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Molecular phylogenetic analysis of a bacterial community in Sulphur River, Parker Cave, Kentucky

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Abstract

Sulphur River in Parker Cave, Kentucky receives sulfurous water (11–21 mg sulfide/L) from the Phantom Waterfall and contains a microbial mat composed of white filaments. We extend a previous morphological survey with a molecular phylogenetic analysis of the bacteria of the microbial mat. This approach employs DNA sequence comparisons of small subunit ribosomal RNA (SSU rRNA) genes obtained from the mat with those from an extensive database of rRNA sequences. Many of SSU rRNA gene clones obtained from the mat are most similar to rRNA sequences from sulfur-oxidizing bacteria (*Thiothrix* spp., *Thiomicrospira denitrificans*, and ''*Candidatus* Thiobacillus baregensis''). The Sulphur River SSU rRNA gene clones also show specific affiliations with clones from environmental surveys of bacteria from deep-sea hydrothermal vent communities and subsurface microcosms. Affiliations with sequences from bacteria that are known to have the ability to obtain energy for CO_2 fixation from the oxidation of inorganic compounds (chemoautotrophs), in combination with the environmental conditions surrounding the microbial mat, indicate that chemoautotrophic metabolism of bacteria in this mat may contribute to the biomass of Sulphur River. Cave communities, such as the one identified in Sulphur River, provide sites to study such relatively autonomous chemoautotrophic communities that are much more accessible than similar communities associated with deep-sea hydrothermal vents. Subsurface microbiology and the contribution of microbial activity on cave development are also discussed.

Introduction

Thomas Gold (1992) speculated that a vast ''deep, hot biosphere'' exists in the crust of the Earth based on the widespread presence of biomolecules in sedimentary rocks, calculations of bacterial transport in downward moving fluids, and the amount of pore space available in rocks. He further suggested that there may be as much microbial biomass below the surface of the Earth as there is above ground in the form of macroscopic and microscopic life. Recent findings of microorganisms in sedimentary rocks (Colwell et al. 1997) and in igneous formations (basalt: Stevens and McKinley 1995; granite: Pedersen 1997) have helped to substantiate the existence of a deep, subsurface biosphere. Abundant populations of bacteria also have been found deep below the surface of the ocean, associated with hydrothermal vents (Prieur 1997). Deming and Baross (1993) suggest that ''smokers'' at the site of vent emissions may serve as windows into the subsurface biosphere. Studies of these communities will help us to understand the full scope of the microbial world. On the other hand, caves, which may extend hundreds of meters below the surface of the Earth, can provide a more accessible window into the subsurface world, through which we can learn how microorganisms utilize the chemical energy sources within the crust of the Earth and how they may participate in the dissolution and precipitation of rock.

Some caves, known as hypogenic caves, are formed by the oxidation of hydrogen sulfide to sulfuric acid, which dissolves away limestone replacing it with gypsum that is later removed by solution (Egemeier 1981). The oxidation of the hydrogen sulfide to form hypogenic caves has been regarded as a purely inorganic process (Palmer 1991). The discovery of the deep-sea hydrothermal vent communities, in which some microorganisms are able to fix inorganic carbon and utilize sulfide as an energy source (for review see Prieur 1997), raised the possibility

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that sulfide-based microbial communities may be the basis of some cave ecosystems. Moreover, microbial sulfide oxidation would result in the production of sulfuric acid, and hence carbonate dissolution, contributing to cave passage enlargement.

Although most caves receive indirect input of energy from photosynthesis through the organic debris washed into them, some examples of chemoautotrophic cave communities have been recognized. Movile Cave, in Romania, provides the first published example of a cave ecosystem that does not require allochthonous input of organic carbon. Instead, its ecosystem is based on chemoautotrophic microbial mats (Sarbu et al. 1996). Other examples of sulfide-based microbial communities in caves have been reported throughout the world: in Cueva de Villa Luz in Tabasco, Mexico (Hose and Pisarowicz 1997); in submarine caves at Cape Palinuro, Italy (Mattison et al. 1998); and in Parker Cave, Kentucky (Olson and Thompson 1988; Thompson and Olson 1988).

Sulphur River in Parker Cave provides a rich but secluded hydrogen sulfide habitat within a normal limestone cave. Sulphur River, one of five parallel stream passages within the cave (Fig. 1), receives sulfurous water from the Phantom Waterfall and contains a thick microbial mat of white filaments (Fig. 2). The Phantom Waterfall, a tributary of Sulphur River, is located in a room approximately 6 vertical meters above the stream canyon and 167 m upstream from Sulphur River. The mud floor (pH = 0.13) in the upper room is coated with elemental sulfur, and the ceiling is coated with an acidic layer of slime. Sulphur River itself has a pH of 6.4–7.6 due to buffering by limestone.

Olson and Thompson (1988) hypothesized that bacteria may play a role in sulfur deposition at the Phantom Waterfall by bacterial oxidation of sulfides to elemental sulfur and by trapping the sulfur in inclusions. Phase-contrast microscopy shows that both the Phantom Waterfall (Olson and Thompson 1988) and the ceiling slime (results not shown) harbor communities of microorganisms. The Sulphur River passage has an enriched terrestrial invertebrate fauna in comparison to the rest of Parker Cave (Thompson and Olson 1988), and it includes species of annelids, collembolans, a psocopteran, a staphylinid and a carabid beetle, mites, and a linyphiid spider. The stream itself contains at least 13 genera of protozoans in eight orders (Thompson and Olson 1988). These authors also observed, by microscopy, an extensive bacterial community including *Beggiatoa* spp. and *Thiothrix* spp. This was the first published report of sulfur bacteria found living in cave sulfur deposits (Thompson and Olson 1988).

Traditionally, microorganisms were only studied extensively in isolation, once the conditions for growth in the laboratory were determined. By this approach, much has been learned about the physiological potential of microorganisms and their impact on the biological processes that underpin life on this planet. However, general growth conditions provided in the laboratory tend to impose enormous selective pressures that act against most microorganisms. This process screens out many fastidious organisms, as well as organisms that rely on close interactions with other species for their survival. Typically, less than 1% of all microorganisms from a particular environment can be grown in the laboratory using standard enrichment techniques (for review, see Ward et al. 1992; Amman et al. 1995). This inability to isolate most of the microorganisms from a particular environment has impaired microbial community analyses. However, with the advent of molecular phylogeny (see below), community profiles can now be drawn. Over the past few decades, this approach has revolutionized our traditional views of microbial diversity, confirming the idea that we have only begun to sample the microbial world (e.g., Barns et al. 1994; Hugenholtz et al. 1998).

With any foray into an ecosystem, primary effort is placed on cataloguing the organisms present in the community. Microorganisms provide little morphological information with which to identify and classify them. Molecular phylogenetic approaches infer evolutionary relationships among organisms based on gene sequence comparisons (Zuckerkandl and Pauling 1965). The molecule targeted for community phylogenetic analyses is the small subunit or 16S-like rRNA, or its gene, referred to as ''rDNA'' (for review, see Pace et al. 1986; Ward et al. 1992; Amman et al. 1995). This universally conserved gene is involved so intimately in the fundamental cellular process of protein synthesis that it evolves very slowly. Portions of the gene are so highly conserved that an approach based on a polymerase chain reaction (PCR) can be used to isolate rDNAs from novel organisms without a priori knowledge of their particular rDNA sequence (Giovannoni 1991) and without having to first grow the organisms in the laboratory. The SSU (small subunit) rRNA has become widely accepted as an excellent phylogenetic standard (Woese 1987; Olsen and Woese 1993) and extensive databases of rRNA sequences are now available for phylogenetic comparisons (Maidak et al. 1997; Benson et al. 1998).

The present study extends the preliminary microscopic study of Olson and Thompson (1988) with a molecular phylogenetic analysis of the Sulphur River microbial mat to characterize this community more fully. Many of the SSU rRNA gene sequences derived from this microbial community are most similar to rRNAs of sulfur-oxidizing bacteria and to rDNAs obtained from environmental surveys of bacteria associated with deep-sea hydrothermal vents or subsurface ecosystems. Based on these phylogenetic analyses and the environmental conditions surrounding the mat, particularly the sulfide-rich waters that bathe the mat, we infer that the biomass found in Sulphur River is supported, at least in part, by sulfur-oxidizing bacteria.

Materials and methods

Cave description and sample collection

Parker Cave is located southwest of Park City, Kentucky, and underlies the Sinkhole Plain of the Mammoth

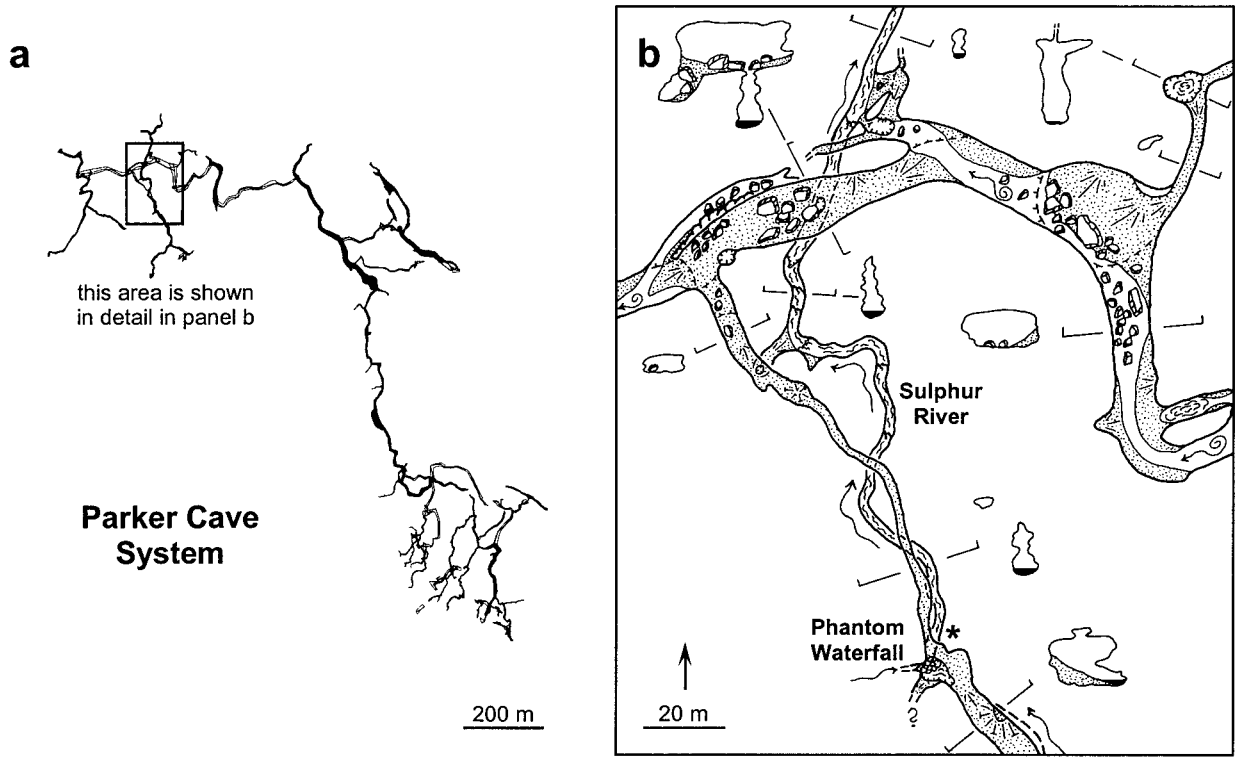

FIGURE 1. (**a**) Plan view of Parker Cave, Kentucky. Streamways are highlighted. (**b**) Enlarged view of the Sulphur River microbial mat area. The location of the microbial mat is indicated by an asterisk (*) to the right of the streamway. Cross-sectional views along the length of the passageway are shown at intervals indicated by brackets perpendicular to the passageway. Maps were drafted by Don Coons and reproduced with his permission.

Cave Region. The cave contains five parallel stream passages, one of which is Sulphur River. The Phantom Waterfall, located upstream of Sulphur River (Fig. 1), is believed to be oil field brine diluted with meteoric waters (Roy 1988; Quinlan and Rowe 1978), and is the probable source of the input of sulfides. Water from the Phantom Waterfall contains 11–21 mg S^{2-}/L (Thompson and Olson 1988). A large, macroscopic microbial mat of white filaments begins at the confluence (0.05 mg S^{2-}/L) of Sulphur River and the Phantom Waterfall (Roy 1988). The temperature of these saline waters (primarily Na and Cl) is approximately 12 °C (Roy 1988). Air in this part of the passage is thick with H_2S and contains as much as 2.8% CO_2 (Quinlan and Rowe 1978). Samples of the Sulphur River microbial mat were collected in April, 1995 (Fig. 2). The samples were immediately placed in a thermos on dry ice and were stored at −70 °C upon returning to the laboratory. Because our study focused solely on a phylogenetic analysis of the Sulphur River microbial community, no samples were collected for chemical analysis. [For information on the geochemistry of the Phantom Waterfall and Sulphur River, see Roy (1988) and Quinlan and Rowe (1978).]

Microscopy

Samples of the microbial mat were applied to poly-L-lysine coated slides and allowed to dry. Cells were stained with the DNA specific dye 4′, 6-diamidino-2-phenylindole (DAPI), 2 μg/mL in phosphate-buffered saline (PBS), for 5 min. The cells were then washed twice with PBS, mounted in 50% glycerol/PBS, and a coverslip was applied. Photomicroscopy was performed using an Olympus BX 60 microscope equipped for phase-contrast and fluorescence microscopy. Images were captured with a MicroMax (Princeton Instruments) cooled charged-coupled device camera driven by MetaMorph software (version 3.0, Universal Imaging). Images were assembled into a figure using Canvas 5.

In situ hybridization with fluorescently labeled, oligonucleotide probes that specifically anneal to rRNA of either Bacteria or Archaea was performed as described previously (DeLong et al. 1989). The domain-specific probes used were Bac 927R (5′-ACCGCTTGTGCGGGCCC-3′, complementary to *Escherichia coli* rRNA positions 943–927) and Arch 915R (5′-GTGCTCCCCCGCCAATTCCT-3′, complementary to the rRNA region equivalent to *E. coli* positions 934–915).

DNA extraction, amplification, and cloning of 16S rRNA genes

Approximately 0.5 mL of the white filament mat sample plus polyadenosine (100 μg/mL) was incubated for 20 min at 37 °C. Sodium dodecyl sulfate (SDS) and pro-

FIGURE 2. Sample collecting from the Sulphur River microbial mat.

teinase K were added to final concentrations of 1% and 1 mg/mL, respectively, and the solution was incubated for 60 min at 50 °C. The SDS concentration was increased to 5% and aminosalicylate was added to 30%; this was further incubated at 70 °C for 10 min. The DNA was extracted three times with equal volumes of phenol-chloroform-isoamyl alcohol (50:49:1), and then precipitated by adding 1/10 volume of 3 *M* sodium acetate and 2 volumes of absolute ethanol. The tube was centrifuged to pellet the DNA. The DNA was washed with 70% ethanol, dried, and suspended in 10 μL of TE (10 m*M* Tris, pH 8.0; 1 m*M* EDTA).

SSU rRNA genes were PCR-amplified with oligonucleotide primers; one that corresponds to nucleotide positions 515–533 of the *E. coli* 16S rRNA (''forward'' primer, 515FPL: 5′-GCGGATCCTCTAGACTGCAGTGCCAGCAGCCGCGGTAA-3′), and another that is the complement of positions 1510–1492 (''reverse'' primer, 1492RPL: 5′-GGCTCGAGCGGCCGCCCGGGTTACCTTGTTACGACTT-3′). Each of these primers has a 5′-end extension that adds multiple endonuclease restriction sites to the PCR product to facilitate cloning. 515FPL and 1492RPL are ''universal primers'' designed to anneal to universally conserved sequences in the rDNA (for review, see Lane 1991). SSU rDNA amplification and cloning were carried out as described previously (Angert et al. 1996).

Sixty-six clones containing inserts of the appropriate size were identified by agarose gel electrophoresis; of these, 58 provided unambiguous sequence information. The clones were screened by single nucleotide sequencing reactions (Angert et al. 1996). Clones representing each of the eighteen unique clone categories identified were sequenced with an Applied Biosystems Inc. automated sequencer.

Phylogenetic analysis

The sequence of each of the clone types was aligned on the *E. coli* SSU rRNA secondary structure (Gutell 1994) to confirm that sequence variation maintained conserved secondary structural features. BLAST searches were performed to identify sequences most similar to the Sulphur River mat rDNA clones (Benson et al. 1998). To ensure that none of the clone sequences used in this analysis were the result of *Taq* polymerase-mediated recombination between rDNAs from disparate organisms (Shuldiner et al. 1989; Pääbo et al. 1990), all sequences were analyzed with the CHECK_CHIMERA program (Maidak et al. 1997). One chimeric sequence was identified and not used in the final analysis. Phylogenetic inferences were made using the SSU rRNA database associated with the ARB software (Ludwig and Strunk, unpublished data, program available from http://www.mikro.biologie.tu-muenchen.de) supplemented with over 500 environmental SSU rDNA sequences obtained from GenBank (Benson et al. 1998). All sequences were inserted into a tree containing over 8000 SSU rRNA sequences using a parsimony insertion tool (Ludwig and Strunk unpublished). Phylogenetic trees were then constructed using distance, maximum parsimony, and maximum likelihood algorithms (Felsenstein 1981; Olsen et al. 1994b). Bootstrap analysis was performed to establish a confidence level for each node.

RESULTS

Microscopy

DAPI-stained microbial mat material was examined by phase-contrast and fluorescence microscopy. The most conspicuous members of the community were bacterial filaments ranging from a few micrometers to more than 1 mm in length. Some of these filamentous bacteria contained phase-bright inclusions of elemental sulfur (Fig. 3). Rod-shaped and coccoid bacteria were also observed. The identification of these organisms as bacteria was based on rRNA in situ hybridization analysis (DeLong et al. 1989) using domain-specific oligonucleotide probes. We found that a bacterial probe hybridized to the rRNA of most of the cells of this community while no hybridization with an archaeal probe was observed (data not shown).

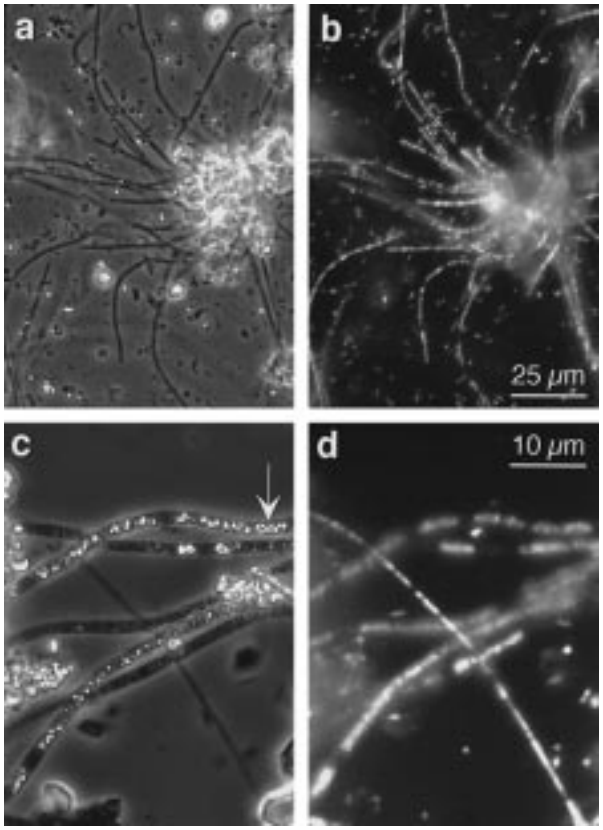

FIGURE 3. Photo micrographs of samples collected from Sulphur River. Two pairs of photographs are shown (**a**–**b** and **c**–**d**), with phase-contrast on the left and DAPI fluorescence on the right. Photo (**c**) shows filamentous bacteria with an arrow indicating phase-bright spherical inclusions, which are probably sulfur granules.

Analysis of the SSU rRNA gene library derived from the microbial mat

Of the clones obtained from the Sulphur River microbial mat rDNA PCR products, 57 were characterized. Based on single nucleotide sequencing patterns, the 57 clones were placed into 17 unique categories. A representative of each of the 17 categories was sequenced entirely. All 17 sequences fell within the bacterial domain (see Olsen et al. 1994a), with 15 sequences in the *Proteobacteria*, one within the *Bacteroides/Flavobacterium/Cytophaga* cluster, and one loosely associated with the newly identified bacterial division OP11 (Hugenholtz et al. 1998). Of the 15 sequences that fall within the *Proteobacteria*, 11 are within the epsilon subdivision and four sequences are within the gamma subdivision. Phylogenetic trees showing the relationship of the 17 unique Sulphur River clones with sequences from some closely related bacteria are shown in Figures 4 and 5.

The clone library was predominated by three clone types or ''phylotypes'' represented by: SRang1.25 (represents 26 of the 57 clones analyzed), SRang1.27 (9 of 57), and SRang1.40 (8 of 57). Since the remaining 14 unique sequences in the library were represented only by a single clone, this survey probably does not completely depict the breadth of diversity in the Sulphur River microbial mat. Representative sequences have been deposited in the GenBank database with accession numbers AF047617 to AF047633.

Phylogenetic distribution

The most abundant phylotype, SRang1.25, together with clones SRang1.23 and SRangD, form a clade within the ϵ-*Proteobacteria* (Fig. 4, Epsilon Group II). The most similar sequence to SRang1.25 derived from a cultivated bacterium is from *Thiomicrospira denitrificans*, a sulfur-oxidizing bacterium isolated from marine tidal flats and subsequently found to be phylogentically distinct from all other *Thiomicrospira* spp. (Timmer-ten Hoor 1975; Muyzer et al. 1995). In addition, three other environmental clone studies have revealed SSU rDNA sequences highly related to clone SRang1.25, with >97% sequence identity. These include: clone groups A14jsn and A15jk obtained from three neutral pH boreholes containing 1.5–3.5 mM SO_4^{2-} (Pedersen et al. 1996); a group of clones represented by BS108 (Goebel et al. 1997) obtained from a microcosm attached to a slightly alkaline borehole (Jw-1) containing 0.215 mM SO_4^{2-} and 36 μm sulfide (Stevens and McKinley 1995); and clones including the predominant phylotype retrieved from a microbial mat associated with a deep-sea hydrothermal vent system known as Pele's Vents (Moyer et al. 1995).

SRang1.27, the second most prevalent phylotype from Sulphur River, along with seven other Sulphur River clones, form another distinct clade within the ϵ-*Proteobacteria* (Fig. 4, Epsilon Group I). A clone recovered from a putative sulfate-reducing bacterium from marine sediment (Devereux and Mundfrom 1994) and rDNA clones obtained from bacterial symbionts of the deep-sea vent-associated invertebrates *Alvinella pompejana* and *Rimicaris exoculata* (Haddad et al. 1995; Polz and Cavanaugh 1995) are included in this monophyletic, bootstrap-supported group. The dense biomass surrounding deep-sea hydrothermal vents relies on the primary production of chemoautotrophic bacteria that are either free-living or symbiotically associated with vent invertebrates (Jannasch and Nelson 1984; Van Dover and Fry 1989). Based on stable isotope analysis and the prevalence of the symbionts at the hydrothermal vent sites, it is believed that the *R. exoculata* ectosymbiont clone and the *A. pompejana* epibiont clones are derived from chemoautotrophic bacteria (Desbruyeres et al. 1983; Haddad et al. 1995; Polz and Cavanaugh 1995; Van Dover et al. 1988).

The third most numerous phylotype from the Sulphur River clone library falls within a clade of rRNAs derived from cultured *Thiothrix* spp. in the gamma subdivision of the *Proteobacteria* (Fig. 5). Clone SRang1.40 is 98% identical to the rRNA of *T. ramosa*, the first isolated *Thiothrix* species that was shown to grow chemoautotrophically (Odintsova et al. 1993; Polz et al. 1996). Among

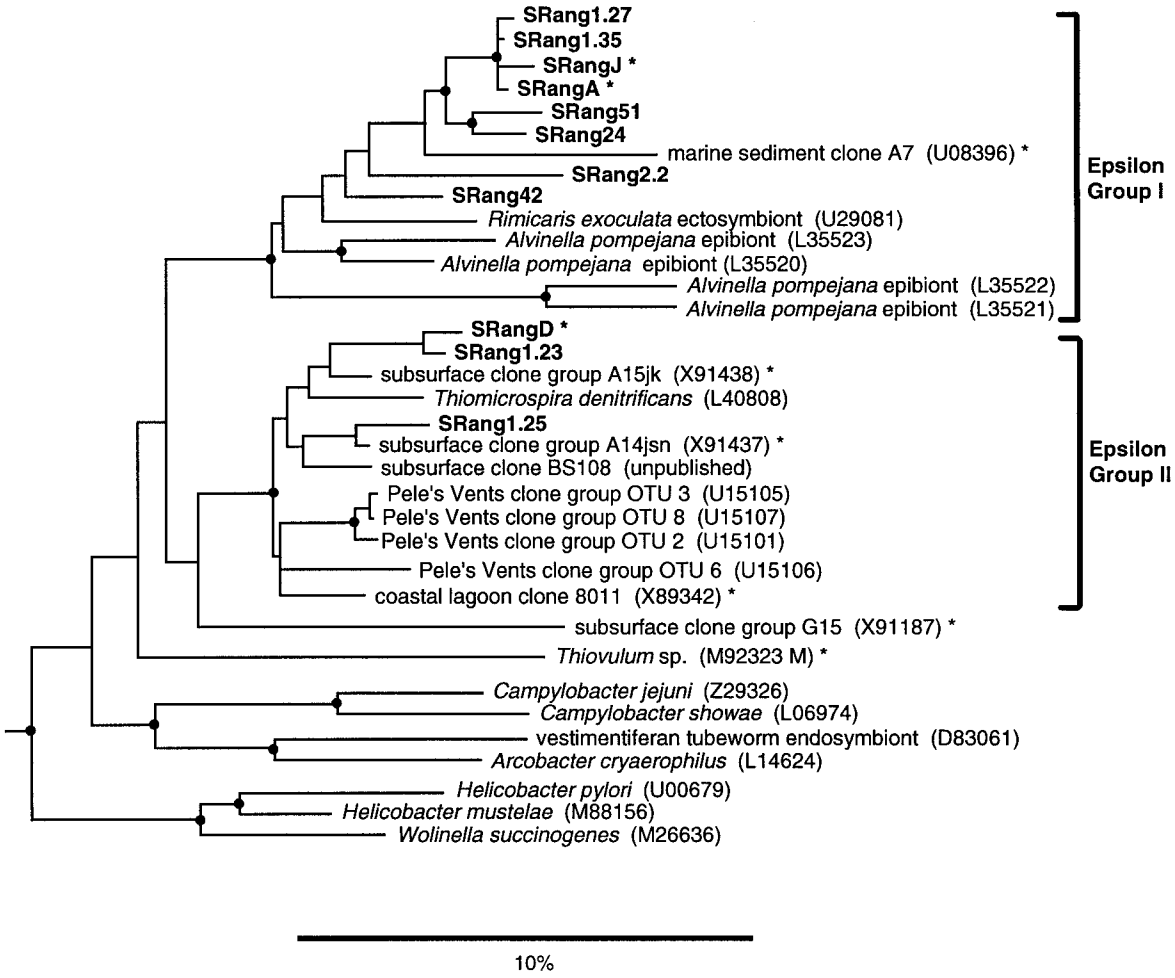

FIGURE 4. Evolutionary distance dendrogram showing the relative positions of SRang-series clones (shown in bold) within the epsilon-*Proteobacteria*. GenBank accession numbers are shown in brackets. An asterisk (*) after a name indicates that the sequence was added to the tree using the parsimony insertion tool of the ARB program (Ludwig and Strunk, unpublished; program can be obtained from http://www.mikro.biologie.tu-muenchen.de/pub/ARB/documentation/arb.ps). Epsilon Groups I and II have been arbitrarily designated for discussion purposes. A solid circle at a branch indicates the clade was supported in >75% of the bootstrap resamplings generated by both Neighbor-Joining distance and Maximum Parsimony analyses. The scale bar represents 10% sequence divergence.

the Sulphur River clones, SRang1.40 shows the strongest affiliation with a cultivated species. Clone BS32, obtained from an alkaline, reducing aquifer in the Columbia River basalt, WA (Goebel et al. 1997), also falls within this bootstrap-supported *Thiothrix* cluster. Members of the genus *Thiothrix* exhibit a distinct morphology of ensheathed filaments that often grow in a rosette (La Rivière and Schmidt 1991; Larkin and Strohl 1983). Such rosettes were identified during the Thompson and Olson (1988) survey of Sulphur River microorganisms. *Thiothrix* spp. are found primarily in streams that contain sulfide and, in such environments, the *Thiothrix* filaments commonly contain inclusions of sulfur (La Rivière and Schmidt 1991; Larkin and Strohl 1983).

Three other Sulphur River clones, SRang2.5, SRang1.33, and SRang1.28, form a monophyletic clade with ''*Candidatus* Thiobacillus baregensis'' in the γ-*Proteobacteria* (Fig. 5). The SSU rDNA sequence from ''*Candidatus* Thiobacillus baregensis'' was obtained from the GenBank database (Benson et al. 1998), but specific details relating to this organism are currently unpublished. From the information listed with the GenBank entry, we infer that this organism is a sulfur-oxidizing bacterium (as it was included in the genus *Thiobacillus*); however, it is yet to be grown in pure culture as it is listed as a ''candidate'' species (Murray and Schleifer 1994).

Clone SRang1.29 groups with the *Bacteroides/Flavobacterium/Cytophaga* division. And finally, SRang2.3 is distantly but specifically affiliated with the SSU rDNA clone koll6 (van der Meer, unpublished data), a clone recovered from a biofilm on a trickling filter that was

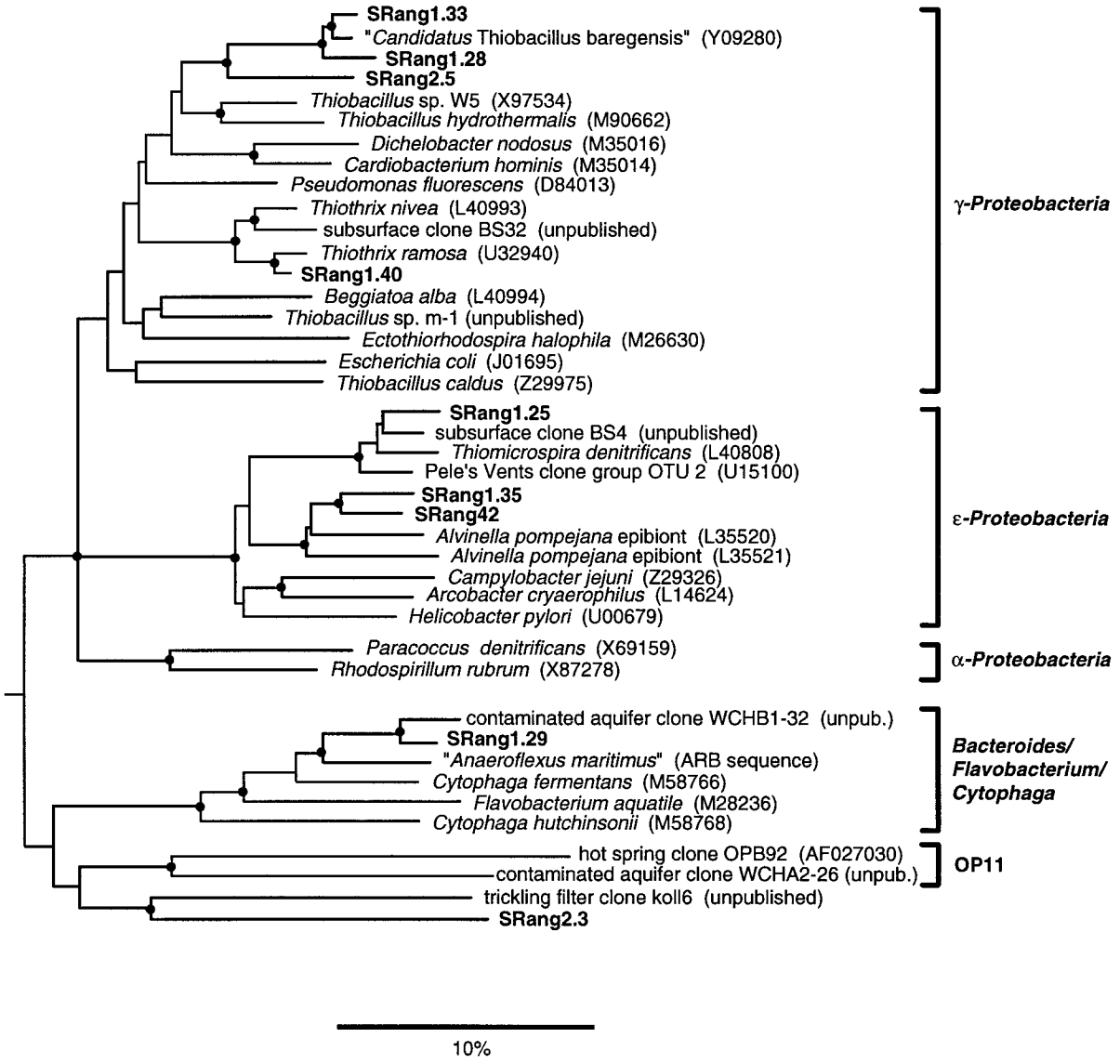

FIGURE 5. Evolutionary distance dendrogram showing the relative positions of representative bacterial SRang-series clones (shown in bold). GenBank accession numbers are shown in brackets. A solid circle at a branch indicates the clade was supported in >75% of the bootstrap resamplings generated by both Neighbor-Joining distance and Maximum Parsimony analyses. The scale bar represents 10% sequence divergence.

used to remove ammonia from water in a treatment facility in Switzerland. Clones koll6 and SRang2.3 form a loose (not supported by bootstrap) affiliation with a newly described phylogenetic division, OP11 (Hugenholtz et al. 1998).

None of the clones examined were specifically affiliated with a SSU rRNA sequence from known *Beggiatoa* spp. (Fig. 5) even though their presence in Sulphur River was suggested by their conspicuous morphology (Thompson and Olson 1988). However, as only two rDNAs from *Beggiatoa* spp. are currently available, forming a monophyletic clade within the γ-*Proteobacteria* (Teske et al. 1995), it is likely that these sequences do not fully represent the diversity of this group of bacteria (Teske et al. 1995). Other approaches, such as in situ hybridization studies, will be required to fully elucidate the identity of the *Beggiatoa*-like organisms of Sulphur River.

DISCUSSION

The conspicuous microbial mat found in Sulphur River is isolated from phototrophically derived organic input because it is deep within the Parker Cave system. However, despite this secluded location the Sulphur River microbial community thrives. Phylogenetic analysis of clones obtained from the Sulphur River microbial mat revealed that many of the predominant phylotypes have

close relatives that are known or presumed sulfide-oxidizing bacteria belonging to the ε- or γ-*Proteobacteria.* However, physiological predictions based on SSU rRNA sequence comparisons must be made with caution. Genes encoding rRNAs evolve slowly relative to most of the genome, including metabolic genes (Woese 1987). Consequently even small differences in SSU rRNA sequences (ca. 4–5%) may accompany significant metabolic diversity. In this instance, however, the assertion that many of the Sulphur River SSU rDNA clone sequences originated from bacteria involved in sulfur metabolism is also supported by the environmental conditions restricted to the area surrounding the mat. The high sulfide levels in the water and air (Roy 1988; Olson and Thompson 1988) could support a community of sulfur-utilizing bacteria. Moreover, the high sequence identity (>97%) between many of the Sulphur River clone sequences and SSU rRNA sequences from sulfur-oxidizing bacteria (''*Candidatus* Thiobacillus baregensis,'' *Thiothrix* spp., and *Thiomicrospira denitrificans*) provides a strong argument that many of the clone sequences originated from bacteria that oxidize sulfur.

Other, more extensive cave communities that are apparently supported by the activity of chemoautotrophic bacteria are currently being explored. An initial analysis of Movile Cave, in Romania, reported stable isotope ratios that show its ecosystem to be supported by chemoautotrophic bacteria growing in the cave (Sarbu et al. 1996). This support extends to the dozens of species of aquatic and terrestrial invertebrates restricted to the cave (Sarbu et al. 1996). Cueva de Villa Luz, Tabasco, Mexico contains streams partly fed by warm sulfur springs (Hose and Pisarowicz 1997). Abundant microbial life is found associated with the sulfurous streams within the cave. Although the cave supports large populations of bats, providing some rich organic input from bat guano, an important source of organic material appears to be sulfur-utilizing bacteria. This microbial community is so vigorous that it can contribute to the support of a large population of cave-adapted fish (Hose and Pisarowicz 1997; Hose and Pisarowicz, unpublished manuscript). Parallels have been drawn between these terrestrial chemoautotrophic communities isolated from phototrophically derived organic carbon sources and deep-sea hydrothermal vent communities (Sarbu et al. 1996). Our study indicates closely related species of bacteria may serve as the metabolic cornerstone of both of these communities.

In addition to being sites for the study of chemoautotrophic communities, caves may serve as our window into the world of subsurface microbiology. Pioneering studies into this realm have indicated the potential for extensive subsurface microbial communities (Chapelle and Lovley 1990; Colwell et al. 1997; Krumholz et al. 1997; Pedersen 1997; Stevens 1997). While many of the communities studied rely on buried organic matter, others appear to rely on various chemoautotrophic metabolisms (Stevens and McKinley 1995). Sampling and in situ experiments in caves may facilitate the examination of subsurface microbial communities by allowing the researcher more control in the choice of sampling site and the various methods used in the analysis.

Clearly the assertion that chemoautotrophic metabolism of Sulphur River bacteria supports a vast microbial and invertebrate community deep within Parker Cave warrants more rigorous physiological analyses. However, the experimental approach reported here can provide an overall view of community structure and serve as an excellent launching point for the design of future analyses. In addition, it provides a meter with which different communities within the cave, or in other caves, can be compared. The Parker Cave system could provide an excellent study site for determining the impact of microbial activity on cave evolution. Sulphur River is apparently the only stream in the Parker Cave system provided with a rich supply of sulfurous water (Quinlan and Rowe 1978). Circumstantial physical evidence points to the production of sulfuric acid by microbial oxidation of hydrogen sulfide in this portion of the cave. For example, extremely low pH values have been recorded for the mud surrounding Sulphur River that is inhabited densely with microbes. With a complement of laboratory and field investigations, such as those used to study bacteria involved in acid mine drainage (Schrenk et al. 1998; Edwards et al. 1998), those microbes responsible for acid production may be identified. It seems likely that a geochemically equivalent site, perhaps upstream of the Phantom Waterfall, which is isolated from the influx of sulfide, could be identified within this cave system. Long-term comparative analyses of the microbial communities, their physiology, regional geochemistry, and cavern expansion at these sites could determine the contribution of microbial activity to limestone dissolution and sulfur deposition.

Acknowledgments

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