Complex Microbial Communities Inhabiting Sulfide-rich Black Mud from Marine Coastal Environments

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ABSTRACT

We have used 16S rRNA phylogenetic analysis to investigate the microbial diversity of sulfur-rich black mud marine sediments. Two distinct environments were examined: mud from a hypersaline marsh and mud from a brackish marsh. Only two out of one hundred cloned 16S rDNA sequences were identical, indicating a very complex community of microbial species. Both environments exhibited significant sulfate-reducing δ-*Proteobacteria*, indicating that these organisms are likely the primary source of reduced forms of sulfate required by other species. Since seawater has abundant quantities of sulfate, microorganisms that reduce this molecule are important for the cycling of sulfur in the global ecosystem. PCR of the 16S rDNAs extracted directly from the mud without culturing revealed that most of the population was less than 95% identical to known 16S rDNAs, and eight of the one hundred sequences analyzed were only 80-85% identical to known sequences. Four sequences from this more divergent group were identified as new candidate divisions. Although sulfate-reducing bacteria dominated both ecosystems, the hypersaline black mud also harbored organisms related to known halophiles. A search for *Archaea* uncovered a diverse collection of halophilic species that showed taxonomic affiliation with the *Halobacteriales*. From our sampling of 100 rDNA clones, we estimate that the diversity of organisms in these black mud samples is approximately 10,000 species – greater than the approximately 6000 *Archaea* and *Bacteria* currently recognized by culture studies. Additionally, we note a lack of predominant species in our sample. This suggests a modestly ambitious DNA sequencing effort of rDNAs from black mud would yield thousands of new species and many new candidate divisions.

INTRODUCTION

Over one hundred years ago, Sergei Winogradsky studied the microbial organisms inhabiting sulfide-rich black mud ecosystems and pioneered our understanding of chemolithotrophy through his experiments with sulfate and nitrate reducing organisms. Although many of the microbes from black mud environments (which are often used to inoculate so-called "Winogradsky columns") have been studied for decades, the vast majority of the microorganisms present in natural black mud ecosystems remain unknown to microbiologists. While the physiology and biochemistry of microorganisms are best studied in pure cultures, this may prove very tedious in the case of black mud microbes, because the complex community has co-evolved for millions of years. Syntrophy -- the requirement of a microbe to associate with another microbial species for metabolites -- is probably critical to the survival of most of the species within this complex environment.

It is generally accepted that less than 1% of soil microbes have been successfully cultured by standard techniques (Amann *et al.,* 1995; Pace, 1997). The identification of rRNA genes from the environment by amplification of genomic rDNA extracted directly from mixed microbiota alleviates the requirement for cultivation. Thus, this microbial community can now be inventoried and individual species can be placed into taxonomic groups based on comparisons of their 16S rRNA sequences. Molecular analyses based on 16S rRNA genes have proved successful in determining the species composition of mixed microbial communities from several environments (Hugenholtz *et al.*, 1998; Pace, 1996).

Abundant research has surveyed a myriad of soil types around the globe (Coates *et al.,* 1998; Devereux *et al.*, 1994; Kuske, *et al.*, 1997; Ravenschlag *et al.,* 1999; Wise *et al.*, 1997; Zhou *et al.*, 1997), but there have been no detailed 16S rRNA analyses of sulfide-rich black mud. This is truly ironic, considering that sulfide-rich black mud is a preferred inoculum for the

Winogradsky column and that, beginning with the very early studies of Winogradsky it was clear that this particular ecosystem must be highly complex. In the present study, we describe the microbial community of anaerobic sulfide-rich black mud from two different ecosystems.

MATERIALS and METHODS

Sample collection and site description. The anaerobic layer of sulfide-rich black mud was collected by uncovering the thin top layer of oxidized mud (about 1 cm) followed by removal of the underlying black mud layer. These samples were placed into 500 ml centrifuge bottles. Samples were transported to the lab and small aliquots (about 0.5-1 ml) were immediately processed. Two distinct sources were examined. The first was brackish standing water adjacent to the East San Francisco Bay. The mud at this first site had a temperature of 23°C, pH of 7.5 $\pm$ 0.5, and a salinity of 2.6% $\pm$ 0.2% (slightly less than seawater). The second site was a hypersaline marsh located in the South San Francisco Bay. Black mud at this second site had a temperature of 25°C, pH of 8.5 $\pm$ 0.5, and a salinity of 16.2% $\pm$ 0.2%.

DNA extraction. Community genomic DNA was extracted with a Soil DNA Kit (MoBio Laboratories, Inc.) according to the manufacturer's instructions. In brief, the kit supplies reagents for lysing microbial cells and for binding the DNA on a solid support and thus DNA can be washed and then eluted. This process removes potential PCR inhibitors so that the DNA can be used immediately for PCR.

PCR and cloning. PCR amplification was performed in 50-μl reactions consisting of buffer reaction mix (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 0.001% [w/v] gelatin), 200 M deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, 4 μM primer, and 1 to 5 μl of template DNA. Reactions were run for 35 cycles of amplification. After an initial denaturation at 94°C for 10 min to activate the Ampli*Taq* Gold DNA polymerase (Perkin-Elmer), cycles of 1 min at 94°C, 1 min at 50°C, and 1.5 min at 72°C were run. A final 10 min extension step at 72°C was included to ensure complete extension for efficient cloning. Each reaction contained 2.5 units of Ampli*Taq* Gold.

Clone libraries were constructed with the following primers: 515F (5'-GTGCCAGCMGCCGCGGTAA-3'), 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), 21F (5'-TTCCGGTTGATCCTGCCGGA-3'), 312F (5'-TCCTACGGGAGGCAGCAGCT-3'), and 1492R (5'-GGYTACCTTGTTACGACTT-3'). Degenerate positions are designated: M = A+C and Y = C+T. Primer pairs employed in this study were: 515F-1492R (universal), 27F-1492R (specific for *Bacteria*), 21F-1492R (specific for *Archaea*), and 312F-1492R (specific for spirochetes). PCR products were purified by a QIAquick PCR purification kit (Qiagen Inc.) and cloned with the TOPO TA cloning kit pCR2.1-TOPO vector (Invitrogen Corporation), according to the manufacturer's recommendations.

Sequencing and phylogenetic analysis. PCR products were sequenced on an ABI Prism 377XL automated DNA sequencer. 16S rDNA sequences were compared to known sequences in GenBank (Benson *et al.,* 2000) with the advanced gapped BLAST (basic local alignment search tool) algorithm (Altschul *et al., 1997*). The sequences were compiled in Chromas version 1.3 (Conor McCarthy; Griffith University, Brisbane, Queensland, Australia), aligned with the genetic database environment alignment editor, and then placed into a phylogenetic tree containing approximately 8,000 rDNA sequences. The neighbor-joining tree was generated on the ARB application (Strunk *et al.,* 1999) running under a Linux operating system. 16S rDNA sequences were examined for chimeras by checking for inconsistent secondary structures and

employing the CHECK-CHIMERA program (Maidak *et al.*, 1999). To test for contaminating 16S rDNA sequences (Tanner *et al.,* 1998), we performed control PCR experiments that lacked environmental sample, but contained all the extraction and PCR solutions that were used for the black mud experiments. No contaminating 16S rDNA sequences were found in the black mud library (data not shown).

RESULTS

Sulfide-rich black mud was collected from two sites, a brackish marsh and a hypersaline marsh both from the San Francisco Bay. Upon examination under a light microscope, we could visualize a diversity of organisms with sizes ranging from small roundworms (nematodes) to microbes less than 1 μm in diameter. Many of the organisms were highly motile and the population included protozoans, diatoms, and numerous organisms with sulfur granules (*Bacteria* or *Archaea*) stored inside their cell boundaries, indicative of a sulfide-rich environment. This study focuses on *Bacteria* and *Archaea* as described below.

Phylogenetic analysis

A total of 100 clones or purified PCR products were partially sequenced to analyze their 16S rRNA genes. Only two out of one hundred 16S rRNA gene sequences were identical, probably indicating that they were derived from the same species. Coverage of the black mud environment was determined to be 1% based on the equation $C = 1 - (n/N) \times 100$, where n is the number of unique clones and N is the total number of clones (Good, 1953). Thus, by this estimate, there were at least 10,000 species present in the black mud samples. This diversity estimate could be higher if some species were actually more abundant than others in the population, hence this number should be considered a lower limit of the diversity estimate. As sequencing of new isolates proceed and more duplicates are found, the Good equation will yield a better estimate of diversity.

DNA sequencing and phylogenetic analysis revealed that most 16S rDNA sequences obtained from black mud were less than 95% identical to any of the sequences within GenBank, and some were less than 90% identical to known sequences. Furthermore, eight sequences could not be placed within a known division based on their phylogenetic position within the bacterial line of descent. 18% of the 16S rDNA sequences were between 95% and 99% identical to sequences in GenBank, whereas none were 100% identical to any sequence in GenBank. All three domains of life were represented in this environment (*Bacteria* and *Archaea* were determined by molecular methods and *Eucarya* were observed by microscopy).

The most prevalent bacterial types, based on 16S rDNA analysis, were the δ-*Proteobacteria*, a subclass of mostly sulfate-reducing organisms (Table 1). γ *Proteobacteria* and *Cytophagales* also were abundant in the black mud and are typical of marine microorganisms (see Tables 1 and 2). To examine the black mud for microbes that are potentially present in low numbers, we designed specific probes. We chose to search for *Archaea* and spirochetes, both well known to reside in anaerobic soil and marine sediments. A PCR signal was apparent when we tested the hypersaline black mud for anaerobic *Archaea*. The resulting clones were sequenced and found to cluster within the *Halobacteriales* order of *Euryarchaeota*. All were novel and represent new genera, and one cluster may represent a new family (see Fig. 1). Since spirochetes are common in anaerobic mud ecosystems, a specific spirochete library also was examined from both the brackish and hypersaline environments, and based on their 16S rDNA sequences all appear to be new phylotypes of uncultivated spirochetes (Fig 2).

Table 1. Select clones identified as *Proteobacteria* from the sulfide-rich black mud.

Clone type	Database match (≥95%)	Percent identity	Accession number*	Division	Salinity type
CE105	Desulfosarcina variabilis	98	M34407	δ *Proteobacteria*	Brackish
CE24	*Desulfobacterium catecholicum*	97	AJ237602	δ *Proteobacteria*	Brackish
CE49	Uncultured δ proteobacterium Sva0485	97	AJ241001	δ *Proteobacteria*	Brackish
CE53	Uncultured δ proteobacterium Sva0081	97	AJ240975	δ *Proteobacteria*	Brackish
CE48	Unidentified eubacterium RFLP25	95	AF058007	δ *Proteobacteria*	Brackish
CE101	*Desulfonema magnum*	95	U45989	δ *Proteobacteria*	Brackish
CE52				δ *Proteobacteria*	Brackish
CE46				δ *Proteobacteria*	Brackish
CE98				δ *Proteobacteria*	Brackish
CE103				δ *Proteobacteria*	Brackish
MT24	*Desulfobulbus* sp. McCal 25m	96	AF132868	δ *Proteobacteria*	Hypersaline
MT21	Benzene mineralizing consortium clone SB-9	95	AF029042	δ *Proteobacteria*	Hypersaline
MT67				δ *Proteobacteria*	Hypersaline
MT22				δ *Proteobacteria*	Hypersaline
MT25				δ *Proteobacteria*	Hypersaline
MT23				δ *Proteobacteria*	Hypersaline
MT27				δ *Proteobacteria*	Hypersaline
MT28				δ *Proteobacteria*	Hypersaline
MT58	*Rhabdochromatium marinum*	95	X84316	γ *Proteobacteria*	Brackish
CE47				γ *Proteobacteria*	Brackish
CE100				γ *Proteobacteria*	Brackish
CE102				γ *Proteobacteria*	Brackish
MT44	*Marinobacter* sp. DS40M8	96	AF199440	γ *Proteobacteria*	Hypersaline
CE57	*Methylophaga marina*	96	X95459	γ *Proteobacteria*	Hypersaline
MT33				γ *Proteobacteria*	Hypersaline
CE104	Uncultured ORGANIC-12 bacterium	95	AF142914	α *Proteobacteria*	Brackish
MT68	*Roseovarius tolerans*	99	Y11551	α *Proteobacteria*	Hypersaline
MT30	*Sulfitobacter mediterraneus*	98	Y17387	α *Proteobacteria*	Hypersaline
CE58	*Roseobacter algicola*	97	X78315	α *Proteobacteria*	Hypersaline

* Listed accession numbers are GenBank entries for the closest database match of >95% identity to the 16S rDNA sequence.

Table 2. Select clones representative of different divisions from sulfide-rich black mud.

Clone type	Database match (≥95%)	Percent identity	Accession number*	Division	Salinity type
CE59				*Verrucomicrobia*	Hypersaline
MT60				*Cytophagales*	Brackish
MT69	*Cytophaga fermentans*	97	M58766	*Cytophagales*	Hypersaline
CE22				*Cytophagales*	Hypersaline
CE54				*Cytophagales*	Hypersaline
CE55				*Cytophagales*	Hypersaline
CE60				*Cytophagales*	Hypersaline
MT57				*Spirochetes*	Brackish
MT62a				*Spirochetes*	Hypersaline
MT56	*Odontella sinensis* chloroplast	97	Z67753	*Cyanobacteria*	Hypersaline
MT36				*Cyanobacteria*	Hypersaline
MT35				Low G+C gram positive	Hypersaline
MT38				Low G+C gram positive	Hypersaline
MT61	Subsurface clone H1.4.f	96	AF005748	Green non-Sulfur (subdivision 2)	Brackish
CE61, MT70				Candidate division KSA1	Hypersaline
MT2				Candidate division KSA2	Hypersaline
MT63, CE99				Candidate division KSB1	Brackish
CE45				Candidate division KSB2	Brackish
MT59				Candidate division KSB3	Berkeley
CE50				Candidate division KSB4	Brackish

* Listed accession numbers are GenBank entries for the closest database match of >95% identity to the 16S rDNA sequence. The four clones -- two in candidate division KSA1 and two in candidate division KSB1 -- were tested for their branch point support (i. e. likelihood of forming a single coherent group). A neighbor-joining algorithm was performed on 1000 trees resampled by bootstrap analysis. Both KSA1 and KSB1 scored bootstrap values above 80% indicating a strong likelihood of being monophyletic groups.

From the universal library, we identified four 16S rDNA sequences that could not be placed into known divisions and probably represent uncultivated members of as yet unrecognized divisions (Table 2). Two of these candidate divisions were represented by at least two distinct 16S rDNA sequences. The secondary structure of the rRNA sequences from these candidate divisions appears to fold into a structure that is consistent with the known secondary structure of bacterial rRNA, thus ruling out possible chimera formation during PCR.

DISCUSSION

Microbial diversity from sulfide-rich black mud was examined by 16S rRNA phylogenetic analysis. Based on the results of restriction fragment length polymorphism (data not shown) and sequence analyses, this ecosystem appears to be highly complex. Greater than 80% of the microbes were found to belong to unknown taxonomic groups at the genus level or higher. This degree of diversity is comparable to that observed in soil environments and marine sediments (Hugenholtz *et al.,* 1998). However, the distribution of microorganisms at the division and subdivision taxonomic levels can vary greatly depending on the environment. For example, bacterial phylotypes from Wisconsin agricultural soil consisted mainly of *Cytophagales*, low G+C Gram positives, and *Proteobacteria*, in which the β-subdivision of *Proteobacteria* was the most prevalent subdivision (Borneman *et al.,* 1996). Bacterial phylotypes from an Amazon forest soil indicated the presence of several divisions including *Verrucomicrobium*, *Plactomycetes*, low G+C Gram positives, *Proteobacteria*, and some unclassified groups (Borneman & Triplett, 1997). Additionally, the Amazon forest soil harbored *Archaea*, whereas this domain of microorganisms was not detected in the Wisconsin soil study. This type of variation has been observed in different marine sediment studies and from the present study.

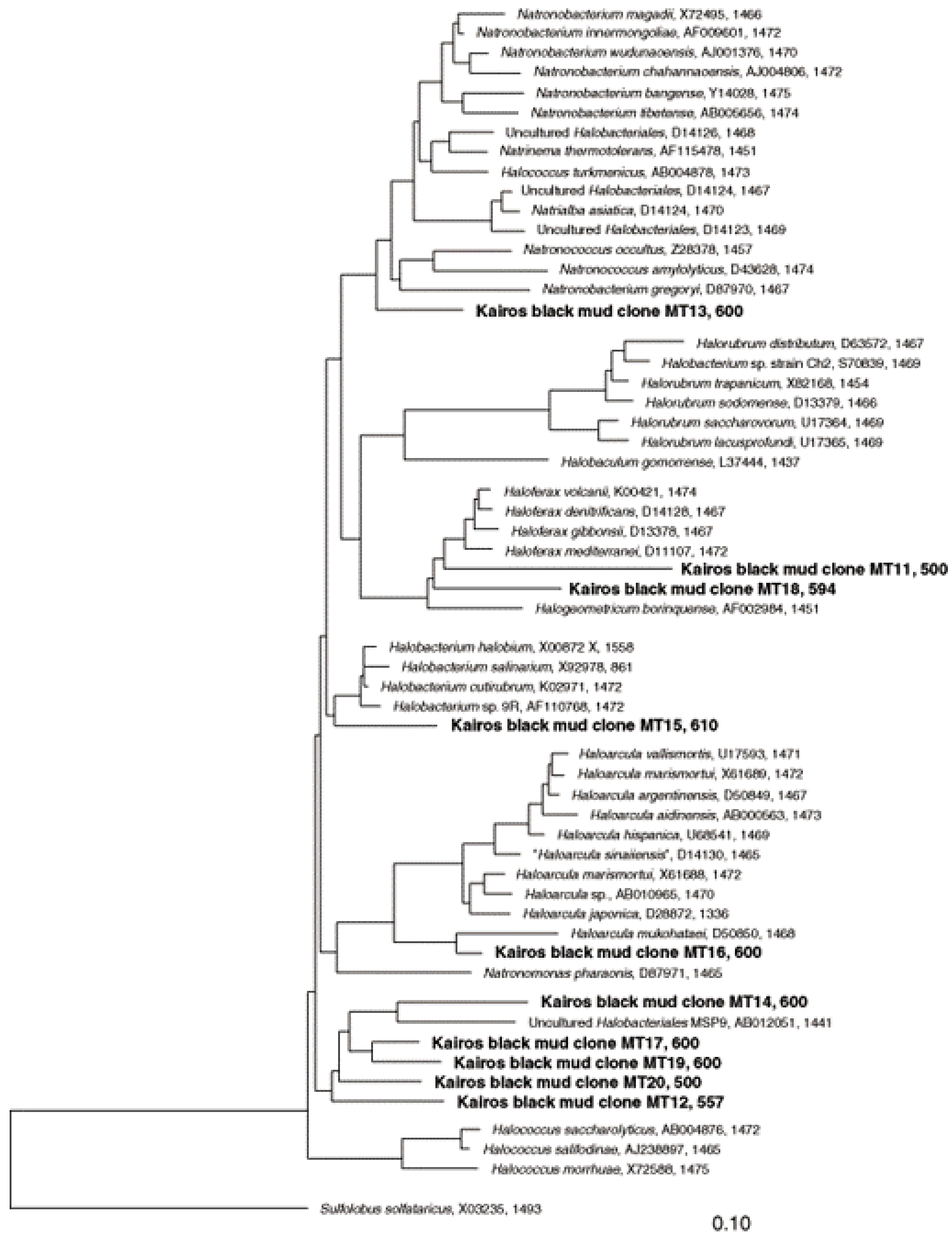

Fig. 1. 16S rDNA phylogenetic tree represents the *Halobacteriales* order of *Euryarchaeota*. All known species are halophilic *Archaea* typically isolated from hypersaline environments. Species or clone names are listed with the GenBank accession number and the number of nucleotides analyzed. All clones in this tree originated from the hypersaline black mud sample. Tree topology was determined by neighbor joining (Strunk *et al.,* 1999) and *Sulfolobus solfataricus* was used as an outgroup. The bar at the bottom indicates nucleotide changes per site.

Taxonomic distribution

Although the microbial inhabitants of black mud consisted of all three domains of life, the sulfate-reducing δ-*Proteobacteria* were the most prevalent microbial types detected. Since sulfur metabolism is the defining process of the black mud ecosystem, it was not surprising to find that a variety of different sulfate-reducing bacteria constituted a high percentage of the community. More than half of the δ-proteobacterial 16S rDNA sequences were less than 95% identical to known sequences and may represent undiscovered genera or families of sulfate-reducing bacteria. In a recent evaluation of the microbial species from samples of permanently cold marine sediments, Ravenschlag et al., (1999) investigated the diversity of sulfate-reducing bacteria. Their study indicated the marine sediment was dominated by species of δ-*Proteobacteria* of different genera including species closely related to those identified in the black mud of the present study. For example, the uncultured δ-proteobacterium clone Sva0081 was closely related to clone CE53 (97% identity). Additionally, sulfate-reducing bacteria from a sandy marine sediment (Devereux *et al*., 1994) and iron-sulfide containing magnetotactic bacteria (DeLong *et al*., 1993) were in this cluster of δ-*Proteobacteria*, suggesting the possibility of distinct genera of uncultured marine δ-*Proteobacteria* (Fig. 3).

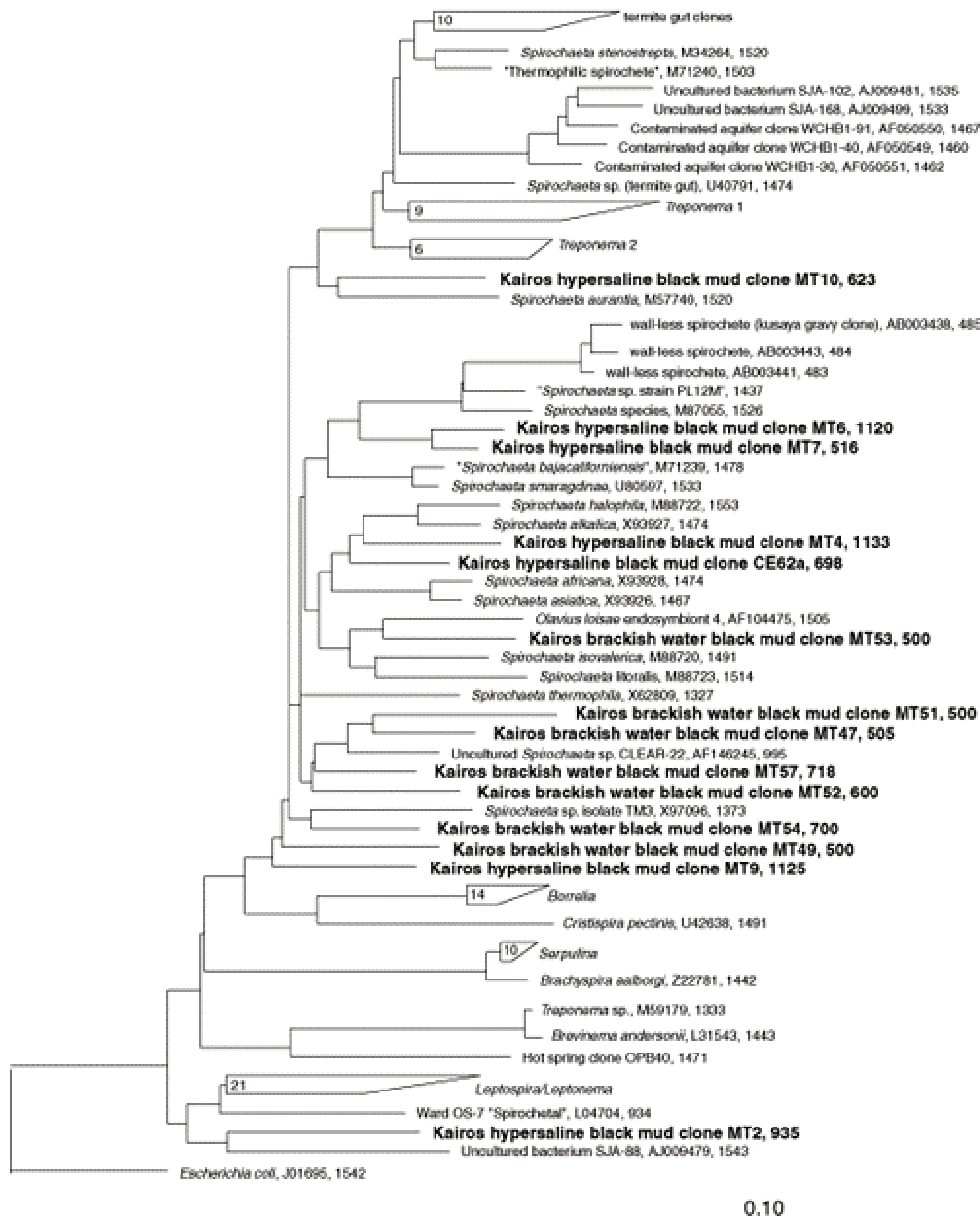

Fig. 2. Neighbor-joining tree of the spirochete division highlighting clones from both the brackish and the hypersaline black mud ecosystems. Genera listed alongside trapezoids indicate monophyletic groups of sequences analyzed, but individual species are hidden from view to conserve space. Numbers inside the trapezoids show the total number of sequences that make up the group. Branch depth for the group is determined by the difference in length of the horizontal lines of the trapezoid. Other features are the same as described in the legend to Fig. 1.

Proteobacteria also were identified within the γ- and α-subdivisions, whereas β-*Proteobacteria* were not detected in the clone library. This suggests that the anaerobic sulfide environment may inhibit the growth of many β-*Proteobacteria*. *Rhodoferax* and *Rhodocyclus*, members of the β-*Proteobacteria*, are able to metabolize low levels of sulfide, but higher concentrations can be toxic. A few 16S rDNA sequences were closely related to marine *Proteobacteria*, such as *Roseovarius tolerans* - an organism isolated from a hypersaline lake in Antarctica (Labrenz *et al.,* 1999) and nearly identical to the 16S rDNA sequence of clone MT68 (Table 1). Another clone, CE57, is related to the 16S rDNA sequence of *Methylophaga marina*, a known halophile.

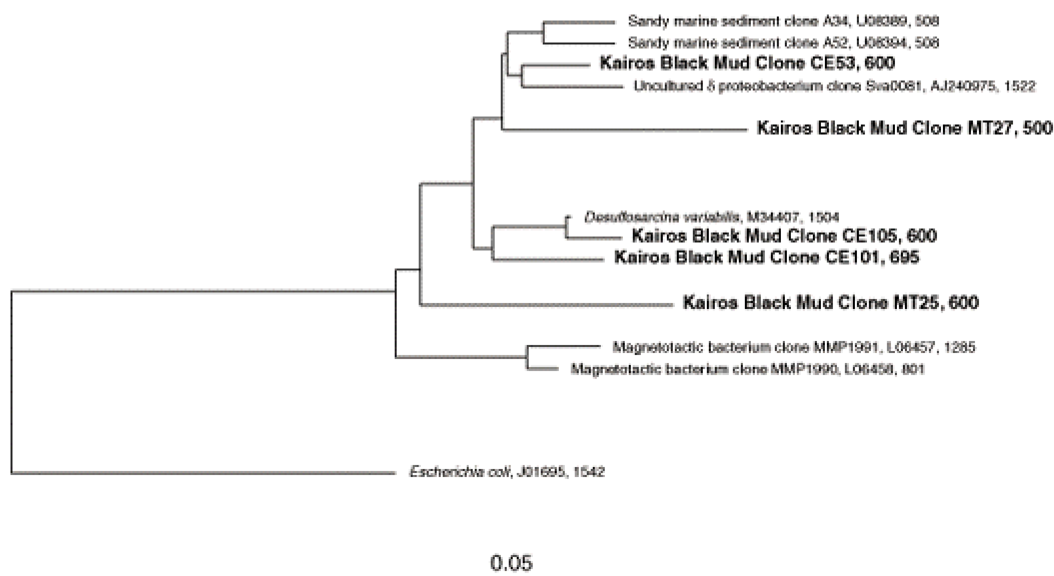

Fig. 3. Phylogenetic tree of a portion of the δ-*Proteobacteria* highlighting uncultivated organisms from marine environments and those from this study. Tree topology was determined in the same manner as for Fig. 1.

Several recognized divisions of bacteria were present in the black mud, most notably three clones of *Verrucomicrobia*. Two were from the hypersaline environment and one was from the brackish environment. Clones MT29 and MT56 were phylogenetically related to recently described uncultivated organisms forming subdivision 5, a subdivision that was first discovered in a hydrocarbon and chlorinated solvent-contaminated aquifer (Dojka *et al.,* 1998) and that has now expanded to include a total of five different environmental sources (Fig 4). None of the microbes have been cultivated at the time of writing. Presently, this subdivision of *Verrucomicrobia* consists of microbes either from contaminated soil (Dojka *et al.,* 1998) or marine sediments (Li *et al.,* 1999; Ravenschlag *et al.*, 1999). It will be of interest to determine if the subdivision is more widespread. Since one of the 16S rDNA sequences from subdivision 5 originated from this study's hypersaline black mud, this subdivision may consist of microbes with the ability to inhabit diverse environments.

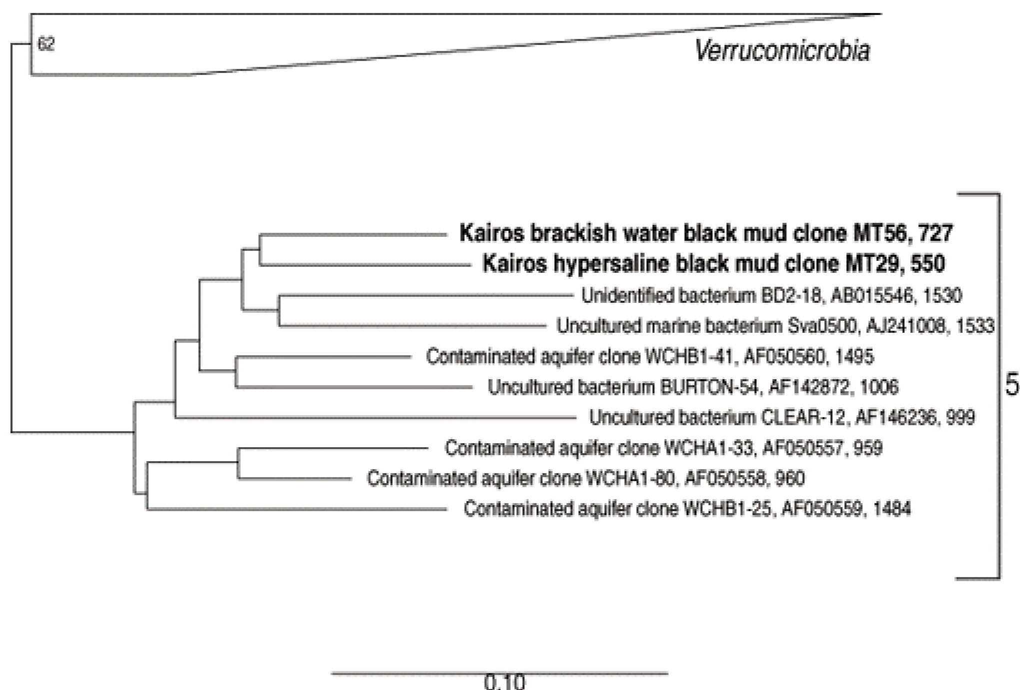

Fig. 4. Tree of the *Verrucomicrobia* division with emphasis on subdivision 5. All members of this subdivision are known only from 16S rDNA sequences. The tree was determined by the same methods used to generate Fig. 1. Accession number for MT56 and MT29 are AF211330 and AF211331, respectively.

New candidate divisions also were determined by the 16S rDNA phylogenetic analyses (Table 2). These 16S rDNA sequences have approximately 80-85% identity to known sequences. Candidate divisions KSA1 and KSB1 show a weak association with the *Cytophagales*-Green Sulfur-Marine Group A cluster of divisions.

Primers specific for *Archaea* indicated the presence of a diverse assemblage of *Halobacteriales* from the hypersaline black mud environment. New species and genera within the order *Halobacteriales* were apparent, since most of the sequenced clones had identities of only 90-95% to known species. Extremely halophilic *Archaea* have been isolated from the Dead Sea (Arahal *et al.*, 1996), soda lakes (Jones *et al.,* 1998), and a saltern (Grant *et al.,* 1999). A group of five clones from the present study form a monophyletic lineage along with an uncultured *Halobacteriales* from an East African saltern (Grant *et al.,* 1999). This group of microbes probably represents a distinct genus with no cultivable representatives to date.

Spirochetes made up approximately 1% of the bacteria based on the recovery of 16S rDNA sequences from the universal library. However, when the mud was examined with primers specific for the spirochete division, highly diverse collections of spirochete 16S rDNA sequences were uncovered. After performing the phylogenetic analyses of the spirochete 16S rDNA sequences, we noticed that two clones were deeply rooted within the spirochete tree (Fig. 2) and included clone MT2 and previously described uncultured bacterial clone SJA-88 from an anaerobic trichlorobenzene-transforming community (von Wintzingerode *et al.,* 1999). These uncultivable bacteria may be early predecessors to the spirochete line of descent, thus representing a potentially deeply rooted subdivision.

Physiological characteristics based on taxonomy

Black mud can be found in a variety of physical environments, but it generally contains sulfur rich compounds -- both inorganic and organic. This environment can be further characterized as containing abundant iron compounds, and a black precipitate forms when the sulfide reacts with ferrous iron to produce insoluble ferrous sulfide. Sulfate-reducing microbes mediate the buildup of this black precipitate. Sulfur compounds are either oxidized by microbes as an energy source or reduced as a final electron acceptor. The most oxidized form of sulfur (SO_4^{2-}) is prevalent in sea water and is reduced by the diverse assemblage of sulfate-reducing microorganisms to sulfite and eventually hydrogen sulfide. This contributes to the sulfurous-like odor near the black mud communities. With the buildup of reduced forms of sulfur, microbial oxidation can take place to generate cellular biomass. The black mud community is abundant with sulfate-reducing microorganisms in the anoxic layer. Physiology of the remaining organisms from this study are unknown, with a few exceptions. A 16S clone was identified as a close relative (98% identity) to *Sulfitobacter mediterraneus*, a recently described sulfite-oxidizing bacterium (Pukall *et al.,* 1999). These microbes benefit directly from the by-products of sulfate reduction and are probably dependent on their neighbors for reductants. Much of the sulfur oxidation probably occurs in the upper layer where higher concentrations of oxygen are present. *S. mediterraneus* is an aerobic bacterium and it could be living at an interface between the oxidized surface and the anaerobic layer of the black mud, assimilating the reduced products from the lower anoxic zones.

When comparing the hypersaline environment to the brackish water sample, we found most microorganisms (except for the archaeal halophiles) fall into the same phylogenetic clusters within the tree of life. However, at the species level, there were no commonalties between the two environments. One possible explanation, is that these two environments were alike at one time and the black mud organisms adapted to the hypersaline environment or vice versa, altering their genetic makeup. From the phylogenetic analyses, it appears that there are many recent common ancestors to the two black mud communities.

We have sampled the microbial diversity of two contrasting environments, hypersaline and brackish black mud, and have found that both harbor large numbers of microbial phylotypes. Sulfate-reducing bacteria were present in both ecosystems, with most species presently uncultured. The hypersaline black mud was home to uncharacterized halotolerant *Bacteria* and *Archaea*. Based on a sample coverage of approximately 1%, this study suggests that further sequencing will yield thousands of new species and perhaps many new candidate divisions.

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