and crenarchaeotal sequences. All euryarchaeotal sequences were closely related (~96-99%) to clones retrieved from methane hydrate-bearing or methane-rich habitats, including the Black Sea, California continental margin, Eel River, Cascadia Margin, Hydrate Ridge, Guaymas Basin, Gulf of Mexico, Nankai Trough, Peru Margin, and R/V Cape Hatteras Blake Ridge (Fig. 3). Within crenarchaeota, the MCG sequences were closely related to those from Okhotsk coastal subseafloor sediment and the Gulf of Mexico and the MBG-B sequences were closely related to those from Peru Margin sediments, Cascadia Margin

sediments associated with gas hydrates, or other locations in the Gulf of Mexico. However, none of our crenarchaeotal sequences were closely related to marine group I of the planktonic crenarchaeota (*e.g.*, DeLong, 1998), suggesting we sampled an *in-situ* sedimentary community.

#### DISCUSSION

#### Archaeal lipids associated with gas-hydrate hosting sediments

The GDGT profiles from the Gulf of Mexico cores GC 233 NBP and MC 118 core 1 (Fig. 2a,b and Table 2) are similar to those observed in other warmer areas such as the Arabian Sea and Cariaco Basin (*e.g.*, Schouten *et al.*, 2002; Zhang *et al.*, 2006). These observations are consistent with the generally-accepted interpretation that GDGTs in normal marine sediments mostly are contributed by the settling and sedimentation of planktonic crenarchaeota from the water column (Schouten et al., 2000). Although separated by over 300 km in distance, the similarity in GDGT profiles between GC 233 NBP and MC 118 core 1 suggests that the planktonic crenarchaeota in this region are homogeneously distributed in the upper water column.

In contrast, the unusually high relative abundances of GDGT-1, GDGT-2, and GDGT-3 in MC 118 cores 8 and 9 on top of the hydrate mound suggest significantly enhanced biomass of methane-oxidizing archaea (Table 2). Qualitatively, clone libraries of archaeal 16S rRNA genes suggest that euryarchaeaota responsible for these enhanced concentrations of GDGT-2 may be dominated by the ANME-1 group (Fig. 3; Blumenberg *et al.*, 2004). Some of the anomalous GDGTs also may come from the *Thermopasmatales*, particularly in the 5-15 cm and 15-30 cm intervals in which sequences of this group were found (Fig. 3); however, the physiology of the non-

thermophilic Thermoplasmatales is unknown and caution needs to be excised when evaluating the contribution of these organisms to GDGTs in the marine environments. On the other hand, the enhanced biomass of the ANME-1 methane-oxidizing archaea can be reasonably linked to syntrophic sulfate-reducing bacteria. This AOM process commonly is observed near gas hydrates or in cold seep environments (e.g., Boetius et al., 2000; Boetius & Suess, 2004; Orphan et al., 2002; Orcutt et al., 2004; Lloyd et al., 2006). Indeed, sulfate in core 9 was significantly depleted (Table 1); whereas sulfate in core 1 (Table 1) and GC 233 NBP (Formolo et al., unpublished data) showed no appreciable decrease with depth. Other methane-oxidizing archaea, in particular the identified ANME-2 group (Fig. 3), also may have contributed to the observed sulfate reduction in core 9. It must be noted that sulfate reduction can also be coupled to oxidation of petroleum hydrocarbons and the process of AOM can some times equate only a small portion of sulfate reduction (Formolo et al., 2004; Orcutt et al., 2005; Lloyd et al., 2006). Thus, a precise correlation cannot be made between sulfate reduction rates, methane oxidation, and quantification of GDGTs putatively associated only with ANME-1 archaea.

# Impact of gas hydrates on phylogenetic distributions of archaea in marine sediments

Extensive research has demonstrated that normal marine sediments are dominated by non-thermophilic crenarchaeota and non-ANME groups of euryarchaeota whereas shallow sediments affected by cold seeps and gas hydrates are dominated by ANME-groups of euryarchaeota (*e.g.*, Inagaki *et al.*, 2003; Lanoil *et al.*, 2005; Wang *et al.*, 2005; Biddle *et al.*, 2006; Inagaki *et al.*, 2006; Sorensen & Teske, 2006; Webster *et al.*, 2006; Kendall *et al.*, 2007). In particular, marine benthic group B and *Thermoplasmatales* 

appear to be cosmopolitan in normal marine sediments (Orphan *et al.*, 2001a; Teske, 2006). Crenarchaeota also dominate the normal marine sediments in the Gulf of Mexico as observed using denaturing gradient gel electropherisis (Huang et al., manuscript in preparation).

AOM communities are ubiquitous in sediments of cold seeps and gas hydrates, although the relative distribution of ANME-1 and ANME-2 varies between locations. For example, ANME-1 is predominant in the Black Sea, whereas ANME-2 is predominant at Hydrate Ridge (Knittel *et al.*, 2005; Nauhaus *et al.*, 2005). In the Gulf of Mexico, both ANME-1 and ANME-2 are conspicuous (Fig. 3). In GC 185 and GC 234 (Mills *et al.*, 2003, 2005) both ANME-1 and ANME-2 appeared to be widely distributed (Table 4). However, in GB 425 (Lanoil *et al.*, 2005) and the Florida Escarpment (Reed *et al.*, 2006), ANME-2 is predominant; whereas, in GC 205 (Lloyd *et al.*, 2006) and MC 118 (this study), ANME-1 is predominant (Table 4). In particular, Lloyd *et al.* (2006) observed that ANME-1 at GC 205 was represented by a single sub-group (ANME-1b).

A number of factors have been proposed to affect the distribution of ANME-1 and ANME-2, which include concentration of methane (Blumenberg *et al.*, 2004, 2005; Girguis *et al.*, 2005), sensitivity to oxygen (Knittel *et al.*, 2005), salinity (Lloyd *et al.*, 2006), and temperature (Nauhaus *et al.*, 2005). For example, ANME-2 is less sensitive to oxygen and thus predominant in the Hydrate Ridge sediments overlain by oxic seawater, while ANME-1 is more sensitive to oxygen and thus predominant in the anoxic Black Sea (Knittel *et al.*, 2005). The difference, however, also could be explained by temperature-dependent variations in rates of sulfate reduction in association with AOM. In the Black Sea, sulfate reduction rates increase with increasing temperature, whereas in Hydrate

Ridge, sulfate reduction rates decrease with increasing temperature (see Figure 1 of Nauhaus et al., 2005). It must be noted, however, rates of sulfate reduction were overall 10-30 times higher in the Black Sea than in the Hydrate Ridge (Nauhaus et al., 2005); thus the effect of temperature on rate of sulfate reduction must be viewed in the context of local habitat. It is possible both oxygen and temperature affect the distribution of ANME groups. The effect of methane is best demonstrated in well-controlled microcosms, which show that ANME-1 out-competes ANME-2 under high methane-flow conditions (Girguis *et al.*, 2005). In natural environments, however, methane flux may vary considerably spatially and temporally and both ANME-1 and ANME-2 may be able to live on a wide range of methane concentrations (Lloyd *et al.*, 2006). An effect of salinity also has been proposed, based on the observation that high salinity (2,200 mM) is associated with the ANME-1b subgroup at GC 205, where all other ANME subgroups did not occur, suggesting that ANME-1b may have adapted to this extreme environment (Lloyd *et al.*, 2006).

Samples from core 9 at MC 118 represent normal salinity and the bottom water temperature is about 4.6-5.7°C (Higley, Sassen, Sleeper, personal communications). Methane concentration in the pore-water of core 9 was not measured thus the effect of methane on ANME group distribution cannot be evaluated. While salinity does not seem to be a major factor affecting the community structure of methane-oxidizing archaea at MC 118 (see Lloyd *et al.*, 2006), the lower-end of temperature range (4.6°C) at this site is closer to that observed in the Black Sea and it is tempting to suggest that the enhancement of ANME-1 group is related to the cold temperature. It is also intriguing to observe that GC 205 and MC 118 have similar water depths (876 m and 890 m, respectively) and similar water temperature (5.8°C at GC 205; Lloyd *et al.*, 2006) and both sites are dominated by ANME-1 (Table 3), thus further supporting the role of temperature in affecting ANME-1. Other unknown variables may also affect the distribution of ANME groups associated with gas hydrates.

In summary, glycerol dialkyl glycerol tetraethers showed distinct patterns between non-hydrate and hydrate-hosting cores at MC 118. Non-hydrate samples were dominated by GDGT-0 and crenarchaeol, which normally accounted for greater than 70% of total GDGTs. Methane-influenced samples had significantly higher ratios of GDGT-1, GDGT-2 and GDGT-3, with correspondingly decreasing relative amounts of crenarchaeol. Cloning of the archaeal 16S rRNA genes from the hydrate samples indicated the predominance of ANME-1 subgroups, which may have contributed to the enhanced production of GDGT-1 to GDGT-3. Our results demonstrated the effectiveness of integrating lipid biomarkers and molecular DNA in characterizing archaeal community structures impacted by gas hydrate-hosting or methane-rich sediments.

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Sample	$SO_4^{=}$ (mM)	$\Sigma H_2 S(\mu M)$								
Core 1_5-10 cm	25.0	0.6								
Core 1_15-20 cm	26.0	0.6								
Core 1_25-30 cm	35.0	0.9								
Core 1_100-105 cm	28.6	0.6								
Core 1_184-189 cm	30.4	2.2								
Core 1_200-205 cm	24.6	0.2								
Core 9_15-30 cm	0.2	979.4								
Core 9_167-182 cm	0.1	461.8								
Core 9_476-486 cm	0.0	27.8								

Table 1. Pore-water chemistry of sulfate and hydrogen sulfide for core 1 and core 9 at MC 118.
	Relative abundance $(\%)^{\dagger}$							
Depth (cm)	GDGT-0	GDGT-1	GDGT-2	GDGT-3	Crenarchaeol	Cren-isomer		
GC 233 NBP								
0~4	33	10	10	1	43	4		
12~14	31	10	11	1	42	5		
24~26	33	8	10	2	42	5		
MC 118 core 1								
5~10	31	10	8	3	43	4		
15~20	35	9	10	2	42	3		
25~30	33	9	10	3	41	4		
100~105	30	8	10	2	45	5		
105~110	37	9	7	2	44	2		
184~189	39	8	13	5	33	2		
200~205	35	7	7	3	45	4		
205~210	35	8	9	$ND^*$	44	4		
MC 118 core 8								
5~15	30	10	11	3	43	3		
20~30	34	12	21	3	28	2		
105~115	26	20	31	5	17	2		
130~138	26	23	33	7	10	1		
MC 118 core 9								
5~15	16	16	39	9	18	2		
15~30	16	18	39	10	13	3		
30~35	15	17	46	8	13	2		
100~110	36	10	11	3	38	2		
167~182	33	11	15	3	34	4		
200~210	31	7	10	4	42	6		
310~320	34	10	9	NA	43	5		
476~486	30	12	8	3	43	4		
500~510	27	9	18	5	39	3		

Table 2. Relative abundance of glycerol dialkyl glycerol tetraethers (GDGTs) in sediment cores collected from GC 233 and MC 118 in the Gulf of Mexico.

<sup>†</sup>The standard error of the relative abundance is  $\sim \pm 2\%$ . not detected.

	Number of clones/OTUs				
	GoMT (5-15 cm)	GoMM (15-30 cm)	GoMD (30-35 cm)		
Total clones sequenced	44	42	43		
Euryarchaeota					
ANME-1a	27	30	29		
ANME-2a		1			
ANME-2c	2		7		
TMEG		1			
Thermoplasmatales	10	10			
Crenarchaeota					
MCG			3		
MBG-B	5		4		
Number of OTUs*	11	9	8		
Number of unique OTUs <sup>†</sup>	7	4	3		
Coverage (%)	82.5	90.4	95.0		

 Table 3: Phylogenetic affiliations of archaeal 16S rRNA genes retrieved from MC 118

 core 9 in the Gulf of Mexico.

\*Determined by DOTUR with a cutoff value of 3%.

notaries from different locations in the Out of Mexico.							
Sample	<sup>a</sup> GC185+GC234	<sup>b</sup> GC234	°FE <sup>*</sup>	<sup>d</sup> GC205	°GB425	<sup>f</sup> AT425	<sup>g</sup> MC118
Location	27°46'N, 27°44'N	27 <sup>°</sup> 44'N	26°01.8'N	27.°43.0'N	27 <sup>°</sup> 33'N	27 <sup>°</sup> 34.1'N	28°51.5'
	91°30'W, 91°13'W	91°13'W	84 <sup>°</sup> 54.9'W	90°32.0'W	92°32'W	88°29.7'W	88 <sup>°</sup> 29.5'
Water depth	~650 m	575 m	3,288 m	876 m	600 m	~1295 m	890 m
Total clones	125	100	68	310	134	93	129
Euryarchaeota							
ANME-1	20	58	6	59	0	17	67
ANME-2a	2	0	34	0	41	3	1
ANME-2b	0	0	22	0	39	0	0
ANME-2c	30	13	34	0	0	0	7
ANME-2d	2	2	0	0	0	0	0
Methanomicrobiales	14	13	0	4	0	0	0
Methanosarcinales	15	8	0	9	0	78	0
Thermoplasmatales	6	1	0	20	6	0	16
TMEG	0	0	0	0	0	0	1
Unclassified Euryarch.	0	3	0	0	4	1	0
Crenarchaeota	10	2	3	8	10	0	9

Table 4: Distribution of different phylogenetic groups (in percentage of total clones) in archaeal clone libraries from different locations in the Gulf of Mexico.

Crenarchaeota10238100Note: a, Mills et al., 2003; b, Mills et al., 2005; c, Reed et al., 2006; d, Lloyd et al., 2006; e, Martinez et al., 2006; f, Lanoil et al., 2001; g, this work.\*FE = Florida Escarpment.

## **Figure captions:**

Fig. 1. Map of site MC 118 in the Gulf of Mexico, modified from Woolsey *et al.* (2005). Colors of the background were obtained using multibeam image by C&C Technologies; the yellow and brown colors indicate shallower water and the light green, light blue, and deep blue indicate deeper water (see insert).

Fig. 2. HPLC-MS chromatograms of intact GDGTs from Gulf of Mexico sediments. A) surface sediment from GC 233 (NBP), B) surface sediment from MC 118 core 1, C) sediment from about 1 m depth in MC 118 core 8, and D) shallow sediment from MC 118 core 9. Shown GDGT series are: I, crenarchaeol and the regio-isomer of crenarchaeol VI, II = GDGT-0, III = GDGT-1, IV = GDGT-2, V = GDGT-3.

Figure 3. Neighbor-joining tree (partial sequences, ~700 bp) showing phylogenetic relationships of eurarychaeota and crenarchaeota using archaeal 16S rRNA gene sequences retrieved from core 9 of the GoM sediment to closely related sequences from the GenBank. One representative clone type within each phylotype is shown and the number of clones within each phylotype is shown at the end (after the GenBank accession number). Number is not shown if there is only one clone within a given phylotype. Clone sequences from this study are coded as follows, with GoMD22 as an example: GoM: Gulf of Mexico; D, bottom; 22, clone number. T stands for top and M for middle in GoMT and GoMM, respectively. Scale bars indicate Jukes-Cantor distances. Bootstrap values of >50% (for 500 iterations) are shown. *Aquifex pyrophilus* is used as the outgroup.



Fig. 1. Pi et al., 2007



Fig. 2. Pi et al., 2007



Fig. 3. Pi et al., 2007