Molecular phylogenetic analysis of a bacterial mat community, Le Grotte di Frasassi, Italy

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Senior Integrative Exercise
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Abstract

The Frasassi Caves are a currently forming limestone karst system in which biogenic sulfuric acid may play a significant role. High concentrations of sulfide have been found in the Frasassi aquifer, and gypsum deposits point to the presence of sulfur in the cave. White filamentous microbial mats have been observed growing in shallow streams in Grotta Sulfurea, a cave at the level of the water table. A mat was sampled and used in a bacterial phylogenetic study, from which eleven 16S ribosomal RNA (rRNA) gene clones were sequenced. The majority of 16S clones were affiliated with the δ-*proteobacteria* subdivision of the *Proteobacteria* phylum, and many grouped with 16S sequences from organisms living in similar environments. This study aims to extend our knowledge of bacterial diversity within relatively simple geochemical environments, and improve our understanding of the biological role in limestone corrosion.

Georef Keywords: Sulfide oxidation, sulfate reduction, caves, bacteria, phylogeny, karst

Introduction

Karst is the name given to large-scale limestone porosity—caves, sinkholes, and other effects of significant limestone dissolution. Karst usually occurs as water percolates through limestone. The water dissolves calcium carbonate, $CaCO_3$, and reacts with it to form bicarbonate, HCO_3^-; eventually pockets and large cavities form. However, karst processes are not as simple as this popular explanation. In many cases, geologists have found evidence of sulfur in limestone karst cave systems—often as gypsum crystallized on the cave walls (Northrup and Lavoie, 2001). Some of the largest limestone caves in the world, including Cueva de la Villa Luz in Tabasco, Mexico and Lechuguilla Cave of Carlsbad Caverns National Park in New Mexico, seem to have been formed through processes involving both bicarbonate and biogenic sulfuric acid (Cunningham et al., 1995; Northrup and Lavoie, 2001).

Sulfuric acid, as a strong acid, can dissolve limestone more effectively than can bicarbonate or its protonated cousin, carbonic acid. Sulfuric acid forms through the oxidation of hydrogen sulfide, H_2S, to sulfate, SO_4^{2-} (Fig. 1a). When sulfate reacts with water, it forms sulfuric acid, H_2SO_4. When sulfuric acid reacts with calcium carbonate, $CaCO_3$, it makes gypsum, $CaSO_4$ (Fig. 1b). Water and carbon dioxide released through the formation of gypsum react with calcium carbonate to make bicarbonate or carbonic acid (Fig. 1c) (Galdenzi et al., 1999).

The Frasassi cave in Italy is a well-known and unusual karst limestone system, in that, unlike most other well-known limestone caves, it is currently undergoing formation. Observations of microbial biofilms covering the limestone surfaces within the cave and of macroscopic microbial mats floating in cave streams have led researchers to postulate that H_2S oxidation is the driving factor behind karstification (Galdenzi et al., 1999).

Understanding the role of microorganisms—in particular, sulfur and sulfide oxidizers and sulfate reducers—in the Frasassi cave may unearth biogeochemical interactions with a broader application to karst systems.

A valuable aspect of working in Frasassi is that the geochemistry is simpler than in many other environments (such as a lake or soil sample), and microbial metabolism is limited by the absence of sunlight and organic material from the surface. Stable isotope ratio analysis conducted by Galdenzi et al. on organic samples found a distinct fractionation signature with consistently lighter isotopes of carbon and nitrogen in the sulfidic sections of the caves compared with those at the caves' entrances and the surface (1999). This suggests that all organic compounds present in the cave are autochthonous, and is consistent with our picture of the cave as an ecosystem completely isolated from surface nutrients and photosynthesis. For this reason Frasassi is an ideal environment that allows us to see discrete links between in situ organisms and the cave's geochemistry.

Given the geochemical and metabolic requirements of organisms such as those in Frasassi, it is difficult to follow the traditional microbiological method of culturing organisms in the lab in order to study their morphologies. This technique places heavy selective pressure on organisms, with the result that what grows is often out of proportion with the original microbial mat. Angert et al. note that typically less than 1% of all microbes from a particular environment can be grown in the lab using standard enrichment techniques (1998). In particular, organisms that rely on symbiosis or syntrophy, whereby a substance is catabolized through the metabolic efforts of two or more organisms that could not catabolize the substance on their own, may not grow at all

in the lab (Madigan et al., 2003). These two relationships often produce microbial mats with intricate layering according to molecule-scale gradients of substances such as oxygen and hydrogen sulfide (Stal, 2001). Therefore, it is important to rely not only on culturing but on molecular phylogenetic work to identify organisms and determine evolutionary relationships.

Some genes change over time faster than others depending on the necessity of their function to the organism. Genes that code for the most important functions in an organism are highly conserved and thus are used for molecular work. The 16S rRNA gene codes for ribosomal RNA, a molecule that plays an important structural and functional role in the ribosome (Madigan et al., 2003). For this reason it is highly conserved and is the gene most often used for phylogenetic work. Using divergences within this one gene, researchers can create phylogenetic trees that show evolutionary relationships. On these trees, internal nodes represent common ancestors, and the lengths of branches correspond to evolutionary changes, or more specifically, base pair substitutions per site. Widespread use of 16S rDNA sequences for phylogenetic analysis has resulted in a systematic and reliable way to diagram evolutionary relationships and survey microorganisms present in natural environments (Hall, 2001a).

This paper presents a molecular phylogenetic analysis of 16S rDNA from bacteria growing in a cave stream microbial mat in the Frasassi Grotta Sulfurea. Results suggest that the mat contains organisms closely related to known sulfur oxidizing and sulfur reducing bacteria. These bacteria likely play a significant role in the production of sulfuric acid and subsequent karstification.

Methods
Cave description and sampling

Le Grotte di Frasassi (Frasassi Caves) are located in the Marche region of Italy,

approximately 150 km NE of Rome, in the Sentino river gorge near the village of Genga.

The cave, at about 200-360 m above sea level, includes four main layers within the

massive Calcare Massiccio limestone formation (Galdenzi and Maruoka, 2003). Frasassi

includes over 100 caves in a network of about 35 cave passages (Fig. 2). The highly

permeable Massiccio (which is 600-1000 m thick) rests on the Anidriti del Burano, an

evaporite formation estimated to be 2000 m thick (Galdenzi and Maruoka, 2003). This is

thought to be the sulfur source for the Frasassi caves (Galdenzi and Maruoka, 2003).

Sodium, chloride and sulfur ions (i.e., sulfate and sulfide) are brought to the cave by

groundwater (Fig. 3) (Galdenzi et al., 1999). Sulfide concentrations of up to 0.4 mmol/l

and sulfate concentrations of up to 2.5 mmol/l have been found in the Frasassi aquifer

(Galdenzi et al., 1999). The cave streams keep a constant temperature of 13˚ C and

range in depth from 10 cm to several meters, with flow rates ranging from 0.5 to 50 l/s

(Vlasceanu et al., 2000). Galdenzi et al. found that conductivity of the streams was 1200-

1900 μS and that limestone dissolution occurred both in the streams and in the air at a

rate of 50 μm/yr over the study period of 5 years (1999).

In 2002, Jenn Macalady[1] collected samples ("GS tm") from a macroscopic

filamentous white mat in a cave stream of Grotta Sulfurea, at about 200 m elevation (the

water table) (Fig. 4). Samples were frozen at -20˚ C until transport to the U.S. on dry ice.

Upon arrival in the laboratory, they again were stored at -20˚ C.

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

Total community DNA was extracted from approximately 30 g (wet wt) frozen sample using a MoBio Ultraclean Mega Prep Soil DNA kit, following the manufacturer's procedure. Presence of DNA was confirmed using agarose gel electrophoresis (1% agar) with a secondary ethidium bromide staining solution. 16S rRNA genes were amplified using the polymerase chain reaction (PCR) with 8-27 forward (5′-AGAGTTTGATCCTGGCTCAG) bacterial and 1510-1492 reverse (5′-GGTTACCTTGTTACGACTT) universal primers (Fig. 5). The PCR cycle was as follows: initial denaturation at 94° C for 5 min., denaturation at 94° C for 1 min., annealing at 45° C for 45 sec., extension at 72° C for 1.5 min., final extension at 72° C for 20 min. and storage at 4° C; the PCR ran for 30 cycles. Tests were run against archaeal and blank controls, and against bacterial DNA extracted from a local stream microbial mat as a positive control. Clones were transformed with a TOPO TA Cloning[®] Kit for Sequencing, according to the manufacturer's procedure. I used chemically competent *Escherichia coli*, which were cultured in Ampicillin LB broth overnight and then inoculated onto agarose plates.

Colonies from these smear plates and from later streak plates were sampled randomly. Plasmids were extracted from cells in each colony and 16S rDNA inserts purified using a Wizard[®] Plus SV Minipreps DNA Purification System (Promega). I checked for concentration using agarose gel electrophoresis with 3.6 mL culture, following the manufacturer's procedure (Fig. 6). PCR with 27f and 1492 primers was used to amplify the inserts, with the following PCR conditions: initial denaturation at 4° C for 10 min., denaturation at 94º C for 30 sec., annealing at 47º C for 1 min., extension

at 72º C for 2 min., final extension at 72º C for 10 min. and storage at 4º C; the PCR ran for 30 cycles.

The 16S gene inserts were sequenced at the University of Wisconsin-Madison's Biotechnology Center, using cycle sequencing with thermostable polymerases and fluorescently labeled dideoxy terminators. The primers used were T3 (5'-ATTAACCCTCACTAAAGGGA) and T7 (5'-TAATACGACTCACTATAGGG).

Phylogenetic analysis

The T3 reverse-complement and T7 ends of each sequence were matched manually using the multiple alignment program Se-Al (Rambaut, 1996). Sequences were submitted to the Ribosomal Database Project's (RDP) online CHECK_CHIMERA software to test for possible PCR artifacts (sequences recombined from more than one organism's 16S gene) as mediated by *Taq* polymerase (Maidak et al., 1997). In order to compare the BK clones with evolutionarily similar sequences, my advisor Jenn Macalady and I searched the ARB 16S rDNA database, which contains over 5000 bacterial sequences (Ludwig and Strunk, unpublished data). Sequences similar to each of the BK clones were used for automatic and manual alignments in Seaview and BioEdit (Galtier et al., 1996; Hall, 2001b; Ludwig and Strunk, unpublished data). Aligning sequences well is crucial to creating reasonable phylogenies, as poor alignments can lead to misleading or meaningless trees (Hall, 2001a). I used the Blast program on the website for the National Center of Biotechnology Information (NCBI) to calculate percent similarity between cultivated species and my clones (Altschul et al.).

Tree-building methods vary depending on their assumptions about genetic evolution. Since we as scientists do not know exactly how evolution works, it is

important to use multiple methods to construct trees and analyze phylogenetic relationships. I used both the neighbor-joining algorithm in ARB and Bayesian analysis to analyze my sequences (Huelsenbeck and Hall, 2000; Ludwig and Strunk, unpublished data). Neighbor-joining uses a distance matrix derived from a multiple alignment. The matrix records the calculated distance, or fraction of differences, between each pair of sequences or lineal groups. In this way it can give a value and a corresponding branch length to the calculated evolutionary distance (Hall, 2001a). Neighbor-joining is the only practical tree-building algorithm to use with large data sets, such as the one in ARB. Large data sets are valuable because they provide a broad context for the placement of new sequences.

My sequences were aligned using the ARB aligner, checked manually, and added to the database using ARB's "QuickAdd" parsimony method. Parsimony is a "minimum change" method that assumes that the most likely tree is the one with the least changes between related taxa (Hall, 2001a). It produces trees that show approximate placement of the organisms and is useful when adding a limited number of sequences to a neighbor-joining tree. The ARB tree was rooted using several archaeal species.

Trees specific to each bacterial lineage were created from the ARB libraries using Bayesian inference in MrBayes and were formatted graphically using PAUP* (Huelsenbeck and Hall, 2000; Swofford, 1999). Bayesian analysis uses posterior probabilities to find the best set of trees for the given sequence alignment and a postulated model of evolution that maximizes the probability of observing the given data. Rather than arriving at a single *best* tree, Bayesian inference samples trees using the Markov chain Monte Carlo method, which produces a set of converging *consensus*

trees—trees for which accepting or rejecting any particular change becomes essentially random. The frequency of any particular tree being sampled is related to the probability that it is the best tree for the data. Bayesian analysis is quite powerful and is becoming increasingly popular among phylogeneticists (Hall, 2001a).

I used MrBayes to run Bayesian inference searches for 10^5 cycles with four Markov chains to check for convergence. Trees were saved every 100 generations; the first 350 trees from each cold chain (the Markov chain used for sampling) were discarded. These were rooted using monophyletic bacterial outgroups.

Results
Sampling environment and mat structure

On the sampling expedition of October 2003 to Grotta Sulfurea, Jenn Macalady and I measured the geochemistry of the shallow flowing cave stream containing the white microbial mat sampled in 2002. We found cave water temperature to be 13.9-14.6° C. The water had pH = 7.0 ± 0.03. The white filamentous microbial mat was floating on or below the surface of the stream, and in places was directly on top of the black sulfidic mud that lined the streambed. The strong "rotten egg" smell of hydrogen sulfide was quite recognizable.

Phylogenetic analysis of 16S rRNA genes derived from mat

From the Grotta Sulfurea cave microbial mat samples, eleven 16S rRNA clones were sequenced. None of the sequences were positively identified as chimeric using the RDP software CHECK_CHIMERA. Two sequences (clones BK9 and BK111) were over 99.5% identical and so only one of these (BK9) was used for phylogenetic work. The remaining ten sequences fit into four phyla, all in the Bacterial domain (Fig. 7): *Proteobacteria* (6 sequences), *Cytophaga/Flavobacteria/Bacteroides* (CFB) (2 sequences), WS6 (1 sequence), and Termite Group 1 (1 sequence). Of the bacteria in the

Proteobacteria phylum, 1 fit in the *γ-proteobacteria* subdivision, and the remaining 5 in the *δ-proteobacteria* subdivision (Table 1). Preliminary results of further phylogenetic work on clones from cave wall microbial films in Grotta Sulfurea ("GSgm") are also shown on ARB trees (Figs. 8, 10, 12, and 14).

Clone BK1 fit well with the *γ-proteobacterium "Thiobacillus baregensis,"* a sulfur oxidizing bacterium found in sulfurated thermal waters in Bareges, France (Hedoin et al., unpublished 1996). It also grouped with a number of Sulphur River clones, clones isolated from a white filamentous microbial mat in Sulphur River, Parker Cave, Kentucky (Angert et al., 1998). Clone BK1 grouped most notably with clone SRang 2.5, which itself grouped closely with "*T.barengensis*" (Figs. 8 and 9).

The clones BK4, BK5, BK7, BK9, and BK10 grouped in the *δ-proteobacteria*, a lineage of predominantly sulfur-reducing bacteria (Figs. 10 and 11). Clones BK4 and BK10 clustered together, with their closest cultivated neighbor being *Desulfosarcina singaporensis*, a sulfate-reducing bacterium isolated from sulfide-rich black marine mud (Lie et al., 1999). Clone BK5 was closely related to *Geobacter sulfurreducens* and to *Geobacter metallireducens*, both metal ion reducers (Methe et al., 2003). Clone BK7 fit near *Desulfonema ishimotoei*, a sulfate reducer isolated from organic-rich sulfidic marine and freshwater sediment (Fukui et al., 1999). Clone BK9 was similar to *Syntrophobacter wolinii*, a sulfate reducer isolated from a culture enriched from anaerobic granular sludge (Harmsen et al., 1993).

Clone BK2 and a partial sequence of BK108 (T3) fit in the CFB group (Figs. 12 and 13). Clone BK2 was most similar to *Cytophaga fermentans*. Clone BK108T3 was closely associated with the Sulphur River clone SRang 1.29, the contaminated aquifer

clone WCHB1.32, and the Cesspool Cave clone CC.8. The only cultivated bacteria in the same group as clone BK108T3 were the fermenter *Bacteroides putredinis* and the termite gut bacterium *Rikenella microfusus*, both anaerobes (Madigan et al., 2003; Ohkuma et al., 2002).

Clone BK6 fit in the WS6 phylum, near the OP11 clone OPB92 (Figs. 14 and 15). Clone BK8 fit in Termite Group 1 (Figs. 15 and 16). Since I had only a partial sequence of Clone BK8 (T3), I could not do rigorous phylogenetic work on it. The phyla WS6 and Termite Group 1 are newly defined and contain no cultivated species.

Discussion

The macroscopic filamentous white microbial mat found in the cave stream of Grotta Sulfurea represents a distinct community isolated from photosynthetically derived carbon. The observed high sulfide and sulfate levels in the water and air are sufficient to support a sulfide-metabolizing community (Madigan et al., 2003). Given the sequence similarities of 88-94% between the Grotta Sulfurea BK clones and known sulfur/ide oxidizers and sulfate reducers (Table 1), it is reasonable to believe that my 16S clone sequences came from respective sulfur/ide oxidizers and sulfate reducers, and that the microbial community in turn includes both these types of bacteria. Since there is no input of organic material from the surface to Grotta Sulfurea, chemolithoautotrophy likely forms the base of the food chain. Sulfur oxidizing autotrophic bacteria thus likely comprise a significant portion of the base-level biomass, a biomass capable of supporting fifteen invertebrate species, of which seven are endemic to Frasassi (Galdenzi et al., 1999).

A sulfur-rich environment such as Frasassi would be expected to support both types of metabolism—especially where sulfur/ide oxidizers and sulfate reducers may

form close symbiotic relationships. This has been seen in hydrothermal vent communities, where bacteria (especially sulfur/ide oxidizers and sulfate reducers) play an important role in mineralization of vent chimneys (Lovley et al., 2000; McCollom and Shock, 1997). In fact, sulfur isotope ratios analyzed by Galdenzi and Maruoka (2003) suggest that sulfate reducing bacteria are present in Frasassi. They found sulfur isotope values of $\delta^{34}S \sim -19.60$ ‰ in gypsum deposits in the cave, $\delta^{34}S \sim -14$ in H_2S and $\delta^{34}S \sim +21.00$ ‰ in sulfate within sulfidic groundwater (Galdenzi and Maruoka, 2003). Galdenzi and Maruoka (2003) propose that the size of these isotope fractionations can best be explained by the sulfur cycling through sulfate reducing bacteria, which are known to produce high fractionations. The presence of sulfate reducers, shown through phylogenetic work, fits with the sulfur isotope analysis.

Not all clones from similar microbial mats in sulfidic streams have this split between sulfur/ide oxidation and sulfate reduction. The Sulphur River clones from a white filamentous mat in Parker Cave, Kentucky are predominantly ε- and γ-*proteobacteria*, both lineages known for their sulfur-oxidizing metabolism (Angert et al., 1998). Similarly, clones from a white filamentous microbial mat in the sulfidic waters of Lower Kane Cave, Wyoming are almost entirely from the ε-*proteobacteria* (Engel et al., 2003). It would seem that these results would be more expected for the Frasassi Grotta Sulfurea BK clones given the similarities in limestone geology and microbial mat morphology. There was one γ-*proteobacteria*, but the majority of clones (5) were δ-*proteobacteria,* putative sulfate reducers.

The differences between the Frasassi Grotta Sulfurea clones and those of Sulphur River and Lower Kane Cave may be due to a difference in sampling technique or an

introduced experimental bias. In the DNA extraction there may be lysing bias, whereby certain cells are more easily opened and their DNA extracted. Similarly, the PCR primers may anneal with certain sequences better than others. This latter potential source of difference can be eliminated entirely from the Lower Kane Cave clones, as they were PCR amplified using the same primers as for the BK clones (27f, 1492r) (Engel et al., 2003). The Sulphur River clones were amplified using a 515 forward primer and the same1492 reverse primer (Angert et al., 1998). It is also possible that differences in the PCR programs used could have affected the results.

Even considering these possible sources of disparity between the Grotta Sulfurea BK clones and the Sulphur River and Lower Kane Cave clones, a difference between the phylogenetic results found in each location is evident. Why were there no sulfate reducers found in either the Sulphur River or the Lower Kane Cave clones? Since sulfur oxidizers produce sulfate, it would seem that their presence would provide the sulfate necessary for sulfate reduction metabolism. This apparent difference between the sulfur-cycling species present in each environment needs to be investigated further.

The presence of only Bacteria in my clone libraries can be attributed to the use of bacterial PCR primers, which amplify only the bacterial 16S rRNA gene. We would need to use archaeal primers and sequence a greater number of bacterial clones in order to explore the full diversity of the Grotta Sulfurea stream microbial mat. Since 16S genes evolve slowly relative to most other genes, they often do not keep up with changes in the genes that code for proteins such as those involved in metabolism (Woese, 1987). It would therefore be germane to sequence protein-coding genes—and even better, entire genomes. Another important and ongoing aspect of this research is Fluorescence In-Situ

Hybridization (FISH), which will help us visualize the population numbers and distribution of organisms between the two prokaryotic domains.

This study expands the known diversity of the Frasassi Grotta Sulfurea cave stream microbial mat community based on phylogenetic analysis, and is an excellent launching point for further physiological and phylogenetic study. The high percent similarities between my 16S clones and the 16S genes of known sulfur/ide oxidizers and sulfate reducers point to a biological source of sulfuric acid through biogenic sulfur oxidation, which implies a crucial role played by bacteria in carving the Frasassi caves. The presence of both sulfur/ide oxidizers and sulfate reducers fits with sulfur isotope fractionation data and testifies to the complex sulfur cycle of the Frasassi cave system.

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[3] Department of Geological Sciences, University of Vienna.

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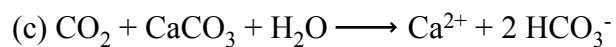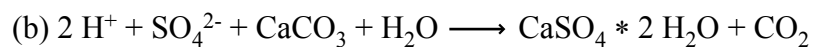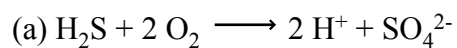
(a) $H_2S + 2\ O_2 \longrightarrow 2\ H^+ + SO_4{}^{2-}$

(b) $2\ H^+ + SO_4{}^{2-} + CaCO_3 + H_2O \longrightarrow CaSO_4 * 2\ H_2O + CO_2$

(c) $CO_2 + CaCO_3 + H_2O \longrightarrow Ca^{2+} + 2\ HCO_3{}^-$

Figure 1. Various pathways for limestone corrosion: (a) oxidation of sulfide to sulfate, which can bond with water to form sulfuric acid, H_2SO_4; (b) formation of gypsum, which frees CO_2 to form bicarbonate; (c) typical karst-style formation of bicarbonate. This can become protonated to form carbonic acid, H_2CO_3. Adapted from Galdenzi et al., 1999.
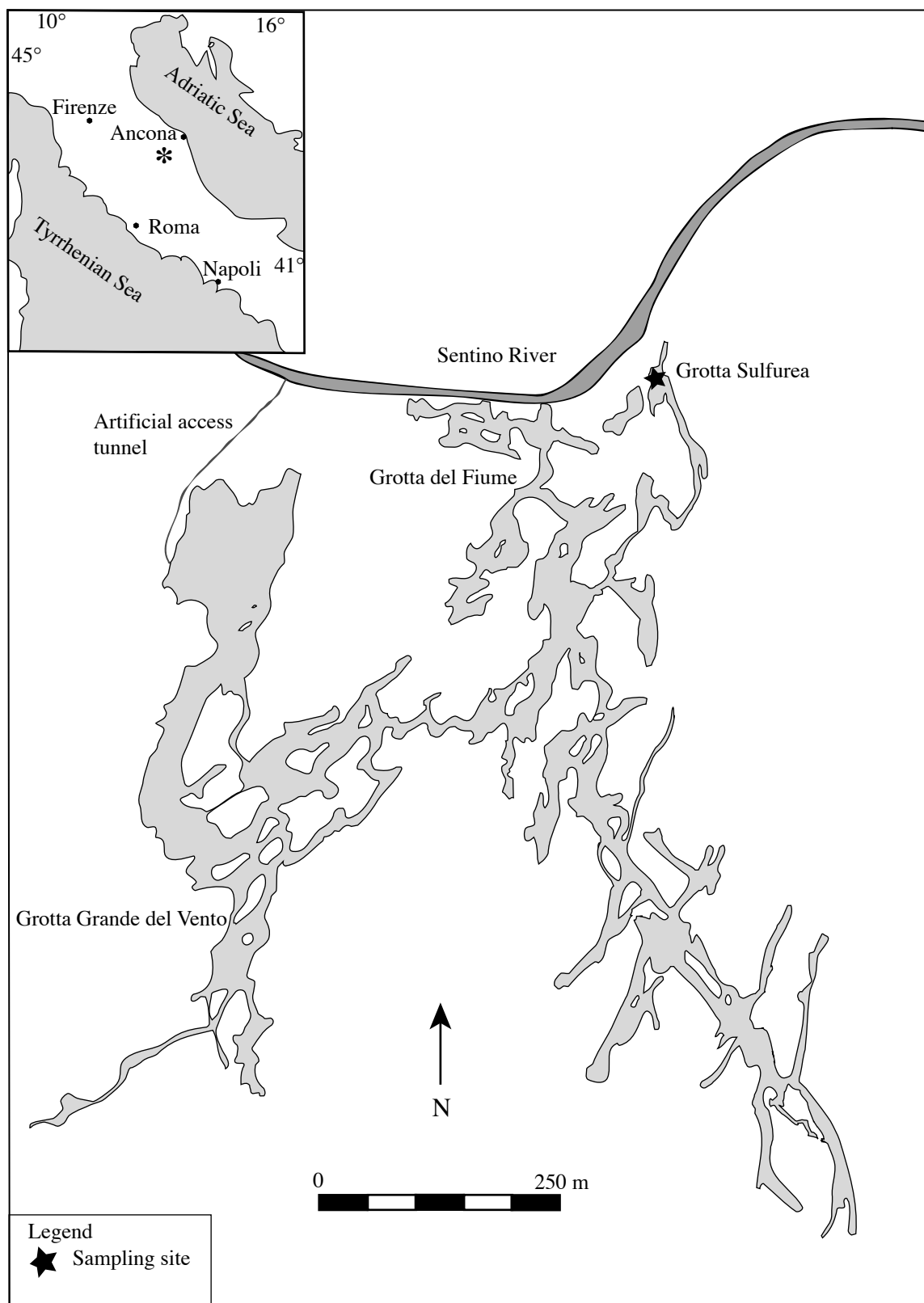
Figure 2. Map of Frasassi Caves showing sampling site in Grotta Sulfurea. Adapted from Vlasceanu, et al., 2000.

Low permeability rocks

Limestone

Frasassi Gorge

H_2O

H_2O

water table

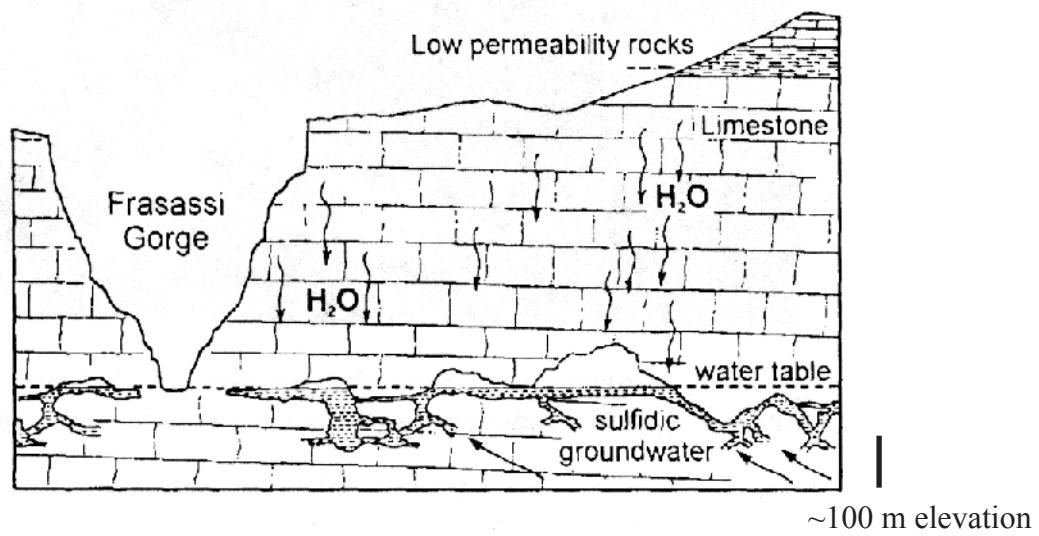sulfidic groundwater

~100 m elevation

Figure 3. Schematic cross section of Frasassi Gorge showing water table and movement of ground water. Adapted from Galdenzi et al., 1999.

Figure 4. Photographs from Frasassi Gorge and Caves: a) entrance to Grotta Sulfurea from SS 76 in Genga; b) Sandro Galdenzi lowering the rope into the first chute of Grotta Sulfurea; c) sampling the white mat; d) white mat (scale bar is about 0.3 m); e) measuring pH of drips from wall gypsum crystals. Photos by Jenn L. Macalady.

Table 1. Summary of 16S rDNA results.

Clone name	Division (Subdivision)	Nearest taxon* (% identity**)	Inferred physiology
BK1	Proteobacteria (gamma)	"Thiobacillus baregensis" (94%)	Sulfur/ide oxidizer
BK4	Proteobacteria (delta)	Desulforhopalus singaporensis (93%)	Sulfate reducer
BK5	Proteobacteria (delta)	Geobacter sulfurreducens (95%)	Sulfate reducer
BK7	Proteobacteria (delta)	Desulfonema ishimotoei (88%)	Sulfate reducer
BK9 (BK111)	Proteobacteria (delta)	Syntrophobacter wolinii (93%)	Sulfate reducer
BK10	Proteobacteria (delta)	Desulforhopalus singaporensis (94%)	Sulfate reducer
BK2	CFB	Cytophaga fermentans (88%)	Anaerobe
BK108T7 (partial)	CFB	Sulphur River clone SRang 1.29 (92%)	Anaerobe
BK6	WS6	OP11 clone OPB92 (79%)	
BK8T3 (partial)	Termite Group 1	Termite gut clone TG13 (79%)	Anaerobe

*Nearest cultivated taxon found in public databases
**Percent identity calculated using number of base pair matches per alignment in the NCBI Blast program
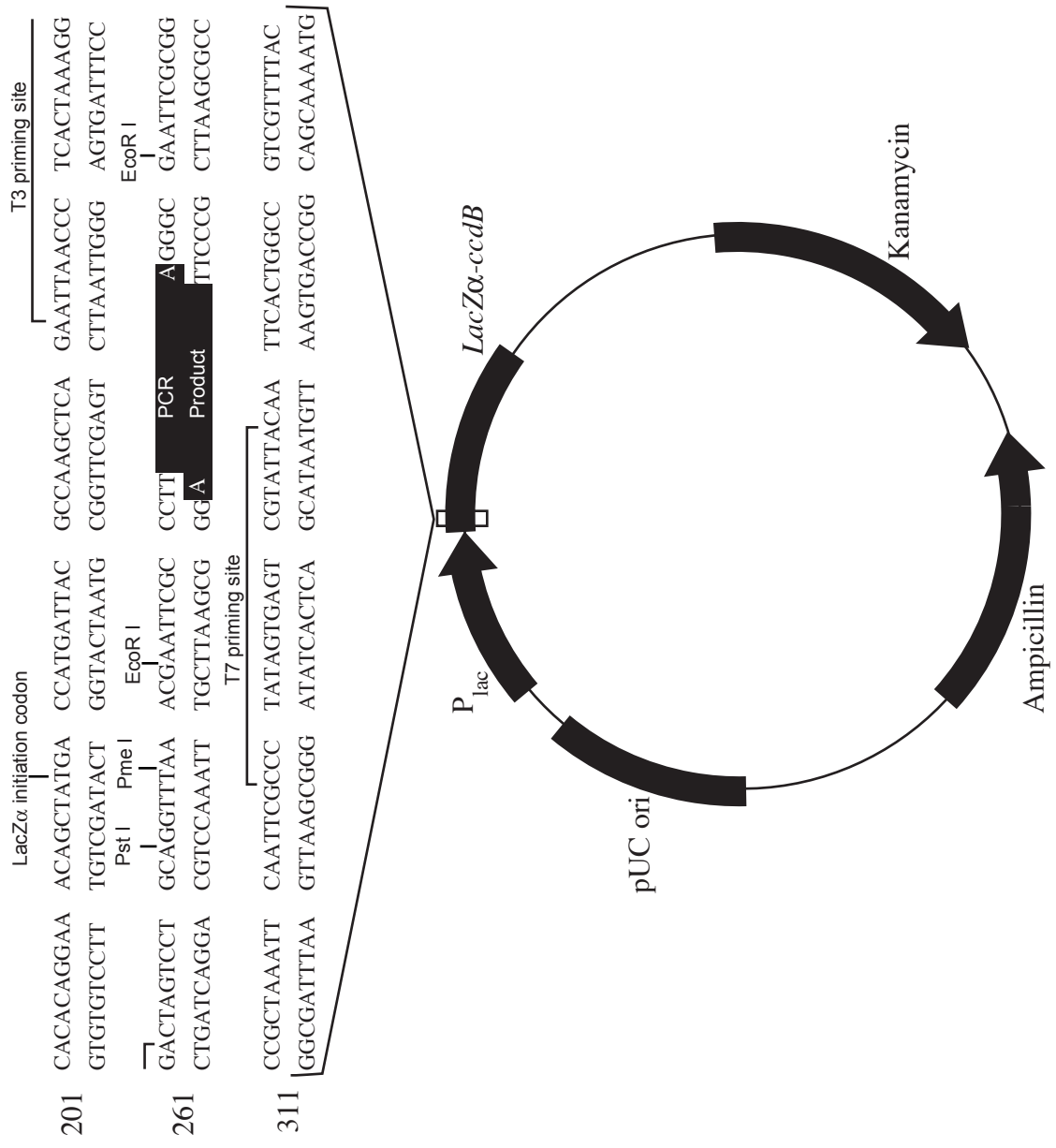(Altschul, S.F., et al.)

LacZα initiation codon

T3 priming site

```
201  CACACAGGAA ACAGCTATGA CCATGATTAC GCCAAGCTCA GAATTAACCC TCACTAAAGG
     GTGTGTCCTT TGTCGATACT GGTACTAATG CGGTTCGAGT CTTAATTGGG AGTGATTTCC
                  Pst I    Pme I              EcoR I

261  GACTAGTCCT GCAGGTTTAA ACGAATTCGC CCTT│ PCR  │AGGGC GAATTCGCGG
     CTGATCAGGA CGTCCAAATT TGCTTAAGCG GG A│Product│TTCCCG CTTAAGCGCC
                            EcoR I                          EcoR I
                  T7 priming site

311  CCGCTAAATT CAATTCGCCC TATAGTGAGT CGTATTACAA TTCACTGGCC GTCGTTTTAC
     GGCGATTTAA GTTAAGCGGG ATATCACTCA GCATAATGTT AAGTGACCGG CAGCAAAATG
```

P_lac

LacZα-ccdB

Kanamycin

Ampicillin

pUC ori

Figure 5. Map of pCR® 4-TOPO® plasmid, showing nucleotide sequence surrounding the cloning site with base pair numbers given on the left. Plasmid contains 3954 base pairs without the PCR product. Adapted from Invitrogen (2003).
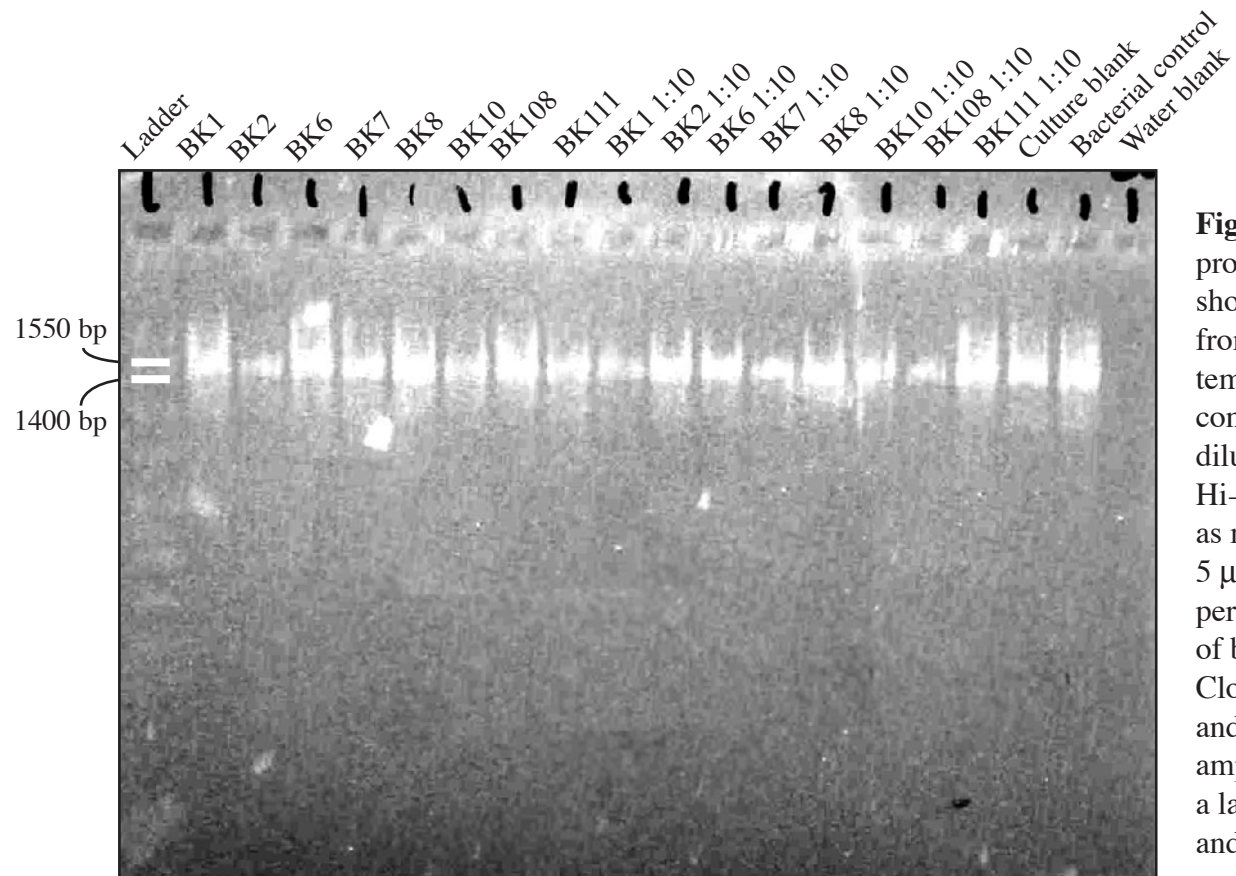
Figure 6. Gel of PCR product (27f, 1492r) showing BK clones from plasmid prep template at full concentration and 1:10 dilution. I used 10 μl Hi-Lo DNA Marker as my ladder, with 5 μl DNA template per well. Note size of band at ~1500 bp. Clones BK4, BK5, and BK9 were amplified during a later PCR run and are not shown.
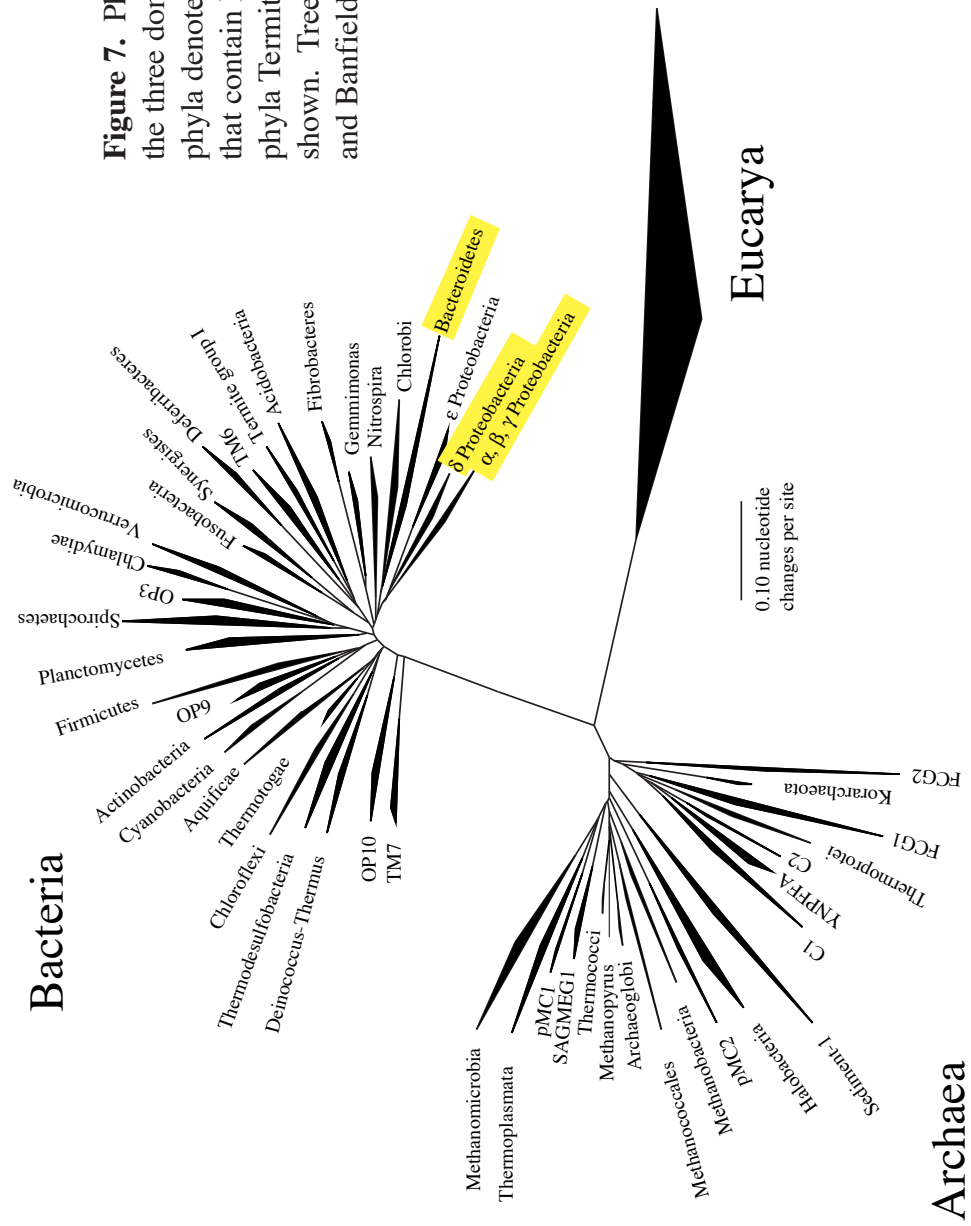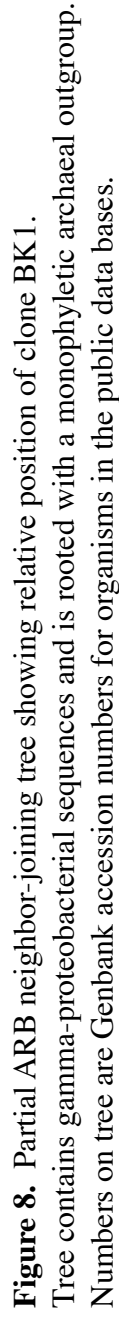
Figure 7. Phylogenetic tree showing the three domains of life. Highlighted phyla denote clades in the Bacteria that contain BK clones. Candidate phyla Termite Group 1 and WS6 not shown. Tree adapted from Macalady and Banfield, 2003.
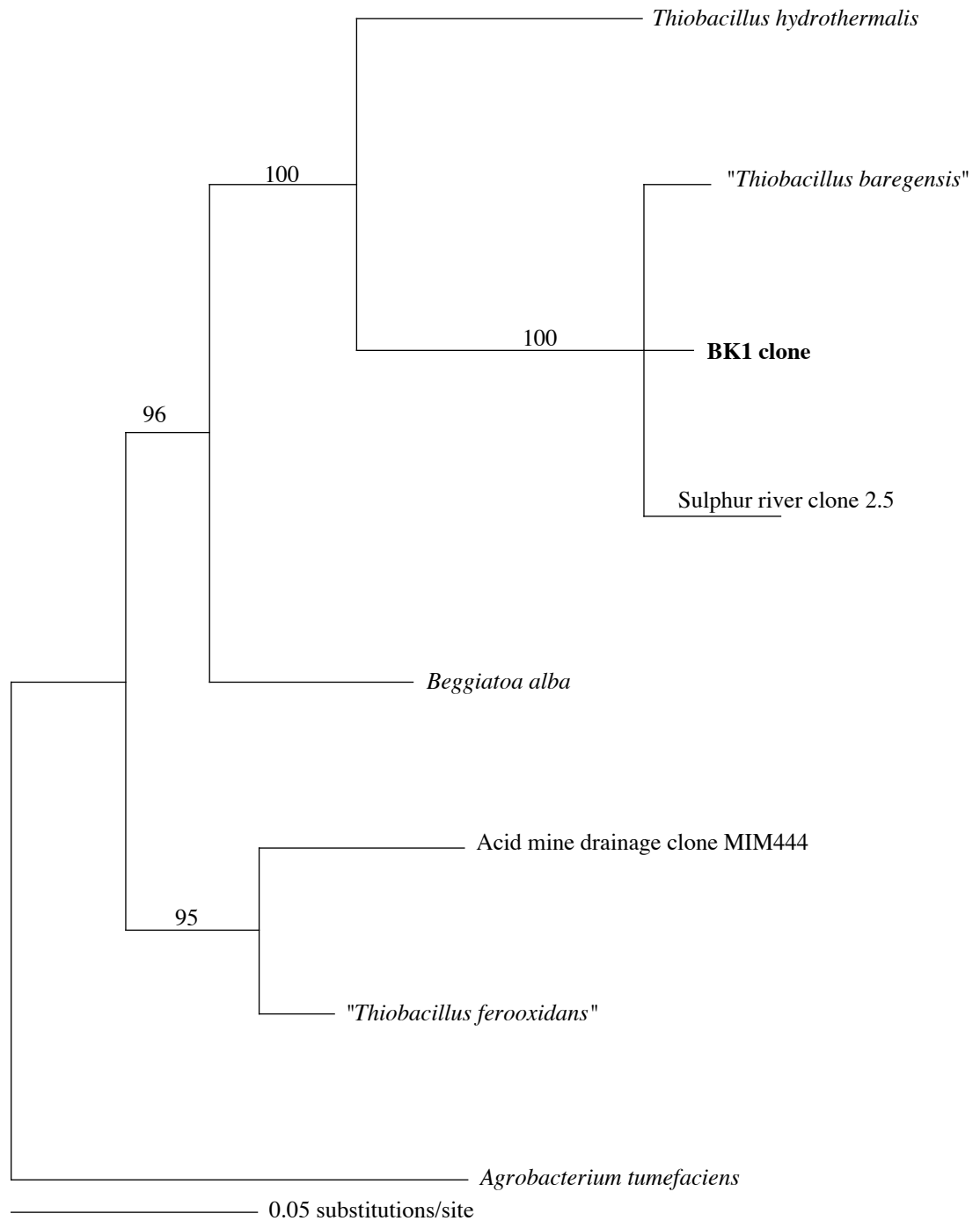

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Figure 8. Partial ARB neighbor-joining tree showing relative position of clone BK1.
Tree contains gamma–proteobacterial sequences and is rooted with a monophyletic archaeal outgroup.
Numbers on tree are Genbank accession numbers for organisms in the public data bases.

Figure 9. Bayesian inference phylogram showing relative position of clone BK1. Tree rooted with *Agrobacterium tumefaciens*. Bootstrap values displayed as percentages of 1000 replications.
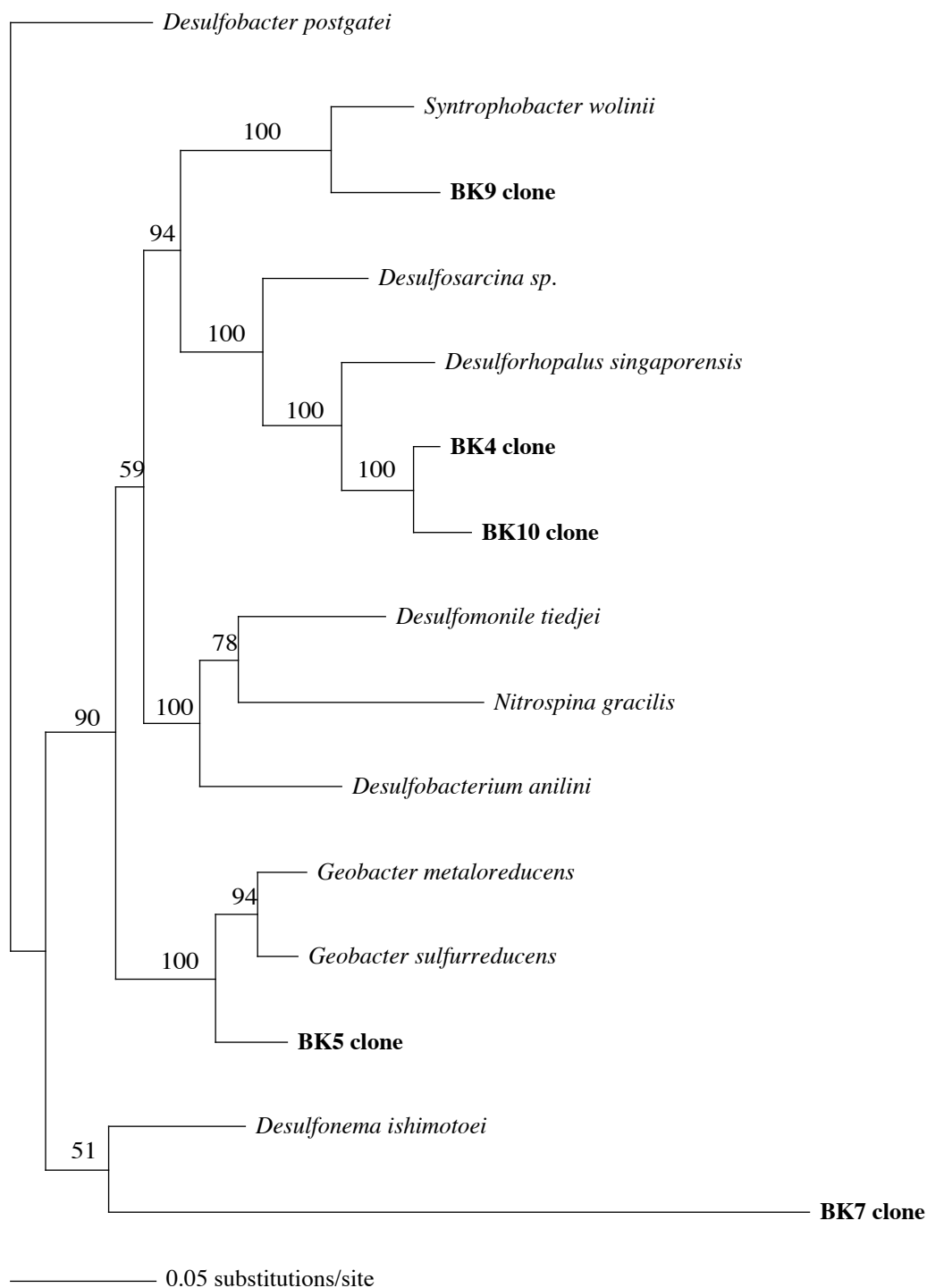
Figure 10. Partial ARB neighbor-joining tree showing relative positions of clones BK4, BK5, BK7, BK9, and BK10 as members of the delta-proteobacteria. Tree rooted with monophyletic archaeal outgroup. Numbers shown are Genbank accession numbers for organisms in the public databases.

Figure 11. Bayesian inference phylogram showing relative positions of clones BK4, BK5, BK7, BK9, and BK10. Tree rooted with *Desulfobacter postgatei*. Bootstrap values displayed as percentages of 100 replications.
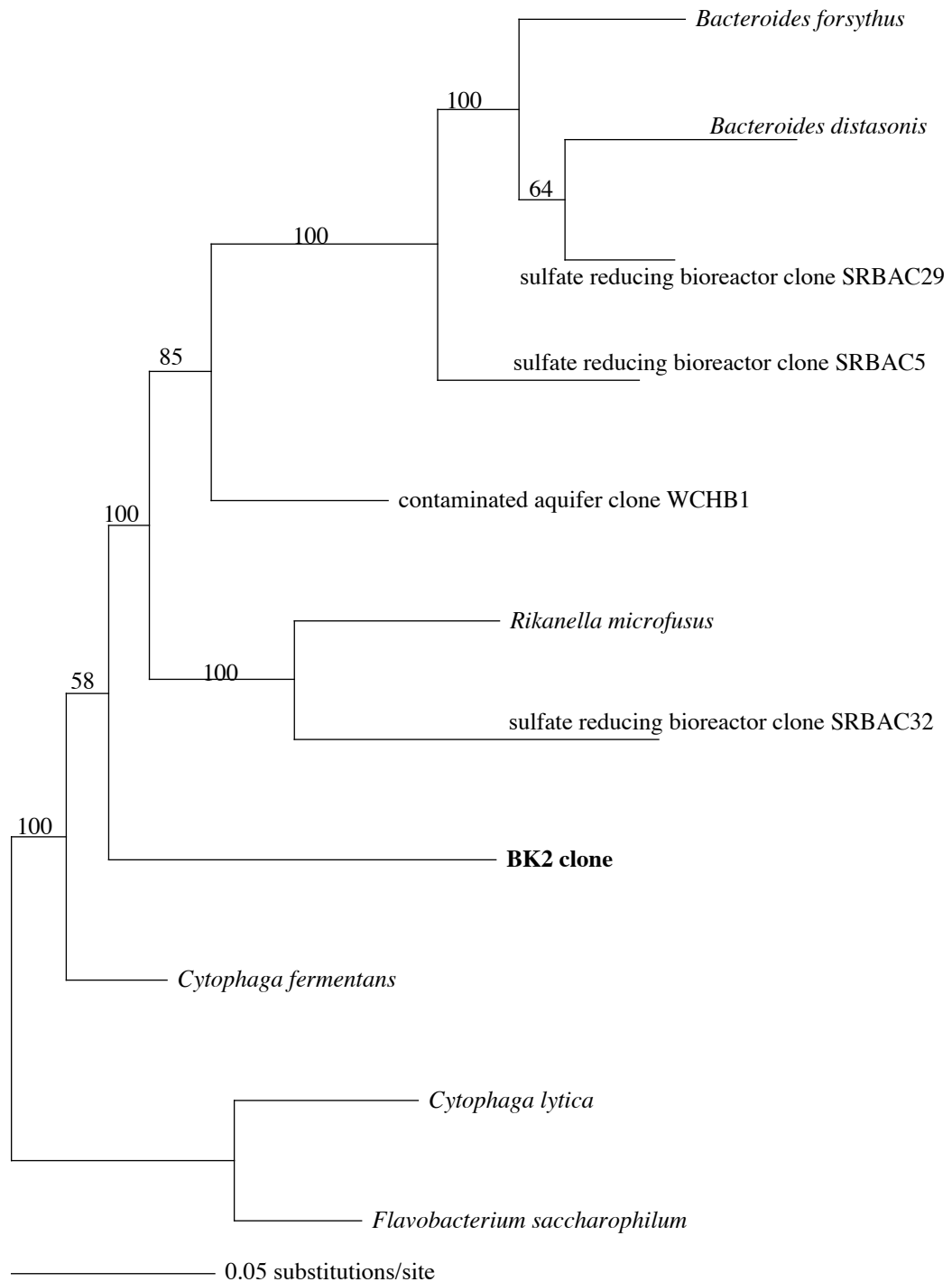
Figure 12. Partial ARB neighbor-joining tree showing relative positions of clones BK2 and partial BK108T3 as members of the Cytophaga/Flavobacteria/Bacteroides. Tree rooted with monophyletic archaeal outgroup. Numbers shown are Genbank accession numbers for organisms in the public databases.

Figure 13. Bayesian inference phylogram showing relative position of clone BK2. Tree rooted with *Cytophaga lytica* and *Flavobacterium saccharophilum a*s monophyletic sister clade. Bootstrap values displayed as percentages of 1000 replications.
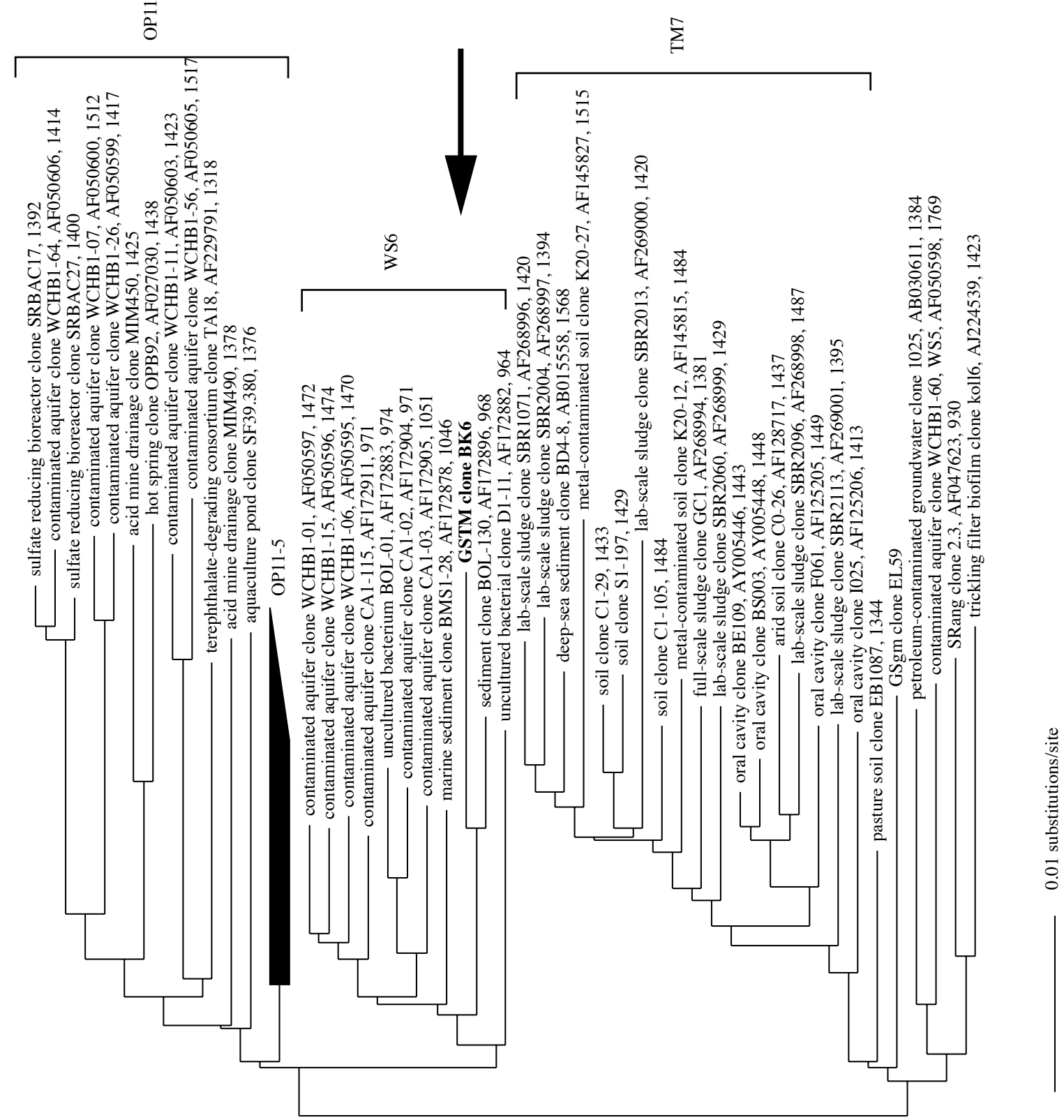
Figure 14. Partial ARB neighbor-joining tree showing relative position of clone BK6 as member of the WS6. Tree rooted with monophyletic archaeal outgroup. Numbers shown are Genbank accession numbers for organisms in the public databases.
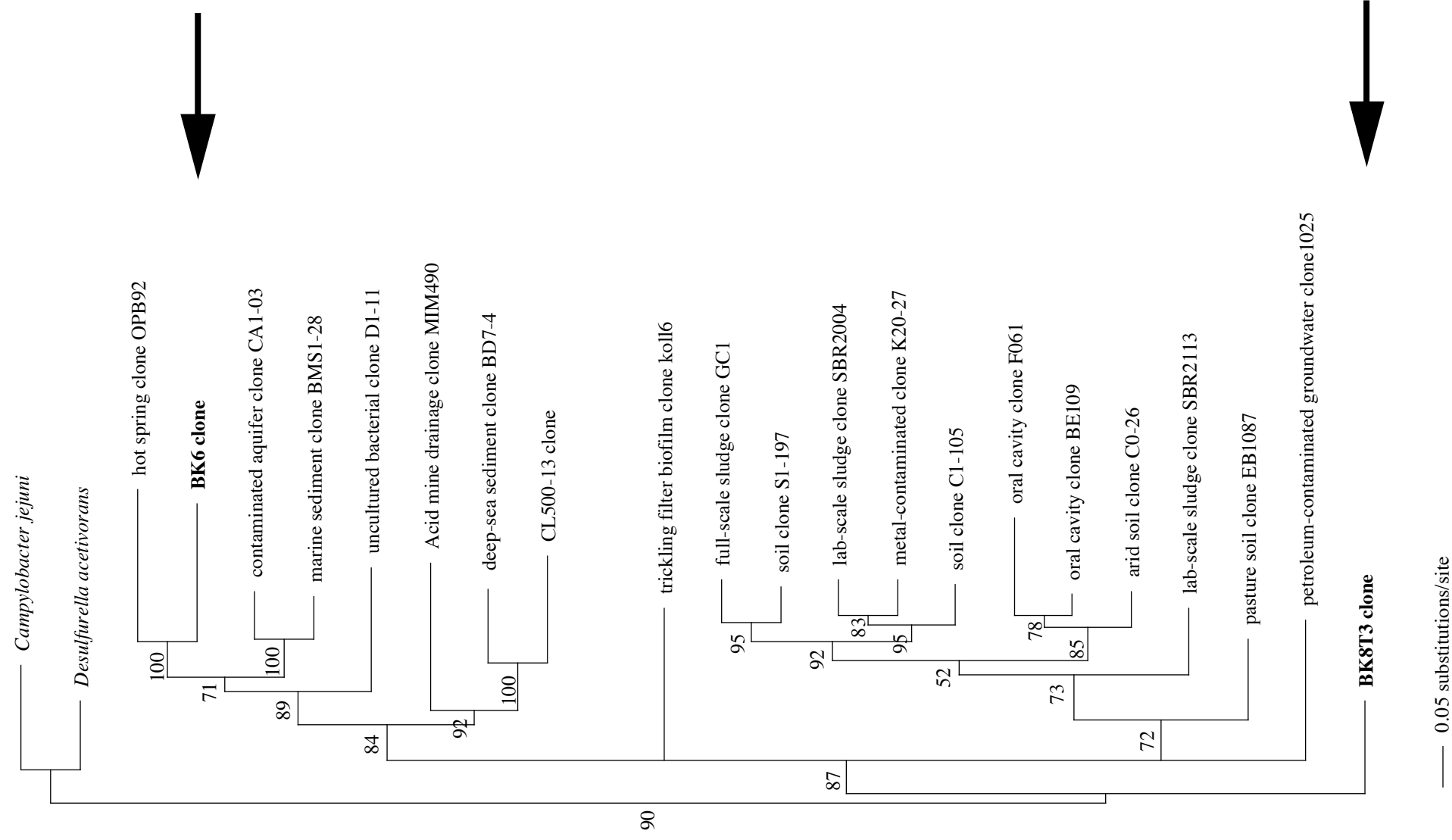
Figure 15. Bayesian inference phylogram showing relative positions of clones BK6 and BK8T3. Tree rooted with *Campylobacter jejuni* and *Desulfurella acetivorans* as monophyletic sister clade. Bootstrap values displayed as percentages of 1000 replications.
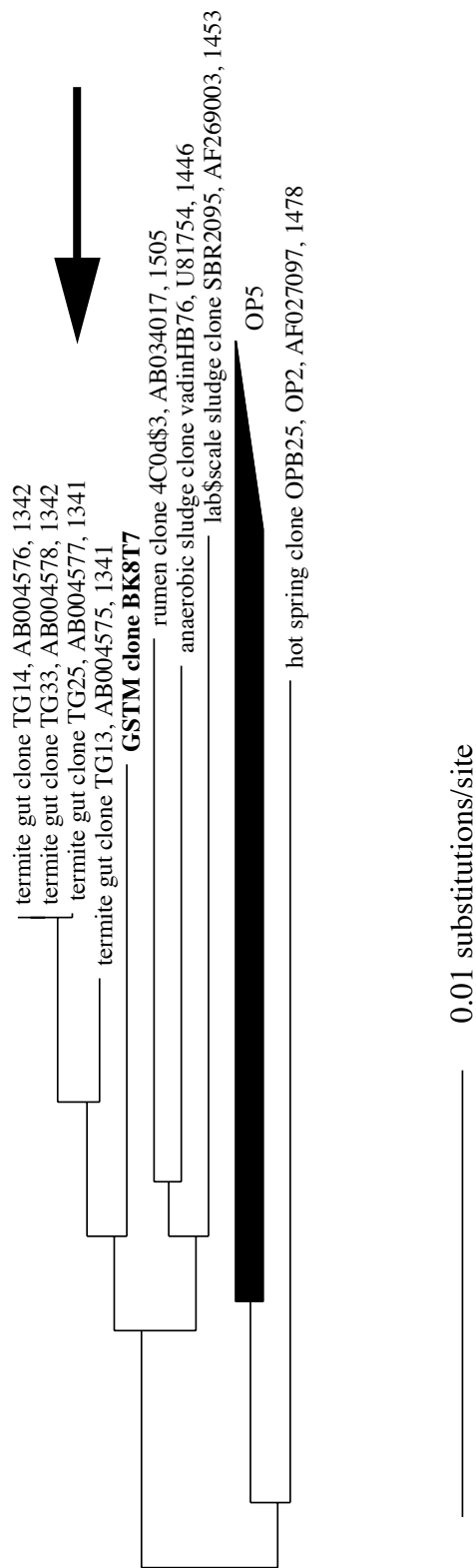
Figure 16. Partial ARB neighbor-joining tree showing relative position of partial clone BK8T7 as member of the Termite Group 1 phylum. Numbers shown are Genbank accession numbers for organisms in the public databases.