Niche separation among sulfur oxidizing bacterial populations in cave waters

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Abstract

The Frasassi cave system affords a unique opportunity to investigate niche relationships among sulfur-oxidizing bacteria, including Epsilonproteobacterial clades with no cultivated representatives. Oxygen and sulfide concentrations in the cave waters range over more than two orders of magnitude as a result of seasonally and spatially variable dilution of the sulfidic groundwater. Full cycle rRNA methods were used to quantify dominant populations in biofilms collected in both diluted and undiluted zones. Sulfide concentration profiles within biofilms were obtained *in situ* using microelectrode voltammetry. Populations in rock-attached streamers showed a strong dependence on the sulfide/oxygen supply ratio of bulk water ($r = 0.97$, $p < 0.0001$). Filamentous Epsilonproteobacteria dominated at high sulfide to oxygen ratios (>150), whereas *Thiothrix* dominated at low ratios (<75). In contrast, biofilms at the sediment-water interface were dominated by *Beggiatoa* regardless of sulfide and oxygen concentrations or supply ratio. Microelectrode sulfide measurements revealed sub-millimeter scale variations as large as those among sampling sites. However, the geochemical variability at small spatial scales did not generally result in layered or mixed-species biofilms. Our results highlight the versatility and ecological success of *Beggiatoa* in diffusion-controlled niches, and demonstrate that high sulfide/oxygen ratios in turbulent water are important for the growth of filamentous Epsilonproteobacteria.

Keywords

Beggiatoa/ Epsilonproteobacteria/ Frasassi Cave/ microelectrode voltammetry/ *Thiothrix*/ *Thiovirga*

Introduction

The Frasassi cave system hosts a rich, sulfur-based lithoautotrophic microbial ecosystem (Jones *et al.*, 2007; Macalady *et al.*, 2007; Macalady *et al.*, 2006; Sarbu *et al.*, 2000; Vlasceanu *et al.*, 2000). Previous studies of the geochemistry of the cave waters have revealed that they are mixtures of slightly salty, sulfidic groundwater diluted 10-60% by oxygen-rich, downward-percolating meteoric water (Galdenzi *et al.*, 2007). Initial observations of the abundant biofilms in cave streams and pools suggested that they respond dynamically to seasonal and episodic hydrologic changes. In particular we noted that changes in specific conductivity (tracking freshwater dilution) and water flow characteristics correspond with morphological differences in the biofilms. Our initial observations motivated a systematic, multi-year study of the population structure of biofilms collected from cave waters with a wide range of hydrological and geochemical characteristics. The goal of the study was to identify environmental factors controlling competition among the biofilm-forming microorganisms. Unraveling the effects of changing hydrologic conditions is not trivial because dilution of the sulfidic groundwater has multiple effects relevant to microbial metabolism. The input of meteoric water increases water depth and flow rates, dilutes dissolved species in the sulfidic aquifer, and adds dissolved oxygen. Water flow conditions that increase turbulence also increase sulfide degassing from the water and oxygen transport into the water from the oxygenated cave air. We found that sulfide/oxygen ratios and physical water flow characteristics are both important for determining the distributions of sulfur-oxidizing groups.

Materials and methods

Field site, sample collection and geochemistry

The Grotta Grande del Vento-Grotta del Fiume (Frasassi) cave system is forming in Jurassic limestone in the Appennine Mountains of the Marches Region, Central Italy. The waters of the cave system are near-neutral (pH 6.9-7.4) and have specific conductivities ranging from 1200 - 3500 uS/cm, or roughly 4-5% of average marine salinity. The major ions are Na^+, Ca^{2+}, Cl^-, HCO_3^- and SO_4^{2-} (Galdenzi *et al.*, 2007). Electron donors and acceptors other than sulfur species and oxygen are present in relatively low concentrations with the exception of ammonium (30-175 µM) and methane (1-20 µM). Dissolved iron and manganese concentrations are below 0.1 µM and .04 µM respectively. Nitrate and nitrite have not been detected at any sample site (< 0.7 and < 2.0 µM respectively). Organic carbon concentrations range between 0.16 and 4.5 mg/L.

Biofilms from cave springs and streams were collected at water depths ranging from 5 to 40 cm at sample locations shown in Figure 1 in May (wet season) and August (dry season) in 2005, 2006 and 2007 . Biofilms were harvested using sterile plastic transfer pipettes into sterile tubes, stored on ice, and processed within 4-6 hours of collection. Subsamples for FISH were fixed in 4% paraformaldehyde and stored at -20 °C. Samples for clone library construction were preserved in 4:1 (volume) RNAlater (Ambion).Water samples were collected in acid-washed polypropylene bottles and stored at 4 °C until analyzed. Conductivity, pH, and temperature of the waters were measured in the field using probes (WTW, Weilheim, Germany). Dissolved sulfide (methylene blue method) and oxygen (indigo carmine method) concentrations were measured in the field using a portable spectrophotometer according to the manufacturer’s instructions (Hach Co., Loveland, CO). Duplicate sulfide analyses were within 1%. Replicate oxygen analyses were within 20%. Nitrate, nitrite, ammonium and sulfate were measured at the Osservatorio Geologico di Coldigioco Geomicrobiology Lab using a portable spectrophotometer within 12 hours of collection according to the manufacturer's instructions (Hach Co., Loveland CO). Light microscopy was performed on live samples within 8 hours of collection on a Zeiss Model 47-30-12-9901 optical microscope (1250x) at the Osservatorio Geologico di Coldigioco Geomicrobiology Lab.

Microelectrode voltammetry

Voltammetric analyses in the field were accomplished using a DLK-60 potentiostat powered with a 12V battery and controlled with a GETAC ruggedized computer. To protect the electrochemical system from drip waters and high humidity, the potentiostat was contained inside a storm case© containing dryrite humidity sponges and modified with rubber stripping to allow the communication ribbon cable and electrode cables to go outside the case while keeping the inside sealed. Electrodes were constructed after the methods of Brendel and Luther (1995). Briefly, 100 μm diameter 99.99% pure gold wire was soldered to a copper lead wire and sealed inside a 5 mm glass tube drawn to a 500 mm diameter tip. The electrodes were polished with successive diamond pastes (15, 6, 1, and ¼ μm), plated with a Hg thin film from a 0.01 M $Hg(NO_3)_2$ 0.1 M HNO_3 solution by applying a potential of 0.1 V (vs. Ag/AgCl) and finally polarized at -9.0 V in a 1.0 M NaOH solution for 60 seconds to fully amalgamate the Hg and gold. Ag/AgCl reference electrodes and Pt counter electrodes were also constructed in the lab by soldering Ag or Pt wire to the copper lead and encasing the connection in epoxy with the respective wires extending approximately 1 cm from the terminus. Ag/AgCl reference electrodes are plated with AgCl by applying a potential of +9.0 V to the electrode in saturated KCl for 90 seconds and inserting the wire inside a Teflon heat shrink tube with a vycor frit on one end.

Voltammetric analyses in the cave system involved placing working (gold-amalgam) electrodes into a narishege 3-axis micromanipulator held above each biofilm sampling site with a 2-arm magnetic base on a steel plate. The reference and counter electrodes were placed in the flowing water near the biofilm. The working electrode was lowered to the air-water interface, then to the biofilm-water interface and subsequently lowered in increments to profile the biofilm. Voltammetric scans utilized both cyclic voltammetry between -0.1 and -1.8 V (vs. Ag/AgCl) at scan rates from 200 to 2000 mV/second with a 2 second conditioning step, and square wave voltammetry between -0.1 and -1.8 V (vs. Ag/AgCl) at scan rates from 200-1000 mV/sec, with a pulse height of 25 mV. Analyses were done at least in sets of ten sequential scans at each sampling point in space, with the first 3 scans discarded as sulfide at these levels can plate to the Au-amalgam surface without any applied potential, and it requires 2-3 scans before reproducible results can be obtained.

Clone library construction

Environmental DNA was obtained using phenol-chloroform extraction as described in (Bond *et al.*, 2000) using 1x Buffer A instead of PBS for the first washing step. Small subunit ribosomal RNA genes were amplified by PCR from the bulk environmental DNA. Libraries were constructed from each sample using the bacteria-specific primer set 27f and 1492r. Each 50 µL reaction mixture contained: environmental DNA template, 1.25 U ExTaq DNA polymerase (TaKaRa Bio Inc., Shiga, Japan), 0.2 mM each dNTPs, 1X PCR buffer, 0.2 µM 1492r universal reverse primer (5'-GGT TAC CTT GTT ACG ACT T-3') and 0.2 µM 27f primer (5'-AGA GTT TGA TCC TGG CTC AG-3'). A universal library was constructed from sample FS06-12 using universal forward primer 533f (5'- GTG CCA GCC GCC GCG GTA A -3') and 1492r. Thermal cycling was as follows: initial denaturation 5 min at 94 °C, 25 cycles of 94 °C for 1 min, 50 °C for 25 sec and 72 °C for 2 min followed by a final elongation at 72 °C for 20 min. PCR products were cloned into the pCR4-TOPO plasmid and used to transform chemically competent OneShot MACH1 T1® *E. coli* cells as specified by the manufacturer (TOPO TA cloning kit, Invitrogen, Carlsbad, CA). Colonies containing inserts were isolated by streak-plating onto LB agar containing 50 µg/mL kanamycin. Plasmid inserts were screened using colony PCR with M13 primers (5'-CAGGAAACAGCTATGAC-3' and 5'-GTAAAACGACGGCCAG-3'). Colony PCR products of the correct size were purified using the QIAquick PCR purification kit (Qiagen Inc., USA) following the manufacturer's instructions. Full length sequences for between 70 and 80 clones from each bacterial library were obtained, in addition to 60 sequences from the universal library constructed from sample FS06-12.

Sequencing and phylogenetic analysis

Clones were sequenced at the Penn State University Biotechnology Center using T3 and T7 plasmid-specific primers. Sequences were assembled with Phred base calling using CodonCode Aligner v.1.2.4 (CodonCode Corp.) and manually checked for ambiguities. The nearly full-length gene sequences were compared against sequences in public databases using BLAST (Altschul et al., 1990) and submitted to the online analyses CHIMERA_CHECK v.2.7 (Cole *et al.*, 2003) and Bellerophon 3 (Huber *et al.*, 2004). Putative chimeras were excluded from subsequent analyses. Non-chimeric sequences were aligned using the NAST aligner (DeSantis et al., 2006) , added to an existing alignment containing >150,000 nearly full length bacterial sequences in ARB (Ludwig *et al.*, 2004), and manually refined. Alignments were minimized using the Lane mask (1286 nucleotide positions). Phylogenetic trees were computed using neighbor joining (general time reversible model) with 1000 bootstrap replicates. Neighbor joining trees were compared with maximum likelihood trees (general time reversible model, site specific rates and estimated base frequencies). Both analyses were computed using PAUP* 4.0b10 (Swofford, 2000).

Probe design and fluorescence in situ hybridization (FISH)

Probes were checked against all publicly available sequences using megaBLAST searches of the nonredundant databases at NCBI. Samples and isolates grown in the lab for use as control cells were fixed in 3 volumes of freshly prepared 4% (w/v) paraformaldehyde in 1X PBS for 3-4 hours and stored in 1:1 PBS/ethanol solution at –20°C. FISH experiments were carried out as described in Hugenholtz et al. 2001 using the probes listed in Table 1. Briefly, fixed samples (homogenized by vortexing and pipetting) and control cells were applied to multiwell, Teflon-coated glass slides, air-dried, and dehydrated by successive immersion in 50%, 80% and 90% ethanol washes (3 min. each). Hybridizations were carried out in 8 uL/well of buffer containing 0.9 M

NaCl, 20 mM Tris/HCl pH 7.4, 0.01% sodium dodecyl sulfate (SDS), 25-50 ng of each oligonucleotide probe, and formamide concentrations given in Table 1. Oligonucleotide probes were synthesized and labeled at the 5’ ends with fluorescent dyes (Cy3, Cy5, FLC) at Sigma-Genosys (USA). Slides were incubated for 2 hours at 46 °C in chambers equilibrated with the hybridization buffer, then immersed in wash buffer (20 mM Tris/HCl pH 7.4, 0.01% SDS, 5 mM EDTA and NaCl concentrations determined by the formula of Lathe (Lathe, 1985)) for 15 min. at 48 °C. Slides were then rinsed with distilled water, air-dried, counterstained with 4’,6’-diamidino-2-phenylindole (DAPI), mounted with Vectashield (Vectashield Laboratories, USA) and viewed on a Nikon E800 epifluorescence microscope. Images were collected and analyzed using NIS Elements AR 2.30, Hotfix (Build 312) image analysis software. The object count tool was used to measure areas covered by cells hybridizing with specific probes. Ten images were collected for each sample, taking care to represent the sample variability, and a total DAPI-stained area of approximately $3*10^4$ μm^2 (equivalent to $5*10^4$ *E. coli* cells) was analyzed for quantitation.

Statistical analyses

The program MINITAB (Minitab Inc., State College, PA, USA) was used for all statistical analyses. A two-sided Student’s *t*-test was used to compare sulfide/oxygen ratios associated with *Thiothrix* and Epsilonproteobacteria, and correlations between parameters were analyzed using the Pearson method.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences determined in this study have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers EF467442-EF467519 and EU101023-EU101289.

Results and Discussion

Field observations and geochemistry

Two common biofilm morphologies were observed in collections from within the cave system over a 3-year period (Fig. 1). Streamers 1 to 5 mm thick and 5 to 20 cm long were attached to rocks in quickly flowing or turbulent water (Supplementary Fig. 1 upper panel). Sediment surface biofilms < 1 mm thick were present in eddies or stream reaches with slower flow, at the interface between the water column and fine gray sediment (Supplementary Fig. 1 lower panel). At many sample sites, we observed that the two biofilm types coexist in a patchwork corresponding to spatial variations in water flow characteristics. All of the biofilms contained abundant extracellular elemental sulfur particles, as evidenced by observations under phase contrast microscopy and by rapid dissolution of the particles in ethanol. Dissolved ion concentrations in the cave waters were strongly correlated with specific conductivity ($0.89 < r < 0.98$, $p < 0.0001$), consistent with previous work showing that the cave water geochemistry is controlled by mixing between slightly salty groundwater and downward percolating meteoric water (Galdenzi *et al.*, 2007). In contrast, dissolved oxygen concentrations were not strongly correlated with specific conductivity ($r = -0.39$, $p = 0.04$). Total dissolved sulfide and oxygen concentrations for each biofilm sample collected in the study are plotted in Fig. 2.

In situ gold-amalgam microelectrode voltammetry was used to investigate the spatial variability of sulfide and other redox-active species within biofilms and the surrounding bulk water. Voltammetric signals are produced when dissolved or colloidal species interact with the working electrode surface. The electron flow resulting from redox half-reactions at the 100μm diameter tip is registered as a current that is proportional to concentration (Skoog *et al.*, 1998; Taillefert and Rozan, 2002). The gradients associated with microbial metabolism in biofilms are thus readily measured using this technique. Aqueous and colloidal species that are electroactive at gold-amalgam electrode surfaces include: H_2S, HS^-, S_8, polysulfides, $S_2O_3^{2-}$, $S_4O_6^{2-}$, HSO_3^-, Fe^{2+}, Fe^{3+}, $FeS_{(aq)}$, Mn^{2+}, O_2, and H_2O_2 (Druschel *et al.*, 2003; Druschel *et al.*, 2004; Glazer *et al.*, 2006; Glazer *et al.*, 2004; Luther *et al.*, 1991; Luther *et al.*, 2001; Taillefert *et al.*, 2000; Xu *et al.*, 1998). Iron species were not detected (Fe^{2+} and Fe^{3+} < 5 μM, FeS < ~ 0.5 μM as FeS monomer), consistent with total dissolved Fe concentrations < 0.1 μM measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Oxygen concentrations were at or below 15 μM (voltammetry detection limit) for all waters analyzed, as expected based on values obtained from spectrophotometric tests at the same sites. Microelectrode profiles of sulfide concentrations through biofilms are discussed below.

16S rDNA clone libraries

Clone libraries were constructed to investigate evolutionary relationships among the most abundant biofilm populations and to facilitate the evaluation of 16S rRNA probes. We recently described the phylogeny of clones from two Frasassi stream biofilms dominated by *Beggiatoa* species (Macalady *et al.*, 2006). Four additional biofilms were selected for 16S rDNA cloning in order to capture a wide range of geochemical conditions and biofilm morphologies (Figure 2, cloned samples indicated by large circles). Libraries were constructed using bacteria-specific primers because FISH analyses indicated that the biofilms contained few archaea (described below). Sample FS06-12 was also cloned using universal primers, but only bacterial sequences were retrieved.

The taxonomy of clones from each library is summarized in Fig. 4 and Supplementary Table 1. Between 25 and 97% of the clones in each library were associated with known or putative sulfur oxidizing clades within Gamma-, Beta- and Epsilonproteobacteria (Fig. 4). Gammaproteobacterial clones (Fig. 5) include representatives of two major sulfur-oxidizing groups, *Beggiatoa* (86-92% identity), *Thiothrix* (92-99% identity), and an unnamed clade containing "Thiobacillus baregensis" (94-99% identity) and the recently described sulfur-oxidizing lithoautotroph *Thiovirga sulfuroxydans* (86-93% identity) (Ito *et al.*, 2005). Most Betaproteobacterial clones were related to species of the sulfur-oxidizing genera *Thiobacillus* (> 97% identity) or *Thiomonas* (90-99% identity). *Beggiatoa* clones were retrieved from four sample locations (Fig. 4) and form a coherent clade most closely related to non-vacuolate, freshwater *Beggiatoa* strains (Ahmad *et al.*, 2006).

Epsilonproteobacterial clones (Fig. 6) were phylogenetically related to *Arcobacter* species or to members of the Sulfurovumales, Sulfuricurvales, and 1068 groups, which have few or no cultivated representatives. The majority of the clones were associated with the Sulfurovumales clade (Fig. 4) and were distantly related to cultivated strains including the named species *Sulfurovum lithotrophicum* (88-94 % identity). Sulfuricurvales group clones were rare and shared 96-97% identity with *Sulfuricurvum kujiense*. Frasassi clones in both Sulfurovumales and Sulfuricurvales were most closely related to clones from other sulfidic caves and springs (98-99% identity), including filaments from Lower Kane Cave Groups I and II (Engel *et al.*, 2003). *Arcobacter* clones were diverse and only distantly related to the closest cultivated strains (91-94% identity). The 1068 group has no cultivated representatives and contains clones from deep subsurface igneous rocks, sulfidic caves and springs, groundwater, and wetland plant rhizospheres. Frasassi clones associated with the 1068 group included phylotypes that shared less than 92% identity with each other, and add significantly to the known diversity within this clade. There was support in both neighbor joining and maximum likelihood phylogenies for the placement of the 1068 group at the base of the Epsilonproteobacteria (Fig. 6).

Biofilm morphology and population structure

Twenty-eight biofilms, including those selected for 16S rDNA cloning, were homogenized and examined using epifluorescence microscopy after fluorescence *in situ* hybridization (FISH). Probes and hybridization conditions are listed in Table 1. Probe BEG811 was designed to bind specifically to *Beggiatoa* populations in environmental samples from Frasassi (Macalady *et al.*, 2006), and matches new *Beggiatoa* clones retrieved in this study (Fig. 5). Probe EP404 targeting Epsilonproteobacteria has no mismatches with Frasassi clones from this or previous studies (n > 120), with the exception of 7 clones within the *Arcobacter* and 1068 groups (Fig. 6). The EP404 probe does not match any publicly available sequences outside the Epsilonproteobacteria.

The FISH experiments revealed three major biofilm types, as shown in Fig. 2. The dominant group in each biofilm sample accounted for more than 50% of the total DAPI cell area (Fig. 2, colored symbols) with one exception (PC05-11). Sediment surface biofilms (n = 15) were dominated by 5-8 µm diameter *Beggiatoa* filaments with abundant large sulfur inclusions and gliding motility. Streamers (n = 13) were dominated either by 1.5 µm diameter gammaproteobacterial filaments with holdfasts and sulfur inclusions (*Thiothrix*), or by filamentous Epsilonproteobacteria with holdfasts and no sulfur inclusions (1-2.5µm diameter). Non-filamentous cells targeted by EP404 made up less than 5% of the EP404-positive cell area in each sample. As reported previously (Macalady *et al.*, 2006), the 23S rDNA probe GAM42a produces no signal from Frasassi *Beggiatoa* filaments at 35 % formamide concentration. GAM42a-positive filaments with holdfasts and sulfur inclusions did not bind with probes EP404 or Delta495a, and were assumed to be members of the *Thiothrix* clade. Archaeal cells in the biofilms were rare or not detected using the probe ARC915. Consistent with this result, bacterial cell area measured using the EUBMIX probe was consistently within 15% of the area measured using the nucleic acid stain DAPI. Representative FISH photomicrographs of the three major biofilm types are shown in Supplementary Fig. 2.

Niches of sulfur-oxidizing populations

Figure 2 shows that sulfide and oxygen concentrations are important niche dimensions for sulfur-oxidizing populations in the cave system. Filamentous

Epsilonproteobacteria colonize waters with high sulfide and low oxygen, and *Thiothrix* colonize waters with low sulfide and high oxygen. A similar pattern was suggested by 16S rDNA clone frequencies in a study of Lower Kane Cave (Engel *et al.*, 2004), but has not been demonstrated until now. Figure 2 also shows that either sulfide or oxygen concentrations alone are poor predictors of biofilm compositions. All three of the dominant sulfur-oxidizing populations tolerate very low oxygen concentrations (<5 μM). Furthermore, *Beggiatoa*-dominated biofilms colonize the entire range of sulfide and oxygen concentrations measured in the cave waters.

The role of sulfide and oxygen concentrations in determining the composition of the biofilms is most clearly demonstrated in Fig. 7, showing biofilm community composition plotted against sulfide/oxygen ratios. Probe EP404 was hybridized with all samples in order to provide a quantitative metric for biofilm composition. We observed a strong linear correlation between filamentous Epsilonproteobacterial area % and the sulfide/oxygen ratio of water hosting streamers (Fig. 7, $r = 0.97$, $p < 0.0001$). Correlations between Epsilonproteobacterial area % and either sulfide or oxygen concentrations alone were weaker, with r values of 0.82 ($p = 0.002$) and -0.80 ($p = 0.003$) respectively. *Beggiatoa* biofilms not included in the correlation had <20% filamentous Epsilonproteobacteria. Including *Beggiatoa* biofilms in the correlation between Epsilonproteobacterial area % and sulfide/oxygen ratio resulted in a slightly lower r value (0.95).

In sharp contrast to streamer populations, *Beggiatoa* filaments colonized the entire range of sulfide, oxygen, and sulfide/oxygen ratios observed in the cave waters (Figs. 2 and 7). Consistent with this result, *Beggiatoa* biofilms were observed immediately adjacent to both *Thiothrix* and filamentous Epsilonproteobacterial streamers *in situ*, always in less turbulent or more slowly flowing water. Although changes in turbulence and water depth have the potential to alter gas exchange and therefore water chemistry, our data suggest that physical effects of turbulence are more significant for microbial growth under the conditions present in the cave system. Because *Beggiatoa* filaments lack holdfasts, they can be washed out by flows which are too strong to allow the accumulation of fine sediment. Likewise, non-motile *Thiothrix* or Epsilonproteobacteria filaments may become buried below the zone where oxidants are available in stream reaches that are accumulating sediment.

Our results support the idea that morphological and behavioral adaptations to physical constraints are responsible for the separate niches colonized by large, filamentous bacteria (Preisler *et al.*, 2007; Schulz and Jorgensen, 2001). As reported in other environments, *Beggiatoa* mats at Frasassi inhabit diffusion-controlled sediment niches, and can respond to changing geochemical conditions by gliding vertically in the sediment column. Sulfide concentration profiles through *Beggiatoa* mats reflect diffusion-controlled transport (Fig. 3), although Frasassi sediments differ from typical marine or lacustrine sediments in that sulfide diffuses both from water above and sediment below the biofilms. Non-motile filaments with holdfasts (*Thiothrix*, filamentous Epsilonproteobacteria) colonized niches with strong currents and a narrower supply ratio of turbulently mixed sulfide and oxygen (Fig. 7). Interestingly, vacuolated marine *Beggiatoa* with holdfasts have recently been identified at cold seeps (Kalanetra *et al.*, 2004). Frasassi clones are only distantly related (~88% identity) to attached *Beggiatoa* species identified to date (Ahmad *et al.*, 2006). Based on the versatility of *Beggiatoa* with respect to sulfide and oxygen concentrations in our study, they could potentially capture more of the available chemical energy if they had the ability to attach. Holdfasts in *Beggiatoa* may have evolved only once in a marine population, or freshwater-adapted attached *Beggiatoa* (yet undiscovered) may be outcompeted by other attached bacteria for reasons not evident in the present study.

Among streamer populations, we observed a strong niche separation between *Thiothrix* and filamentous Epsilonproteobacteria based on sulfide/oxygen ratios. Microbial activity within biofilms modifies sulfide/oxygen ratios on a sub-mm scale due to oxygen consumption at the biofilm surface and sulfide production from sulfate reduction or sulfur disproportionation deeper in the biofilm. High sulfate concentrations (1-3 mM), the presence of deltaproteobacterial cells detected using probes Delta495a and SRB385 ((Macalady *et al.*, 2006) and additional data not shown), and abundant clones from sulfate reducing and sulfur disproportionating clades (Fig. 4, Supplementary Table 1) strongly suggest that sulfide is produced *in situ* within Frasassi stream biofilms. Sulfide concentrations based on voltammetry varied up to several-fold with depth in individual biofilms, typically reaching the highest values in the center. A representative sulfide concentration profile through biofilm sample PC06-110 is shown in Fig. 3 (right panel). Sulfide concentrations within *Thiothrix* streamers could not be profiled due to their small size and rapid motions in the stream flow. The sulfide concentration just outside biofilm RS05-21 was approximately 200 µM, compared to 350 µM in the interior. Based on these values, the approximate range of conditions inside the *Thiothrix* biofilm are shown as a blue dashed box in Figure 2, and clearly overlap with those permitting the growth of filamentous Epsilonproteobacteria. Nonetheless, *Thiothrix*-

dominated biofilms contained at most 3.6 area % Epsilonproteobacterial filaments. This can be explained if rock surface area available for attachment is a limiting resource, or if other antagonistic interactions such as antibiotic production preclude the growth of the two species in layered biofilms.

Although much work remains to be done on the physiology of Frasassi biofilm populations, we can speculate about the mechanisms responsible for niche separation between *Thiothrix* and filamentous Epsilonproteobacteria based on our current data. Whereas both groups tolerate extremely low oxygen (< 3 µM) and low sulfide (< 50 µM) concentrations, *Thiothrix*-dominated biofilms do not occur at sulfide concentrations above 210 µM, suggesting that sulfide toxicity may play a role in excluding them from high-sulfide environments. The absence of Epsilonproteobacterial filaments in waters with oxygen concentrations above 3 µM is also striking, suggesting that oxygen toxicity may be limiting the Epsilonproteobacteria. Functional genomic studies provide some evidence that Epsilonproteobacteria may be uniquely sensitive to oxygen compared to other Proteobacteria inhabiting sulfidic and microoxic environments due to electron transport proteins with the potential to produce mM levels of superoxide anions during oxidative stress (St. Maurice *et al.*, 2007). Further work will be required to determine whether this explanation is generalizable to filamentous Epsilonproteobacteria in the cave environment. Filamentous Epsilonproteobacteria are also apparently unable to store elemental sulfur intracellularly, an attribute that may limit their ability to consume toxic levels of oxygen in the absence of high sulfide concentrations. The inability to store intracellular S° may also be a disadvantage in permanently or transiently low-sulfide environments such as those where *Thiothrix* thrive.

The extremely robust positive correlation between sulfide/oxygen ratios and the area % of filamentous Epsilonproteobacteria strongly suggests that there is selection in the cave environment based on this niche dimension. We cannot rule out the possibility that other factors are also partially responsible for the niche separation between *Thiothrix* and filamentous Epsilonproteobacteria. Since waters with the highest sulfide/oxygen ratios in the study are also generally the least diluted with meteoric water, other aspects of the water chemistry may be important. For example, the least diluted waters (e.g. Pozzo di Cristalli, Fissure Spring) typically contain 10-20% higher dissolved inorganic carbon than the most diluted waters (e.g. Ramo Sulfureo, Cave Spring) (Galdenzi *et al.*, 2007). Adaptations such as facultative anaerobic or heterotrophic metabolism, or differing strategies for uptake of limiting nutrients such as Fe or P, could also potentially play a role.

Filamentous Epsilonproteobacteria have previously been described from Lower Kane Cave (LKC), Wyoming (Engel *et al.*, 2003). The LKC Epsilonproteobacterial filaments are associated with 2 major clades, each incorporating bacterial sequences with approximately 85% nucleotide identity. The taxonomy of the Epsilonproteobacteria is currently in revision due to a large number of unaffiliated environmental clones. For the purposes of this study, the two clades containing LKC filament groups are designated according the Hugenholtz taxonomy employed in the greengenes online workbench (DeSantis *et al.*, 2006). Both clades fall within the provisional Thiovulgaceae family proposed by Campbell et al. (2006). Sulfuricurvales (includes LKC group I) and Sulfurovumales (includes LKC group II) are both present in Frasassi clone libraries (Fig. 4). Frasassi sequences differ significantly from LKC clones, and do not hybridize with previously published oligonucleotide probes LKC59 and LKC1006 targeting environmental groups (Engel *et al.*, 2003). LKC waters have an order of magnitude lower sulfide concentrations than those hosting filamentous Epsilonproteobacteria at Frasassi (Fig. 2). Nonetheless, Fig. 7 shows that the LKC biofilms colonize waters within the filamentous Epsilonproteobacterial niche defined by high sulfide/oxygen supply ratios. As in Lower Kane Cave, no sulfur inclusions were observed in Epsilonproteobacterial filaments, suggesting that this is a consistent physiological attribute. Competition between Sulfuricurvales- and Sulfurovumales-group Epsilonproteobacterial filaments with respect to sulfide, oxygen, and other factors that may influence their growth are currently the subject of further investigation.

We have shown that *Thiothrix*, *Beggiatoa*, and filamentous Epsilonproteobacteria are the major biofilm forming populations within the Frasassi cave system, and that both water flow and geochemical factors affect their spatial and temporal distributions within the cave waters. The biofilms host a wide variety of other known or putatitive sulfur oxidizing taxa as shown in Figure 4. We note that close relatives of "Thiobacillus baregensis" are present in all clone libraries analyzed to date, sometimes comprising the majority of clones (e.g., sample FS06-12). Although the physiology of this novel clade within the Gammaproteobacteria remains speculative, circumstantial evidence suggests that they are sulfur oxidizers (Elshahed *et al.*, 2003; Ito *et al.*, 2005). We commonly observed abundant GAM42a-positive cells with a uniform, rod morphology in FISH experiments, especially associated with filamentous Epsilonproteobacteria. Experiments designed to test the hypothesis that the GAM42a-positive rods are "Thiobacillus baregensis" relatives, and to enrich this population in the laboratory are under way.

Arcobacter relatives in the clone libraries were diverse and relatively abundant. The data presented above preclude the possibility that *Arcobacters* are dominant components of the biomass in Frasassi streams, but *Arcobacter* relatives likely play important roles in some biofilms or niches. A marine strain (*Candidatus* Arcobacter sulfidicus) that grows attached to solid substrates via filamentous sulfur strands in high-flow, microoxic, sulfidic environments has recently been described (Wirsen *et al.*, 2002). Related bacteria or their filamentous sulfur products have since been observed in a wide variety of marine coastal and vent environments, suggesting that they are widespread and biogeochemically relevant. This study is the first to our knowledge to retrieve abundant *Arcobacter* clones from a freshwater environment. Sulfide concentrations for growth of the marine *Arcobacter* (400-1200 μM) are broadly consistent with the Frasassi environment. Further work will be required to evaluate the ecological role of *Arcobacter* and other sulfur cycling populations in the cave waters.

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Supplementary information is available at The ISME Journal's website.

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Figure Legends

Fig. 1.

Map of the Frasassi cave system showing sample locations (open circles). Major named caves are shown in different shades of gray. Topographic lines and elevations in meters refer to the surface topography. Base map courtesy of S. Mariani.

Fig. 2

Dissolved oxygen and total sulfide concentrations for waters hosting Frasassi biofilm samples. Concentration field for Lower Kane Cave (Engel et al. 2003) is shown in gray for comparison. Symbols are colored if more than 50% of the biofilm cell area is composed of a single population or group based on FISH. Colored squares with error bars show the mean ± 1 standard deviation for each major biofilm type. Samples analyzed by 16S rDNA cloning are circled. The open diamond symbol represents a filamentous Epsilonproteobacterial biofilm from Lower Kane Cave reported in Engel et al. 2004. The range of conditions inside the RS05-21 biofilm based on voltammetry is indicated by the blue dashed box.

Fig. 3.

Vertical sulfide concentration profiles measured using *in situ* Au-amalgam microelectrode voltammetry. Zero depth corresponds to the upper surface of the biofilms. Dissolved oxygen concentrations were <15 µM (detection limit) for all points. Marine sediment curve is from a *Beggiatoa* mat described in (Jorgensen and Revsbech, 1983).

Fig. 4.

Taxonomy of 16S rDNA clones in Frasassi stream biofilms. Colored wedges represent known or putative sulfur oxidizing clades, with Gamma(beta)proteobacteria in blue/green tones and Epsilonproteobacteria in red/brown tones. Deltaproteobacteria associated with sulfate-reducing clades are shown in black. White wedges include all other clones (see Supplementary Table 1). GS02-zEL and GS02-WM clone libraries are described in Macalady et al. 2006.

Fig. 5.

Neighbor-joining phylogenetic tree showing Gamma(beta)proteobacteria. Frasassi clones are shown in bold followed by the number of clones represented in each phylotype. Neighbor joining bootstrap values > 50% are shown. Filled circles indicate nodes present in the maximum likelihood phylogeny. Sequences identical to the probe BEG811 are indicated by the dashed line.

Fig. 6.

Neighbor-joining phylogenetic tree showing Epsilonproteobacteria. Frasassi clones are shown in bold followed by the number of clones represented in each phylotype. Neighbor joining bootstrap values > 50% are shown. Filled circles indicate nodes present in the maximum likelihood phylogeny. Clades which hybridize with probe EP404 are indicated by the dashed line.

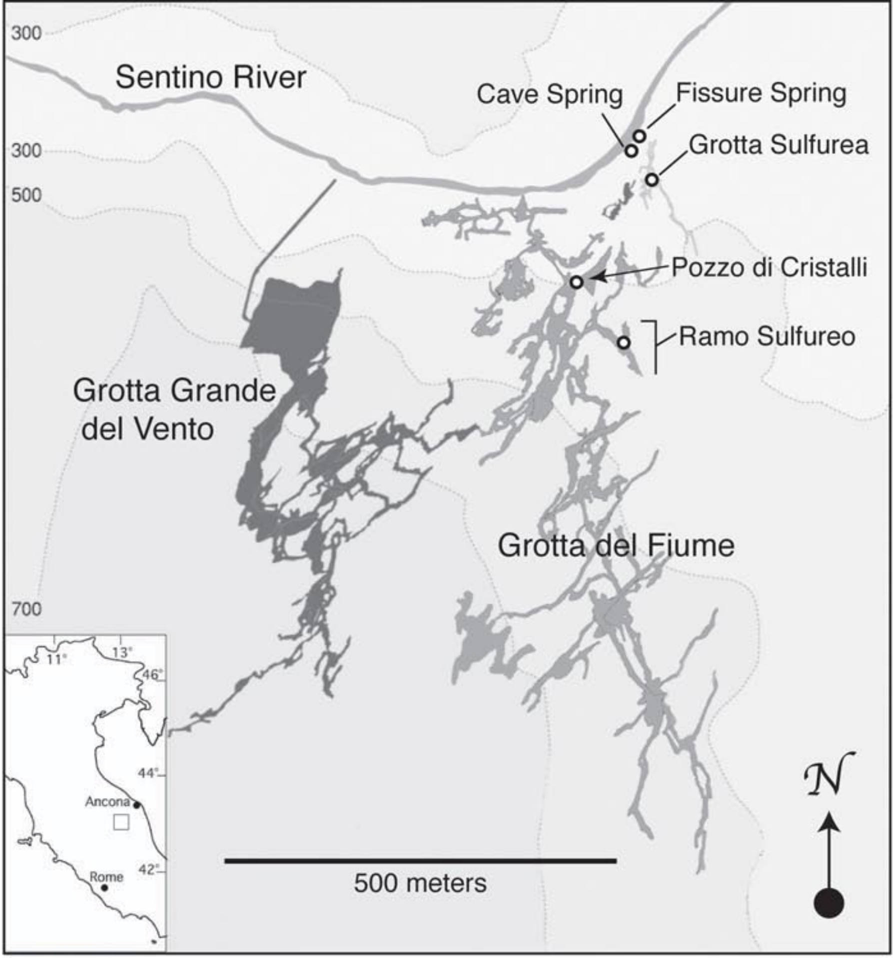
Fig. 7.

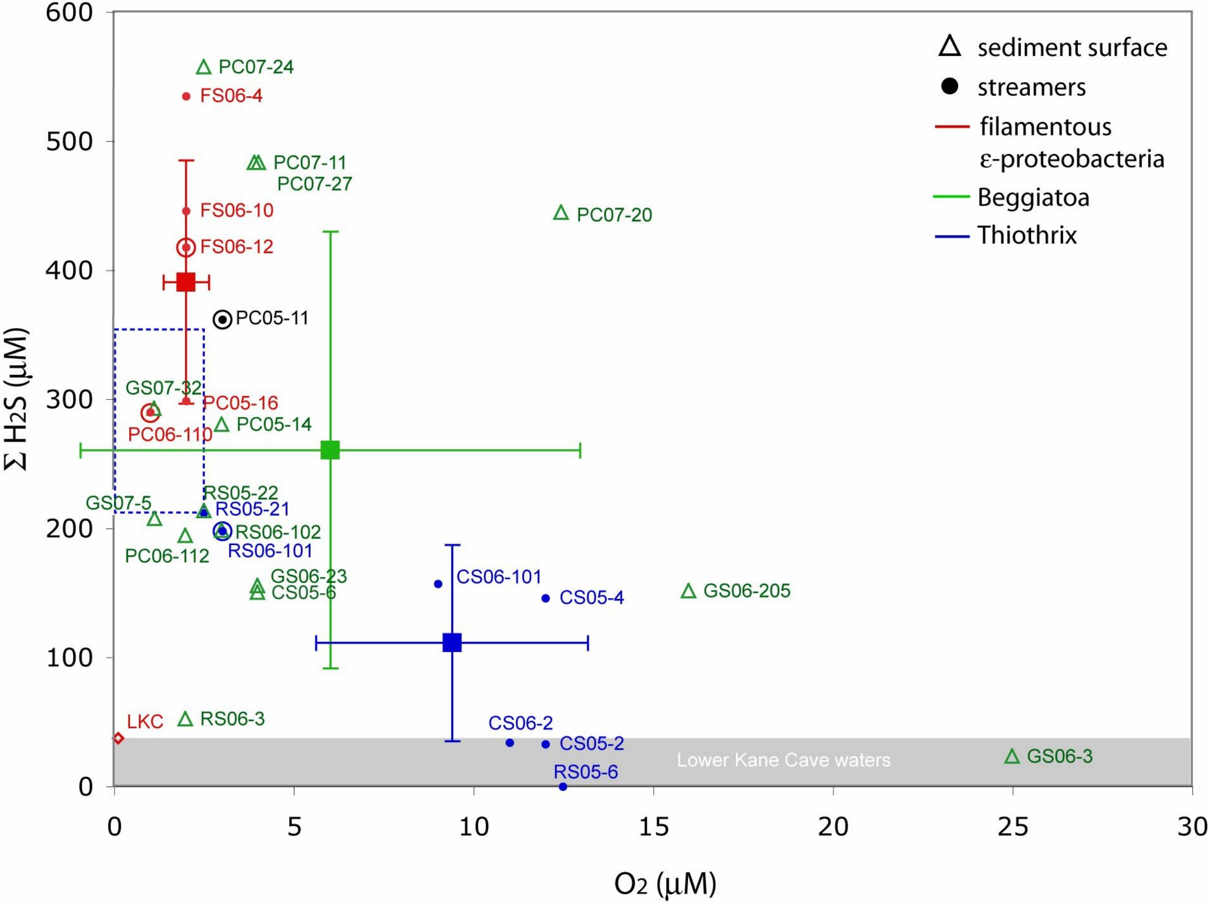
Sulfide/oxygen ratios for Frasassi biofilms analyzed using FISH. The upper panel shows a linear correlation ($p < 0.0001$) between sulfide/oxygen ratio and the microbial composition of streamers. The open diamond (not included in correlation) represents a biofilm from Lower Kane Cave (Engel et al. 2004) and assumes 0.2 µM O_2 (detection limit). The dashed arrow shows how the sulfide/oxygen ratio for the sample would change assuming an O_2 concentration of 0.1 µM. The lower panel shows the distribution of biofilm types compared based on sulfide/oxygen ratios. Colored boxes and associated bars show the average ± 1 standard deviation for each major biofilm type. Mean sulfide/oxygen ratios associated with *Thiothrix* and filamentous Epsilonproteobacteria habitats are significantly different ($p = 0.0004$).

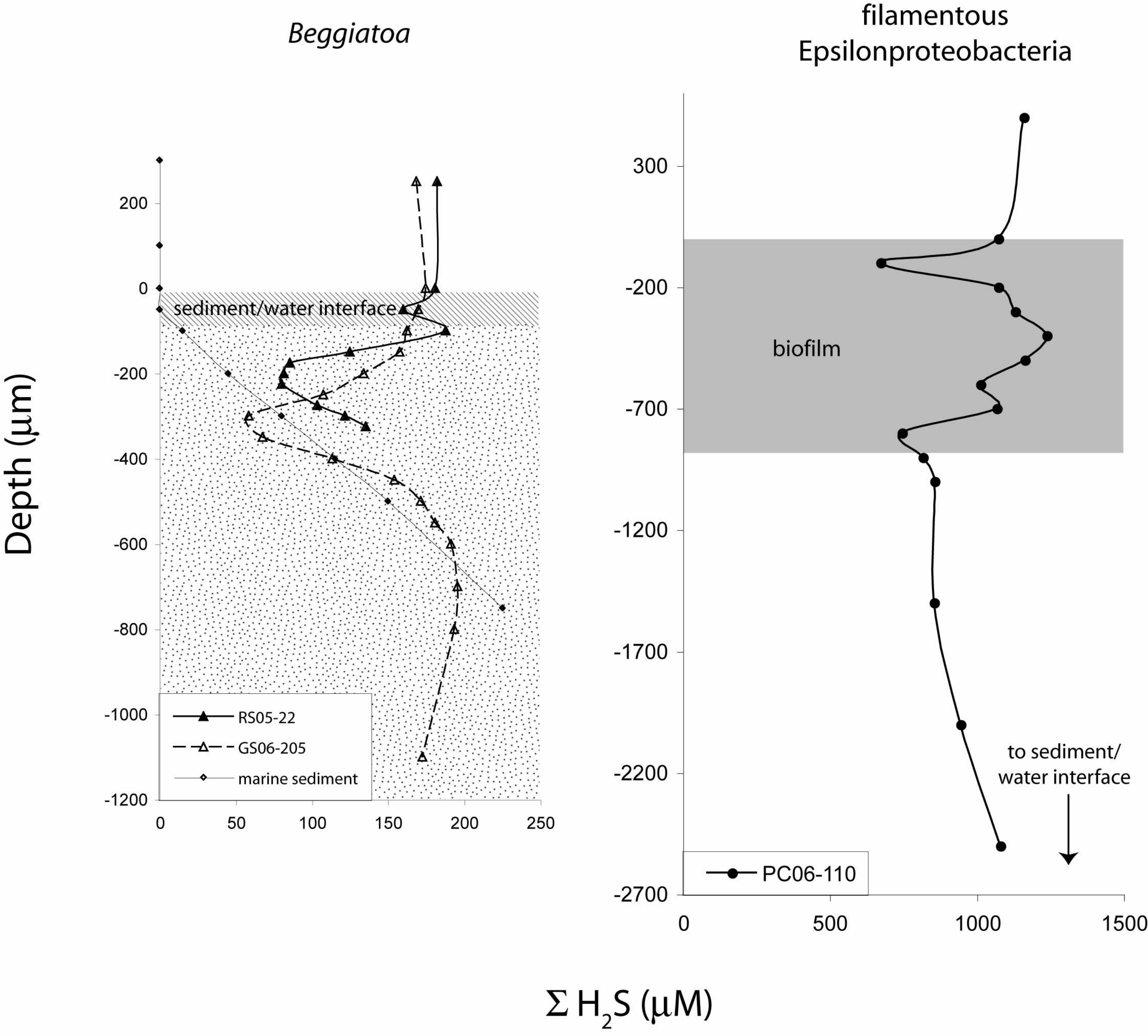
Table 1. Oligonucleotide probes used in this study.

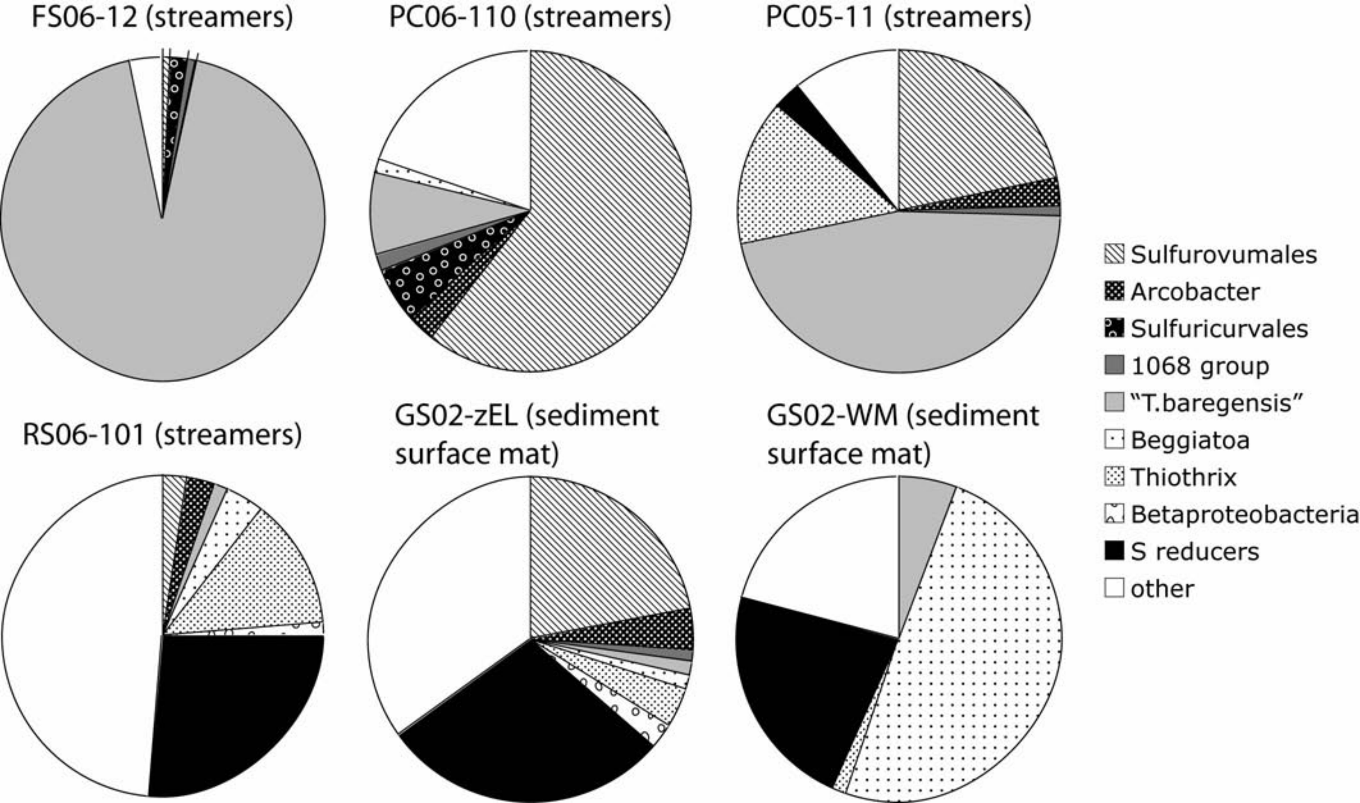
Probe	Target group	Sequence (5’ → 3’)	% formamide	Target site	Reference
[§]EUB338	most Bacteria	GCT GCC TCC CGT AGG AGT	0-50%	16S (338-355)	(Amann et al., 1990)
[§]EUB338-II	Planctomycetales	GCA GCC ACC CGT AGG TGT	0-50%	16S (338-355)	(Daims et al., 1999)
[§]EUB338-III	Verrucomicrobiales	GCT GCC ACC CGT AGG TGT	0-50%	16S (338-355)	(Daims et al., 1999)
ARCH915	Archaea	GTG CTC CCC CGC CAA TTC CT	20%	16S (915-934)	(Stahl and Amann, 1991)
GAM42a	γ-Proteobacteria, including Frasassi *Thiothrix* clones	GCC TTC CCA C**A**T CGT TT	35%	23S (1027-1043)	(Manz et al., 1992)
cGAM42a	competitor	GCC TTC CCA C**T**T CGT TT	35%	23S (1027-1043)	(Manz et al., 1992)
DELTA495a	most δ-proteobacteria, some Gemmatimonas group	AGT TAG CCG GTG CTT C**C**T	45%	16S (495-512)	(Macalady et al., 2006)
cDELTA495a	competitor	AGT TAG CCG GTG CTT C**T**T	45%	16S (495-512)	(Macalady et al., 2006)
SRB385	some δ-proteobacteria, some Actinobacteria and Gemmatimonas group	CGG CGT CGC TGC GTC AGG	35%	16S (385-402)	(Amann et al., 1990)
EP404	ε-proteobacteria	AAA KGY GTC **A**TC CTC CA	30%	16S (404-420)	(Macalady et al., 2006)
EP404mis	negative control for EP404	AAA KGY GTC **T**TC CTC CA	30%	16S (404-420)	(Macalady et al., 2006)
BEG811	Frasassi *Beggiatoa* clade	CCT AAA CGA TGG GAA CTA	35%	16S (811-828)	(Macalady et al., 2006)

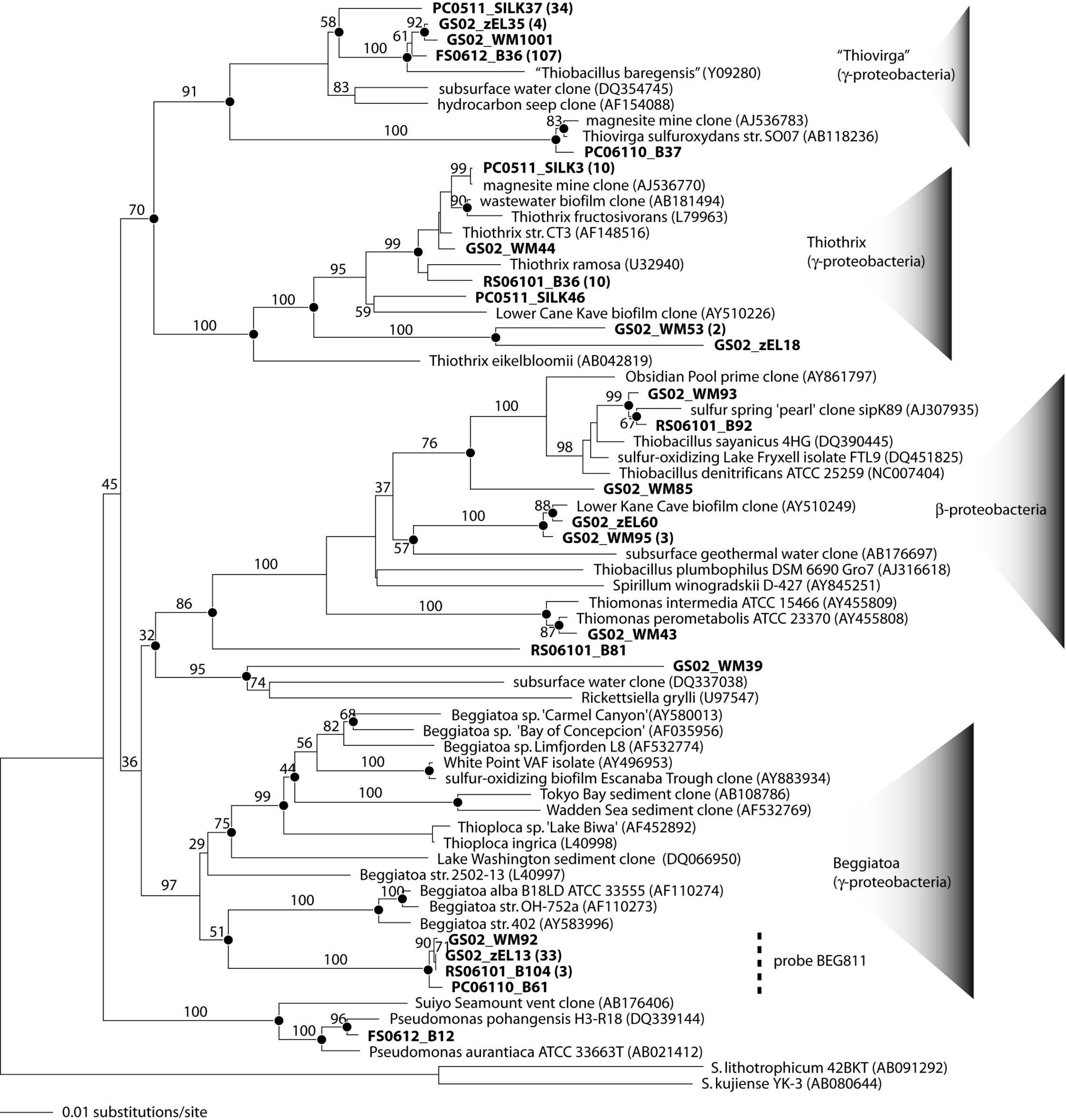
[§] combined in equimolar amounts to make EUBMIX


300
Sentino River
Cave Spring
Fissure Spring
300
Grotta Sulfurea
500
Pozzo di Cristalli
Ramo Sulfureo
Grotta Grande
del Vento
Grotta del Fiume
700
11°
13°
46°
44°
Ancona
42°
Rome
500 meters
N


600
500
400
300
200
100
0
Σ H2S (μM)
0
5
10
15
20
25
30
O2 (μM)
△ sediment surface
● streamers
filamentous ε-proteobacteria
Beggiatoa
Thiothrix
PC07-24
FS06-4
PC07-11
PC07-27
FS06-10
PC07-20
FS06-12
PC05-11
GS07-32
PC05-16
PC06-110
PC05-14
RS05-22
GS07-5
RS05-21
RS06-102
PC06-112
RS06-101
GS06-23
CS05-6
CS06-101
CS05-4
GS06-205
LKC
RS06-3
CS06-2
CS05-2
Lower Kane Cave waters
GS06-3
RS05-6


Beggiatoa
filamentous Epsilonproteobacteria
Depth (μm)
200
0
-200
-400
-600
-800
-1000
-1200
sediment/water interface
RS05-22
GS06-205
marine sediment
0
50
100
150
200
250
300
-200
-700
-1200
-1700
-2200
-2700
biofilm
to sediment/ water interface
PC06-110
0
500
1000
1500
ΣH_2S (μM)


FS06-12 (streamers)
PC06-110 (streamers)
PC05-11 (streamers)
RS06-101 (streamers)
GS02-zEL (sediment surface mat)
GS02-WM (sediment surface mat)
Sulfurovumales
Arcobacter
Sulfuricurvales
1068 group
"T.baregensis"
Beggiatoa
Thiothrix
Betaproteobacteria
S reducers
other


PC0511_SILK37 (34)
GS02_zEL35 (4)
GS02_WM1001
FS0612_B36 (107)
"Thiobacillus baregensis" (Y09280)
subsurface water clone (DQ354745)
hydrocarbon seep clone (AF154088)
magnesite mine clone (AJ536783)
Thiovirga sulfuroxydans str. SO07 (AB118236)
PC06110_B37
"Thiovirga"
(γ-proteobacteria)
PC0511_SILK3 (10)
magnesite mine clone (AJ536770)
wastewater biofilm clone (AB181494)
Thiothrix fructosivorans (L79963)
Thiothrix str. CT3 (AF148516)
GS02_WM44
Thiothrix ramosa (U32940)
RS06101_B36 (10)
PC0511_SILK46
Lower Cane Kave biofilm clone (AY510226)
GS02_WM53 (2)
GS02_zEL18
Thiothrix eikelbloomii (AB042819)
Thiothrix
(γ-proteobacteria)
Obsidian Pool prime clone (AY861797)
GS02_WM93
sulfur spring 'pearl' clone sipK89 (AJ307935)
RS06101_B92
Thiobacillus sayanicus 4HG (DQ390445)
sulfur-oxidizing Lake Fryxell isolate FTL9 (DQ451825)
Thiobacillus denitrificans ATCC 25259 (NC007404)
GS02_WM85
Lower Kane Cave biofilm clone (AY510249)
GS02_zEL60
GS02_WM95 (3)
subsurface geothermal water clone (AB176697)
Thiobacillus plumbophilus DSM 6690 Gro7 (AJ316618)
Spirillum winogradskii D-427 (AY845251)
Thiomonas intermedia ATCC 15466 (AY455809)
Thiomonas perometabolis ATCC 23370 (AY455808)
GS02_WM43
RS06101_B81
β-proteobacteria
GS02_WM39
subsurface water clone (DQ337038)
Rickettsiella grylli (U97547)
Beggiatoa sp. 'Carmel Canyon'(AY580013)
Beggiatoa sp. 'Bay of Concepcion' (AF035956)
Beggiatoa sp. Limfjorden L8 (AF532774)
White Point VAF isolate (AY496953)
sulfur-oxidizing biofilm Escanaba Trough clone (AY883934)
Tokyo Bay sediment clone (AB108786)
Wadden Sea sediment clone (AF532769)
Thioploca sp. 'Lake Biwa' (AF452892)
Thioploca ingrica (L40998)
Lake Washington sediment clone (DQ066950)
Beggiatoa str. 2502-13 (L40997)
Beggiatoa alba B18LD ATCC 33555 (AF110274)
Beggiatoa str. OH-752a (AF110273)
Beggiatoa str. 402 (AY583996)
GS02_WM92
GS02_zEL13 (33)
RS06101_B104 (3)
PC06110_B61
Beggiatoa
(γ-proteobacteria)
probe BEG811
Suiyo Seamount vent clone (AB176406)
Pseudomonas pohangensis H3-R18 (DQ339144)
FS0612_B12
Pseudomonas aurantiaca ATCC 33663T (AB021412)
S. lithotrophicum 42BKT (AB091292)
S. kujiense YK-3 (AB080644)
0.01 substitutions/site
58
92
61
100
83
91
83
100
99
90
99
95
100
59
100
100
70
45
100
99
67
98
76
37
88
100
57
100
100
87
86
32
95
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71
100
100
96
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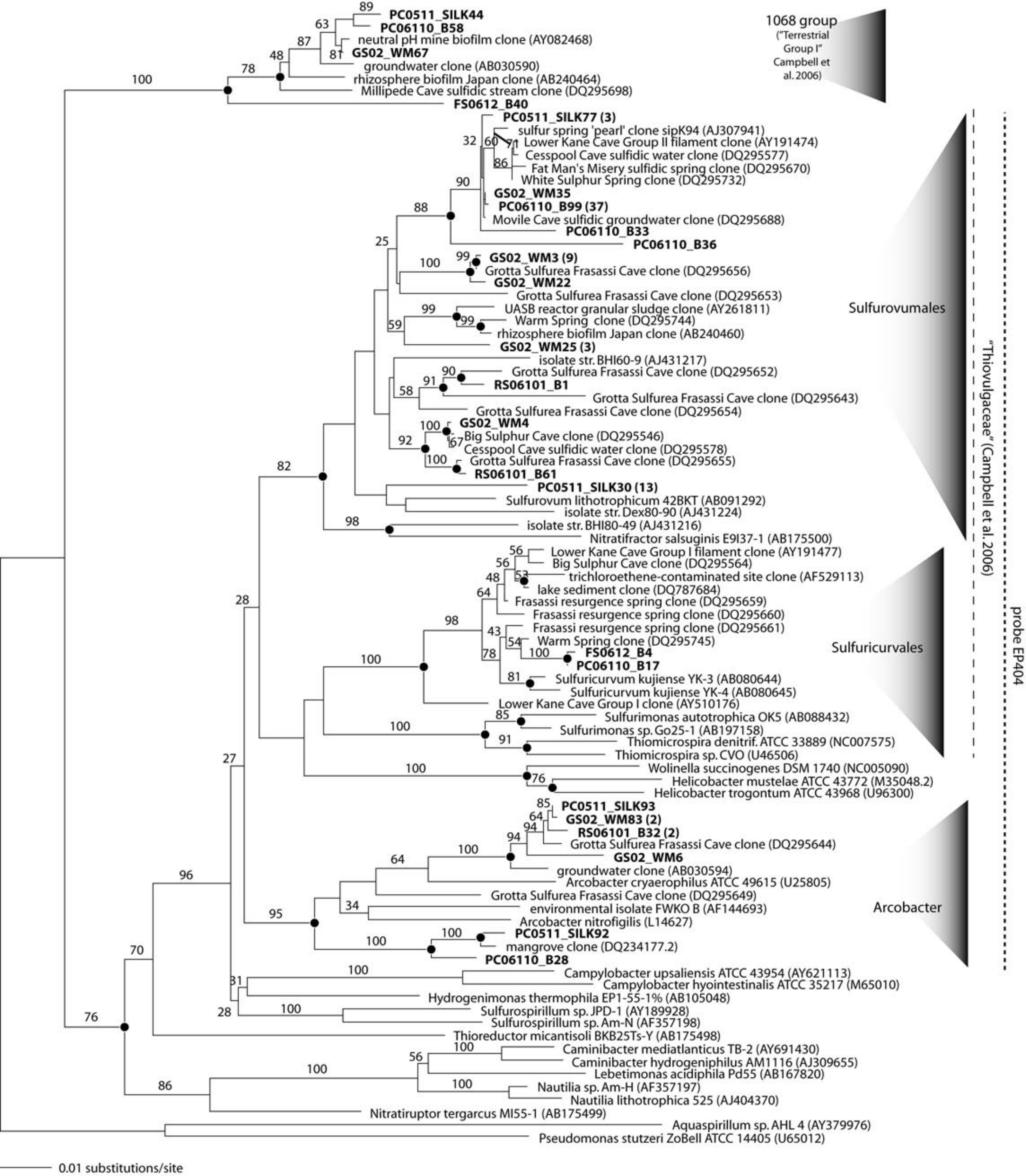
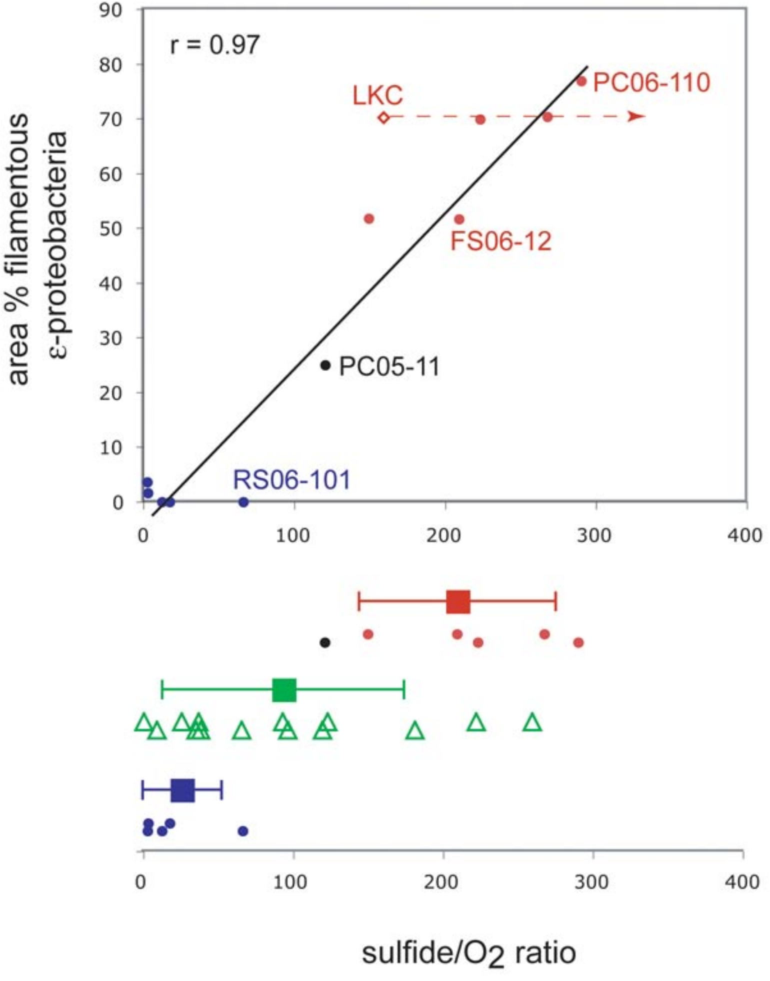

89
PC0511_SILK44
63
PC06110_B58
87
neutral pH mine biofilm clone (AY082468)
81
GS02_WM67
48
groundwater clone (AB030590)
78
rhizosphere biofilm Japan clone (AB240464)
100
Millipede Cave sulfidic stream clone (DQ295698)
FS0612_B40
1068 group
("Terrestrial Group I" Campbell et al. 2006)
PC0511_SILK77 (3)
sulfur spring 'pearl' clone sipK94 (AJ307941)
32
60
71
Lower Kane Cave Group II filament clone (AY191474)
Cesspool Cave sulfidic water clone (DQ295577)
86
Fat Man's Misery sulfidic spring clone (DQ295670)
White Sulphur Spring clone (DQ295732)
90
GS02_WM35
PC06110_B99 (37)
88
Movile Cave sulfidic groundwater clone (DQ295688)
PC06110_B33
PC06110_B36
25
99
GS02_WM3 (9)
100
Grotta Sulfurea Frasassi Cave clone (DQ295656)
GS02_WM22
Grotta Sulfurea Frasassi Cave clone (DQ295653)
99
UASB reactor granular sludge clone (AY261811)
99
Warm Spring clone (DQ295744)
59
rhizosphere biofilm Japan clone (AB240460)
GS02_WM25 (3)
isolate str. BHI60-9 (AJ431217)
90
Grotta Sulfurea Frasassi Cave clone (DQ295652)
91
RS06101_B1
58
Grotta Sulfurea Frasassi Cave clone (DQ295643)
Grotta Sulfurea Frasassi Cave clone (DQ295654)
100
GS02_WM4
Big Sulphur Cave clone (DQ295546)
67
92
Cesspool Cave sulfidic water clone (DQ295578)
100
Grotta Sulfurea Frasassi Cave clone (DQ295655)
82
RS06101_B61
PC0511_SILK30 (13)
Sulfurovum lithotrophicum 42BKT (AB091292)
isolate str. Dex80-90 (AJ431224)
98
isolate str. BHI80-49 (AJ431216)
Nitratifractor salsuginis E9I37-1 (AB175500)
Sulfurovumales
"Thiovulgaceae" (Campbell et al. 2006)
probe EP404
56
Lower Kane Cave Group I filament clone (AY191477)
56
Big Sulphur Cave clone (DQ295564)
53
trichloroethene-contaminated site clone (AF529113)
48
lake sediment clone (DQ787684)
64
Frasassi resurgence spring clone (DQ295659)
28
Frasassi resurgence spring clone (DQ295660)
98
Frasassi resurgence spring clone (DQ295661)
43
54
Warm Spring clone (DQ295745)
100
FS0612_B4
78
PC06110_B17
100
81
Sulfuricurvum kujiense YK-3 (AB080644)
Sulfuricurvum kujiense YK-4 (AB080645)
Lower Kane Cave Group I clone (AY510176)
85
Sulfurimonas autotrophica OK5 (AB088432)
100
Sulfurimonas sp. Go25-1 (AB197158)
91
Thiomicrospira denitrif. ATCC 33889 (NC007575)
Thiomicrospira sp. CVO (U46506)
Sulfuricurvales
27
100
Wolinella succinogenes DSM 1740 (NC005090)
76
Helicobacter mustelae ATCC 43772 (M35048.2)
Helicobacter trogontum ATCC 43968 (U96300)
85
PC0511_SILK93
64
GS02_WM83 (2)
94
RS06101_B32 (2)
94
Grotta Sulfurea Frasassi Cave clone (DQ295644)
100
GS02_WM6
64
groundwater clone (AB030594)
Arcobacter cryaerophilus ATCC 49615 (U25805)
Grotta Sulfurea Frasassi Cave clone (DQ295649)
34
environmental isolate FWKO B (AF144693)
95
Arcobacter nitrofigilis (L14627)
Arcobacter
100
PC0511_SILK92
100
mangrove clone (DQ234177.2)
PC06110_B28
96
100
Campylobacter upsaliensis ATCC 43954 (AY621113)
31
Campylobacter hyointestinalis ATCC 35217 (M65010)
Hydrogenimonas thermophila EP1-55-1% (AB105048)
28
100
Sulfurospirillum sp. JPD-1 (AY189928)
70
Sulfurospirillum sp. Am-N (AF357198)
76
Thioreductor micantisoli BKB25Ts-Y (AB175498)
100
Caminibacter mediatlanticus TB-2 (AY691430)
56
Caminibacter hydrogeniphilus AM1116 (AJ309655)
100
Lebetimonas acidiphila Pd55 (AB167820)
86
100
Nautilia sp. Am-H (AF357197)
Nautilia lithotrophica 525 (AJ404370)
Nitratiruptor tergarcus MI55-1 (AB175499)
Aquaspirillum sp. AHL 4 (AY379976)
Pseudomonas stutzeri ZoBell ATCC 14405 (U65012)
0.01 substitutions/site


r = 0.97
LKC
PC06-110
FS06-12
PC05-11
RS06-101
area % filamentous
ε-proteobacteria
0
10
20
30
40
50
60
70
80
90
0
100
200
300
400
sulfide/O2 ratio